Mycotoxin Reduction in Grain Chains

John F. Leslie & Antonio F. Logrieco

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Contents

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Preface

Mycotoxins are fungal secondary metabolites that may be found in human foods and animal feeds. These compounds belong to families of organic molecules that share no significant properties other than the ability to confer death, disease, and misery on humans and other animals that consume them. Mycotoxins, and the fungi that synthesize them, frequently are detected by discoloration or other symptoms of fungal growth, but insidious contamination where the infected foodstuff appears normal to the eye is a significant problem. In developed countries, food safety regulatory systems are effective and efficient at identifying contaminated materials and removing them from primary food and feed channels. Rare incidents where animals are killed following consumption of mycotoxin-contaminated pet foods make national headlines, and most consumers know neither the names of the toxins nor the consequences of their consumption.

In less developed countries, especially in rural areas, the food commonly consumed often is a miasmatic mixture of inferior grain consistently contaminated with one or more mycotoxins, yet outright toxic outbreaks with multiple deaths are not common. Continuous sub-acute exposure to mycotoxins, however, suppresses immune system activity and retards the normal mental and physical development of children, and is likely responsible for far more human debilitation than are the much more sensational outbreaks where deaths occur. The physical separation of contaminated and uncontaminated food is a prelude to discarding the contaminated material in the developed world, but merely increases the exposure of those in less developed countries who are too poor to be able to afford anything else. As long as food security remains a significant issue, the threat of starving tomorrow will always be more significant than the threat of cancer in some 10–20 years. Trade regulations that lead to rejection of food lots containing any mycotoxins, effectively reduce food safety in developing countries as only the best quality food makes it to international trade and all of the rest remains for local consumption in the country of origin. Climate change and the pressure to expand production into marginal areas to be able to feed 9 billion people will only increase mycotoxin contamination problems, which are most common in stressed plants growing in less-than-optimal environments.

Most mycotoxin exposure is a result of consuming one or more of the world's five most commonly grown grains—wheat, maize, rice, barley, and sorghum—although some people also may be exposed to high levels of toxins following the consumption of peanuts. In this volume mycotoxin contamination is evaluated for these five grains, with emphases on wheat (and barley) and maize. For wheat and maize the entire grain chain from breeding to storage to food processing is considered, while for rice and sorghum, where mycotoxin problems are much less important, a literature review is provided. In many cases the authors look at current status as well as to future needs and desirable innovations. Developing rapid, simple techniques for detecting mycotoxins remains a major challenge as does breeding host plants that are resistant to the fungal diseases and depress accumulation

of the toxins the fungi can produce. Proper post-harvest storage can retain grain quality and prevent the synthesis of new toxins even if toxin-producing fungi are present. While HAACP-type protocols are not yet available to prevent mycotoxin contamination many of the critical parameters are known, and an important goal is to increase the efficacy of processes and protocols already identified as significant.

This book is unusual in that it looks at all five of the world's major grains and evaluates the entire grain chain from planted seed to processed food. We hope that it provides answers to many questions about the problems posed by and the controls available for mycotoxins, and that some readers will find all they need to know (and perhaps more than they want to know) within these pages. At the same time we know that there are many who will likely find this book as a first comprehensive introduction to these compounds and to the broadly interdisciplinary research that is needed to understand their implications, impacts, and costs in today's world. For them we hope this book, supported by a European Union FP 7 project—MycoRed, provides a good guide for where to go next. It is clear that mycotoxins are a significant problem that is becoming more widely recognized as such, and that the broadly integrated efforts of a variety of life, physical and social scientists is needed to effectively address it.

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1 An Introduction to the MycoRed Project

Antonio F. Logrieco and Angelo Visconti

Abstract

To comply with the needs of the European Union and to address global strategies for mycotoxin reduction, a 4-year large collaborative project, termed MycoRed (www.mycored.eu), was approved within the European FP7—"Food, Agriculture and Biotechnologies" Work Program. MycoRed's goal is to develop strategic solutions to reduce contamination by mycotoxins of major concern in economically important food and feed chains. Novel methodologies, efficient food handling procedures, and information dissemination and educational strategies are considered in a context of multidisciplinary integration of know-how and technology to reduce global exposure to mycotoxins. In this context, this volume brings together for the first time eminent scientists (many of them involved in the MycoRed project) on subjects relevant to mycotoxin production and accumulation in the cereal and maize food chains and ways that these toxins can be reduced or eliminated in the global food supply.

Keywords: global trade; international research collaboration; mycotoxin contamination reduction; food safety; industry competitiveness; training

Introduction

The main objective of the European Commission in the food sector is to create a knowledge-based bio-economy (KBBE). New models, tools, and methods—developed by science, industry, and other stakeholders—are required to assure the sustainable production of high-quality food and feed. To reach a KBBE, the vision for 2020 of the European Technology Platform (ETP), Food4Life, is the "effective integration of strategically focused, transnational, concerted research in the nutritional, food and consumer sciences and food chain management so as to deliver innovative, novel and improved food products for, and to, national, regional and global markets in line with consumer needs and expectations."

Protection of human health and the environment are important aspects of a KBBE. Reducing mycotoxin contamination in the worldwide food and feed chains is a major challenge to improve human and animal health. Mycotoxins are responsible for a variety of noxious problems in humans, including the induction of cancer, and digestive, blood, kidney, and nerve problems. One quarter of the world's food crops, including many basic foods, are potentially contaminated by mycotoxin-producing fungi. The mycotoxin problem is particularly important for human health in tropical

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areas, e.g., Sub-Saharan Africa, where crops are particularly susceptible to contamination with the carcinogenic aflatoxins and fumonisins. Globalization of trade has complicated the way we deal with mycotoxins in that regulatory standards often become bargaining chips in world trade negotiations (FAO, 2004; van Egmond *et al.*, 2007). While developed countries have numerous mycotoxin regulations and a well-developed infrastructure for enforcing food quality standards, people in developing countries are not protected by food quality monitoring or by the enforcement of safe standards within their countries. Food commodities that enter international commerce are expected to comply with *CODEX Alimentarius* standards. This requirement may indirectly increase the risk of mycotoxin exposure in developing countries because the best quality foods leave the country, while the lower quality food is consumed, often by humans, and not discarded. The consequences of this requirement to comply with the needs of the European Union and its consequences for global strategies for mycotoxin reduction need further consideration.

Due to numerous stringent mycotoxin regulations, particularly in Europe, the export/import agro-food industries must improve handling procedures during storage and processing, and utilize low-cost, user-friendly, multi-analysis detection kits (Leslie *et al.*, 2008; Piñeiro, 2008). One objective of the MycoRed (www.mycored.eu) project is to develop new practical technological solutions that may impact mycotoxin detection and management, e.g., micro-systems for ambient intelligence, new biomarkers, and multi-mycotoxin analyses.

Multidisciplinary integration of know-how and technology is required to address the broad requirements for reducing mycotoxins in agro-food chains. The project is based on the integration of specific technologies in the whole food/feed chain with respect to wheat, maize, grapes, nuts, and dried fruits. The main consumer demands posed in the agro-food sector drive research and technological developments in MycoRed. The need to improve prevention to minimize mycotoxins in products at different critical steps of the food chain, i.e., raw materials, storage, feed supply, food processing, and final products, is addressed by MycoRed through vertical (across food and feed chains) and horizontal (among methodologies and procedures) integration of experiences to develop a set of systems with clear breakthrough solutions to specific mycotoxicological problems. Additionally, dissemination of information and best practice education strategies to enhance the involvement of operators at all levels along the food and feed chains, facilitating participation and cooperation at the European and global levels, commensurate with the interest of the European Union community, is also a MycoRed priority.

MycoRed Objectives

A set of mycotoxin reduction targets of vital importance have been identified by some international food organizations, e.g., FAO, CIMMYT, EFSA, IITA, and SAFE consortium, European Union reports, and relevant food industry representatives (Barug *et al.*, 2006; CAST, 2003; Logrieco, 2001; Logrieco and Visconti, 2004; Visconti and Logrieco, 2005). In this respect, the knowledge and reduction of aflatoxins, trichothecenes (deoxynivalenol, nivalenol, T-2 and HT-2 toxins, etc.), zearalenone, fumonisins, and ochratoxin A are the most important issues addressed in the project. Aflatoxin B_1 is a proven carcinogen for humans. It is immunotoxic and causes stunted growth in children and growth retardation in animals. *Fusarium* toxins, especially fumonisins, are neurotoxic and possible carcinogens; trichothecenes are immunotoxic; and zearalenone is estrogenic. Ochratoxin A is a nephrotoxin, possibly carcinogenic to humans, and associated with Balkan endemic nephropathy. Mixtures of mycotoxins probably have at least an additive, if not synergistic, egregious effects. These toxins are primary sources of both yield losses and increased management

Table 1.1 Food/feed chains and relative mycotoxins and fungi studied under the MycoRed program

Plant	Chain	Toxin	Fungal genus
Maize	Food/feed	Fumonisins	*Fusarium*
		Aflatoxins	*Aspergillus*
Wheat	Food/feed	Trichothecenes	*Fusarium*
		Zearalenone	*Fusarium*
		Ochratoxin A	*Penicillium*
Grapes—including raisins and sultanas	Food and wine	Ochratoxin A	*Aspergillus*
Nuts and dried fruits—peanuts, figs, pistachios, and almonds	Food	Aflatoxins	*Aspergillus*

costs worldwide. MycoRed has an integrated vision of a reduction system as a horizontal task ensuring dissemination of different technological solutions developed by the research activities in the project.

MycoRed targeted strategic solutions for reducing contamination by the mycotoxins of major concern in economically important selected food and feed chains. The following toxins and commodities have been focused on in the project: trichothecenes, fumonisins, and aflatoxins in wheat/maize-based food and feed chains; ochratoxin A in the grape, wine and wheat chains; and aflatoxins in the nut and dried fruit chain (Table 1.1). Novel methodologies, efficient handling procedures, and information dissemination and educational strategies were considered in a context of a multidisciplinary integration of know-how and technology to reduce mycotoxins worldwide from the field to the market.

Wheat and maize, in particular, have major mycotoxin safety concerns worldwide and are being studied up and down their food and feed chains. Research on mycotoxins in wheat has been much more intensive than it has been recently in maize, even though maize is at least as significant toxicologically as wheat. In Europe there generally are no data on the economic costs of mycotoxins. One exception is Hungary, where the direct and indirect losses attributable to a 1998 wheat epidemic were estimated at €100 million. In the United States alone, the mean economic annual costs at the farm gate of cereal crop losses due to aflatoxins, fumonisins, and trichothecenes combined are estimated to be $932 million (CAST, 2003), which dwarfs the USDA budget for research on this problem by some 600×.

The overall objectives of MycoRed are:

- to develop novel, solution-driven methodologies and handling procedures to reduce both pre- and postharvest mycotoxin contamination in selected feed and food chains; and
- to generate and disseminate information and education strategies to reduce mycotoxin risks at a global level. High-risk areas receive major attention through cooperation with international agriculture and food organizations and through applications of the results of all of the technical work packages in the project.

The multidisciplinary approach and the numerous existing collaborations/networks involving MycoRed partners that are already in place have ensured that European, American, African, and Australian scientists are actively engaged with one another and have resulted in synergistic advances in the development of practical solutions with applications in both developed and developing countries. The integration of multidisciplinary expertise in chemistry, microbiology/plant pathology, genetics, plant breeding, agronomy, toxicology, plant and animal physiology, molecular biology,

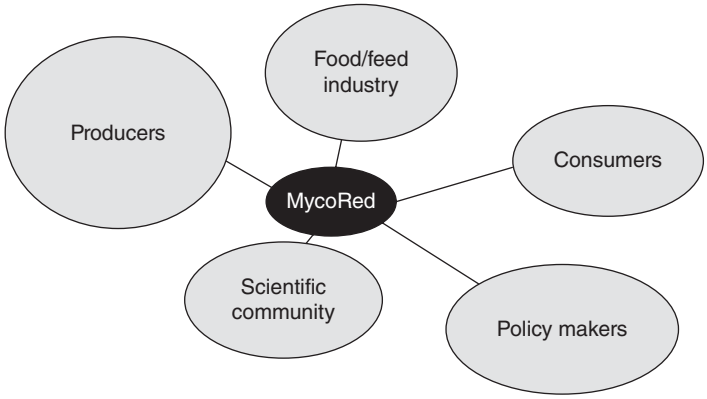

Figure 1.1 MycoRed stakeholders. The size of the circle indicates the relative importance of the user group. The relative distance of the circle to the user group indicates the relative strength of the communication with that group, with shorter lines indicating stronger communications than longer ones.

and food technology will have a synergistic effect on the development of practical solutions to minimize mycotoxins in the food and feed chains. Some of the proposed solutions are generic and will have direct application to other food/feed chains. The numerous dissemination events have ensured the widespread distribution of the information and technologies generated and encouraged scientific cross-fertilization between different disciplines.

Thus, MycoRed's goals are to increase mycotoxin awareness in five user communities (Figure 1.1) with interests in mycotoxins:

- *For consumers*—Increased food safety for humans and feed safety for animals, i.e., minimize health risks from mycotoxin-contaminated food and feed, especially in high-risk areas.
- *For agro-food producers* (farmers and animal and plant breeders)—Provide tools and demonstrate advantages of pre- and postharvest solutions to reduce and control mycotoxin contamination in plant products and in the consequent food/feed chains. To obtain agro-industrial products with major added value that results in increased economic development in rural areas.
- *For food/feed industries*—Generate opportunities for new applied research and development. Use current research results for developing novel preventive and curative (bio)control measures against toxigenic fungi. Reduction of mycotoxin contamination by improved handling procedures during storage and processing. Availability of new mycotoxin and mycotoxigenic fungi detection kits.
- *For policy makers and legislators*—Provide improved information and know-how for evaluating real and potential mycotoxin risks, including possible outcomes of climate change and increased international trade. Provide indicators for assessing the risk of contaminated commodities.
- *For the scientific community*—Increase advanced knowledge, e.g., fungal and plant genomics, metabolomics, and proteomics, of the mycotoxin-producing fungi and their hosts. Improve global communications, international networking, and dissemination of innovative research results with the support of learned societies, e.g., the International Society for Mycotoxicology, and dedicated scientific journals, e.g., *Food Additives and Contaminants* and the *World Mycotoxin Journal*, that help knit the interdisciplinary mycotoxin research community together.

MycoRed Structure

The MycoRed approach uses global, multidisciplinary, and integrated strategies, that are effectively applied along the food and feed chains and linked to decision-making bodies and consumers through effective mycotoxin risk assessment and information and education programs. Five work packages develop novel solution-driven strategies and handling procedures to reduce both pre- and postharvest contamination in feed and food chains; two work packages have horizontal disciplinary foci on detection; and a final work package focuses on communication and outreach:

- *Work Package 1*—optimization of plant resistance and fungicide use;
- *Work Package 2*—biological control to reduce toxigenic fungi in cropping systems;
- *Work Package 3*—modeling and developing decision support systems;
- *Work Package 4*—postharvest and storage practices;
- *Work Package 5*—application of new food-processing technologies;
- *Work Package 6*—develop methodologies for advanced diagnostics and quantitative detection of toxigenic fungi;
- *Work Package 7*—rapid and multi-analyte detection and quantification of mycotoxins and relevant biomarkers;
- *Work Package 8*—information, education, dissemination, and demonstration activities to reduce mycotoxin risks worldwide based on the results generated by the other seven work packages and other knowledge of methodologies and handling procedures actually in use for particular crops in various geographical areas.

The project has a solid technical foundation due to the involvement of well-regarded European groups who developed collaborative programs through previous European projects funded in Framework 5 and Framework 6 in the area of mycotoxins and toxigenic fungi. In particular, MycoRed partners have previously been involved in 22 projects funded by the European Commission and have coordinated 8 others that are scientifically linked to MycoRed. This project offers one of the best opportunities for reducing mycotoxin contamination in food and feed chains worldwide.

The direct involvement of ICPC countries, i.e., Russia, Egypt, and Argentina, and International Organizations, i.e., CIMMYT and IITA, that focus on developing countries in Africa, South and Central America, and Asia will benefit areas of the world where mycotoxin problems are critically important for human health and trade. These countries and institutions are of particular importance for MycoRed information/dissemination/education activities, and scientific conferences sponsored by MycoRed have been held in all of these regions.

Strategic alliances with major public research institutions in the United States (three USDA centers and four universities), Australia, South Africa, and Malaysia are of particular value and strengthen the project by sharing experience and resources from numerous past and current mycotoxin projects at a global level. Mutual interest in this intercontinental collaboration has resulted in mutual benefits from joint efforts in national, regional, and international research and development programs. For example, the results of ongoing mycotoxin research programs involving three USDA partner locations (with an annual investment of US $3.5 million) is being shared with corresponding European researchers working in the same area.

An External Advisory Board, composed of scientists from concerned disciplines and related initiatives, industrialists from the agro-food sector and food regulatory bodies, and representatives from the ETP Food4Life program, provides an external perspective to the project, advises the

consortium on issues of knowledge transfer and exploitation, and recommends effective actions to reach the project goals.

Social and Economic Impact

A significant reduction of mycotoxins in the food and feed industries is one of the key challenges for increased sanitary quality in the European food market. MycoRed has addressed this challenge by developing and utilizing innovative, multidisciplinary strategic solutions that provide higher value added and are changing the agro-food industry as it adapts to the requirements of the future European consumer society.

The objective-oriented approach of this project addresses a strong potential socioeconomic impact. We expect the MycoRed solutions to significantly reduce mycotoxin contamination in food and feed chains in Europe and, for some targets, worldwide. The reduction of these principal mycotoxins by novel, multidisciplinary, integrated strategies through the maize, wheat, grape, and dried fruit chains will produce the following socioeconomic impacts:

- A significant decrease in the number of acute and chronic pathologies in Europe and in ICPC countries due to the consumption of mycotoxin-contaminated products. This reduction will directly reduce costs in the human and animal health-care systems.
- A decrease in the costs of rejection of contaminated raw crop materials, especially dried fruits and cereals, and processed products. This impact will be very important for farmers, food/feed producers, and retailers. Over the past years, mycotoxin regulations in the European Union have changed rapidly and proliferated, posing significant challenges to more sectors of the food industry than ever before. Failure to comply with the latest standards can have major economic consequences for exporters and importers, ranging from costly retesting and reprocessing to impounded, rejected, or destroyed shipments.
- Improved ability to reduce safety problems due to mycotoxin contamination beginning at any stage in the production chain—pre-, postharvest, and processing.
- Increased safety of feed and food stuffs due to the application of various innovative technical solutions developed through research sponsored by MycoRed. Ultimately there will be full and effective interconnections and communication between sensing systems and decision-making bodies. The earlier in the food chain that corrective actions can be taken, the better, simpler, and easier the storage/shipment and production processes.
- An increase in the competitiveness of European agro-food industries resulting from the application of research results, e.g., safety of food/feed, predictive models, antagonist formulations, and new detection kits, to relevant problems.
- Promotion of new spin-off small and medium-sized enterprises (SMEs) in Europe and ICPC countries that serve the agro-food application markets. Training activities carried out under MycoRed have helped to satisfy the demands for skilled employees in the area of food safety.

Thus, the MycoRed project has a potentially huge economic impact on both the international agro-food community (farmers, animal and plant breeders, etc.) and the European agro-food industries. There are no European economic analyses that have evaluated the scientific improvements and altered strategies that have resulted from research sponsored by MycoRed and targeted at the reduction of global mycotoxin contamination. MycoRed is now sponsoring such a study to identify

additional areas in which new technologies and improved handling solutions could have an economic impact on food safety.

Conclusions

MycoRed plays an important role in European and global mycotoxin food/feed safety through its integrated research projects on mycotoxin reduction. It shares the information gathered through training and dissemination programs and through cooperative efforts with other similar initiatives, e.g., ISM and MoniQa. MycoRed enables the sharing of data and knowledge for harmonizing good agricultural practices, good management practices, and good storage practices in diverse geographic areas and multiple food/feed chains, through publications such as this book.

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Part I
The Maize Grain Chain

2 Identification of Toxigenic *Aspergillus* and *Fusarium* Species in the Maize Grain Chain

Deepak Bhatnagar, Gary Payne, Maren Klich, and John F. Leslie

Abstract

Many of the most economically important mycotoxins are synthesized by fungi in one of two fungal genera: *Aspergillus* (aflatoxins) and *Fusarium* (fumonisins, zearalenone, and trichothecenes). Even within these genera, many of the species do not produce economically significant quantities of any known mycotoxins. Morphology of spores and cultures growing on defined media have traditionally been used to distinguish these species. Molecular analyses of conserved regions of the genome have increased the putative number of species, with the number of isolates of many newly delineated taxa often too limited to determine their economic importance. Molecular analyses hold the promise of greater taxonomic stability across these genera, but inconsistencies based on morphology, sexual cross-fertility, and phylogenetic methods remain in some critical cases. Mycotoxin biosynthetic genes for individual toxins are usually found in clusters that are recognizable as such in both toxin-producing species and non-toxin-producing related species. Insertions, deletions, and rearrangements within these clusters may affect the amount and exact structure of the mycotoxin produced. Genes within pathways that produce mycotoxins often are well understood, but the regulation of these pathways in response to environmental factors remains poorly understood and is a major target for future research

Keywords: aflatoxins; biological species; corn; deoxynivalenol; fumonisins; gene clusters; heterothallic; homothallic; morphology; nivalenol; phylogenetic species; population structure; zearalenone

Introduction

Natural toxins occurring in food and feed can be categorized as mycotoxins, bacterial toxins, and phycotoxins, all of which are produced by living organisms in food or transferred through the food chain. In addition, there are phytotoxins and zootoxins, which are inherent components of plants or animals and are harmful to humans and domesticated animals. Mycotoxins are natural products produced by fungi that evoke a toxic response in higher vertebrates and other animals when ingested at low concentrations (Bhatnagar *et al.*, 2002; Richard and Payne, 2003). These compounds are low-molecular weight, secondary metabolites derived primarily from amino acids, shikimic acid or malonyl CoA. Mycotoxins generally are produced in the mycelia of filamentous fungi, but can accumulate in specialized structures of fungi, e.g., conidia or sclerotia, as well as in the surrounding environment.

Mycotoxin Reduction in Grain Chains, First Edition. Edited by John F. Leslie and Antonio F. Logrieco.

A specific mycotoxin may be produced by members of several fungal species, members of a single fungal species, or a specific subset of strains within a fungal species. The toxic effects of these mycotoxins are as diverse as the number of fungal species that make them. Some mycotoxins have acute toxic effects, especially when ingested at high concentrations, whereas others have toxic effects only after long-term exposure to lower doses, i.e., chronic effects. Over 300 mycotoxins have been identified, and almost all of these are produced by strains from three genera, i.e., *Aspergillus*, *Fusarium*, and *Penicillium*. The former two genera are those found most commonly in the maize grain chain throughout the world and produce the mycotoxins aflatoxins (*Aspergillus flavus* and *Aspergillus parasiticus*), fumonisins (by *Fusarium verticillioides* and *Fusarium proliferatum*), and trichothecenes and zearalenone (*Fusarium graminearum* and *Fusarium culmorum*). The identification of these fungi is discussed in this chapter.

Morphological Identification of Aflatoxin-Producing *A. flavus* and *A. parasiticus*

The three fungal genera, *Aspergillus*, *Fusarium*, and *Penicillium*, responsible for most agronomic mycotoxin problems, reproduce asexually, forming mitospores called conidiospores or conidia) on specialized structures called conidiophores (Figure 2.1). The conidia of *Aspergillus* and *Penicillium*

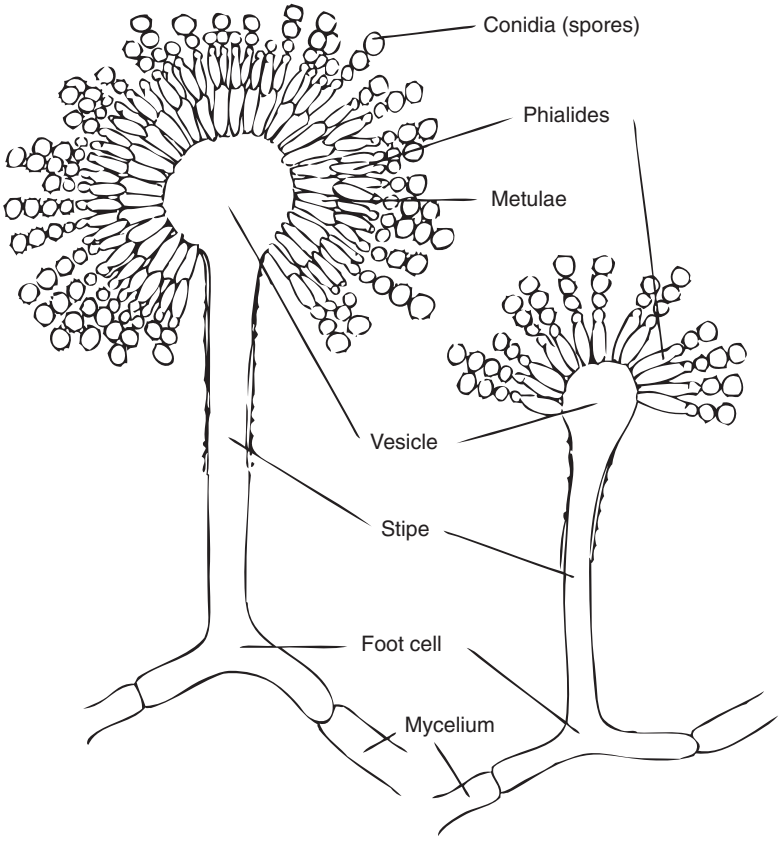

Figure 2.1 Basic morphological structure of *Aspergillus* showing (i) biseriate and (ii) uniseriate conidial heads.

are single-celled spheres or ellipsoids. In contrast, *Fusarium* species typically produce falcate multicelled macroconidia and may produce smaller one- to two-celled microconidia as well as long-lived chlamydospores. The asexual state of these fungi is called the anamorph, or the imperfect stage. These genera are very large, so they are broken down into subgenera and sections. *Aspergillus flavus*, for instance, belongs to subgenus *Circumdati* and section *Flavi.* Some members of these genera also produce a sexual state called the teleomorph, or the perfect stage. The sexual stage may have a different name; for example, the sexual state of *A. flavus* is *Petromyces flavus* (Horn *et al.*, 2009b). This "dual nomenclature" has been the subject of much taxonomic debate since molecular methods now enable the linkage of sexual and asexual stages even if there are no clear, common morphological characters. Beginning January 1, 2013, the Botanical Code was amended to allow only one name per fungal species. The arguments are now shifting to which names should be kept and/or abandoned. Changes in genus name for at least some *Aspergillus* species seem all but certain, while changes in genus names for most *Fusarium* species appear unlikely (Geiser *et al.*, 2013).

Traditionally, species of *Aspergillus* have been distinguished from one another by using morphological characters and gross physiological characters, e.g., growth rate or pigment production. Some species, however, cannot be distinguished based solely on these characters, so metabolite production and/or molecular data often are used as descriptive characters as well, an approach termed "polyphasic" taxonomy.

To date, all validated aflatoxin-producing fungi belong to the genus *Aspergillus* or one of its teleomorphic states (Varga *et al.*, 2009). Most of these species belong to *Aspergillus* section *Flavi*, including *A. arachidicola*, *A. bombycis*, *A. flavus*, *A. minisclerotigenes*, *A. nomius*, *A. parasiticus*, *A. parvisclerotigenus*, and *A. pseudotamarii* (Varga *et al.*, 2009). Two rarely recovered aflatoxin-producing species *A. ochraceoroseus* and *A. rambellii* are in their own section, section *Ochraceorosei* (Frisvad *et al.*, 2005). Three fungi in section *Nidulantes* produce aflatoxins: *A.* (*Emericella*) *venezuelensis*, *A.* (*Emericella*) *astellata*, and *A.* (*Emericella*) *olivicolor* (Frisvad and Samson, 2004; Frisvad *et al.*, 2004; Zalar *et al.*, 2008).

The aflatoxin-producing members of sections *Ochraceorosei* and *Nidulantes* have no known economic importance. Members of these two sections can readily be distinguished from those in section *Flavi.* Unlike the aflatoxigenic species in section *Flavi*, members of section *Ochraceorosei* do not grow at 37°C. The three aflatoxigenic species in section *Nidulantes* readily produce red stellate ascospores in ascocarps surrounded by highly refractive Hulle cells. The sexual stages of species in section *Flavi* described to date are all in the teleomorphic genus *Petromyces*, which is characterized by hard, sclerotia-like ascocarps. The *Petromyces* states of both *A. flavus* and *A. parasiticus* are known (Horn *et al.*, 2009a, 2009b) and may arise from the sclerotia of these species over time when strains with different mating types are crossed, i.e., these species are heterothallic (Horn *et al.*, 2009c). *Aspergillus* species with known sexual stages are homothallic, i.e., they can form ascospores with only one strain present. *Aspergillus flavus* and *A. parasiticus* are not the only economically important heterothallic species, as the human pathogen, *Aspergillus fumigatus*, is also heterothallic, with a sexual stage in the genus *Neosartorya* (O'Gorman *et al.*, 2008).

In general, *A. flavus* and *A. parasiticus* are presumed to be the major economically important aflatoxin producers, but the data available for more recently described species are insufficient to determine their true economic importance. A number of these species would have been identified as *A. flavus* or *A. parasiticus* until just a few years ago (Geiser *et al.*, 2007). Before the new sibling species were described, *A. flavus* and *A. parasiticus* were relatively easy to distinguish from one another morphologically by observing the spores under the light microscope at 40× magnification. At that magnification, the spores of *A. flavus* appear to be smooth and those of *A. parasiticus*

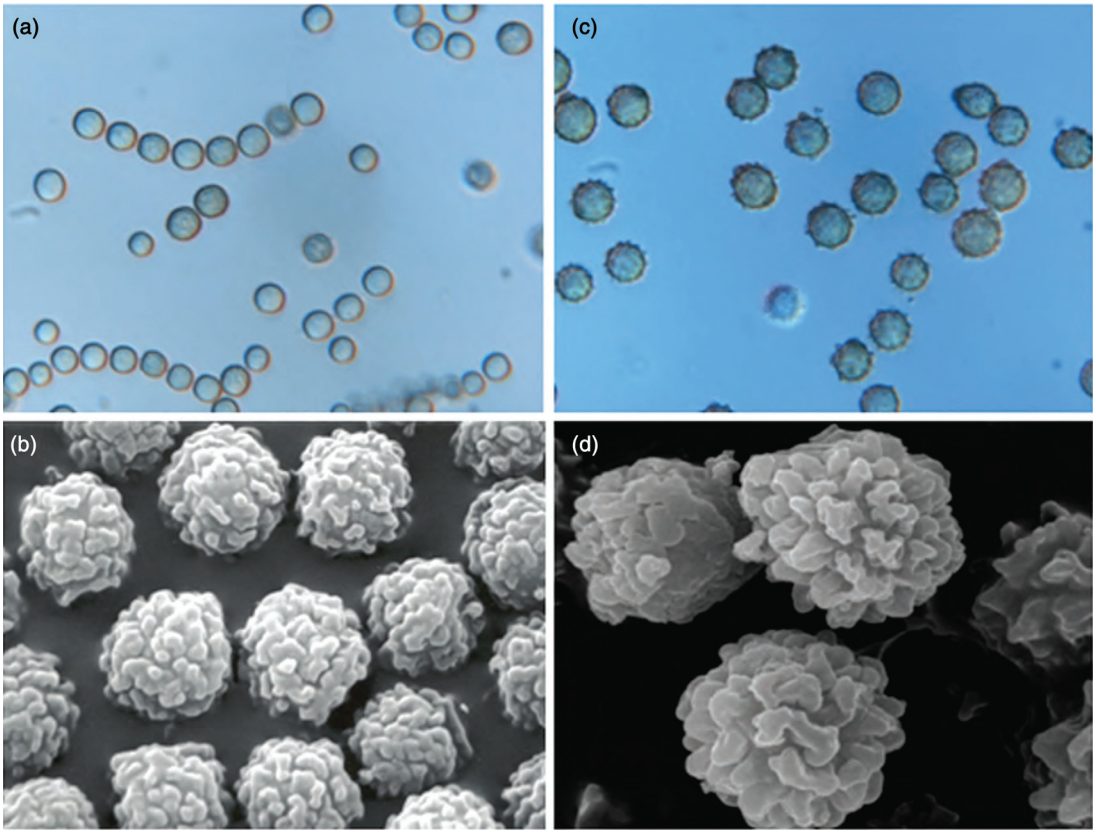

Figure 2.2 Scanning electron micrograph of conidia for *Aspergillus flavus* (left panels a and b) and *Aspergillus parasiticus* (right panels c and d).

appear to be rough (Figure 2.2, Klich, 2002). Seriation, the number of cell layers between the conidiophore vesicle, i.e., the swollen region at the end of the stipe (Figure 2.1), also has been used to distinguish these species, even though it is not a consistent character in section *Flavi* (Klich and Pitt, 1988).

Most aflatoxin-forming species can be identified by using only morphology and metabolite data. Among the described aflatoxin-producing species in section *Flavi*, *A. flavus* and *A. pseudotamarii* produce only B series aflatoxins if they produce any aflatoxins, while the other species in this section can produce both B and G series aflatoxins. Of the species producing both B and G series aflatoxins, *A. bombycis* is unique in that it grows to only about 15 mm in a week when incubated at 37°C (Peterson *et al.*, 2001), with members of the other species forming much larger colonies under those conditions. Both *A. parvisclerotigenus* and *A. minisclerotigenes* produce cyclopiazonic acid, but only *A. parvisclerotigenus* produces versicolorins (Pildain *et al.*, 2008; Varga *et al.*, 2009). Of the remaining three B and G aflatoxin producers, only *A. nomius* produces nominine. *Aspergillus parasiticus* and *A. arachidicola* are very similar morphologically and are best separated by differences in their β-tubulin gene sequences (Pildain *et al.*, 2008).

Morphological Identification of Toxin-Producing *Fusarium* Species

Toxins produced by *Fusarium* species can cause major problems for the health of both humans and domesticated animals. These fungi can produce thousands of different secondary metabolites with diverse chemical structures and physiological effects (Desjardins, 2006; Vesonder and Goliński, 1989). In most of the world, the trichothecenes deoxynivalenol and nivalenol and the polyketide-derived fumonisins and zearalenone are the major toxins of interest. Other toxins, including some on the US government list of select agents for bioterrorism (www.selectagents.gov/Select%20Agents%20and%20Toxins%20List.html), include diacetoxyscirpenol, T-2 toxin, beauvericin, fusaproliferin, moniliformin, and fusarenone-X. Diacetoxyscirpenol and T-2 toxin, the two deadliest toxins produced by any *Fusarium* species, are not common contaminants at high levels in commodities grown outside of climates that are cold and often humid, e.g., Russia, the Scandinavian peninsula, and eastern Canada. Deoxynivalenol, nivalenol, fumonisins, and zearalenone can each be synthesized by several different *Fusarium* species (Desjardins, 2006; Leslie and Summerell, 2006; Marasas *et al.*, 1984), although no species synthesizes all four of these metabolites. Widespread production of these toxins at high levels usually implicates one of three species—*F. graminearum* (*Gibberella zeae*), *F. proliferatum* (*Gibberella intermedia*), or *F. verticillioides* (*Gibberella moniliformis*).

Fusarium taxonomy has been a major research topic for much of the 200 years since the genus was first described by Link in 1809 (Link, 1809). By the early 1900s, there were thousands of *Fusarium* species names with little definition to back them up and identification often based on the host from which the fungus was isolated. Wollenweber and Reinking (1935) simplified the taxonomy by defining 16 sections, 65 species, and a number of subspecific taxa based on the morphology of fungal cultures and independent of the host from which the fungus was isolated. The Wollenweber and Reinking taxonomic analysis brought order and routine to the identification and description of *Fusarium* species and remains the basis for most of the current taxonomic systems in the genus. Biological and phylogenetic species definitions usually result from adding characters to the Wollenweber and Reinking species definitions or some modification thereof, e.g., Booth (1971), Gerlach and Nirenberg (1982), Nelson *et al.* (1983), and Leslie and Summerell (2006). We recommend the use of the criteria presented in the Leslie and Summerell (2006) manual for making routine species identifications.

From the late 1940s through the early 1980s, Snyder and Hansen (1940, 1941, 1945, 1954) proposed that all *Fusarium* isolates could be placed into one of only nine species. This reduction made identification much simpler, but also minimized and confounded the information conveyed by the name to such a degree that many of the studies done by researchers following the Snyder and Hansen naming conventions are difficult, if not impossible, to relate to current studies because the true identity of the fungi being evaluated is not known. Two of the species that were redefined by Snyder and Hansen, i.e., *Fusarium roseum* and *Fusarium moniliforme*, have been abandoned because they have too many different meanings, e.g., Seifert *et al.* (2003), and should no longer be used. *Fusarium graminearum* was a part of the *F. roseum* group and *F. verticillioides* and *F. proliferatum* were both a part of the *F. moniliforme* group. Two of the Snyder and Hansen species, *Fusarium solani* and *Fusarium oxysporum*, are still in wide use, although most researchers agree that these are not single species, but rather are species complexes containing tens if not hundreds of species that are in need of further resolution.

For identification purposes, *Fusarium* is not usually identified to species based on morphology observed directly on the infected host. Instead colonies are recovered from infected plant material or soil placed on a medium that contains PCNB, which is semi-selective for *Fusarium* spp. These colonies are purified through a single conidial spore subculture (Figure 2.3) and then transferred to carnation leaf agar and potato dextrose agar, and perhaps other media depending on the particular

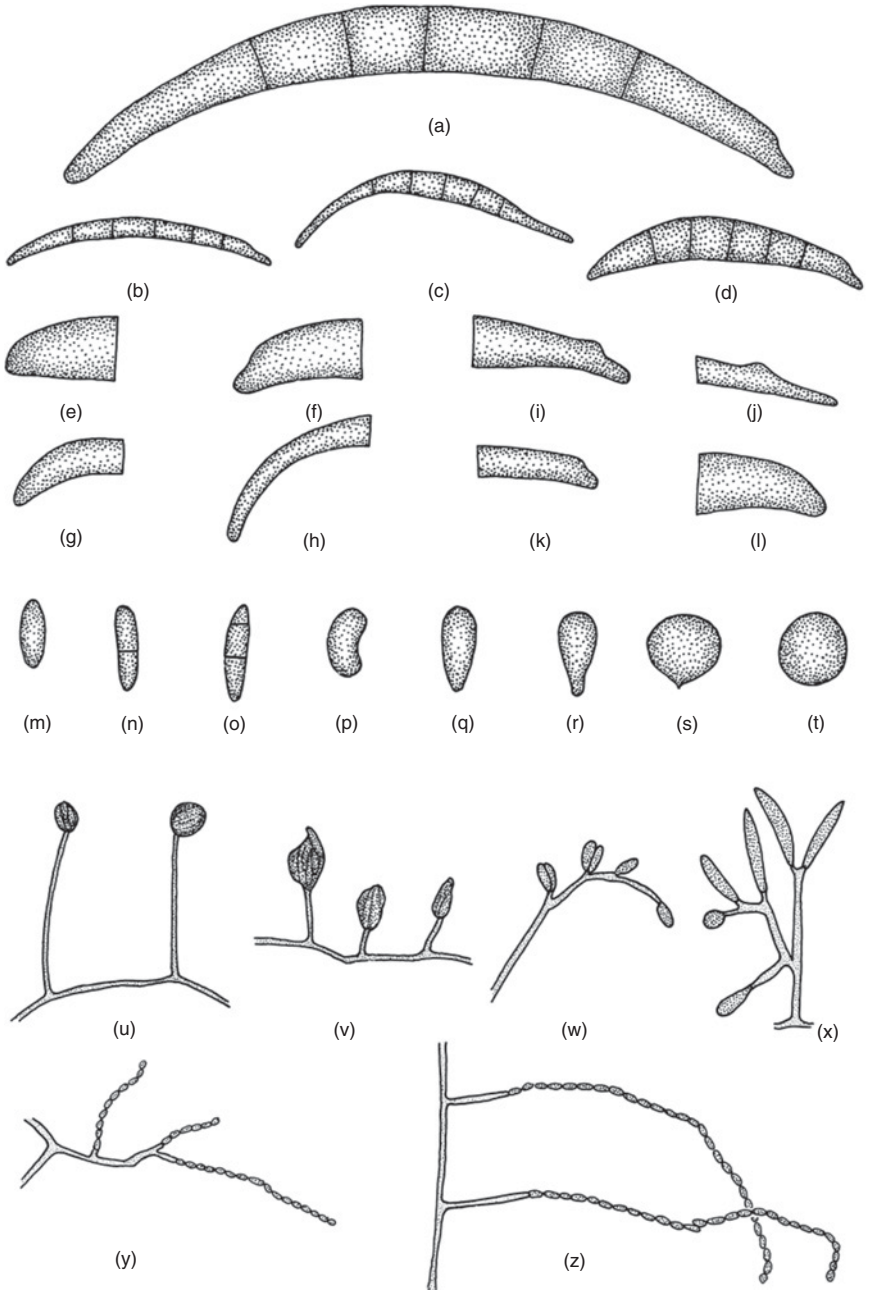

Figure 2.3 Spore morphology characters used in the identification of *Fusarium* species. Drawings are idealized and not necessarily to the same scale. (a–d) Macroconidial shapes. (a) Typical *Fusarium* macroconidium. Apical cell on left, basal cell on right. (b) Slender, straight, almost needle-like macroconidium, e.g., *F. avenaceum*. (c) Macroconidium with dorsoventral curvature, e.g., *F. equiseti*. (d) Macroconidium with the dorsal side more curved than the ventral, e.g., *F. crookwellense*. (e–h) Macroconidial apical cell shapes. (e) Blunt, e.g., *F. culmorum*. (f) Papillate, e.g., *F. sambucinum*. (g) Hooked, e.g., *F. lateritium*. (h) Tapering, e.g., *F. equiseti*. (i–l) Macroconidial basal cell shapes. (i) Foot shaped, e.g., *F. crookwellense*. (j) Elongated foot shape, e.g., *F. longipes*. (k) Distinctly notched, e.g., *F. avenaceum*. (l) Barely notched, e.g., *F. solani*. (m–t) Microconidial spore shapes. (m) Oval. (n) Two-celled oval. (o) Three-celled oval. (p) Reniform. (q) Obovoid with a truncate base. (r) Pyriform. (s) Napiform. (t) Globose. (u–x) Phialide morphology. (u) Monophialides, e.g., *F. solani*. (v) Monophialides, e.g., *F. oxysporum*. (w) Polyphialides, e.g., *F. polyphialidicum*. (x) Polyphialdes, e.g., *F. semitectum*. (y–z) Microconidial chains. (y) Short chains, e.g., *F. nygamai*. (z) Long chains, e.g., *F. verticillioides*. From Leslie and Summerell (2006); used with permission.

characters to be observed (see Leslie and Summerell, 2006 for media recipes, isolation protocols, and species descriptions). If the morphological characters are inadequate for identification, then sexual crosses with standard tester strains on carrot agar (Klittich and Leslie, 1988; Leslie, 1991), or DNA sequences, most commonly of the *tef-1* gene encoding transcription elongation factor 1-α, are used to complete the identification. When comparing sequences for identity with those already known, GenBank can be used, but the database of Geiser *et al.* (2004) is preferable as this database contains only sequences from cultures that are publicly available and whose morphological identity has been confirmed.

Fusarium graminearum produces only macroconidia, no microconidia, in sporodochia on carnation leaf agar. Chlamydospores may form occasionally, often within the macroconidia. The macroconidia are thick-walled, five- to six-septate, slender with modest to no curvature, and have a tapered apical cell and a basal cell with a distinct foot shape (Leslie and Summerell, 2006). In 1977, Francis and Burgess separated *F. graminearum* into two groups. Group 1 was the primary cause of wheat crown rot and individual strains never produced perithecia under laboratory conditions. Individual Group 2 isolates could produce perithecia under laboratory conditions, i.e., they were homothallic, and are associated with Fusarium head blight (FHB) of wheat and red Fusarium ear rot of maize. Members of Group 2 also produced more zearalenone and either deoxynivalenol or nivalenol than did members of Group 1. In 1999, Group 1 was shown to be heterothallic and phylogenetically distinct from Group 2 and renamed *Fusarium pseudograminearum* (*Gibberella coronicola*) (Aoki and O'Donnell, 1999a, 1999b) with the homothallic Group 2 retaining the *F. graminearum* name. This split is well accepted across the *Fusarium* research community. The lack of microconidia, the ability to form the sexual stage as a homothallic, and the distinctive shape of the macroconidia are the most useful morphological diagnostic characters.

More recently, a number of phylogenetic species have been proposed within *F. graminearum*. These phylogenetic species were first described as phylogenetic lineages (O'Donnell *et al.*, 2000; Ward *et al.*, 2002) and later raised to species rank (O'Donnell *et al.*, 2004) with additional phylogenetic species described in later publications (O'Donnell *et al.*, 2008; Starkey *et al.*, 2007; Yli-Mattila *et al.*, 2009). Currently, these 16 phylogenetic species are diagnosable by their clustering pattern on a phylogenetic tree and by a multi-locus genotype test that relies on 13 different DNA sequences to discriminate the species (Yli-Mattila *et al.*, 2009). Neither the most commonly used DNA sequence for resolving species in filamentous fungi, i.e., the internally transcribed spacer of the ribosomal RNA (ITS-rRNA) repeat region, nor the most commonly used DNA sequence for resolving species within *Fusarium*, i.e., the region encoding transcription elongation factor 1-α, is included in the set of discriminatory DNA sequences for the *F. graminearum* "clade." No single DNA sequence in the set of 13 suffices to discriminate all of the species, although two sequences—*MAT* and *URA-Tri101-PHO*—each suffices to resolve at least 11 of the 16 species. The only practical way to distinguish these phylogenetic species at this time is to use a multi-locus genotyping test available through the USDA-ARS National Center for Agricultural Utilization Research in Peoria, Illinois (Yli-Mattila *et al.*, 2009). To complicate matters further, the value and validity of the phylogenetic species remain in question as there are no clear differences in morphology or pathogenicity associated with them and members of different lineages/species are cross-fertile (Leslie and Bowden, 2008).

Fusarium verticillioides and *F. proliferatum* were both a part of the *F. moniliforme* species complex of Snyder and Hansen. Both species produce numerous microconidia, no chlamydospores, and relatively few macroconidia. They differ in the manner in which the microconidia are produced in that *F. verticillioides* produces microconidia in long chains from monophialides, while *F. proliferatum* produces microconidia in shorter chains, with occasional false heads, from both monophialides and polyphialides. Many of the species in the *Gibberella fujikuroi* species complex, also known as

Fusarium section *Liseola*, have morphological characters that are similar to those described for *F. proliferatum* and *F. verticillioides*, but most produce little more than trace levels of fumonisins (Fotso *et al.*, 2002; Nelson *et al.*, 1992). The best way to distinguish *F. proliferatum* from *F. verticillioides* is to sexually cross them with standard mating-type strains (available from the Fungal Genetics Stock Center, University of Missouri-Kansas City). There are a number of differences in sequenced genes that can be used to separate *F. verticillioides* and *F. proliferatum*. However, *F. proliferatum* is a very diverse species and cross-fertility should be used to confirm the identity of strains carrying novel alleles.

Both *F. proliferatum* and *F. verticillioides* are capable of synthesizing large amounts of fumonisins and are widely distributed in most places where maize is grown. *Fusarium verticillioides* can be seedborne and grow as an endophyte within maize plants (Bacon *et al.*, 2008; Lee *et al.*, 2009b; Oren *et al.*, 2003), which means that grain from healthy looking plants can still be contaminated with high levels of fumonisin. The biosynthetic gene cluster in both species is very similar, but the flanking regions are quite different suggesting that the cluster's chromosomal location is not conserved in these species (Waalwijk *et al.*, 2004).

Cladal Relationship and Organization of the Toxin Biosynthetic Gene Cluster

Mycotoxigenic fungi have complex genomes, with species differences often reflected in the organization of the genes responsible for the synthesis of secondary metabolites, including mycotoxins. Health concerns associated with mycotoxin contamination have focused research on the mycotoxin biosynthetic pathways and the identification of the producing fungi. Species in the genera *Aspergillus* and *Fusarium* have received the most attention because they produce families of mycotoxins and are among the most common fungi recovered from developing and mature seeds. The discovery that the genes responsible for mycotoxin biosynthesis often are clustered (Bhatnagar *et al.*, 2006; Cary, 2004; Proctor *et al.*, 2004; Waalwijk *et al.*, 2004), along with the development of new tools for analyzing population biology (Bowden *et al.*, 2008; Lee *et al.*, 2012; Leslie and Klein, 1996; Schmale *et al.*, 2006; Zeller *et al.*, 2004), has provided tremendous insight into the complexity of the population structure of mycotoxigenic fungi. It is now possible to examine the association between genetic rearrangements of the mycotoxin biosynthetic gene clusters, the species producing the mycotoxin, and the structure of the fungal population. An interesting question, which has not yet been answered, is whether mycotoxin phenotype drives adaptation and speciation.

Aspergillus

Peterson (2008) provided insights into phylogenetic analysis of *Aspergillus* species using DNA sequences from four loci. Alternatively, insight into the role of chemotype in niche adaptation can be obtained by studying the organization and stability of the mycotoxin biosynthetic gene clusters. Two critical questions are "How are clusters formed?" and "Are clusters evolving in response to positive selection?" When five sequenced aflatoxin biosynthetic cluster homologs were evaluated (Carbone *et al.*, 2007a, 2007b), there was a high level of gene duplication and seven modules representing putative aflatoxin cluster orthologs (*aflA*/*aflB*, *aflR*/*aflS*, *aflX*/*aflY*, *aflF*/*aflE*, *aflT*/*aflQ*, *aflC*/*aflW*, and *aflG*/*aflL*) were identified. An explanation for these gene pairs is the duplication of a single gene and retention of both genes to form modules. These modules can assemble into biosynthetic clusters for secondary metabolites. There also were syntenic partial clusters for five

genes (*aflC*, *aflS*, *aflR*, *aflX* and *aflY*) in *A. fumigatus* and *A. terreus* and the overall gene order was similar to that for the sterigmatocystin gene cluster in *A. nidulans*. The sterigmatocystin cluster contains all but one of the genes required for aflatoxin biosynthesis, but the genes are not in the same order as in the aflatoxin clusters found in *A. flavus* and *A. parasiticus*. The aflatoxin biosynthetic genes in section *Flavi* are under stronger positive selection than are their homologs in non-section *Flavi* species (Carbone *et al.*, 2007a, 2007b).

Most aflatoxin-producing species of *Aspergillus* are in section *Flavi*, which includes *A. flavus*, *A. parasiticus*, *A. nomius*, and *A. bombycis* (Cary *et al.*, 2005). Aflatoxin production is not limited to this section of the genus, however, as aflatoxins are also produced by *A. astellata* (Cary *et al.*, 2005; Frisvad *et al.*, 2004) and *A. venezuelensis* (Frisvad and Samson, 2004) in section *Nidulantes* and by *A. rambellii* and *A. ochraceoroseus* (Cary *et al.*, 2005) in section *Ochraceroesi* (Cary *et al.*, 2009; Frisvad *et al.*, 2005; Klich *et al.*, 2003). The conservation of the structure and content of the aflatoxin cluster across all of these species argues for a biological role of its aflatoxin product.

The most similar aflatoxin-producing strains are *A. flavus* and *A. parasiticus*. The aflatoxin gene clusters in these species share 96% DNA identity and the cluster genes are in the same chromosomal order (Cary and Ehrlich, 2006; Yu *et al.*, 2004). The most striking difference is the absence of *norB* and *cypA* from the *A. flavus* cluster. These genes are both required for the biosynthesis of the G series aflatoxins (Cary and Ehrlich, 2006; Ehrlich *et al.*, 2004). With the exception of the S clade, *A. flavus* produces only B series aflatoxins while *A. parasiticus* produces both B and G series aflatoxins. The S clade of *A. flavus* has the same chemotype as *A. parasiticus*. Until recently, *A. flavus* and *A. parasiticus* were both thought to be asexual with little genetic recombination. With the description of the sexual stages of each species (Horn *et al.*, 2009a, 2009b, 2009c), questions regarding the possibility of genetic exchange between these species and the extent of their cross-fertility can be asked and hopefully answered.

An important debate is whether *Aspergillus oryzae* is an ecotype of *A. flavus* rather than a different species. Unlike *A. flavus*, which is both a plant and an animal pathogen, *A. oryzae* is commonly used in food fermentation and has GRAS (Generally Regarded As Safe) status. Morphologically these two species are very similar, but they can be distinguished by careful examination (Hesseltine *et al.*, 1970; Klich and Pitt, 1985; Thom and Church, 1921). Regardless of the morphological features, no strains of *A. oryzae* can produce aflatoxin. This phenotype may be due to a total lack of the aflatoxin biosynthetic cluster or to mutations in one or more of the genes in the cluster. The lack of toxin production may result from genomic changes such as deletions, insertions, frameshift mutations, and base-pair substitutions (Lee *et al.*, 2006). Aflatoxin-nonproducing strains can also be found in *A. flavus*, and many of these strains have deletions within the 75 kb cluster region that are similar to those found in *A. oryzae*. Both phylogenetic and genomic analyses show these two fungi are very similar and argue that *A. flavus* and *A. oryzae* are ecotypes of a common species. Geiser *et al.* (2000) found that both species were polyphyletic, and Chang *et al.* (2006) reported that several *A. oryzae* strains—RIB 40, SRRC 2044, SRRC 2098, and SRRC 2103 were in a clade that contains isolates of *A. flavus*.

Aspergillus flavus and its close relative *A. parasiticus* are the most commonly occurring aflatoxin-producing species. More is known about the population structure of *A. flavus* than of any other *Aspergillus* species, and these populations are genetically and genotypically diverse. Within these species there are at least two economically important clades (Atehnkeng *et al.*, 2008; Chang *et al.*, 2006) described as either S or L based on the size of their sclerotia (Cotty, 1989; Duran *et al.*, 2007; Horn and Dorner, 1999), with different chemotypes. L strains do not produce G series aflatoxins, but most produce B series aflatoxins, whereas some S strains produce B and G series aflatoxins (Geiser *et al.*, 2000). The aflatoxin biosynthetic gene clusters of strains from the L and S clades

are 99% identical at the nucleotide level (Cary and Ehrlich, 2006). The proportion of each clade in the population differs geographically (Cardwell and Cotty, 2002; Cotty, 1997; Cotty and Cardwell, 1999; Donner *et al.*, 2009; Horn and Dorner, 1999). Thus, strains of *A. flavus* may produce only the B series of aflatoxins, both the B and the G series of aflatoxins, or no aflatoxin at all. Aflatoxin production is not always stable in *A. flavus*. Aflatoxin production by *A. parasiticus* is stable in culture and the strains most commonly isolated produce both the B and the G series aflatoxins, although the ratio of B to G produced may differ (Carbone *et al.*, 2007a, 2007b). There also are naturally occurring strains that produce no aflatoxins but produce *o*-methylsterigmatocystin instead (Horn *et al.*, 1996). Strains in other *Aspergillus* sections also produce various metabolites derived from functional portions of the aflatoxin biosynthetic pathway. *Aspergillus nidulans*, e.g., produces only sterigmatocystin and there are populations of *A. rambellii* and *A. ochraceoroseus* that produce both aflatoxin and sterigmatocystin (Cary *et al.*, 2009).

Fusarium

The genus *Fusarium* also has a complex population structure with numerous species adapted to a wide range of hosts and habitats worldwide (Leslie and Summerell, 2006; Summerell *et al.*, 2001) and by the production of a diverse array of mycotoxins (Desjardins, 2006). The most studied mycotoxins are the trichothecenes, the fumonisins, and zearalenone.

FHB is a devastating disease of wheat and barley worldwide on cereals that is caused by a complex of species that includes the phylogenetic species/lineages within *F. graminearum* as well as other related species such as *F. pseudograminearum*, *F. culmorum*, and *F. avenaceum* (Francis and Burgess, 1977; O'Donnell *et al.*, 2004, 2008; Starkey *et al.*, 2007; Yli-Mattila *et al.*, 2009). Members of all of these species produce trichothecenes and/or zearalenone. The trichothecene deoxynivalenol has a role in wheat pathogenicity, which may be the reason this toxin is commonly produced by strains recovered from Fusarium-blighted wheat heads (Proctor *et al.*, 2002). Curiously, the genes for trichothecene biosynthesis do not have the same phylogeny as do the neutral genes used to infer the biological species (Ward *et al.*, 2002). To complicate the taxonomy of *F. graminearum* even further, the biological species concept is not concordant with the phylogenetic species concept, with only a single species described based on cross-fertility criteria (Fuentes-Bueno, 2012; Leslie and Bowden, 2008). The lineages/phylogenetic species of *F. graminearum* also do not differ markedly in terms of pathogenicity, at least toward wheat (Goswami and Kistler, 2005), although there may be other alterations that reflect differences in the ecological niches occupied by these fungi, e.g., Lee *et al.* (2009a). Alignment of the physical map of *G. zeae* with a recombination-based map developed from a cross between members of two different lineages/phylogenetic species, suggests that there are relatively few genomic rearrangements among the various lineages (Lee *et al.*, 2008). From the work to date it is clear that (i) there are consistent differences between strains in different lineages/phylogenetic species of *F. graminearum*; (ii) differences in mycotoxin profiles are correlated with lineage/phylogenetic species, but these correlations are not 100%; (iii) the lineages/phylogenetic species are more closely related to one another than most other *Fusarium* species are to their next nearest phylogenetic relative; and (iv) until a significant consistent critical parameter, e.g., mycotoxin profile or pathogenicity, among the lineages/phylogenetic species is identified the differences between the lineages/phylogenetic species can probably be ignored by most diagnosticians.

Another agriculturally important *Fusarium* clade with a complex population structure is the *G. fujikuroi* species complex, which contains numerous closely related *Fusarium* species, both

described and undescribed (Leslie and Summerell, 2006). The most important toxins produced by these fungi are the fumonisins (Fotso *et al.*, 2002; Gelderblom *et al.*, 1988; Leslie *et al.*, 1992; Rheeder *et al.*, 2002), which also are produced by some strains of *F. oxysporum* (Sewram *et al.*, 2005). Although fumonisins are phytotoxic (Glenn *et al.*, 2008), it is not clear whether these toxins are essential for the plant diseases caused by the fungi in this species complex (Proctor *et al.*, 2002). Some other secondary metabolites, e.g., moniliformin, are made at very high levels by some of the fungi in this species complex, but their role, if any, in plant diseases and in toxicoses of humans and domesticated animals is not well understood.

The biosynthesis of fumonisins requires 15 genes that reside in a cluster (Xu and Leslie, 1996). The distribution of these genes among 44 strains from 27 species of *Fusarium* was discontinuous (Proctor *et al.*, 2004). Fumonisins were detected in cultures of *F. fujikuroi*, *F. globosum*, *F. nygamai*, *F. proliferatum*, *F. verticillioides*, and *F. oxysporum*, which was the only fumonisin producer that was not a member of the *G. fujikuroi* species complex. The inability to produce fumonisins usually was due to the absence of a gene within the cluster rather than to the presence of a nonfunctional gene.

Conclusions

Species of *Aspergillus* and *Fusarium* produce some of the most important mycotoxins found in maize and in wheat, including, aflatoxins, fumonisins, trichothecenes (deoxynivalenol and nivalenol), and zearalenone. All of these toxins are regulated in international trade, can result in serious losses to farmers, and are threats to the health of humans and domesticated animals. Identifying these fungi can be done with some accuracy based solely on morphology, but a more detailed examination with molecular markers often is required to discriminate sibling species that may differ in toxin production (both the spectrum and the quantity) and host preference. The genetics of the biosynthetic pathways for these toxins are now well understood, but the molecular regulators of production, for the most part, remain to be elucidated. These regulators are of critical importance for understanding the conditions under which toxins may be synthesized. Identifying these regulators and understanding their mode of action along with more sophisticated and user-friendly molecular species identification protocols will be critical areas of research in the coming years.

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3 Determination of Mycotoxins in Maize

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Abstract

The determination of major mycotoxins occurring in maize, aflatoxins and fumonisins, is the primary focus of this chapter. Depending on the purpose of the analysis, a range of analytical methods are available, including official methods for food control purposes and rapid methods for screening raw materials and products. The former typically rely on chromatography, usually high-performance liquid chromatography, for separation of individual toxin analogs from one another and from other matrix-related compounds. Rapid methods, which rely on anti-mycotoxin antibodies, have been developed in a wide range of formats, including quantitative enzyme-linked immunosorbent assays and qualitative dipsticks and flow-through devices that usually provide a single pass/fail value for the sampled material.

Keywords: aflatoxins; analytical methods; ELISA; fluorescence polarization; fumonisins; high performance liquid chromatography; mass spectrometry; multi-toxin analyses; rapid tests; sampling plans; thin layer chromatography

Introduction

The mycotoxigenic fungi that infect maize can produce a wide range of mycotoxins, of which the carcinogenic aflatoxins and fumonisins are the most important. Aflatoxins are produced primarily by *Aspergillus flavus* and *Aspergillus parasiticus*. These fungi are common saprophytes and opportunistic pathogens that occur extensively in the tropics and semitropics on a wide range of agricultural commodities and food matrices, including maize. Of these two species, *A. flavus* produces aflatoxin B_1 (AFB_1) and aflatoxin B_2 (AFB_2), whereas *A. parasiticus* produces these B aflatoxins, as well as aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2). The fumonisins in maize are produced primarily by two *Fusarium* species, *Fusarium verticillioides* and *Fusarium proliferatum*. Both of these species produce fumonisin B_1 (FB_1, the major naturally occurring analog), fumonisin B_2 (FB_2), and fumonisin B_3 (FB_3). These two species, and a number of other *Fusarium* species that co-occur on maize, can collectively synthesize a wide range of other toxins, including moniliformin (MON), trichothecenes, such as deoxynivalenol and nivalenol, zearalenone, fusaproliferin, and beauvericin (CAST, 2003).

Analytical methods for mycotoxins are required to satisfy a wide range of demands and can consequently be categorized into three groups. First, official analytical methods, which are

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collaboratively validated under the auspices of various international bodies such as AOAC International or European Committee for Standardization (CEN), are developed to meet these regulatory demands. Second, rapid screening methods, usually in the form of commercial kits, have been developed to provide a rapid semiquantitative analysis for situations where time is a priority. Lastly, there are the research methods developed and applied by scientists that use novel approaches and cutting-edge technology in analytical science. The aim of this chapter is to provide an overview of methodologies applicable to mycotoxin analysis in the maize chain, with an emphasis on the most important mycotoxins in maize, aflatoxins and fumonisins. A number of other mycotoxins (deoxynivalenol, zearalenone, and ochratoxin A) that occur in maize also are important in wheat, and relevant methods will be addressed in the corresponding section on the wheat grain chain (Gilbert and Pascale, 2014). In most cases, the methods that are applicable to wheat also are applicable to maize. Analytical methods for mycotoxins such as moniliformin, fusaproliferin, and beauvericin, which are not regulated, are usually used only in research environments and are described in detail in the specialized scientific literature (Munimbazi and Bullerman, 2001; Sewram *et al*., 1999), but will not be detailed in this chapter.

Aflatoxin

Sampling

Mycotoxin contamination of cereal grains and nuts results from infection with mycotoxigenic fungi, which frequently occurs only on individual grains or nuts, resulting in a heterogeneous distribution of mycotoxins within a lot. Some samples from a lot can have very high mycotoxin levels, even though the majority of the samples from the same lot have little or no detectable mycotoxin. The implication of this skewed distribution is that "hot spots" of contamination occur and that samples from a single batch could vary greatly in contamination, leading to uncertainty about the true mean of the batch and whether the batch meets a regulatory limit. Aflatoxin contamination of a range of commodities provides some of the worst examples of this problem.

The total error associated with mycotoxin testing can be partitioned into three independent terms: (i) the sampling error (introduced by withdrawing a random sample from the batch to be tested); (ii) sampling preparation error (introduced by milling and subsampling to generate the laboratory aliquot); and (iii) analytical error (introduced by the analytical process itself). These individual variances were determined by high-performance liquid chromatography (HPLC) from a batch of shelled maize contaminated at 20 μg/kg aflatoxin, using a 1.13 kg sample, a Romer mill for grinding, and a 50 g laboratory aliquot (Johansson *et al*., 2000a). The percentage of the total error associated with sampling, sample preparation, and sample analysis was 78%, 20%, and 2%, respectively.

The problem of sampling is addressed by sampling plans, which are based on statistical evaluations to balance consumer protection (by not accepting contaminated batches) and producer protection (by not rejecting clean batches) (Johansson *et al*., 2000b; Whitaker, 2006). For official food safety testing, sampling plans, which specify commodity type, the number of samples taken from a batch, the size of the samples, the location in the batch from which the sample was taken, and total size of aggregate sample, frequently are specified by government regulation. For regulatory purposes, the specified sampling plan must be followed; for other purposes, these plans can provide a guide. The European Commission has mandated a variety of sampling plans whose use depends on the mycotoxin/commodity combinations. In the case of aflatoxins in cereal lots larger than 50 tons, 100 samples of 100 g each are taken across the lot to yield an aggregate sample of 10 kg (European

Commission, 2006). The large sample size mandated in this regulation helps reduce the error associated with the sampling stage.

An important aspect of a sampling plan is the operating characteristics curve, which is generated from statistical evaluation of the mycotoxin distribution and the analytical variation. In these curves, the probability of a batch's acceptance is plotted against the mycotoxin level. As the contamination level increases and approaches the regulated limit, the probability of its rejection increases as well. The probability that an acceptable lot will be rejected is known as the producer risk. Similarly, lots contaminated at increasing levels above the regulated level, have a diminishing probability of being accepted. The probability of contaminated lots being accepted is known as the consumer risk. The ultimate goal of a good sampling plan is to reduce both risks, although neither can be completely eliminated. A published operating characteristics curve for aflatoxins in shelled maize (Johansson *et al*., 2000c) indicates that with a 5 kg sample and a regulatory limit of 20 μg/kg, lots containing 10 μg/kg will be accepted 90% of the time and those containing 30 μg/kg will be rejected approximately 75% of the time. If no characterized sampling plan is available, then analysts must work to obtain as representative a sample as possible. Thus, much more is required of analysts than the performance of the final analytical steps, since the history and context of the sample may significantly alter the reliability of the analytical results.

Extraction and Cleanup

Extraction of aflatoxins from maize and maize-based foods generally involves shaking or blending with aqueous mixtures of polar organic solvents, e.g., methanol, acetone, or acetonitrile. Extraction with chlorinated solvents, e.g., used in a few older methods, such as the CB method (Trucksess, 2000), is no longer common. The most common approach for aflatoxin analysis is to extract with methanol:water, usually in an 80:20 or 70:30 ratio, although one enzyme-linked immunosorbent assay (ELISA) method uses a ratio of 55:45 (Trucksess, 2000). Published methods often recommend the addition of NaCl to the weighed sample or the use of 0.1M HCl rather than pure water. Methanol is preferred to acetone or acetonitrile when an immunoaffinity column (IAC) is used for cleanup. These columns, which contain antibodies for binding aflatoxin from the sample extract, tolerate relatively higher amounts of methanol than of other organic solvents. In addition, interactions have been noted between the food matrix and the acetone and acetonitrile aqueous mixtures (Stroka *et al*., 1999). The extraction of dry sample material can result in compositional changes in the extraction mixture due to water absorption by the dry matrix and overestimation of the aflatoxin levels (Stroka *et al*., 1999). Aqueous acetonitrile solutions may separate into two layers when NaCl is added or if a food product contains sucrose. Thus, aqueous methanol is the generally accepted extractant for aflatoxins from maize and maize-based foods.

Other than in some newly described HPLC methods with tandem mass spectrometry (HPLC-MS/MS) determination (Spanjer *et al*., 2008), sample extracts for aflatoxin analysis require purification prior to quantification. The original cleanup methods, which used glass columns packed with silica and required large volumes of organic solvents, have been supplanted by solid phase extraction (SPE) methods, multifunctional columns containing specific adsorbents, and IACs (Brera *et al*., 2007; Malone *et al*., 2000; Sobolev, 2007). The latter two techniques were developed as commercial methods in which the column eluate is derivatized and total fluorescence measured. IACs have become widely accepted and are now incorporated in internationally validated methods of aflatoxin measurement in a wide range of food commodities, including infant food and maize (Brera *et al*., 2007; Stroka *et al*., 2001).

Thin Layer Chromatography

Thin layer chromatography (TLC) was the first chromatographic method to be applied to aflatoxin determination and is still in routine use in many laboratories, especially in developing countries. Aflatoxins are readily separated on silica gel TLC plates with any of a number of solvent mixtures, including acetone:chloroform (10:90), benzene:methanol:acetic acid (90:5:5), ether:methanol:water (96:3:1), or chloroform:isopropanol (99:1) (Trucksess, 2000). The aflatoxins separated on silica gel plates are readily identified as spots under long-wave (365 nm) UV light. Visual comparison with analytical standards yields semiquantitative results, but when densitometry is combined with high-performance TLC then the precision, accuracy, and sensitivity are comparable to that obtained with HPLC (Tosch *et al*., 1984). A TLC method sensitive enough to meet European Union regulatory requirements for aflatoxin in food (Stroka *et al*., 2000a) has been developed by combining IAC cleanup with densitometry.

High-Performance Liquid Chromatography

Aflatoxins are low molecular mass polar compounds that are ideally suited to separation and quantification by HPLC on reversed-phase columns. Mobile phases of water:methanol containing AFB_1, AFB_2, AFG_1, and AFG_2 have long run times and broad peaks, whereas water:acetonitrile mixtures do not result in full separation (Stroka *et al*., 2000b). Tailored mixtures of water:methanol:acetonitrile are generally satisfactory. Some researchers rely on UV detection of aflatoxins, but most researchers now rely on HPLC determinations based on fluorescence. Aflatoxin analogs fluoresce naturally, although in reversed-phase solvents the fluorescence of AFB_1 and AFG_1 is quenched, necessitating their derivatization. Multiple derivatizations are possible that rely on various chemical reactions with the 8,9-double bond of the dihydrofuran moiety. Reaction of aflatoxins with trifluoroacetic acid prior to HPLC injection hydrates the reaction site and forms the hemiacetals, AFB_{2a} and AFG_{2a}.

Pre-column derivatization has not been widely adopted due to the instability of the derivatives and the advantages that post-column derivatization offers with respect to automation. Reaction with a saturated iodine solution mixed with the HPLC column eluate and then passed through a reaction coil (typically 10 m long with 0.34 mm internal diameter) at 60–75°C was suitable for detecting AFB_1 down to 20 pg/injection (Thiel *et al*., 1986). Although widely used, this system has several disadvantages including the need for a post-column pump and reaction coil and potential problems with iodine crystallization in poorly operated HPLC systems. A more user-friendly system incorporates a post-column electrochemical generator, a Kobra cell, that generates bromine from KBr dissolved in the HPLC mobile phase. The post-column bromine derivatization system is simpler and more efficient than post-column iodination and also results in a better detector response than iodine (O'Riordan and Wilkinson, 2009). An alternative to the Kobra cell for bromine derivatization is to add pyridinium bromide perbromide to the column eluate via a post-column pump, followed by passage through a short reaction coil at room temperature. These bromination methods have been widely adopted and validated in a number of collaborative studies of aflatoxin detection, including those for infant food and maize (Brera *et al*., 2007; Stroka *et al*., 2001).

A more economic post-column derivatization method is photochemical derivatization achieved by passing the HPLC eluate through a reaction coil wound around a UV lamp at room temperature. In a comparison of the iodination and bromination protocols with the UV protocol, the protocols were analytically equivalent for groundnuts, but the UV protocol gave a slightly high result for maize (Waltking and Wilson, 2006). Another alternative to chemical derivatization is the incorporation

of cyclodextrins into the HPLC mobile phase. These cyclic oligosaccharides provide a cavity into which some molecules can incorporate and subsequently enhance their fluorescence properties. A method using either β-cyclodextrin or succinyl-β-cyclodextrin has been developed and tested in animal feed. The added cyclodextrin did not markedly alter the retention times of aflatoxin analogs and increased the natural fluorescence of AFB_1 and AFG_1 by at least 10-fold (Chiavaro *et al*., 2001).

The coupling of HPLC to mass spectrometry (LC-MS) via atmospheric pressure ionization techniques such as electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) has resulted in a range of new methods for individual mycotoxins, for mycotoxin groups, e.g., aflatoxins, and for true multi-toxin analyses (Zollner and Mayer-Helm, 2006). The advantages of LC-MS or of tandem LC-MS (LC-MS/MS) include (i) lower detection limits, (ii) compound identification confirmation provided by the mass spectral fragmentation patterns, and (iii) the ability to filter out by mass any impurities that interfere with spectrophotometric detection. Various ionization techniques and instrument types have been used to detect aflatoxins following reversed-phase separation (reviewed in Zollner and Mayer-Helm, 2006). In general, ESI is the most commonly used ionization technique. During method validation, sample cleanup techniques were developed that reduce matrix effects on signal intensity. In addition to the methods developed solely for the determination of aflatoxins, LC-MS/MS methods have been developed for simultaneous determination of a wide range of mycotoxins, including aflatoxins (Spanjer *et al*., 2008; Sulyok *et al*., 2006). These methods utilize a single extraction solvent, a dilution of the extract without cleanup prior to HPLC injection, and matrix-matched standards to overcome the signal enhancement or suppression that occurs in maize and in corn flakes.

Immunological Methods

Numerous immunological assay formats are available, but all rely on the recognition of a hapten, aflatoxin, by a specific anti-aflatoxin antibody. The development of immunological methods for aflatoxin analysis is an area of intense research activity. However, for practical applications, the vast majority of laboratories rely on commercially available products such as ELISAs, lateral flow strips, or flow-through immunoassays. Depending on the kit, the ELISAs can provide screening results or quantitative measures within their range of application and for the specified matrices for which they are validated. Lateral flow strips and flow-through immunoassays are usually designed to give a yes/no result at a prespecified decision level. The essential principle of ELISA is that an antibody or antigen is immobilized on a suitable surface and there is a competitive process involving this resource and components of the analytical solution. In the last stage of the analysis, an enzyme color reaction provides an endpoint for optical readout. As the ELISA is usually performed without extract cleanup, there are limitations due to matrix effects caused by co-extracted compounds, e.g., lipids, carbohydrates, tannins, polyphenols or pigments, extract pH, and solvent composition. These effects may elevate or reduce the analytical results. Measures to overcome these effects include extract dilution, the use of detergents, and extract purification.

Lateral flow devices or dipsticks have been commercialized as rapid tests at a preset mycotoxin level. The sample extract is added to the base of the stick containing colloidal gold-conjugated anti-aflatoxin antibodies. The aflatoxin in the extract binds to the antibodies, and both bound and residual unbound antibodies move along the membrane of the strip. At a test line on the dipstick containing bound aflatoxin, unbound colloidal gold-conjugated antibodies are immobilized by the bound aflatoxin and become visible as a pink line. The appearance of this line indicates that the aflatoxin level in the sample is below the decision level of the test, whereas absence of the

line indicates an aflatoxin level above the decision level. The performance of the dipstick is checked by a control line, consisting of anti-antibodies set in a control line above the test line, which checks the migration of colloidal gold-conjugated antibodies along the dipstick. This basic technology has also been converted into a commercial quantitative method for rapid testing of AFB_1 in maize up to a level of 150 μg/kg. In flow-through rapid tests, antibodies are bound to a flow-through membrane along a line across the membrane contained in the device. The test is run by the sequential addition of diluted sample extract to bind aflatoxin, enzyme-labeled aflatoxin to bind residual anti-aflatoxin antibodies on the membrane, and the final addition of an enzyme substrate to yield a color line, which indicates that the aflatoxin level in the original sample is less than the cutoff level for the rapid test.

Immunoassays all involve liquid–solid surface interactions, but fluorescence polarization occurs entirely in solution and measures fluorescence orientation rather than total fluorescence. The orientation is influenced by the speed of molecular rotation, which depends upon molecular size. The assay is run by setting up a competitive immunoassay in the sample extract between aflatoxin present in the sample and an added fluorescently labeled aflatoxin. When the antibody binds to the fluorescent probe, it increases the size of the probe, reduces its rotation speed, and, hence, increases its polarization signal. As with competitive ELISA assays, the lower the aflatoxin level in the sample, the greater the binding of the labeled analogue and the greater the test signal (Nasir and Jolley, 2002). This test is commercially available for rapid qualitative testing of maize at aflatoxin levels $\geq$ 10 μg/kg.

Fumonisins

Sampling

As with aflatoxins, the distribution of fumonisins in maize is heterogeneous and careful attention must be paid to sampling issues. Bulk lots of fumonisin-contaminated shelled maize have been sampled and analyzed to determine sampling, sample preparation, and sample analysis variances (Whitaker *et al*., 1998). At a contamination level of 2 mg/kg of fumonisins with a 1.1 kg sample milled in a Romer mill to yield a 25 g subsample for a single analysis by HPLC, the sampling variance accounted for 61% of total testing variability, the sample preparation variance accounted for 18%, and the sample analysis accounted for the remaining 21% of the total variance. These variances follow the same pattern as those observed for aflatoxin testing, so the European Commission's official method for sampling cereals and cereal products is the same for fumonisins as it is for aflatoxins (European Commission, 2006). Based on an operating characteristics curve generated for fumonisin in maize (Whitaker *et al*., 2001), for a 1.1 kg sample, ground in a Romer mill to yield a 25 g test aliquot for HPLC analysis and a 2 mg/kg accept/reject limit, a farmer's lot at 3 mg/kg would have a 20% probability of acceptance, whereas a lot at 1 mg/kg would have an 8% chance of rejection. Increasing the sample size from 1.1 to 2.2 or 4.4 kg reduces the chance of the 3 mg/kg lot being accepted to 10% and 3%, respectively.

Extraction and Cleanup

Fumonisins usually are extracted with methanol:water (75:25) or methanol:acetonitrile:water (25:25:50) mixtures by shaking or homogenization (Sydenham *et al*., 1996; Visconti *et al*., 2001).

As with some matrices for aflatoxin extraction, the separation of acetonitrile:water mixtures into two layers precludes the use of this binary mixture (De Girolamo *et al*., 2001). In some cases (Zoller *et al*., 1994), better recovery results with acidified extractants, whereas a fluorescence polarization assay (Maragos *et al*., 2001) used phosphate-buffered saline (pH 7.2) and completely avoided the organic solvents that could affect the activity of the antibody required for the analysis. Determination of fumonisins in Mexican tortillas requires the inclusion of EDTA in the extraction mixture to complex the calcium added during the nixtamalization process. Unless being analyzed by an immunological method, e.g., ELISA or fluorescence polarization, maize extracts are cleaned up on reversed-phase (C18) or strong anion exchange (SAX) SPE cartridges, or on IACs containing specific anti-fumonisin antibodies. Depending on the method, the solvent must be adjusted to pH $>$ 6.0 for SAX cleanup, to ensure that fumonisin is in the anionic form, or appropriately diluted for C18 or IAC cleanup, to ensure retention on the cleanup medium. Of these cleanup methods, C18 gives the poorest recovery, whereas IACs, which are the most costly, yield a relatively pure solution and have been commercialized as a direct fluorometric method (Duncan *et al*., 1998).

The extractants and cleanup methods discussed above perform satisfactorily for maize meal and have been tested in successful international collaborative studies (Sydenham *et al*., 1996; Visconti *et al*., 2001). However, a number of fumonisin extraction and cleanup issues remain unsettled, especially for processed foods. A method applicable to many substrates has been proposed that uses the ternary mixture methanol:acetonitrile:water (25:25:50) in a double extraction with an IAC cleanup for samples such as maize, corn flakes, extruded maize, muffins, and infant formula (Visconti *et al*., 2001). The composition of the matrix of maize-based infant foods can radically alter the fumonisin recovery following SAX cleanup of a variety of possible extractants (Sewram *et al*., 2003). The reuse of IACs for fumonisin analysis, after appropriate regeneration, has been advocated by some researchers, e.g., Fazekas *et al*. (1999). Other analysts have highlighted problems that can arise if extracted impurities interfere with antibody activity and cause underreporting of fumonisin levels (Castegnaro *et al*., 2006). The use of pure methanol to elute fumonisins from IACs also has been questioned, and better recoveries were demonstrated from naturally contaminated maize meal and cornflakes with methanol:water (80:20) (Oh *et al*., 2009).

Thin Layer Chromatography

A limited number of TLC methods, mostly based on reversed-phase, have been published. Fumonisin levels in maize were determined by visual comparison with standards after separation on reversed-phase plates with 96:4 methanol:aqueous potassium chloride as the developing solvent (Rottinghaus *et al*., 1992). Successive sprays of alkaline borate buffer, fluorescamine, and boric acid were used to identify the fumonisins. Cleanup for this method was with reversed-phase SPE and/or SAX SPE, with the latter giving less background. The results were comparable with those obtained with HPLC in the 1–250 mg/kg range (Schaafsma *et al*., 1998). TLC detection limits can be lowered by using IACs for cleanup to reduce background interference. Combining cleanup of maize extracts with IACs and reversed-phase TLC separation followed by fluorescamine spray and densitometry for quantification of FB_1 resulted in a fumonisin detection limit of 0.1 mg/kg (Preis and Vargas, 2000). Maize extracts cleaned up on SAX SPE cartridges contained an impurity that co-chromatographs with FB_1 on reversed-phase TLC plates and leads to significant overestimation of FB_1 at low levels of contamination (Shephard and Sewram, 2004). This problem can be overcome by derivatizing a cleaned-up maize extract with fluorescamine and separating the fumonisin B_1 derivative by reversed-phase TLC with 96:4 methanol:aqueous 4% potassium chloride mobile

phase. Based on visual comparisons, the quantification limit of the method in maize is approximately 0.5 mg/kg.

High-Performance Liquid Chromatography

Fumonisins are widely determined by HPLC and methods for their determination in maize have been validated by international collaborative studies (Sydenham *et al*., 1996; Visconti *et al*., 2001). Fumonisin analogs are readily separated from each other and impurities remaining in the cleaned-up sample by reversed-phase HPLC, usually on columns packed with C18-modified silica, although C8 and phenylhexyl packing materials have also been used (Pagliuca *et al*., 2005; Sydenham *et al*., 1992). Mobile phases, which need to be tailored to the individual HPLC column, consist of approximately 80% methanol and 20% phosphate buffer adjusted to pH 3.35 to reduce the ionization of the carboxylic acid moieties of the fumonisins and hence provide acceptable HPLC peak shapes. The HPLC separation is usually performed isocratically, although gradients have been used for complex samples such as animal feeds (Holcomb *et al*., 1993). The fumonisins lack useable UV absorption, so derivatization is necessary for fluorescence detection at the low levels commonly found in naturally contaminated maize. Various pre-column derivatization techniques involving reaction of the primary amine group have been reported. The most widely used derivatization is with *o*-phthaldialdehyde (OPA) and 2-mercaptoethanol (Sydenham *et al*., 1996). This reaction is not only rapid and reproducible at room temperature in a borate buffer (pH 9–10), but also produces unstable reaction products. This problem is usually countered by carefully timing the HPLC injection. Alternatively, a more stable reaction product can be produced by replacing the 2-mercaptoethanol with *N*-acetyl-cysteine (NAC) (Stroka *et al*., 2002), or the HPLC system can be modified to automatically perform post-column derivatization by pumping an OPA NAC–borate reaction mixture into the HPLC column eluate (Akiyama *et al*., 1998).

Many authors have used LC-MS or LC-MS/MS, mostly with ESI in the positive ion mode, without derivatization to detect and quantify fumonisins in a number of matrices (Zöllner and Mayer-Helm, 2006). Confirmation of fumonisin is achieved in MS/MS mode as the fumonisin molecule readily fragments into a characteristic pattern of product ions (Musser *et al*., 2002). As with aflatoxins, some researchers have confined their work to fumonisin analogs, whereas others have used the versatility of MS to develop multi-toxin methods. The multi-toxin methods utilize extract dilution and direct injection (Spanjer *et al*., 2008; Sulyok *et al*., 2006, 2007) or extract cleanup with either a multi-toxin IAC (Lattanzio *et al*., 2007) or with conventional SPE cartridges (Cavaliere *et al*., 2005). Many of the developed methods for direct injection use matrix-matched standards to overcome potential matrix effects in the ionization process (Sulyok *et al*., 2007).

Immunological Methods

As with aflatoxins, various antibody-based analytical methods have been commercialized for the determination of fumonisins in maize. These methods include ELISAs, lateral flow strips, and flow-through immunoassays aimed at appropriate levels of contamination. One competitive direct ELISA has been granted AOAC official method status for use at total fumonisin levels above 1 mg/kg (Bird *et al*., 2002). None of the immunological methods should be used outside the range and matrix for which the assay was validated. IACs not only are widely used for purification of extracts for fumonisin determination by HPLC, but also have been incorporated into a direct fluorometric method

for total fumonisins in which the eluate from the IAC is derivatized and total fluorescence measured (Duncan *et al*., 1998). This method compares favorably with HPLC. Total fumonisins also can be analyzed by fluorescence polarization (Maragos *et al*., 2001). This test is extremely rapid, requires only a few minutes after sample extraction, and is performed with a minimum of reagents and no washing steps. A commercial version of this test is available for qualitative analysis of maize at or above levels of 1 mg/kg.

Discussion and Conclusions

The methods discussed above are those currently in wide use for the determination of the relevant mycotoxins in the maize grain chain. Both the purpose of the analysis and the available instrumentation must be considered when selecting a method for use. Although current sophisticated instrumentation, e.g., HPLC-MS/MS, can yield multi-toxin analytical results, such results may not be applicable for all mycotoxin analyses. Targeted HPLC analysis of specific mycotoxins with conventional spectrophotometric detectors probably suffices for most laboratories. Certainly, in many developing countries, where the costs of purchasing, operating, and maintaining scientific instruments are high, more reliance is placed on TLC or ELISA methods. Similarly, rapid screening methods have an important role to play in providing an analytical result when analysis time is an important factor.

In addition to the choice of analytical method, a second critical factor in mycotoxin analysis is quality control of the analytical process. Irrespective of the method chosen or whether it is an official method, the method must be validated in the laboratory where the analysis is being performed. Quality assurance programs, in the form of certified reference materials for some mycotoxin/matrix combinations, and proficiency testing schemes are commercially available. A third critical issue is the purity of the mycotoxin standards used to calibrate the analysis. Aflatoxin standard purity can be checked by measuring the molar absorptivity by UV spectrophotometry. Fumonisins, which lack a useful UV absorption band, should be checked for chromatographic purity (Trucksess, 2000).

Mycotoxin analytical methods have developed over time, and usually follow improvements in general analytical chemistry. Thus, the original widespread use of TLC gave way to HPLC and the original spectrophotometric detectors have either improved in sensitivity or given way to advances in the development of diode array detection for UV or the coupling of HPLC to MS. In recent years, much interest has been generated in developing new rapid testing formats based on anti-mycotoxin antibodies and the commercial demand for such tests should continue to drive their availability, reduce the price, and shorten the time from grinding a sample to obtaining a result.

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4 Breeding Maize for Resistance to Mycotoxins

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Abstract

Maize can host several fungi that produce mycotoxins. Despite decades of research, preventing the infection of maize kernels by toxigenic fungi remains a challenging problem. One approach to reducing mycotoxins in maize grain is to identify germplasm with increased resistance to fungal infection and lower levels of accumulated toxins. We review field and laboratory techniques that measure these two aspects of the disease and the application of these techniques to the identification of resistant maize genotypes and to the evaluation of quantitative inheritance of resistance to ear rot and mycotoxin accumulation. Sources of resistance to *Fusarium verticillioides*, *Fusarium graminearum*, and *Aspergillus flavus* have been identified and incorporated into public and private breeding programs. Recently described genes and quantitative trait loci providing resistance to fungal infection and their application to breeding for resistance to mycotoxin accumulation are reviewed. Genomic and transgenic approaches to preventing mycotoxin accumulation in maize also are discussed.

Keywords: aflatoxins; *Aspergillus flavus*; fumonisins; ear rot; *Fusarium graminearum*; *Fusarium verticillioides*; infection techniques; molecular fungal host interactions; QTL mapping; resistant host germplasm; stress response pathways; transgenic plants

Introduction

Fungi cause numerous ear and kernel rots in maize, which may be severe and result in losses in grain yield and quality. Many ear rot fungi produce mycotoxins that can affect the feed value and marketability of the grain. Colonization of the grain by ear rot fungi is usually enhanced by insect damage and stalk lodging. Kernels infected in the field may rot and accumulate mycotoxins during storage (White, 1999).

In surveys performed since the 1980s, *Fusarium verticillioides* has been reported as the most common causal agent of ear rot in most maize growing areas of the world (Battilani *et al.*, 2008; Bottalico, 1998). In the southern part of Europe, *F. verticillioides* is the most common *Fusarium* species in maize fields (Folcher *et al.*, 2009; Logrieco *et al.*, 2002). *F. verticillioides* ear rot is more severe when hot, dry weather occurs (Eller *et al.*, 2008a). Symptoms vary greatly depending upon genotype, environment and disease severity. Usually, individual or groups of infected kernels are

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scattered randomly across the entire ear. Whitish pink to lavender fungal growth on kernels and/or silks is typical. Infected kernels also may exhibit a "starburst" symptom, i.e., white streaks radiating from the point of silk attachment at the cap of the kernel or from its base. *F. verticillioides* produces fumonisins, which are of particular concern because they are suspected to be carcinogenic and are linked with neural tube defects in humans (Missmer *et al.*, 2006). They also cause a variety of toxicoses in domesticated animals (Morgavi and Riley, 2007). Fumonisins are commonly found at biologically significant concentrations in maize grain produced in Europe and the United States (Bottalico, 1998; Eller *et al.*, 2008a; Pietri *et al.*, 2004).

Other ear and kernel rots accompanied by mycotoxin contamination problems include (i) *Gibberella* ear rot caused by *F. graminearum*, which is more severe in cool, humid areas, and characterized by a reddish mold and zearalenone and nivalenol or deoxynivalenol contamination (Bottalico, 1998); zearalenone is a pseudo-estrogen, while deoxynivalenol and other trichothecenes are potent protein inhibitors (CAST, 2003). (ii) *Aspergillus* ear rot, which is more serious in locations with high temperatures and droughty environments (Payne, 1998), is caused by the related species *Aspergillus flavus* and *Aspergillus parasiticus* and is characterized by the production of the potent carcinogen, aflatoxin (CAST, 2003). Less common ear and kernel rots, which are not usually associated with mycotoxin contamination, include *Diplodia* ear rot, which is caused by *Stenocarpella maydis*, and is more severe when maize follows maize and when wet weather occurs shortly after silking; as well as *Nigrospora oryzae*, which can shred the cob; *Penicillium oxalicum*, which produces a powdery green or blue-green mold; *Trichoderma viride*, which produces dark green fungal growth; *Cladosporium* spp., which results in dark, greenish-black kernels; and *Botryosphaeria zeae*, *Botryodiplodia theobromae*, and *Rhizoctonia zeae* (White, 1999).

In this review, *Fusarium* ear rot, *Gibberella* ear rot, and *Aspergillus* ear rot will be used to describe rot caused by *F. verticillioides, F. graminearum*, and *A. flavus*, respectively. There are agronomic practices that reduce the growth of toxigenic fungi, but the best control strategy is to plant resistant hybrids. Research and breeding efforts aimed at improving resistance to ear rots and mycotoxin contamination should focus on (i) accurate techniques for phenotyping ear rot and mycotoxin contamination; (ii) identification of novel host germplasm sources and resistance traits; (iii) development of genomic technologies for marker-assisted selection (MAS) programs; and (iv) application of biotechnological approaches to breeding for reducing susceptibility to ear rot and mycotoxin contamination.

Techniques for Phenotyping Ear Rot and Mycotoxin Contamination

Natural and Artificial Infection Techniques

Maize breeders do not have defined selection criteria for ear rot resistance. Resistance is selected by planting maize hybrids in areas with a known high incidence of ear rot and then rating the plants for ear rot at maturity. *Fusarium verticillioides, F. graminearum*, and *A. flavus* can overwinter in the soil and may be spread by wind (*F. verticilliodes* and *A. flavus*), rain splash (*F. verticillioides* and *F. graminearum*), and insect larvae (*F. verticillioides* and *A. flavus*; Munkvold, 2003). To ensure equal distribution of the pathogen for all of the plants in the field, artificial inoculation is needed (Munkvold and Desjardins, 1997). For all three fungal species, kernel infection through the silks is more important than infection through the seeds, stalk, crown, or roots; therefore, silk inoculation is the best technique for evaluating genetic resistance to *Fusarium* ear rot (Munkvold and Desjardins, 1997). However, not all ear and silk inoculation techniques are equally effective.

Inoculation methods can be divided into two types: with (type 1) and without (type 2) mechanical assistance. Type 1 methods include toothpick inoculation methods and their derivatives, e.g., 10–15 days after midsilking, ears are inoculated with nail punches (or similar items) through the husk perpendicular to the ear axis and midway between the base and ear tip, where the punches remain until harvest. Maximum infection and the onset of fungal growth and toxin accumulation occurs around 20% kernel moisture, while fumonisin first appears in the dent stage of development at 35–40% moisture (Eller *et al.*, 2008a). Ear rot incidence is positively correlated with the diameter of the inoculation punch. Resistance to this type of inoculation is considered by Reid *et al.* (1996) to be kernel resistance. In a typical type 2 method, a spore suspension is sprayed onto the maize silks with an atomizer or injected into the silk channel near the cob tip, which causes only minor mechanical injury. The silks may remain uncovered or be covered immediately after inoculation with moist paper toweling and then bagged. The best differentiation between resistant and susceptible genotypes was obtained when inoculation occurred a week after silking (Reid *et al.*, 1992). Later inoculations, e.g., 19, 21, and 23 days, resulted in significantly less severe disease symptoms, while very early inoculations, e.g., 4–6 days, increased disease severity. Sufficient moisture in the silk and the kernel is needed for spore germination and the subsequent invasion of the kernel.

A comparison of four type 2 techniques found that only inoculum injection through the husk significantly increased disease severity and mycotoxin levels. This technique also effectively differentiated host lines on the basis of susceptibility and resistance (Clements *et al.*, 2003a). Robertson *et al.* (2006) conducted a similar study comparing five inoculation techniques for determining resistance or susceptibility of a variety. Two inoculation techniques, inoculation by penetrating husks with pin bars and injecting inoculum down the silk channel, resolved different levels of resistance to fungal infection and fumonisin accumulation.

From the points of inoculation, infection spreads to the neighboring kernels. Mesterhazy *et al.* (2000) measured the total ear rot for different isolates and also the amount of spread per day. The disease severity at harvest was divided by the number of days from inoculation until the grain reached 28% water content. The highest values were 2–3% and the lowest were 0.1–0.3% per day, depending on the fungal strain. The severity of ear rot after inoculation is evaluated by using a visual scale to rate individual ears (Emerson and Hunter, 1980).

Resistance to mycotoxigenic fungi in maize has two conceptual components: (i) resistance to initial penetration and (ii) resistance to spreading by the pathogen in host tissue. Type 1 inoculation methods usually screen for only resistance component ii, i.e., spread on the host. These inoculation methods simulate insect attack as they bypass many of the plant's morphological barriers. Type 2 inoculation methods more closely resemble natural infection of a non-wounded host plant. If ear rot or a percentage of infected kernels is scored, then the resultant phenotype may be a combination of type i and type ii resistance components.

Analysis of Fungal and Mycotoxin Contamination

Fungal contamination of grains has been estimated and measured by various methods. Ergosterol, the predominant sterol in fungi, is an essential component of cell membranes and other cellular structures in fungi, but is absent in higher plants, and has been used as a quantitative and qualitative measure of fungal contamination. Since ergosterol is a primary rather than a secondary metabolite, its levels are directly related to fungal growth (Bakan *et al.*, 2002). This molecule is easily detected with high-performance liquid chromatography (HPLC) or thin layer chromatography (TLC). Ergosterol is very stable and its transformation into ergocalciferol (vitamin D_2) is very slow, if it occurs at all,

in grains and flours preserved with normal techniques. However, fungal biomass need not be strictly correlated with mycotoxin content, so measurement of toxins is preferable whenever a toxicological evaluation of maize grain is necessary.

Accurate mycotoxin analyses can be conducted with HPLC, but its high costs render this technique unsuitable for use in large-scale breeding programs. An alternative to HPLC is an ELISA assay, based on antibodies to specific mycotoxins. These assays are available commercially as kits for analyzing the mycotoxin content of ground maize samples as individual samples or in 96 well plates (Eller *et al.*, 2008a). A nondestructive measurement system that accurately monitors, simultaneously, fungal and mycotoxin parameters in real time and with minimum effort and cost is very desirable for breeding purposes.

Many researchers have focused on the potential use of near-infrared spectroscopy (NIR) for the detection of organic compounds under field conditions because of its nondestructive nature, accuracy, rapid response, and ease of use (Siesler *et al.*, 2002). NIR currently is used to measure deoxynivalenol in whole grains via NIR transmittance (Pettersson and Åberg, 2003). When comparative studies of maize collected from naturally contaminated maize and from ears artificially inoculated with *F. verticillioides* were analyzed with both HPLC and NIR (Berardo *et al.*, 2005), NIR accurately predicted the incidence of kernels infected by fungi in general, by *F. verticillioides* in particular, and the quantity of fumonisin B_1 in the meal. The NIR methodology can potentially be applied for large-scale selection of genotypes resistant to fungal contamination and the accumulation of fumonisins.

The presence of *A. flavus* in maize often can be determined qualitatively by observing bright greenish yellow fluorescence (BGYF; Busboom and White, 2004). BGYF is produced when a heat-labile enzyme in the kernel oxidizes kojic acid, a compound produced by *A. flavus*. BGYF can be detected in the germ or endosperm of the kernel when it is irradiated with 365 nm ultraviolet light. BGYF is used as an initial test to identify maize samples that may require analysis for aflatoxin.

Sources and Genetics of Resistance

Genetic variation for resistance to *Fusarium* ear rot exists among inbred lines and hybrids of field maize (Clements and White, 2004; Eller *et al.*, 2008b; King and Scott, 1981). There is no evidence of complete resistance to either ear rot or fumonisin contamination in maize. Shelby *et al.* (1994) reported significant variation among commercial maize hybrids for fumonisin accumulation, but no hybrid was found to be completely resistant. We have been conducting screening trials for both *Fusarium* ear rot and fumonisin concentration using public and private inbred lines (Lanubile *et al.*, 2011). Each plant in these trials was inoculated with a mixture of two strains of *F. verticillioides* with the pin-bar method at 15 days post midsilk. This screen has identified several lines and hybrids with good levels of resistance to both *Fusarium* ear rot and fumonisin accumulation.

Pérez-Brito *et al.* (2001) demonstrated that resistance to *Fusarium* ear rot in two F_2 tropical maize populations is polygenic with relatively low heritability ($h^2 = 0.26$–0.42). Clements *et al.* (2004) screened top crosses of 1589 inbred lines to the susceptible inbred tester FR1064 and found significant genetic variation for both *Fusarium* ear rot and fumonisin concentration, but no complete resistance to either. The alleles for reduced fumonisin accumulation were nearly completely dominant or overdominant in this study. Robertson *et al.* (2006) tested two segregating populations of backcross $(BC)_1F_{1:2}$ and F_7–F_8 families, respectively, derived from the resistant lines GE440 and NC300. Heritability estimated on an entry mean basis ranged from 0.75–0.86 for

fumonisin concentration and from 0.47–0.80 for ear rot resistance. Robertson *et al.* (2006) reported higher heritability values than did Pérez-Brito *et al.* (2001) perhaps because Robertson *et al.* used two artificial inoculations per plant instead of one and three or four evaluation environments instead of two. Moderate to high heritabilities suggest that ear rot resistance and fumonisin accumulation should respond well to selection by using family means.

The phenotypic correlation between the severity of *Fusarium* ear rot and the amount of fumonisin accumulated has been reported to be moderate to low (Clements *et al.*, 2003a, 2004). In these studies, the correlations reported were affected by covariances between genetic, genotype × environment, and experimental error effects on these two traits. Moreover, fumonisin concentration is affected by the presence of asymptomatic kernels (Desjardins *et al.*, 1998). Maize often is colonized endophytically by *Fusarium* spp. (Oren *et al.*, 2003), and a significant amount of fumonisin can be produced in symptomless or only slightly rotten grain (Munkvold and Desjardins, 1997).

The correlations reported between disease severity and toxin accumulation do not necessarily provide an accurate indication of the magnitude of response of fumonisin accumulation that would result when selecting for ear rot resistance. The genotypic and phenotypic correlations between fumonisin concentration and ear rot were estimated by Robertson *et al.* (2006) in two populations of maize. The genotypic correlation was higher than the phenotypic correlation (0.87–0.96 vs. 0.40–0.64) between ear rot and fumonisin accumulation, indicating that genotypic effects on susceptibility to ear rot and fumonisin accumulation are highly correlated, but that genotype × environment interactions and error effects for these traits are not highly correlated. The correlation between *Fusarium* ear rot caused by *F. verticillioides* and fumonisin accumulation suggests that toxin analysis is only rarely needed, if disease severity data are available. Thus, the genetic control mechanisms for resistance to ear rot and to reduced fumonisin accumulation are largely the same, and selection for reduced ear rot should reduce fumonisin accumulation and vice versa. Thus, from a breeder's point of view, selecting against ear rot also should select against genotypes that are likely to accumulate high levels of fumonisin.

Relatively old maize public inbred lines, A632 (Stiff Stalk) and WF9 (Reid-WF9), exhibited intermediate *Gibberella* ear rot resistance (Kovács *et al.*, 1994). Maize germplasm lines with resistance to *Gibberella* ear rot and deoxynivalenol accumulation were developed by Agriculture and Agri-Food Canada: inbreds CO441 (Lancaster heterotic group), CO433 (Minnesota 13), and CO430 (Pioneer 3990 type) possessed both silk and kernel resistance; CO387 (Early Butler), CO379 (Iodent), CO377 (Pioneer 3990 type), and CO373 and CO371 (Minnesota 13) were highly resistant to *Gibberella* ear rot at the kernel level; CO431 exhibited *Gibberella* ear rot resistance after silk inoculation (Reid *et al.*, 2001a, 2001b, 2003, 2009). Elite breeding material adapted to central European climates that has good combining ability with dent lines was obtained at the University of Hohenheim, namely the UH006 and UH007 lines (Martin *et al.*, 2012).

Selection for resistance is positively correlated with a reduced response for mycotoxin accumulation, particularly with respect to *Gibberella* ear rot and deoxynivalenol accumulation (Bolduan *et al.*, 2009). Heritability estimates were found moderate to high and the genotype × environment interaction was usually significant. The authors recommended a multi-stage selection to develop *Gibberella* ear rot resistant hybrids based on artificial inoculation and a visual rating, followed by evaluation of selected materials for mycotoxin concentrations (Bolduan *et al.*, 2010). The performance of an inbred line per se should be evaluated in a single environment followed by testcross evaluations in two or three environments. Only the most promising testcrosses should be tested for deoxynivalenol contamination.

Resistance to *F. verticillioides* and to *F. graminearum* are moderately correlated in European elite maize material ($r = 0.46$–0.65) (Löffler *et al.*, 2010, 2011). The genotypic correlations between fumonisin, deoxynivalenol and zearalenone accumulation are high (0.77 and 0.76, respectively), but separate testing of *Fusarium* ear rot and *Gibberella* ear rot and the accumulation of the corresponding mycotoxins is necessary since genotypes resistant to *Fusarium* ear rot were not necessarily resistant to *Gibberella* ear rot and vice versa. Based on multi-environment inoculation trials, there is good potential to select improved elite inbred lines within the existing germplasm.

Commercial maize hybrids are differentially susceptible to *Aspergillus* ear rot and aflatoxin accumulation; however, hybrids produced by crossing lines selected for reduced aflatoxin accumulation have consistently low levels of accumulated aflatoxin (Warburton *et al.*, 2009). The need for hybrids that accumulate low levels of aflatoxin is particularly obvious under drought stress or high-temperature conditions. Selection of hybrids can be hampered by variation within experiments and by variation between years. The USDA-ARS Corn Host Resistance Research Unit in Mississippi released the first maize germplasm (Mp313E and Mp420) with resistance to kernel infection by *A. flavus* (Scott and Zummo, 1990, 1992). Lines with Mp313E or Mp420 had the lowest levels of aflatoxin contamination (Scott and Zummo, 1998; Windham and Williams, 1998). Other sources of genetic resistance, e.g., CI2, CML176, CML269, CML322, FR2128, LB31, MI82, Mo18W, Mp494, Mp81, Mp92, Mp715, Mp717, Tex6, TX776, SC54, and population GT-MAS:gk (Betrán *et al.*, 2002; Bhatnagar *et al.*, 2003; Campbell and White, 1995; Maupin *et al.*, 2003; McMillian *et al.*, 1993; Scott and Zummo, 1988; Williams and Windham, 2001, 2002, 2006; Williams *et al.*, 2008), are either unadapted or are unacceptable agronomically and cannot be used directly in commercial hybrids.

Heritability for resistance to *Aspergillus* ear rot is extremely variable (12–74%), with high variation within and among genotypes, locations, and years. As many as eight effective genes may be involved in resistance (Clements and White, 2005). Resistance to aflatoxin accumulation is primarily additive, although some dominant effects occur in crosses with LB31 and MI82 as parents. In general, breeding methods that maximize the use of general combining ability should be most effective in enhancing resistance to aflatoxin accumulation.

Traits Providing Resistance to Ear Rots

Fusarium verticillioides may cause a generalized ear rot, which usually is initiated following infection through natural wounds, e.g., those caused by insects (Munkvold, 2003). More commonly infection by *F. verticillioides* causes rotting of individual grains or localized areas of grain on the cob. The infection is internal, usually at the embryo end and asymptomatic, i.e., without causing visible symptoms, which may lead to systemically infected maize plants with no disease symptoms. Internal infection also may occur through the silks from adjacent cob tissues. Infection of the ear via the stalk occurs less frequently than does infection from an airborne or insect-borne source.

Several factors can prevent strains of *Fusarium* and *Aspergillus* from entering through the silk channel opening. First, morphological characters can influence infection. Hybrids with tight, adherent husks and less open apical parts of the ear are more resistant to ear rot (Butron *et al.*, 2006; Warfield and Davies, 1996). Second, physiological factors may influence resistance. Kernels that mature faster shorten the period of susceptibility. Colonization of silks by fungi depends on the conditions of the silks. Symptomatic and asymptomatic infection of kernels by *F. verticillioides* is less common for inbred lines with silks that are green and actively growing at inoculation than it is when the silks are brown.

The seed coat and other areas of the kernels also affect colonization by *Fusarium* spp. and *Aspergillus* spp. (Headrick and Pataky, 1991; Hoenish and Davis, 1994; Scott and King, 1984). The resistance factors of grain to infection are in the pericarp, with thicker pericarp contributing significantly to ear rot resistance (Sampietro *et al.*, 2009). For commercial hybrids in FAO classes 500, 600, and 700 (medium to late season), "slow dry down" hybrids are more prone to fumonisin accumulation, irrespective of their maturity class (Battilani *et al.*, 2011).

A small peptide from maize kernels also inhibits *in vitro* spore germination and hyphal elongation of *F. verticillioides* and *F. graminearum* (Duvick *et al.*, 1992). Kernels of MI82 and Tex6 contain proteins that inhibit fungal growth and aflatoxin accumulation in grain. A 14 kDa protein in kernels of MI82 may limit fungal growth by inhibiting α-amylase production (Chen *et al.*, 1999). Kernels from Tex6 also have β-1,3-glucanase and chitinase activity. These enzymes can degrade fungal cell walls and could minimize fungal infection (Moore *et al.*, 2004).

Several studies, summarized in Snijders (1994), evaluated proteins, sugars, and starches in the endosperm for their role in resistance to infection by *F. verticillioides*, but identified no intrinsic effect of endosperm type on resistance to spreading of the pathogen in the host tissue. Kernel endosperm composition can regulate fumonisin B_1 biosynthesis (Bluhm and Woloshuk, 2005). When a mature maize kernel is colonized, the metabolism of amylopectin induces fumonisin B_1 biosynthesis, possibly through uptake or perception of a 1,6-linked glucoside, e.g., dextrin. The low amounts of amylopectin in the early stages of kernel development and in some maize mutants may be below the threshold required to induce appreciable levels of fumonisin B_1.

Fatty acid composition affects the amount of fumonisins and masked fumonisins accumulated in maize, with higher fumonisin accumulation in hybrids with higher linoleic acid content and more fumonisin masking occurring in hybrids with a higher oleic to linoleic ratio (Dall'Asta *et al.*, 2012). Oleic and linoleic fatty acids can be oxidized to produce as many as 400 biologically active molecules known as oxylipins. Some of these metabolites have signaling activity in biotic stress response pathways (for a review see Christensen and Kolomiets, 2011).

Phenolics also may contribute to the resistance of plants to infection by various *Fusarium* species. These compounds accumulate rapidly during host–pathogen interactions and may mediate disease suppression through the inactivation of fungal enzymes or the strengthening of plant structural components. Flavones in the silks (Reid *et al.*, 1992) and (*E*)-ferulic acid in the kernels (Assabgui *et al.*, 1993; Bily *et al.*, 2003) may be important in inbred lines resistant to *Gibberella* ear rot and less susceptible to mycotoxin accumulation. 4-Acetylbenzoxazolin-2-one (4-ABOA) and hydrocinnamic acids also increase resistance to *Gibberella* ear rot (Cao *et al.*, 2011; Miller *et al.*, 1997). The ears of the brown midrib (*bm*3) mutant of maize, which have a mutated *O*-methyltransferase, can methylate neither caffeic nor 5-hydroxyferulic acids to ferulic and sinapic acids and are much more susceptible to *Fusarium* ear rot than are the isogenic wild-type lines (Vignols *et al.*, 1995).

Benzoxazinones function as allelochemicals and provide resistance to pathogenic fungi, bacteria, and insects (Bacon *et al.*, 2007). The main benzoxazinones produced by maize are 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA) and its dimethoxy derivative 2,4-dihydroxy-6,7-dimethoxy-1,4-benzoxazin-3(4H)-one (DIM2BOA), with the former occurring at higher concentrations. Synthesis and storage of DIMBOA and DIM2BOA, as the glycosides, begins at germination. Maize pathogens, e.g., *F. verticillioides*, can detoxify benzoxazinones to 2-aminophenol (AP), by converting them to *N*-(2-hydroxyphenyl) malonamic acid (HPMA) and 2-acetamidophenol (HPAA). *Bacillus mojavensis*, an endophytic bacterium, is an efficacious antagonist of *F. verticillioides*. This bacterium produces a pigment, 2-amino-3H-phenoxazin-3-one (APO), which interacts with the fungus and blocks the transformation of AP to HPMA. Consequently, higher amounts of toxic APO accumulate and host resistance increases (Bacon *et al.*, 2007).

Genomic Resources for Analyzing *Fusarium* and *Aspergillus*–Maize Interactions

Much progress has been made developing *Fusarium* and *Aspergillus* spp. genomic resources, which have provided researchers with powerful tools to speed the process of identifying new fungal genes and understanding their role in pathogenicity and mycotoxin biosynthesis. Yet, the molecular basis for the interaction between the fungi and the plant is not understood and there is little information about the defense response of maize to *Fusarium* and *Aspergillus* infections.

Genes Providing Resistance to Fungal Infection

Plants can respond to pathogen infection through a complex activation of defense strategies (Delledonne *et al.*, 2001). Following pathogen infection, the defense response changes the expression of a large number of plant genes, which may be up- or downregulated. Pathogenesis-related (PR) protein synthesis and the accumulation of these proteins are a common response to fungal infection. PR proteins are grouped into 17 independent families, some of which have a glucanase or endochitinase activity (Eulgem, 2005). Ca^{2+}-regulated protein phosphorylation often is associated with the activation of the expression of PR genes. A full-length cDNA coding for a calcium-dependent protein kinase (CPK) in the W64A maize inbred, resistant to *F. verticillioides*, has been identified (Murillo *et al.*, 2001). Calcium regulates the activity of these kinases through binding and activating various calcium-binding domains that mediate the plant response to different stimuli.

The *ZmCPK*10 gene, which encodes a specific maize kinase, was transcriptionally activated in an elicitor-treated or fungal-infected germinating maize seed. A strong, cell-type-specific expression of the gene encoding this enzyme was identified through *in situ* mRNA hybridization in infected tissues. Thus, the activation of this kinase was associated with a high level of the PR mRNA (Murillo *et al.*, 2001). In previous work, Murillo *et al.* (1997) showed that the PR maize genes are expressed specifically in some cell types during growth of the *Fusarium*-infected maize radicle, in particular, in the parenchyma cells of the differentiating xylem elements, in the pericycle, and during primordium development of the lateral roots (Murillo *et al.*, 1997). *ZmCPK*10 gene expression always occurred in the cell types and developmental stages in which PR expression occurred. That CPK10 induces the PR gene expression and the response of other genes involved in plant defenses remains to be demonstrated (Murillo *et al.*, 2001).

In several plant–pathogen interactions, the plant defense response is associated with the induction of phenylalanine ammonia lyase and chalcone synthase, and with the accumulation of flavonoids, phenolic compounds, and phytoalexins. Plant secondary metabolites present in maize kernels may play an important role in the plant's resistance to ear rot fungi. However, the amount of phenolics and flavones present in uninoculated silk tissue alone is not a good indicator of maize resistance to *Fusarium* ear rots (Sekhon *et al.*, 2006), even though the production of 3-deoxyanthocyanidins occurs in resistant lines following fungal inoculation and suggests a role for these compounds in resistance to *F. verticillioides.* Decreased accumulation of 3-deoxyflavonoids in susceptible lines could be due to the inhibition of the plant's metabolism by the fungus. There is no direct correlation between the induced expression of flavonoid biosynthesis genes in greenhouse-grown plants and field-based ratings of ear rot severity.

Specific genes putatively providing resistance to *Fusarium* pathogens are known. A defective lipoxygenase (*lox3*) in maize reduces fumonisin B_1 contamination and *F. verticillioides* conidiation and also provides resistance to several other pathogens (Gao *et al.*, 2007, 2009). Six multi-enzyme pathways, collectively termed the lipoxygenase (LOX) pathway, produce oxylipins, a large class

of diverse oxygenated polyenoic fatty acids. Specific host 9-lipoxygens are required for normal pathogenicity and the production of spores and mycotoxins by *F. verticillioides* and *A. flavus* (Christensen and Kolomiets, 2011).

A maize guanylyl cyclase gene is associated with resistance to *Gibberella* ear rot (Yuan *et al.* 2007). Two of the multiple copies of this gene in maize map near quantitative trait loci (QTL) positions for ear rot resistance. Transcription of the genes increases in response to pathogen inoculation, and the more resistant parent has a higher level of expression of the entire gene family (Yuan *et al.*, 2007). If markers consistently associated with the resistant alleles can be identified, then these markers would be ideal tools for implementing MAS on a broad scale for resistance to infection by *Fusarium* in maize breeding programs.

The maize *An2* gene encodes a copalyl diphosphate synthase (CPS)-like protein with 60% amino acid sequence identity with the *An1* gene product that functions in gibberellin biosynthesis. *An2* transcript levels increase following *Fusarium* infection, with an increase in silk, husk, and ear tip tissues as early as 6 hours after inoculation of silk channels with spore suspensions of various *Fusarium* species (Harris *et al.*, 2005). The *Fusarium*-inducible nature of *An2* is consistent with the ability of maize seedlings to produce ent-copalyl diphosphate-derived diterpenes in response to *Fusarium* infection. However, it is not known whether *An2* is involved in defense-related secondary metabolism in addition to gibberellin synthesis.

The defense response by maize to infection by *F. verticillioides* changes the expression of many host genes (Lanubile *et al.*, 2010, 2012a). The gene expression patterns of seeds of susceptible and resistant maize genotypes also differed. In resistant lines, expression of defense-related genes begins before infection, i.e., there is a basal defense response, whereas in susceptible genotypes defense gene expression occurs only after pathogen attack. For example, in resistant seedlings, prior to infection and 5 days after inoculation, ascorbate peroxidase and superoxide dismutase (enzymes that reduce reactive oxygen species) activities remained constant (Lanubile *et al.*, 2012b). These levels were higher than in the susceptible seedlings prior to inoculation. Enzyme levels in the susceptible seedlings increased only after infection by *F. verticillioides*.

Proteomic analyses of plant defense responses to *Fusarium* infection identified numerous changes in plant defense protein changes in germinating W64A maize embryos (Campo *et al.*, 2004). Homology-based searches with MALDI-TOF data in association with nanospray ion-trap tandem mass spectrometry (nESI-IT MS/MS) identified multiple antioxidant enzymes, e.g., Cu/Zn-superoxide dismutase, glutathione-*S*-transferase, and catalase, that protect cells from oxidative damage.

Proteins that induce the synthesis of other proteins or that participate in the protein-folding process and stabilization were also identified in infected embryos and kernels. Eukaryotic translation initiation factor 5A may initiate the translation of mRNAs involved in the plant defense response. Increases in aldolase activity and decreases in glyceraldehyde-3-phosphate dehydrogenase activity suggest that sugar metabolism is altered in infected plants. β-1,3-Glucanases and chitinases are constitutively expressed at high levels. Thus, these enzymes are linked to both pathogen infection and to the normal process of seed germination (Campo *et al.*, 2004; Mohammadi *et al.*, 2011).

Similar studies with *Aspergillus*-infected plants found differences between sensitive and resistant maize lines (Ankala *et al.*, 2011), and genes upregulated in resistant lines were identified (Kelley *et al.*, 2009). Maize resistance to infection by *A. flavus* and reduced aflatoxin accumulation results from the activity of antifungal proteins that restrain fungal growth and/or aflatoxin production. Increased levels or activities of antifungal proteins occur in resistant genotypes relative to those in susceptible lines both before and after infection by *A. flavus* (Kelley *et al.*, 2012). Proteins

with potential antifungal activity include β-1,3-glucanases, chitinases, trypsin inhibitors, PR-10, ribosome inactivating proteins (RIPs), zeamatin, and lectin-like proteins from kernels, calli, embryo, endosperm, and silk (Chen *et al.*, 2007; Guo *et al.*, 1994, 1997, 1998; Magbanua *et al.*, 2007; Peethambaran *et al.*, 2010). Changes also were observed in proteins associated with abiotic stress resistance, such as aldose reductase, glyoxalase I, small heat shock proteins, peroxiredoxin, cold-regulated proteins, anionic peroxidase, storage proteins, e.g., globulins GLB1 and GLB2, and late embryogenesis abundant proteins LEA3 and LEA14 (Guo *et al.*, 2005). The Corn Fungal Resistance Associated Sequences Database (CFRAS-DB; http://agbase.msstate.edu) was created to help identify genes important for aflatoxin resistance (Kelley *et al.*, 2010). The database collates many types of data, e.g., microarrays, proteomics, QTLs, and SNPs, related to the infection of maize by *A. flavus* infection and the subsequent production of aflatoxin by the fungus.

The rachis is an important maize structure that transports essential nutrients to the developing kernels, can serve as a conduit for *A. flavus* dispersion throughout the maize ear, and plays an important role in resistance to *A. flavus* (Pechanova *et al.*, 2011). *Aspergillus flavus* can grow within the rachis and enter the kernels through rachilla (Smart *et al.*, 1990). The fungus spreads more widely within the rachis of a susceptible hybrid than of a resistant hybrid. Young resistant rachises contains higher levels of abiotic stress-related proteins and proteins associated with phenylpropanoid metabolism, whereas susceptible young rachises contain PR proteins, which usually are produced in response to biotic stress. Differential expression of many stress/defense proteins in juvenile, mature, and post *A. flavus* infection rachises are consistent with a hypothesis of constitutive defense, whereas a susceptible rachis is dependent upon inducible defenses.

A developing rachis from an aflatoxin accumulation resistant inbred contains higher levels of heat shock proteins and enzymes that are associated with the abiotic stress response, such as ascorbate peroxidase, dehydroascorbate reductase, and superoxide dismutase. These results are consistent with the hypothesis that *A. flavus* synthesizes aflatoxin in response to oxidative stress (Chen *et al.*, 2004; Kim *et al.*, 2005). When maize is subjected to heat and drought stress in the field, reactive oxygen species may accumulate in ear tissues. If these tissues respond to these stresses by controlling reactive oxygen species levels with antioxidants, they could simultaneously limit aflatoxin biosynthesis. High levels of enzymes from the phenylpropanoid pathway also could contribute to resistance to *Aspergillus* through increased lignification of the cell wall or the production of toxic phenolic compounds. Caffeic acid, gallic acid, tannins, and related compounds all have antimicrobial and/or antiaflatoxigenic properties that inhibit the growth of *A. flavus* and/or aflatoxin biosynthesis (Pechanova *et al.*, 2011).

Molecular Markers and QTL Mapping

High levels of genetic resistance to mycotoxigenic fungi in maize are not available, even though significant progress has been made for resistance to *Aspergillus* ear rot, *Fusarium* ear rot, and *Gibberella* ear rot. There are quantitative differences in resistance among maize genotypes and both traits have moderate to high heritability, so phenotypic selection efficiently increases resistance. Unfortunately phenotypic selection for these traits has numerous practical problems. For example, many diseases can be evaluated during the young plant stage or before flowering, whereas ear rot severity and mycotoxin accumulation can be assessed only with mature plants. In addition, two liquid inoculations with calibrated fungal spore suspensions are required for consistent evaluation of ear rot (Clements *et al.*, 2003a). Assessing mycotoxin accumulation is time consuming and requires expensive toxin assays of precisely weighed and ground grain. Finally, many environmental factors

influence ear rot severity and toxin accumulation and test sites with consistently high disease pressure are required (Shelby *et al.*, 1994).

PCR-based DNA markers linked to resistance genes could be used to select lines resistant to ear rots (Robertson *et al.*, 2005). QTL mapping provides a powerful method to understand the genetic relationships between correlated traits, although meaningful results are hard to obtain for traits with moderate heritability traits (Beavis, 1998). Pérez-Brito *et al.* (2001) showed that resistance to *Fusarium* ear rot is polygenic and that the QTLs were not conserved in just the two populations analyzed. Robertson-Hoyt *et al.* (2006) reached different conclusions in the two segregating populations they evaluated, in which three QTLs for *Fusarium* ear rot severity and two QTLs for fumonisin accumulation resistance were identified and mapped to similar genomic locations in both populations. In particular, QTLs mapped on chromosomes 4 and 5 were consistent for both traits across both populations. Two QTLs on chromosome 3 (bin 3.04) were consistently identified across environments by Ding *et al.* (2008). The QTL with the largest effect on resistance explained 13–22% of the observed phenotypic variation and is flanked by SSR markers *umc1025* and *umc1742*, which are 3.4 cM from each other. These SSR markers will facilitate MAS of resistance to *Fusarium* ear rot in maize breeding programs.

To determine the effectiveness of selection against *Fusarium* ear rot for decreasing fumonisin accumulation several selection procedures can be applied. Advanced BC lines containing alleles from GE440, which has good disease resistance but poor agronomic quality, were introgressed into an elite genetic background (FR1064) that lacks effective resistance (Eller *et al.*, 2008b). The BC lines were more resistant to ear rot, accumulated less fumonisins, and provided grain yields similar to the elite parent (Eller *et al.*, 2010). The advanced BC lines are a valuable resource for developing near-isogenic lines (NILs) for finer-scale genetic analysis of previously mapped QTLs. The results of these experiments provide a better understanding of the genetic relationship between *Fusarium* ear rot severity and fumonisin accumulation.

The pedigree method was used to increase maize resistance to *Gibberella* ear rot and *Fusarium* ear rot (Presello *et al.*, 2005, 2011). Responses to selection were more evident in later generations for both silk and kernel inoculations. Selection after kernel inoculation was more effective than selection after silk inoculation in developing families with stable resistance. Responses to family selection can be accelerated, without increasing operational costs, by increasing selection intensity in later generations and inoculating fewer plants per family. From resistant parent CO387, 11 QTLs for *Gibberella* ear rot resistance that were identified following silk inoculation and 18 QTLs for resistance that were identified following kernel inoculation which account for 6.7–35% of the total phenotypic variation (Ali *et al.*, 2005). Only two of these QTLs were detected in more than one test for silk resistance and only one QTL was detected in more than one test for kernel resistance. Four to six QTLs for *Gibberella* ear rot severity and the accumulation of deoxynivalenol and zearalenone colocalized in bins 1.11 and 2.04, and collectively explained 29–35% of the total genotypic variance (Martin *et al.*, 2011, 2012).

Inbred Oh516 provides useful agronomic traits that can be readily incorporated into commercial inbreds and carries QTLs on three chromosomes that provide resistance to aflatoxin accumulation in grain (Busboom and White, 2004). Inbred Mp313E carries the *afl3* and *afl5* QTLs for reduced aflatoxin accumulation (markers *bnlg371* and *bnlg2291*, respectively), which account for 7–18% of the variation in aflatoxin accumulation (Brooks *et al.*, 2005). In a genetic mapping population of $F_{2:3}$ families developed from Mp715, QTLs with large phenotypic effects also were identified (Warburton *et al.*, 2011a). Mapping populations, association mapping panels, and phenotypic and SNP data for mapping QTLs for resistance to *Aspergillus* ear rot and aflatoxin accumulation are available in the CFRAS database (http://agbase.msstate.edu) (Kelley *et al.*, 2010; Warburton *et al.*, 2011b).

QTLs for resistance to different ear rots and reduced toxin accumulation colocalize on a genetic map providing circumstantial evidence for common genetic mechanisms (Wisser *et al.*, 2006). For example, several QTLs for fumonisin accumulation colocalize with QTLs for aflatoxin accumulation on six different chromosomes (Robertson-Hoyt *et al.*, 2006; Wisser *et al.*, 2006). In a follow-up study, two QTLs that affected both fumonisin and aflatoxin accumulation, one QTL that affected both *Fusarium* ear rot and *Aspergillus* ear rot, and one QTL that affected *Aspergillus* ear rot, *Fusarium* ear rot, and fumonisin accumulation were identified (Table 4.1; Robertson-Hoyt *et al.*, 2007).

Transgenic Approaches to Reduce Ear Rots and Mycotoxin Accumulation

In maize, several approaches can increase genetic resistance to insect feeding, reduce disease severity, and either detoxify or block the synthesis of mycotoxins in seed (Duvick, 2001).

Resistance to Insect Feeding

Insects enable fungal infection by making wounds that fungi can use to enter the plant. In transgenic maize hybrids, genes coding for insecticidal proteins from *Bacillus thuringiensis* (*Bt*) are expressed (Hofte and Whitely, 1989). The *Bt* gene encodes the δ-endotoxins Cry1Ab and Cry1Ac, which protect against spike damage. The target insects are the European corn borer, the Southwestern corn borer, the corn earworm, and the corn root beetle (*Diabrotica virgifera*). Their larvae prefer ear tops and other ear parts where they produce wounds that enable many fungal species to invade the ears and produce various mycotoxins.

The efficacy of *Bt* maize hybrids to indirectly reduce aflatoxin accumulation is not settled (Munkvold, 2003). Studies in Texas and Mississippi, where natural infection by *A. flavus* is common, identified some reduction in aflatoxin accumulation in *Bt* hybrids, but not in a consistent manner. Fumonisin accumulation and damage from European corn borer are correlated (Dowd, 2001), and *Bt* hybrids are associated with reduced fumonisin concentrations in grain when conditions favor European corn borer damage (Clements *et al.*, 2003b; de la Campa *et al.*, 2005). Munkvold and Desjardins (1997) reported small differences between transgenic and non-transgenic hybrids for incidence of *Fusarium* ear rot in non-inoculated control plants. *Bt* hybrids grown in Germany and Canada had modest but significant reductions in deoxynivalenol accumulation (Munkvold, 2003).

The presence of the *Bt* gene does not directly influence fungal populations. The presence of both *Bt*-transformed and non-transformed ears and overwintered stalk residues did not alter the relative frequencies of the *Fusarium* species present (Naef and Défago, 2006). As *F. graminearum* and *T. viride* can degrade the CrylAb protein, a fungal infestation could also decrease the level of expected insect control (Naef *et al.*, 2006). Mycotoxin reduction due to the current level of *Bt* crop planting is economically significant in the United States (Hammond *et al.*, 2004). In less developed countries, the potential mycotoxin reduction attributable to *Bt* crops could have important economic and health impacts (Wu, 2006), even though this form of protection may decrease the exposure to only a few specific *Fusarium* toxins (Papst *et al.*, 2005).

Maize lines and hybrids that are resistant to *Fusarium* and that accumulate lesser amounts of mycotoxins are still needed and the underlying resistance mechanisms remain to be elucidated. Infection through insect-free channels, e.g., silks or cobs, is not reduced in *Bt* hybrids, and *Bt*-resistant insect populations are expected to develop. Magg *et al.* (2002) concluded that the accumulation

Table 4.1 Summary of chromosomal distribution of quantitative trait loci (QTLs) for resistance to ear rots caused by *Fusarium verticillioides* (FER), *Fusarium graminearum* (GER), and *Aspergillus flavus* (AER) and for reduction in accumulation of fumonisins (Fum), deoxynivalenol (Don), zearalenone (Zea), and aflatoxin (Afl) in different populations of maize

Population				Chromosome[b]										
Parents[c]	Size[d]	Type[e]	QTL analysis[a]	1	2	3	4	5	6	7	8	9	10	Reference
Cross 3 × 18	238	$F_{2:3}$	CIM	FER	FER	FER	FER		FER	FER			FER	Pérez-Brito *et al.* (2001)
Cross 5 × 18	206	$F_{2:3}$	CIM	FER		FER	FER	FER	FER	FER				Pérez-Brito *et al.* (2001)
GE440 × FR1064	213	$BC_1F_{1:2}$	CIM	**FER Fum**	**FER Fum**	**Fum**	**FER Fum**	**FER Fum**		**Fum**		**Fum**		Robertson-Hoyt *et al.* (2006)
NC300 × B104	143	$BC_1F_{1:2}$	CIM	**Fum**	**FER**	**FER Fum**	**FER Fum**	**FER Fum**	**FER Fum**		**Fum**			Robertson-Hoyt *et al.* (2006)
87-1 × Zong3	187	RIL	CIM			**FER**		FER			FER		FER	Ding *et al.* (2008)
CO387 × CG62	144	RIL	CIM	GER GER[f]	GER	GER GER[f]	GER	**GER**	GER GER[f]	GER **GER**[f]		GER		Ali *et al.* (2005)
UH006 × UH007	150	DH	CIM MR	**GER Don Zea**	**GER Don Zea**		**GER Zea**	**Don**	**GER Zea**		**GER**		**Don**	Martin *et al.* (2011)
UH007 × D152	180	DH	ANOVA		**GER Don**	**Don GER**		**Don**			**GER Don**			Martin *et al.* (2012)
UH006 × UH009	101	DH	ANOVA		**GER Don**							**GER**	**GER Don**	Martin *et al.* (2012)
UH007 × UH009	227	DH	ANOVA	**GER**	**GER**	**GER Don**	**GER**					**GER**	**GER Don**	Martin *et al.* (2012)
Tex6 × B73	100	BC_1S_1	CIM MR					Afl						Paul *et al.* (2003)
Tex6 × B73	176	$F_{2:3}$	CIM MR			Afl	Afl						Afl	Paul *et al.* (2003)
Oh516 × B73	217	BCS_1	ANOVA	**AER**	**AER Afl**	**Afl**	**AER**	**AER**		**AER Afl**				Busboom and White (2004)
Mp313E × B73	210	$F_{2:3}$	CIM	Afl	**Afl**	**Afl**	**Afl**	Afl	Afl					Brooks *et al.* (2005)
Mp717 × NC300	270	$F_{2:3}$	CIM	Afl	Afl	Afl		Afl		**Afl**	Afl		Afl	Warburton *et al.* (2009)
Mp715 × T173	250	$F_{2:3}$	CIM	**Afl**		**Afl**	Afl	**Afl**				Afl	**Afl**	Warburton *et al.* (2011a)
NC300 × B104	48	RIL	CIM	**Afl**		**AER FER Fum**	**AER**	**AER FER Afl Fum**	**AER Fum**		**FER Afl Fum**	**Afl**	**FER Afl**	Robertson-Hoyt *et al.* (2007)

[a] CIM, composite interval mapping; ANOVA, analysis of variance; CIM MR, composite interval mapping with multiple regression.
[b] Stable QTLs in multiple year–location environments are indicated in bold.
[c] Resistant genotype is indicated in bold.
[d] Number of progeny.
[e] RIL, recombinant inbred line; BC, backcross; DH, doubled haploid; S, self-pollinated.
[f] Silk resistance.

of *Fusarium* mycotoxins was not reduced in grain produced by *Bt* hybrids. Thus, resistance to European corn borer and to *Fusarium* rots are inherited separately, and a separate breeding program is needed for each trait.

Maize Genes with Antifungal Properties

Transgene-enhanced disease resistance has not had the same success as has *Bt* maize. A major limitation is the lack of understanding of the basic biology of the maize–*Fusarium*–*Aspergillus* interactions. Genetic engineering could introduce proteins with antifungal activities to augment pathogen resistance, as reported for several leaf pathogens of dicotyledonous plants (Munkvold and Desjardins, 1997). Unfortunately antifungal proteins must be expressed at high levels to reduce fungal growth in plant tissues. In maize endosperm, a cytosolic albumin, b-32, with a molecular weight of 32 kDa, is synthesized in temporal and quantitative coordination with the deposition of storage proteins. b-32 is homologous to several previously characterized RIPs. Maize plants expressing a chimeric gene containing the *b-32* coding sequence downstream of a constitutive *35SCaMV* are more tolerant to *Fusarium* attack in leaf tissue bioassays (Lanzanova *et al.*, 2009). The maize b-32 protein effectively slows *Fusarium* infection progression in the plant.

Enzymes involved in secondary metabolite synthesis in response to fungal infection have been cloned and can be modified and overexpressed in maize (Gao *et al.*, 2007; Yuan *et al.*, 2007). The primary limitation to this strategy is the possibility that other biosynthetic pathways might be altered as a result of the biosynthesis of novel plant secondary metabolites.

Many experiments focus on detecting changes in expression of the numerous genes simultaneously involved in defense pathways and on their regulation. Although many defense pathways are known in maize (Morris *et al.*, 1998), their role, if any, in defense against *Fusarium* or *Aspergillus* infections and mycotoxin accumulation is not known. These studies are best conducted with developing seed tissues, as defense gene expression can be improved by elicitor application following germination.

Another strategy for conferring resistance to mycotoxigenic fungi through protein engineering/directed evolution is through the identification of new dominant resistance genes (R genes). These genes allow plants to recognize the pathogen in a gene-specific manner through elements secreted by the fungus during plant infection.

Detoxification/Prevention of Mycotoxins Accumulation

Suppressing mycotoxin production or degrading the mycotoxin *in situ* could also reduce mycotoxin accumulation. For example, transgenic plants could produce specific signals to turn off mycotoxin biosynthesis pathways in the pathogen or be defective in some essential signals or metabolic components required for toxin production. For example, fumonisin biosynthesis by *F. verticillioides* is subject to nitrogen repression (Shim and Woloshuk, 1999), but using this mechanism to control fumonisin biosynthesis has other effects on the grain.

Another approach is to synthesize catabolic enzymes to detoxify the mycotoxins *in situ* before they have a chance to accumulate. Many studies have focused on the overexpression of plant genes encoding detoxification enzymes to reduce plant disease or toxin accumulation (Munkvold, 2003). Little is known about fumonisin localization in maize, which could explain why fumonisins appear to be resistant to the activity of known esterases and amine-modifying enzymes. Thus, a search

for new detoxification enzymes is needed. Many fungal species can catabolize fumonisins, e.g., the black yeasts *Exophiala spinifera* and *Rhinocladiella atrovirens*, which can subsist on fumonisin B_1 and produce enzymes that can degrade the C-20 backbone of the molecule (Duvick, 2001). In particular, enzymes that participate in catabolic processes, e.g., de-esterification and oxidative deamination, to detoxify fumonisins have been identified and the genes encoding them have been cloned.

Bacillus mojavensis can inhibit the growth of *F. verticillioides* and reduce fumonisin accumulation (Bacon *et al.*, 2007). This bacterium produces surfactin A, a cyclic lipopeptide, which is a powerful biosurfactant, and toxic to fungi, bacteria, viruses, and mites (Bacon *et al.*, 2012). Thus, the surfactins produced by this bacterium may alter events that result from changes in transient channeling of host plasma membrane in addition to altering events at higher levels of complexity that result in defensive host reactions. Endophytic production of this surfactin and its *in vivo* relationship to plant protection, fungal infection, and plant colonization remain unproven, even as surfactins are being modified to have specific activity.

Future Prospects

Quantitative genetic variation for resistance to ear rots and for reduced mycotoxin accumulation is present in only a few elite inbred lines. Other sources of these traits are needed and may exist given the positive/negative relationship between infection by *Fusarium* and *Aspergillus* species (Lanubile *et al.*, 2011; Marin *et al.*, 1998; Zummo and Scott, 1992). Such results suggest that these fungal species may require similar substances for growth and development and that they interact in a similar way with the host plant. In this case, host plant resistance to one pathogen could result in resistance to the other as well. Robertson-Hoyt *et al.* (2007) estimated the correlation between *Fusarium* and *Aspergillus* ear rot, and fumonisin and aflatoxin accumulation in maize, by inoculating plants with only one of the pathogens. Data obtained from recombinant inbred lines tested in different environments and selected for high and low levels of fumonisin accumulation demonstrated that there is a strong genetic correlation between fumonisin accumulation and the other three traits. These results suggest that selecting for reduced incidence of *Fusarium* ear rot also should reduce susceptibility to *Aspergillus* ear rot and reduce the accumulation of fumonisins and aflatoxins. The hypothesis that common resistance mechanisms to these two fungi exist also is supported by the consistency between QTLs for fumonisin and aflatoxin accumulation (Wisser *et al.*, 2006).

Linkage analysis is the tool most commonly used to dissect complex traits and can be typically used in plants to localize a QTL to a 5–20 cM interval depending upon the mapping populations. Several linkage analysis studies have been conducted to identify QTLs for ear rot resistance (Table 4.1), but only a limited number of maize QTLs have been validated and none of these have been cloned or tagged at the gene level. Recent advances in genomic technology have increased interest in the explanation for natural diversity and development of robust statistical analysis methods has made QTL identification possible and affordable for plant research programs. Association mapping is emerging as a tool to resolve complex trait variation down to the sequence level (Zhu *et al.*, 2008). As an alternative to traditional linkage analysis, association mapping offers three advantages: (i) increased mapping resolution, (ii) reduced research time, and (iii) a greater number of alleles.

A further development is the creation of a large multi-parent population of recombinant inbred lines, which has been termed MAGIC (multi-parent advanced generation inter-cross; Cavanagh *et al.*, 2008). The incorporation of multiple parents ensures that the population segregates multiple QTLs for multiple traits. Precision and power to detect QTLs are greatly enhanced, e.g., an

eight-parent recombinant inbred line population with 1000 progeny has potential mapping resolution in the sub-centimorgan range.

Researchers interested in a specific trait often exploit candidate gene association mapping. Candidate genes are selected based on prior knowledge of biochemical pathways or gene expression analysis of the trait of interest.

Functional genomic analysis is being sped up by the use of recently developed deep expression profiling (Mortazavi *et al.*, 2008). RNA-Seq produces a genome-scale transcription map that consists of both the transcriptional structure and the level of expression for each gene. RNA-Seq is of great value for validating and refining genetic models and can identify new genes not previously annotated. The available maize genome sequences and the high number of unique expressed sequence tags (ESTs) are valuable tools when implementing RNA-Seq approaches.

Successful use of molecular tools in the breeding of resistant maize genotypes requires the ability to integrate phenomics, genomics, transcriptomics, proteomics, and metabolomics data in a system biology approach. In model plants, various omic-based tools were used to dissect complex pathways and to significantly speed up the elucidation of mechanisms contributing to stress resistance (Pitzschke and Heribert, 2010).

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5 Crop Management Practices to Minimize the Risk of Mycotoxins Contamination in Temperate-Zone Maize

Gary Munkvold

Abstract

Maize produced in temperate regions can be contaminated with fumonisins, deoxynivalenol, zearalenone, aflatoxins, and other mycotoxins, as a result of infection by toxigenic fungi, primarily in the genera *Fusarium* and *Aspergillus*. The crop production environment has a profound influence on the risk of mycotoxin contamination. Many aspects of this environment can be altered by management practices to reduce this risk and to produce high-quality grain, low in mycotoxins. In this chapter how crop management practices can be optimized to reduce mycotoxin risk is discussed. Pre-planting decisions including hybrid selection, planting date, crop rotation, tillage, fertilization, and planting density can have significant impacts on infection by toxigenic fungi and resulting mycotoxin contamination; however, the importance of each practice varies by mycotoxin and by geographic location. During the growing season, insect control is the most important practice affecting the risk of mycotoxin contamination. The most effective mycotoxin reductions are accomplished with transgenic insect resistance, but properly timed insecticide applications also can be effective. Irrigation management should be optimized to prevent drought stress and also to avoid excessive moisture. Harvest delays can lead to increased mycotoxin development. Effective decisions about crop management during the season and postharvest can be facilitated by using mycotoxin risk assessment or prediction models that are available in some areas. Management practices can be guided by these models and optimized to best manage the risk of mycotoxin contamination in the field, as part of a comprehensive mycotoxin risk management strategy that extends from pre-plant decision-making through crop utilization.

Keywords: crop rotation; fertilization; grain safety; harvest time; hybrid selection; insect control; irrigation; plant density; pre-harvest; tillage; risk modeling; seed treatment; transgenic plants; weed control

Introduction

Maize produced in temperate regions of the world is subject to infection by a variety of mycotoxin-producing fungi, primarily in the genera *Fusarium* (with teleomorphs in the genus *Gibberella*), *Aspergillus*, and *Penicillium*. The most common economically important diseases are *Fusarium* ear rot (caused primarily by *F. verticillioides*, *F. proliferatum*, and *F. subglutinans*), *Gibberella* ear rot (caused primarily by *F. graminearum*), and *Aspergillus* ear or kernel rot (caused primarily by *A. flavus*) (Logrieco *et al.*, 2002; Munkvold, 2003c). This chapter focuses on toxin-producing species of *Fusarium* and their important mycotoxins (fumonisins, deoxynivalenol and other trichothecenes, and zearalenone) and aflatoxin-producing *Aspergillus* species. The reference list is

Mycotoxin Reduction in Grain Chains, First Edition. Edited by John F. Leslie and Antonio F. Logrieco.

not comprehensive; many older references can be found in more comprehensive review articles or chapters (Abbas *et al.*, 2009; Munkvold, 2003a, 2003b, 2003c; Wilson *et al.*, 2005).

Managing the risks of mycotoxin contamination requires an integrated strategy that extends from the crop production practices, through harvesting and storage, to processing and utilization. While there are strategies to manage mycotoxin development postharvest and to reduce the impacts of mycotoxins already in the grain, an effective integrated strategy starts in the field, before the crop is planted. Therefore, a considerable amount of research continues to be directed at pre-harvest prevention of mycotoxins in maize (Cleveland *et al.*, 2003). The aims of this chapter are to describe how pre-planting decisions and management practices in the field affect the risks of mycotoxin contamination in maize grown in temperate areas and to outline practices that can most effectively reduce those risks.

Managing maize crops to meet the needs of the farmer, consumer, and society in general is a challenging task. The farmer requires value in relation to crop production inputs; the consumer requires quality in relation to cost; and society requires food and energy security along with environmental stewardship. Maize crops in temperate climates typically are grown as cash crops or for on-farm livestock feeding; in fewer cases the crop is for direct human consumption. Thus, many maize fields are managed with the goal of maximizing net revenue, while at the same time providing the environmental stewardship that will support sustainability of crop production on the land and prevent negative off-farm impacts. This balance can be a delicate one, since some intensive crop management practices may have negative environmental consequences that detract from sustainability. This balance is further complicated by the issue of grain safety in relation to mycotoxins. Revenue from a crop is affected by both quantity of yield and value/unit of sale (a reflection of quality).

The relationship between value and mycotoxin content is not straightforward, since acceptable levels of contamination depend on the end-use, with a significant loss of value occurring when the contamination level crosses a threshold. For some end-uses of maize grain, e.g., human food products, contamination thresholds are low, and near-freedom from mycotoxin contamination is a critical component of the crop's intrinsic value. However, for other end-uses, e.g., feed for beef cattle, higher mycotoxin contamination levels can occur before there is a loss of value. In either case, the value of a maize crop is not linearly tied to its mycotoxin content. Therefore, the management goal is to reduce the probability of exceeding the appropriate threshold. Reducing mycotoxin risk may not always contribute to net revenue, and there is uncertainty over the benefit expected from the associated management costs. Therefore, maize crop production is a process of balancing management costs and environmental impacts against their uncertain benefits in terms of crop yield and value, including mycotoxin risk (Wu *et al.*, 2008). Crops that are grown for on-farm use may not be subject to net revenue considerations, but still must be managed to balance these same factors. In fact, mycotoxin content is likely to be more important to the value of grain for on-farm use than it is for grain sold as a commodity. Whether or not the crop is grown as a cash crop, the nonlinear relationship between mycotoxin content and grain value creates a unique framework for crop management decisions.

Pre-planting Management Decisions

Hybrid Selection

This choice is the most important pre-planting decision that affects mycotoxin risk (Hooker and Schaafsma, 2005) and is often the most cost-effective method available. There is a wide range of susceptibility to mycotoxin-producing fungi among commercial maize hybrids (Figure 5.1)

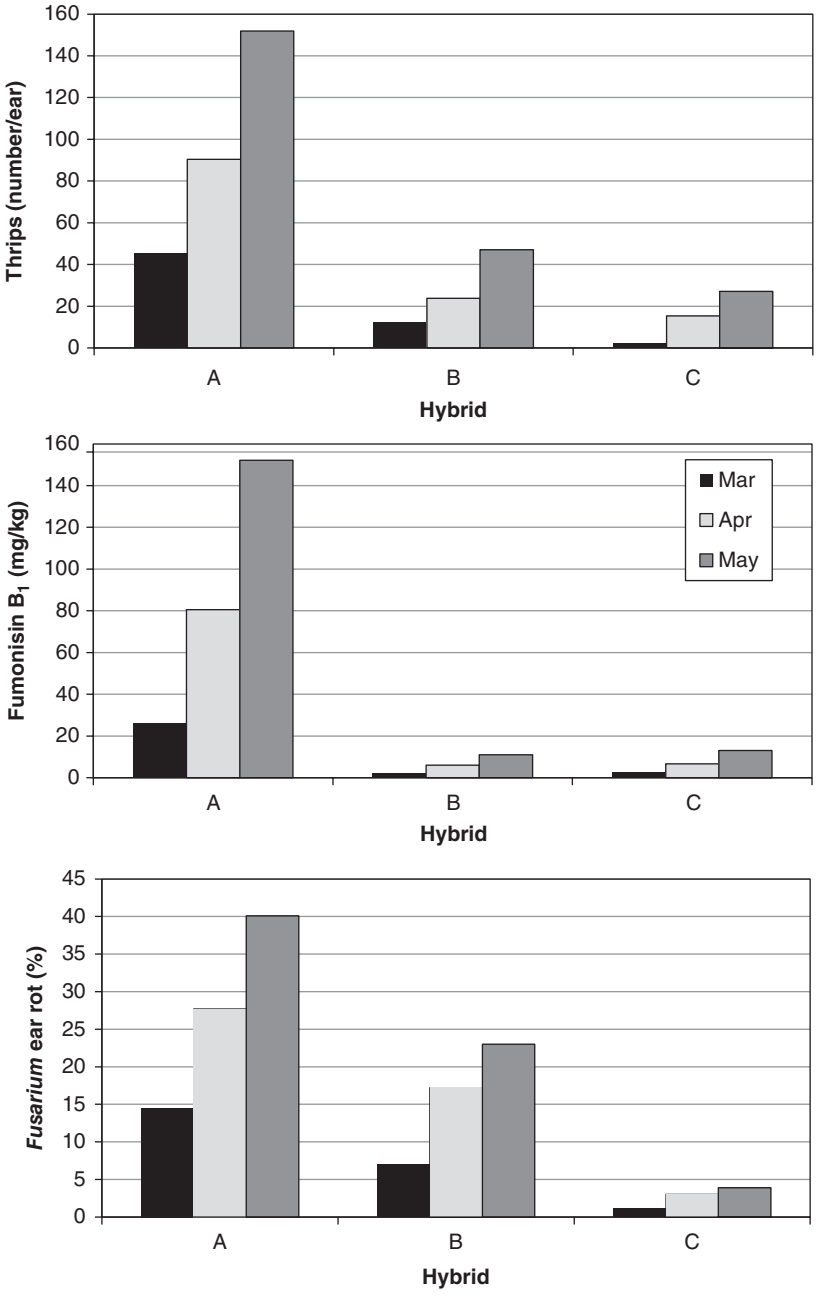

Figure 5.1 Effects of maize hybrid and planting date on populations of thrips in maize ears, 21 days after pollination (top). Fumonisin B_1 concentrations in maize kernels after harvest (middle). Percentage of kernels with *Fusarium* ear rot symptoms at harvest (bottom) in a field experiment conducted for 2 years in California, USA. Hybrids A, B, and C were commercial dent maize hybrids that vary in husk coverage and susceptibility to *Fusarium* ear rot. Planting dates were late March, late April, or late May. Data from Parsons and Munkvold (2010).

(Clements *et al.*, 2004; Cleveland *et al.*, 2003; Lauren *et al.*, 2007; Parsons and Munkvold, 2010), and information should be sought about known levels of resistance to the important ear rot diseases in the area. Universities or government agencies may have independent evaluation data on mycotoxin levels in maize hybrids. Unfortunately, reliable information is not always available for relative resistance/susceptibility of hybrids, especially for *A. flavus* and aflatoxins. Better information is often available for *Fusarium* and *Gibberella* ear rots. Even partially resistant hybrids can accumulate unacceptable levels of mycotoxins if environmental conditions favor fungal infection and disease development. Resistance may be expressed in the form of reduced infection, but some hybrids may have resistance to postinfection mycotoxin development (Lanubile *et al.*, 2014). Even in the absence of information about resistance, planting a variety of maize hybrids that differ in genetic background and maturity usually reduces the risk of widespread mycotoxin contamination. If conditions favor high levels of infection in one hybrid or developmental stage, the damage will be more limited if a variety of hybrids have been planted (Trenholm *et al.*, 1989).

Other than physiological mechanisms for resistance to infection or mycotoxin development, other hybrid characters can also indirectly affect the vulnerability of a hybrid to mycotoxin contamination. Selecting hybrids that are adapted to local conditions is important for at least two reasons: (i) their maturity rating will be appropriate and (ii) they will be less influenced by abiotic stress conditions that can predispose maize plants to infection and mycotoxin contamination. Planting hybrids outside their adapted range leads to higher mycotoxin contamination risk (Shelby *et al.*, 1994). The risk of mycotoxin contamination generally increases with later maturing hybrids, especially if their maturity is outside the normal range for the area, and a delayed harvest results. Earlier maturing hybrids generally have a lower risk for mycotoxin contamination (Abbas *et al.*, 2009; Blandino *et al.*, 2009a; Bruns, 2003) because they mature and dry down more quickly, but this benefit must be weighed against the difference in yield potential between earlier and later hybrids. Abiotic stresses, especially heat and drought stress, can predispose maize plants to mycotoxin contamination (Abbas *et al.*, 2009; CODEX, 2009; Miller, 2001). In areas where the climate or soil conditions favor frequent moisture stress, hybrids with drought stress tolerance will have a reduced risk of aflatoxin or fumonisin contamination.

Morphological characteristics also affect susceptibility to mycotoxin-producing fungi, either directly or indirectly. Hybrids with a thicker kernel pericarp are usually more resistant than those with a thinner pericarp (Hoenisch and Davis, 1994; Sampietro *et al.*, 2009), and other aspects of kernel composition can also contribute to resistance (Warren, 1978). Husk coverage of the ears can be an important determinant for ear rot development. In drier areas or low-latitude growing areas, hybrids with tighter husk coverage and longer husks are less susceptible to fumonisin and aflatoxin development (Warfield and Davis, 1996). Husk coverage indirectly protects against insect injury. In high latitude or high-altitude areas, tighter husks can promote *Gibberella* ear rot and other ear rot diseases because the husk coverage slows kernel drying. Hybrids with ears that tend to remain upright after maturity also have a higher risk for ear rot development. In summary, to reduce mycotoxin risk, hybrid selection criteria should include partial resistance to ear rot diseases, appropriate maturity range, husk coverage characteristics, and adaptation to local conditions of abiotic stress.

Crop Rotation and Tillage Practices

Fusarium and *Aspergillus* species survive in crop residue, and in most cases, the chances for survival and dispersal of these fungi are greatest if the crop residue is left on the soil surface, especially if maize is grown in the same field continuously (Abbas *et al.*, 2009; Hooker and Schaafsma, 2005).

The risk posed by these fungi in crop residue can be reduced by crop rotation and tillage practices that bury the residue, but the impacts of crop rotation and tillage vary substantially by pathogen and by location. In general, these practices have a bigger impact on *F. graminearum* or *F. culmorum* and their mycotoxins, most prominently deoxynivalenol and zearalenone, than on the fumonisin- and aflatoxin-producing fungi.

Although survival characteristics may not fundamentally differ among mycotoxin-producing fungi, their dispersal mechanisms probably influence their responses to residue management. *Fusarium verticillioides* and other fumonisin-producing species produce large numbers of microconidia that are aerially dispersed. Other mycotoxin-producing species, e.g., *F. sporotrichioides* and *A. flavus*, also produce asexually numerous airborne spores, while *F. graminearum* produces both splash-dispersed conidia and airborne ascospores (Schaafsma *et al.*, 2005; Schmale *et al.*, 2006).

Tillage also can alter the water permeability of field soil, which may be beneficial or detrimental, depending on the soil conditions. Tilled fields tend to dry more quickly and, under some conditions, this drying can increase drought stress. Under other conditions, tillage may improve water availability to the plants by disrupting the compacted layers in the soil profile (Wilson *et al.*, 2005).

Numerous studies of the effects of crop rotation and tillage on mycotoxin-producing fungi have been directed at *Fusarium* head blight in wheat. In most studies, the risk of deoxynivalenol contamination was lower if wheat did not follow maize or wheat in the rotation, or if the crop residues were buried by tillage (Beyer *et al.*, 2006; Schaafsma *et al.*, 2005). However, following these practices in individual fields may not have a significant impact on disease and mycotoxin accumulation, because of long-distance dispersal of ascospores from other fields (del Ponte *et al.*, 2003; Schmale *et al.*, 2006). In maize, crop residues also are the most important source of *F. graminearum* inoculum (Sutton, 1982), but there have been fewer studies addressing the effects of crop rotation and tillage in this crop. In general, inoculum levels of *F. graminearum* or *F. culmorum* are higher, and the risks for *Gibberella* ear rot and deoxynivalenol contamination are higher when maize or wheat was the previous crop (Hooker and Schaafsma, 2005) or in fields with minimum tillage (Mansfield *et al.*, 2005; Steinkellner and Langer, 2004). However, in some studies, tillage practices did not affect the incidence of ear rot caused by *F. graminearum* or *F. verticillioides* (Flett and Wehner, 1991; Flett *et al.*, 1998). Managing crop residue through rotation or tillage is considered an important practice for reducing the risk of deoxynivalenol contamination in both wheat and maize (Hook and Williams, 2007; Sutton, 1982; Trenholm *et al.*, 1989), but the benefits of tillage practices must be weighed against their costs and their potential to promote soil erosion.

Crop residue is the most important source of inoculum for *A. flavus* and many *Fusarium* species including *F. verticillioides*, *F. subglutinans*, and *F. proliferatum*, but the dispersal gradients for these fungi probably are sufficiently shallow that within-field residue management alone is inadequate to reduce infection levels or mycotoxin contamination. Inoculum reduction due to crop rotation or tillage is likely to be offset by airborne inoculum arriving from outside the field (Maiorano *et al.*, 2009; McGee *et al.*, 1996). Introduced strains of *F. verticillioides*, *F. proliferatum*, and *F. subglutinans* survived for at least 630 days in maize stalk residue left on the soil surface or buried up to 30 cm deep (Cotten and Munkvold, 1998), suggesting that tillage and crop rotation are unlikely to result in adequate inoculum reductions. Although populations of *A. flavus* are higher in the surface residues under no-till conditions (Abbas *et al.*, 2009), studies directly testing rotation and tillage effects have not demonstrated reductions in *Fusarium* or *Aspergillus* ear rot and fumonisin or aflatoxin contamination of grain (Flett and Wehner, 1991; Flett *et al.*, 1998; Marocco *et al.*, 2008; Wilson *et al.*, 2005). Nevertheless, incorporating crop residues often is recommended for reducing aflatoxin (Abbas *et al.*, 2009) or fumonisin (CODEX, 2009) risk. There may be incremental risk reduction if crop residues are buried, especially if the practice is followed on a regional scale. Crop rotation has

other benefits, e.g., improved yield and plant vigor, which could contribute to reduced abiotic stress in maize plants and make them less vulnerable to toxigenic fungi under some conditions.

Planting Date

Changing the planting date can significantly affect fungal infection and mycotoxin accumulation because these events depend on the co-occurrence of a susceptible plant developmental stage, favorable environmental conditions, and, in some cases, vector activity. The ideal planting date results in pollination and kernel development when insect activity is low and under conditions less favorable for fungal infection and mycotoxin development. In temperate-zone maize production, earlier planting dates usually result in a lower risk, but annual weather fluctuations can alter this relationship.

For fumonisins, earlier planting dates have consistently resulted in the lowest levels of contamination across diverse locations (Abbas *et al.*, 2007; Blandino *et al.*, 2009a, 2009b; Bruns, 2003; Parsons and Munkvold, 2010). Although there may be multiple reasons underlying this decrease, the planting date effect may be largely due to differences in insect activity, as earlier planting dates result in less injury by European corn borers (Figure 5.2; Blandino, 2009b) and lower thrips infestation levels (Figure 5.1; Parsons and Munkvold, 2010). Both insects are connected with *Fusarium* infection and fumonisin contamination. There are fewer studies on the effect of planting date on *Gibberella* ear rot, but earlier planting has reduced ear rot severity and deoxynivalenol content (Blandino *et al.*, 2009b).

Most studies of planting date effects on aflatoxin contamination have also concluded that earlier planting lowers the mycotoxin contamination risk (Abbas *et al.*, 2007, 2009; Jones and Duncan, 1981; Jones *et al.*, 1981). In the southern United States, earlier planted maize usually avoids the

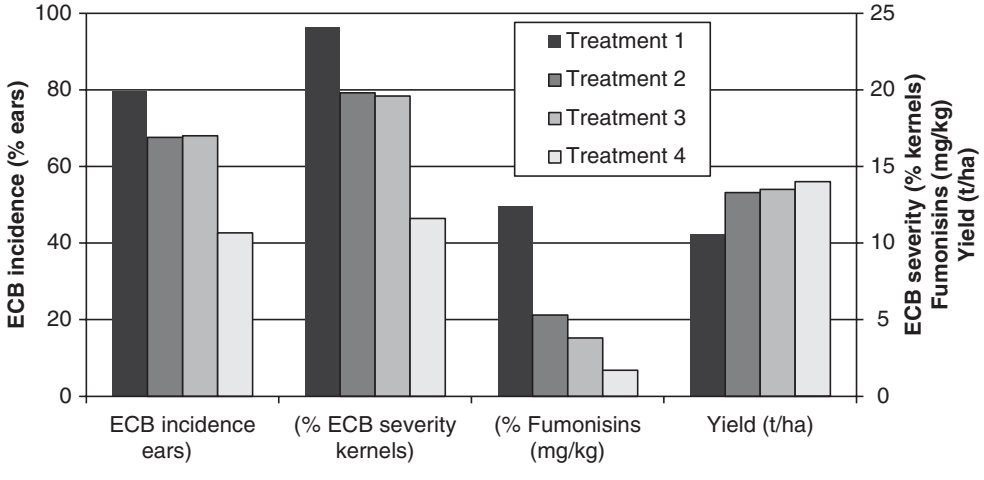

Figure 5.2 European corn borer (ECB) incidence and severity, fumonisin concentrations, and grain yields from three crop management programs compared in field trials at three locations over 2 years in northwest Italy. Treatment 1: May planting, 80,000 plants/ha, 400 kg nitrogen fertilization/ha, no insecticide; Treatment 2: late March to early April planting, 80,000 plants/ha, 400 kg nitrogen fertilization/ha, no insecticide; Treatment 3: late March to early April planting, 65,000 plants/ha, 200 kg nitrogen fertilization/ha, no insecticide; Treatment 4: late March to early April planting, 65,000 plants/ha, 200 kg nitrogen fertilization/ha, insecticide treated for control of ECB. Data from Blandino *et al.* (2009a).

extreme heat stress during grain fill that promotes aflatoxin production and accumulation (Abbas *et al.*, 2009). In Florida, one study (Wiatrak *et al.*, 2005) showed that very late planting presented the lowest risk for aflatoxin contamination; however, these late planting dates would not be commercially feasible because of reduced yield potential. Variation in the planting date/aflatoxin relationship reflects the impact that variable weather conditions after flowering have on aflatoxin accumulation. Under most conditions in temperate-zone maize production, earlier planting reduces the risk for aflatoxin contamination.

Fertilization

Susceptibility of maize plants to mycotoxin contamination can be influenced by soil fertility levels, but the effects are not constant and may depend on other interacting factors. Most studies have focused on nitrogen fertilization. Nitrogen-deficient crops may have elevated mycotoxins levels; therefore, adequate, but not excessive, nitrogen fertilization typically results in the lowest risk. Fumonisin levels may increase when a nitrogen fertilizer is applied (Hassegawa *et al.*, 2008; Marocco *et al.*, 2008), but this effect usually occurs only when rates of N application are excessively high. In a study with a range of rates of nitrogen fertilization, lower rates of application resulted in lower levels of fumonisins, aflatoxins, and ochratoxin A than in the non-fertilized control, although high rates of nitrogen application increased ear rot severity and the amounts of fumonisins, deoxynivalenol, ochratoxin A, and zearalenone accumulated (Blandino *et al.*, 2008b). Similarly, *Gibberella* ear rot was reduced by a moderate N rate (100 kg/ha), but increased at a higher rate (200 kg/ha), while deoxynivalenol levels overall were reduced by N application (Reid *et al.*, 2001). This relationship seems to differ from the effect of N fertilization on *Fusarium* head blight in wheat, where disease symptoms and deoxynivalenol levels tend to increase with N fertilization (Lemmens *et al.*, 2004). Most studies indicate that a nitrogen deficiency results in the greatest risk for aflatoxin contamination, with higher rates of nitrogen fertilization consistently resulting in reduced aflatoxin levels (Abbas *et al.*, 2009; Jones and Duncan, 1981). For aflatoxin risk reduction, like the other mycotoxins, the best recommendation is to use adequate, but not excessive, fertilization (Wilson *et al.*, 2005).

Planting Density

Planting density can affect the risk of mycotoxins in at least two ways: (i) excessive plant densities can stress plants, increasing their susceptibility; and (ii) denser plantings may create denser crop canopies, which hold moisture and create a more favorable environment for fungal infection. In Italy, maize planted at a higher density (82,000 plants/ha) had significantly higher fumonisin levels in 3 of 4 years than did a lower density planting (65,000 plants/ha; Blandino *et al.*, 2008a). In years when deoxynivalenol, zearalenone, and ochratoxin A occurred, they were consistently higher in the higher density plantings. In another study, a combination of lower density planting and lower N fertilization significantly lowered fumonisin accumulation relative to treatments with higher plant densities and higher N fertilization rates (Blandino *et al.*, 2009a). Other research on aflatoxins and fumonisins also indicates that higher plant densities can increase mycotoxin contamination risks (Bruns, 2003), although this relationship is not consistent (Bruns and Abbas, 2005). Current evidence indicates that some degree of mycotoxin risk can be alleviated by planting at densities that are not excessive for the growing conditions and the hybrids planted.

Seed Treatment

The primary contribution of seed treatment to mycotoxin management is to facilitate early planting, which can lower mycotoxin contamination risks because early season soil conditions often favor seedling diseases. However, there is evidence in wheat (Moretti, 2008; Poels *et al.*, 2008) and in maize (Causin *et al.*, 2008; Galperin *et al.*, 2003) suggesting that some seed treatment fungicides may impact kernel infection and contamination by deoxynivalenol or fumonisins by suppressing seed transmission of *Fusarium* species. More research is needed in order to confirm these effects.

Post-planting Management Decisions

Insect Management

Insect management is the most important practice for reducing mycotoxin risk in temperate-zone maize production, especially for fumonisins and aflatoxins, other than human hybrid selection. Associations between insects and toxigenic fungi are well known, including a wide range of insect orders and species (Table 5.1; Dowd, 1998). Insects act as vectors for fungal spores, they cause damage to maize kernels, creating infection sites for toxigenic fungi, and they induce plant stress that may predispose the plants to infection (Munkvold, 2003b). *Fusarium* ear rot and fumonisins are most closely associated with insect injury, followed by *A. flavus* and aflatoxins. *Gibberella* ear rot and its associated mycotoxins are increased by insect injury to a lesser degree. Insects in

Table 5.1 Insects and mites associated with mycotoxin-producing fungi and mycotoxin contamination in maize

Common name	Latin name	Insect order	Distribution
Maize stalk borer	*Busseola fusca*	Lepidoptera	AF
Mites	*Caloglyphys rodriguezi*	–	AM
Sap beetles	*Carpophilus* spp.	Coleoptera	AM, AF
Spotted stem borer	*Chilo partellus*	Lepidoptera	AF
Southwestern corn borer	*Diatraea grandiosella*	Lepidoptera	AM
Corn rootworm adults	*Diabrotica* spp.	Coleoptera	AM, EU
Stink bugs	e.g., *Euschistus* spp.	Hemiptera	AM
Bronze psocid	*Ectopsocopsis cryptomeriae*	Psocoptera	AM
Sugarcane borer	*Eldana saccharina*	Lepidoptera	AF, AM
Western flower thrips	*Frankliniella occidentalis*	Thysanoptera	AM, EU, AF
Picnic beetles	*Glischrochilus* spp.	Coleoptera	AM
Corn earworm	*Helicoverpa zea*	Lepidoptera	AM
Ear borer	*Mussidia nigrivenella*	Lepidoptera	AF
Scarab beetles	Not listed	Coleoptera	AM
European corn borer	*Ostrinia nubilalis*	Lepidoptera	AM, EU
Pink scavenger caterpillar	*Sathrobrota rileyi*	Lepidoptera	AM
Stem borer	*Sesamia calamistis*	Lepidoptera	AF
Pink stem borer	*Sesamia nonagrioides*	Lepidoptera	AF, EU
Maize/rice weevils	*Sitophilus* spp.	Coleoptera	AM, AF
Fall armyworm	*Spodoptera frugiperda*	Lepidoptera	AM
Western bean cutworm	*Striacosta albicosta*	Lepidoptera	AM
Mold mite	*Tyrophagus putrescentiae*	–	AM

Source: Adapted from Dowd (2003).
AF, Africa; AM, Americas; EU, Europe.

the Order Lepidoptera (moths) typically have the biggest impact on mycotoxin-producing fungi, although some species in Orders Coleoptera (beetles) and Thysanoptera (thrips) are equally important in some locations. Lepidopteran insects such as the European corn borer (*Ostrinia nubilalis*), corn earworm (*Helicoverpa zea*), fall armyworm (*Spodoptera frugiperda*), pink stem borer (*Sesamia nonagroides*), southwestern corn borer (*Diatraea grandiosella*), and a few others can be common feeders on maize ears in temperate-zone maize production (Avantaggiato *et al.*, 2002; Dowd, 1998).

The most effective way to manage Lepidopteran insects and reduce the associated mycotoxin risk is with transgenic insect resistance genes. Maize hybrids containing these genes, known as Bt genes, express insecticidal proteins derived from various strains of the bacterium *Bacillus thuringiensis*. Several types of Bt genes have been incorporated into commercial maize hybrids, either singly or in combination. All commercialized Bt genes (or combinations) are very effective against *O. nubilalis* and other *Ostrinia* spp., but vary in effectiveness against other important pests, such as *H. zea*, *S. frugiperda*, and *Striacosta albicosta* (Western bean cutworm) (Catangui *et al.*, 2006; Clements *et al.*, 2003). Most hybrids currently used in the United States employ single or dual Bt genes that together provide good control of the range of Lepidopteran pests. In parts of the world without full access to biotechnology, older Bt genes may be available with a more limited spectrum of control.

Due to the effectiveness of these transgenic traits, Bt maize hybrids have been widely adopted in countries where they can be planted with few restrictions. In 2010, Bt maize was grown in 16 countries around the globe. In the United States in 2012, 67% of the maize-producing area was planted with Bt hybrids, and in some states, more than 75% was Bt maize. Similarly, in 2010, about 77% of the maize crop in Argentina, more than 60% in Canada, 24% of the crop in Spain, 83% of the maize area in Uruguay, 67% of the maize area in South Africa, and more than 50% of the maize area in Brazil was planted with Bt maize (James, 2008). In every country that grows Bt maize, there are studies that document reductions in fumonisins or other mycotoxins in Bt hybrids relative to conventional maize hybrids (Bakan *et al.*, 2002; Clements *et al.*, 2003; de la Campa *et al.*, 2005; Delgado and Wolt, 2010; Dowd, 2003; Hammond *et al.*, 2004; Magg *et al.*, 2002; Masoero *et al.*, 1999; Munkvold, 2003a; Munkvold *et al.*, 1997, 1999; Schaafsma *et al.*, 2002; Windham *et al.*, 1999). In studies in Iowa over a 9-year period, fumonisins were reduced by an average of 80% in Bt hybrids relative to conventional hybrids when European corn borer populations were moderate to high. When European corn borer populations were low, the fumonisin reduction averaged 38%. Other mycotoxins also are reduced with the use of Bt maize, but the effects are smaller and less consistent than those reported for fumonisins (Dowd, 2003).

Insecticides also can effectively reduce the risk of mycotoxin contamination associated with Lepidoptera or other insects. Insecticide application may be an acceptable option in areas where Bt maize is not available or non-Lepidopteran insects are important. In some studies, significant mycotoxin reductions were achieved only when unrealistic numbers of insecticide applications were made (Dowd, 2003; Munkvold *et al.*, 1997) Other successful studies have resulted from well-timed single applications combined with an accurate scouting or sampling method for estimating the intensity and timing of the insect infestation (Figure 5.2; Blandino *et al.*, 2009c; Dowd, 2003; Folcher *et al.*, 2012). For example, a single insecticide application dramatically reduced the fumonisin levels in California, USA, where thrips, primarily *Frankliniella occidentalis*, were facilitating *F. verticillioides* infection (Parsons and Munkvold, 2010). A single insecticide application significantly reduced levels of deoxynivalenol in grain from several locations in a study conducted in France (Folcher *et al.*, 2012). Insecticides also are an important component of integrated programs for aflatoxin and fumonisin management in parts of the United States, Mexico (Cleveland *et al.*, 2003; Dowd, 2003), and Italy (Blandino *et al.*, 2009a).

In organic systems, where transgenes and synthetic insecticides cannot be used, insects may be managed by using cultural or biological control methods. If these methods effectively suppress insect injury, then they will help reduce mycotoxin contamination.

Irrigation Management

Drought-stressed maize crops are thought to be more susceptible to aflatoxin and fumonisin contamination (Abbas *et al.*, 2009; Miller, 2001; Shelby *et al.*, 1994; Wilson *et al.*, 2005). Consequently, most work on irrigation and mycotoxin risk has focused on alleviating drought stress as a tactic to avoid mycotoxin contamination, especially for aflatoxins. Irrigation adequate to prevent drought stress lowers aflatoxin levels in grain (Abbas *et al.*, 2009; Jones and Duncan, 1981; Jones *et al.*, 1981; Payne *et al.*, 1986; Rodriguez-del-Bosque, 1996).

Data on fumonisin contamination are less definitive. Associations between drought stress and *Fusarium* ear rot or fumonisins are based primarily on observations of occurrence under uncontrolled field conditions (de la Campa *et al.*, 2005; Miller, 2001; Munkvold, 2003b) and may be confounded with other factors. In the most detailed study available on irrigation effects and fumonisins, drought stress did not have a consistent impact on fumonisin levels, with insect activity, hybrid, and planting date being much more influential (Parsons and Munkvold, 2010). Similarly, Bruns (2003) did not detect significant differences in fumonisin accumulation between irrigated and nonirrigated maize. The contribution of irrigation to fumonisin risk reduction may be limited, but avoiding drought stress is recommended.

Gibberella ear rot and associated mycotoxins are typically associated with high moisture levels after flowering (Sutton, 1982; Sutton *et al.*, 1980; Vigier *et al.*, 1997). Longer wet periods promote greater silk infection, leading to higher concentrations of deoxynivalenol and zearalenone (Stewart *et al.*, 2002; Sutton, 1982). Thus, irrigation often is used to promote *F. graminearum* infection in hybrid screening efforts. This relationship should also be considered for late season irrigation management. In areas where *Gibberella* ear rot occurs, late season irrigation could increase the risk of mycotoxin contamination, though furrow irrigation probably represents a much lower risk than does overhead sprinkler irrigation.

In irrigated maize crops, the amount and timing of irrigation is typically designed to minimize drought stress and optimize maize yields in relation to irrigation costs. In most cases, this strategy alleviates aflatoxin or fumonisin risks associated with drought stress and usually does not result in the excessive moisture that could increase deoxynivalenol or zearalenone risk. Irrigating to prevent drought stress and optimize yield benefit is recommended as the best practice to avoid mycotoxin risk, unless more detailed information becomes available regarding the precise effects of alternative irrigation strategies on mycotoxins.

Weed Management

Weeds contribute indirectly to mycotoxin risk through their competition with crop plants. This competition can induce the moisture and nutrient stresses that increase the risk of mycotoxin accumulation (Wilson *et al.*, 2005). Some weeds may also serve as alternative hosts for mycotoxin-producing fungi, providing an additional inoculum source for the crop (Trenholm *et al.*, 1989). Numerous studies have been conducted to test the impact of various weed management practices, primarily related to aflatoxins, with mixed results (Wilson *et al.*, 2005). In Italy, weed control resulted in significant reductions in fumonisins and deoxynivalenol compared to an uncontrolled

treatment (Blandino *et al.*, 2007). While weed competition can lead to increased mycotoxins, the response to weed management treatments probably will vary depending on the weed species present (Blandino *et al.*, 2007) and their density. Some herbicides have fungistatic properties, but none are considered effective for directly suppressing infection by mycotoxin-producing fungi. Effective weed control is usually a high priority in maize production, in order to maintain crop yields; therefore, in most temperate-zone maize production systems, uncontrolled weeds are not a major contributor to mycotoxin risk.

Harvest Date and Practices

Fields should be inspected at physiological maturity to determine whether there are severe mold problems and to make decisions about harvest timing, postharvest handling, storage, and marketing strategy. If 10% or more of the ears have 10–20% mold damage, or are lodged, the field should be scheduled for the earliest possible harvest. If a problem is evident, samples also should be collected to test for mycotoxin levels; an adequate, representative sample collection scheme is critical. If a sample is taken from the standing crop, it should be at least 5 kg and should include ears taken individually from throughout the field.

Maize crops left to dry in the field can be exposed to temperature and moisture conditions that are favorable for continued growth of toxigenic fungi and the accumulation of their mycotoxins in grain. Therefore, harvest timing can have a major impact on the final levels of mycotoxins accumulated in the grain. The most appropriate harvest moisture content depends on the weather conditions, harvest method, drying capabilities and costs, and storage conditions. Some *Fusarium* species can continue to produce mycotoxins until the grain dries to ~19% moisture content, while *A. flavus* may continue to grow at grain moistures levels as low as 16% (Munkvold, 2003c). Ideally, grain should be harvested and dried to 15.5% moisture content or less (depending on storage intentions) as quickly as possible. If field conditions promote drying and there are no major ear rot problems, field drying can be safe. On the other hand, wet, cool conditions late in the season will slow drying and lengthen the time during which mycotoxins may be produced. Several studies have demonstrated that greater ear rot infection and higher levels of aflatoxins, deoxynivalenol, nivalenol, or fumonisins may be associated with delayed harvest, both for grain (Jones *et al.*, 1981; Lauren *et al.*, 2007; Munkvold, 2003c; Nordby *et al.*, 2007) and for silage (Mansfield *et al.*, 2007).

Mechanical combines should be adjusted to minimize grain damage with proper cylinder speed and clearance settings (Herum, 1987). The combine's fan settings and screens also should be set to reduce the occurrence of fines, broken kernels, glumes, ear tips, and cob pieces in the harvested grain. Harvested grain can be further cleaned if necessary, by using screens to remove moldy or broken kernels, or other undesirable material.

After harvest, grain left behind in the field or around the field can be a "breeding ground" for toxigenic fungi. Infection of maize kernels in the field is higher in the vicinity of waste piles of discarded grain that were contaminated with *A. flavus* (McGee *et al.*, 1996). Leaving waste piles of grain near fields should be avoided.

Integration of Risk Management Tactics

Several studies have been conducted to demonstrate the best combination of crop production tactics to reduce mycotoxin contamination in maize. Aflatoxin accumulation in commercial maize is influenced by field selection, hybrid selection, fertilization, planting date and density, irrigation,

weed management, crop rotation, tillage, insect and disease management, and harvest practices (Jones and Duncan, 1981; Jones *et al.*, 1981; Payne *et al.*, 1986). Earlier planting and harvest, combined with irrigation or deep tillage to alleviate drought stress, all reduced infection and aflatoxin concentration. In Mexico, a combination of cultural practices (early planting, reduced plant population, and irrigation), hybrid selection, and insect control reduced aflatoxin concentrations to 0–6 ng/g from 63–167 ng/g in late planted, nonirrigated maize at a higher plant population without insect control (Rodriguez-del-Bosque, 1996). Blandino *et al.* (2009a) showed that a combination of early planting, reduced plant density, adequate (but not excessive) N fertilization, and control of European corn borers reduced fumonisins to 1733 ng/g, compared to 12,412 ng/g in a treatment that was planted later, with higher plant density and N fertilization, without insect control (Figure 5.2). Integrated mycotoxin management programs have been developed for parts of the United States, Mexico (Cleveland *et al.*, 2003; Dowd, 2003), the United Kingdom (Hook and Williams, 2007), and Italy (Blandino *et al.*, 2009a).

Risk Assessment or Prediction Modeling

The need for tactics to reduce mycotoxins traditionally has been gauged by using informal (often imprecise) methods of risk assessment. Quantitative, site-specific risk assessments or predictive models for mycotoxin accumulation could contribute significantly to management efficiency. Mycotoxin risk model development has been very active for wheat (de Wolf and Paul, 2014), and some efforts are being made in maize (Battilani and Logrieco, 2014). These studies not only contribute to the ability to predict mycotoxin problems, but also add to our knowledge of the relative importance of environmental and management factors that influence mycotoxin development. Some models are based on laboratory studies describing the effects of environmental conditions, e.g., temperature and water activity, on the production of mycotoxins (Marin *et al.*, 1999) and can be easily adapted for field models.

Other models are based on data collected from field studies or developed by validating laboratory results in the field. Such models have been developed to describe or predict occurrence of aflatoxins, deoxynivalenol, fumonisins, or zearalenone (Battilani and Rossi, 2003; Battilani *et al.*, 2008; Cleveland *et al.*, 2003; de la Campa *et al.*, 2005; Maiorano *et al.*, 2009; Schaafsma and Hooker, 2007; Sutton *et al.*, 1980), while others describe or predict infection by various *Fusarium* species (Stewart *et al.*, 2002; Vigier *et al.*, 1997). Input variables for these models typically include temperature, rainfall and other weather inputs, insect populations, maize hybrid and maturity, previous crop, planting date and other crop production practices, and sometimes economic factors.

Fairly comprehensive fumonisin prediction models have recently been presented, and they confirm the importance of many of the previously mentioned agronomic factors. Battilani *et al.* (2008) found that higher levels of fumonisins were associated with maize as the previous crop, later planting and harvesting, later maturing hybrids, higher levels of nitrogen fertilization, and higher grain moisture content. A negative correlation was found between fumonisins and mid-season rainfall, strengthening the association between drought stress and fumonisin accumulation. This study did not evaluate insect injury, and most (or all) fields were irrigated, so rainfall effects may have been underestimated. Battilani and Rossi (2003) also described the effects of temperature and other weather variables on various portions of the *F. verticillioides* disease cycle.

A similar approach was taken by Maiorano *et al.* (2009), who also included insect injury as an important predicting factor, as have most other modeling efforts. de la Campa *et al.* (2005) identified insect injury as a major predictor of fumonisins, along with high temperatures and low rainfall during

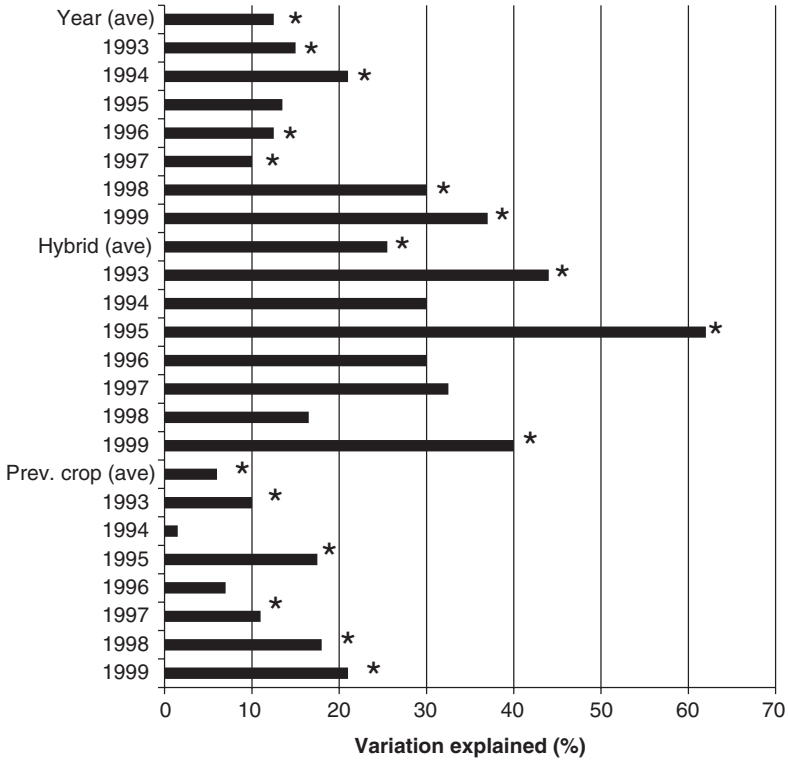

Figure 5.3 Variation in deoxynivalenol concentration attributable to year, maize hybrid, and previous crop in grain from commercial fields in Ontario, Canada, from 1993 to 1999. *indicates statistically significant effects ($p \leq 0.05$). Data from Hooker and Schaafsma (2005).

the period from 2 to 8 days after silking. A model developed by Dowd (2003) for both aflatoxins and fumonisins identified insect injury as an important factor, as well as critical temperatures and rainfall amounts. Schaafsma and Hooker (2007) identified the maize hybrid cultivated as the most important variable affecting variation in deoxynivalenol and fumonisin accumulation. The previous crop also had a significant influence on deoxynivalenol accumulation (Figure 5.3) but not on fumonisin accumulation.

Most of these models are tools for providing an early warning for the risk of unacceptable mycotoxin levels, but depend on within-season weather data. Pre-planting management decisions require stochastic models based on agronomic factors and long-term weather forecasts and/or extensive historical data on mycotoxin occurrence. Pre-planting models have not been developed for mycotoxins in maize. However, it is likely that they will become available in the near future, and prediction models may become widely available as risk assessment tools to assist in mycotoxin management.

Discussion

In this chapter I described a variety of management practices that can all contribute to reducing mycotoxin contamination risks. The impact of the different practices will vary by geographic

Table 5.2 Relative importance of different crop management practices for reducing the risks of mycotoxin contamination in temperate-zone maize

	Fusarium ear rot[a]	*Gibberella* ear rot[b]	*Aspergillus* ear rot[c]
	Fumonisins	Deoxynivalenol, zearalenone	Aflatoxins
Hybrid selection	++	++	++
Insect control	++	+	++
Early planting	++	++	+
Irrigation management	++	+	++
Timely harvest	++	++	++
Crop rotation and tillage	+/?	++	+/?
Plant density	+	+	+
Fertilization	+	+	+
Weed management	+/?	?	+/?
Biological control	?	?	+
Seed treatment	?	?	?
Fungicide application	?	?	?

++, major effect; +, minor effect; ?, uncertain or insufficient evidence.
[a]Caused primarily by *F. verticillioides*, *F. proliferatum*, and *F. subglutinans*.
[b]Caused primarily by *F. graminearum* (*Gibberella zeae*) and *F. culmorum*.
[c]Caused primarily by *A. flavus* and *A. parasiticus*.

location and environmental conditions in any given season. It may not be possible to predict which combination of practices will result in the minimum risk for every situation, but it is possible to prioritize management practices, according to the strength of their influence on infection or mycotoxin contamination (Table 5.2). It may not be appropriate or economical to implement all of these practices in every field, but they can be applied more or less aggressively according to the severity of the mycotoxin risk in a particular field. Any crop management practice must be conducted with a cost/benefit relationship in mind. Unfortunately, the economic benefits of mycotoxin management practices have not been extensively quantified, though some work has been done in this area (Wu *et al.*, 2004, 2008). More information is needed on the economics of mycotoxin management. Some important areas which should be considered to help reduce mycotoxin contamination include:

Hybrid Selection

Know which ear rot diseases and mycotoxins are the most important in your area and choose a hybrid that has partial resistance to the one or two most important ones. This information usually is available through seed dealers or from government agencies that conduct tests. Choose a hybrid that is in the proper maturity range and is well adapted to local conditions, so that there is less chance of environmental stress from drought, temperature, or soil conditions. Plant a mixture of hybrids in order to dilute the risk.

Insect Control

Control insects that feed on maize kernels. The most effective control is with a hybrid that has transgenic resistance to Lepidopteran insects (Bt hybrids), if they are available. If Bt hybrids are

not available, or if other insects must be controlled, a properly timed insecticide application may be needed. Local information about pest populations and available scouting methods should be used to determine if insect populations will be high enough to warrant an insecticide application and to determine the best time for the application.

Planting Date

Plant the crop as soon as conditions are conducive for effective stand establishment. Early planted crops tend to escape peak insect populations and avoid the stressful weather conditions that promote fungal infection and mycotoxin development. Use an effective fungicide seed treatment to facilitate successful early planting. Some seed treatments may also contribute by reducing the systemic infection of the plants by mycotoxin-producing fungi.

Crop Rotation and Tillage Practices

Rotate maize with legumes or other dicot crops to reduce the buildup of fungal inoculum in crop residue. Similarly, if crop residue from a maize or small-grain crop is left on the soil surface, toxin-producing fungi will survive more easily, and there can be a greater source of inoculum for the next crop. Partially burying the residue through tillage can reduce the risk. Although these practices will have less impact on contamination with fumonisins or aflatoxins, there are significant benefits for reducing the risk of deoxynivalenol and zearalenone.

Irrigation Management

If the crop is grown in an irrigated field, be sure to provide adequate water to avoid drought stress. Furrow irrigation is preferable to overhead systems, so that the silks do not become excessively wet. Irrigation must be managed carefully, because excess moisture in the field can raise humidity and promote fungal infection.

Timely Harvest

Scout fields late in the season to identify problem areas. Harvest should not be delayed. If field drying progresses slowly, there is an extended opportunity for infection, fungal growth, and mycotoxin accumulation before the crop is harvested. Drying costs must be weighed against the mycotoxin risk, but earlier harvesting usually results in the lowest mycotoxin levels, as long as the grain can quickly be dried to 15% moisture content or less, for long-term storage.

Plant Density

Use a planting rate that is not excessively high for the hybrid and the growing conditions. If plants are excessively crowded, they compete for water and other resources, leading to stress that can increase the mycotoxin accumulation risk. Consult with seed suppliers and other sources of agronomic information about appropriate plant densities for local conditions.

Other Practices

Other crop production practices, e.g., fertilization and weed management, should be followed carefully to optimize plant health and yield. Balanced fertilization and freedom from weed competition will help reduce mycotoxin accumulation. Additional tactics, e.g., biological control and the application of fungicides, have been evaluated for their ability to reduce infection by mycotoxin-producing fungi. Although there are reports of success in the literature, e.g., Abbas *et al.* (2009), these methods require additional research.

Managing mycotoxin accumulation requires a comprehensive strategy that begins before planting and continues through the final utilization of the grain or grain products. Following the practices described in this chapter will contribute greatly to reducing the risk of mycotoxin contamination, but there is no guarantee that mycotoxin accumulation will be less than any particular regulatory levels, even if all of the recommendations detailed here are followed. Monitoring mycotoxin levels in harvested grain and following best practices for transport, storage, and utilization are also essential components of a comprehensive mycotoxin risk management plan.

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6 Best Stored Maize Management Practices for the Prevention of Mycotoxin Contamination

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Abstract

Mycotoxins are secondary metabolites produced by fungi that commonly belong to the *Aspergillus, Fusarium*, and *Penicillium* genera. These harmful metabolites can cause toxicoses in humans and animals. Fungal infection, and subsequent production of mycotoxins, can occur in the field during plant growth, harvesting, and wet holding and may continue during transportation and grain storage. Preventing fungal infection and subsequent mycotoxin synthesis during crop growth and postharvest handling is the best management practice and is strongly favored over ameliorative treatments of mycotoxin-contaminated grain, food, and feed products. The effective implementation of good agricultural practices (GAPs) and integrated pest management (IPM) in the production process reduces mycotoxins in the maize supply chain. Sanitation, screening, aeration, monitoring, sampling, and testing of stored grain as part of the SLAM (sanitation, loading, aeration, and monitoring)-based strategy are critical to reduce mycotoxins entering grain-based food and feed chains.

Keywords: aeration; ammonification; blue-eye; bulk storage; condensation; drying; grain moisture; postharvest; temperature

Introduction

Fungi are the primary cause of spoilage in stored maize and can cause detrimental changes in appearance, quantity, and quality of stored grain (Ng *et al*., 1998), thereby reducing the end-use value of maize for food, feed, and biofuels. More importantly, some fungal species can produce toxic substances known as mycotoxins. Mycotoxins are fungal secondary metabolites that can cause toxicoses and nutritional diseases in humans and domesticated animals (Bennett and Klich 2003; Hedayati *et al*., 2007; Milicevic, 2007). Preventing these toxins from entering the food and feed chains is a major concern of the global food and feed industry (Jouany, 2007).

Usually, fungal infection and subsequent production of mycotoxins begins in the field during plant growth and may continue through harvesting, handling, storage, and processing. The economic impact of mycotoxins includes loss of human and animal health and life, increased health-care costs, reduced livestock production, disposal costs of contaminated foods and feeds, pre- and postharvest losses in crops, research investment, and regulatory programs aimed at reducing or excluding mycotoxins from end products. Critical factors for fungal postharvest infection and

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subsequent synthesis of mycotoxins include initial grain moisture content, timeliness of harvest, length of wet holding before drying, the amount of broken corn and foreign materials (BCFM), the amount of grain dust, the type and quality of storage structures, grain temperature, the interstitial air relative humidity (RH), headspace condensation, bulk grain moisture movement, and insect infestation.

Regular grain sampling to detect fungal contamination or insect infestation and to monitor aeration and temperature is essential to preserve the quality of stored maize. In general, grain moisture content is a more important determinant of the length of the time for allowable storage than is temperature. Successful storage requires a thorough understanding of best stored grain management practices. Effective monitoring of stored grain and timely inspection are critical because there are few means to overcome problems once mycotoxins are present. In this chapter we discuss the best stored maize management practices for preventing mycotoxin contamination. Although bag storage of maize is common in developing countries, we focus only on bulk storage of maize while acknowledging that many of the basic principles apply to both bag and bulk storage of grain.

Fungal Source

Fungi are ubiquitous in nature. Field soil, air, and irrigation water are all potential sources of inoculum for *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp. Most of these fungi are capable of surviving saprophytically on organic material such as plant debris, animal fodder, dead insects, and stored grain. Usually, fungal infections begin in the field and are carried into storage structures. Field fungi, e.g., *Fusarium* spp., normally grow at grain moisture levels of 20% or higher. Field damaged grain, regardless of the reason for the damage, should not be mixed with good quality grain harvested from uninfected fields. Fungal spores may be present in equipment used for grain harvesting, transportation, handling, and storage including on grain residues not cleaned out from previous handling and storage operations. Fungal spores also may be introduced into the storage structure by air and by pests such as insects and rodents. Under improper storage conditions, a small quantity of spores (as inoculum) introduced by any means can germinate and then begin to grow rapidly, leading to grain spoilage and mycotoxin synthesis.

Storage fungi, e.g., *Aspergillus* spp. and *Penicillium* spp., generally grow at grain moistures of 13–20%, or moistures in equilibrium with 65–90% RH. Fungal infection is greater after kernels have been damaged in the field by birds, insects, mites, early frost, hail, heat, drought, and excess or deficient nutrients. The US Food and Drug Administration (FDA) has established action levels for aflatoxin content in maize-based food and feed products to protect human and animal health (Table 6.1).

Table 6.1 US FDA action levels for aflatoxin in maize based on intended use (FDA, 2009)

Aflatoxin (ppb)	Intended use
<20	Maize-based ingredients intended for dairy animals, for animal species or uses not specified above, or when the intended use is not known
	Maize-based ingredients intended for immature animals
<100	Maize-based products intended for breeding beef cattle, breeding swine, or mature poultry
<200	Maize-based products intended for finishing swine (>100 lb)
<300	Maize-based products intended for finishing (i.e., feedlot) beef cattle

Factors Affecting Fungal Growth and Mycotoxin Biosynthesis

In general, fungi grow at temperatures between 10°C and 40°C, above 0.7 water activity (a_w) and on a substrate with a pH between 4 and 8. It is possible to predict the type of fungal growth and the associated mycotoxin synthesis to some extent depending upon the moisture content and the temperature of the stored grain. However, the conditions for mycotoxin production are usually more limited than are the conditions for normal fungal growth (Magan *et al*., 2014). Factors such as temperature, a_w, pH, carbon and nitrogen source(s), biotic and abiotic oxidative stress, and lipids all can affect the synthesis of aflatoxin by toxigenic *Aspergillus* species (Bhatnagar *et al*., 2014; Yu, 2012).

Previously, fungal growth models that consider factors such as a_w, temperature, nutritional factors (carbon and nitrogen source), and RH have been developed. Baranyi *et al*. (1993) and Gibson *et al*. (1994) used water activity as the primary variable, whereas others have focused on temperature to explain fungal growth and development (Carlile *et al*., 2001; Cuero and Smith 1987; Shanahan *et al*., 2003). Some researchers have used both temperature and a_w to model fungal growth and mycotoxin production (Northolt and Bullerman, 1982; Schindler *et al*., 1967; White *et al*., 1982a, 1982b). The effect of carbon and nitrogen source in aflatoxin biosynthesis has been well established (Bennett *et al.*, 1979; Luchese and Harrigan, 1993). In general, the three main factors that favor fungal growth and mycotoxin biosynthesis in stored grain are high grain moisture, i.e., 16–30%, warm grain temperature, i.e., 25–32°C, and high air RH, i.e., 80–100% (Shanahan *et al.*, 2003). Thus, grain moisture content and grain temperature are the critical factors for determining if maize is being stored safely. These values should be known before storage and should be monitored to determine how long the grain can be safely stored (Table 6.2).

Equilibrium Moisture Content of Maize

A key to understanding safe storage of maize is the equilibrium moisture content (EMC) concept which relates air temperature and RH to grain temperature and moisture content. EMC is an equilibrium that occurs when the grain neither gains nor loses its moisture content. EMC and

Table 6.2 Maximum allowable safe storage days for shelled maize based on 0.5% dry matter loss ($D = 30\%$, $M_D = M_H = M_F = 1$. Times calculated using °C temperature values)

Grain temperature (°C)	Grain moisture (% wet basis)							
	16	18	20	22	24	26	28	30
1.7	1144	437	216	128	86	63	50	41
4.4	763	291	144	85	57	42	33	27
7.2	509	194	96	57	38	28	22	18
10	339	130	64	38	26	19	15	12
13	226	86	43	25	17	13	10	8
16	151	58	29	17	11	8	7	5
18	113	43	22	13	9	7	5	4
21	85	32	16	10	7	5	4	4
24	63	24	12	8	5	4	3	3

Source: Reprinted with permission from ASABE Standard D535: Shelled corn storage time for 0.5% dry matter loss. Revised 2010. ASABE, St. Joesph, Michigan, USA.

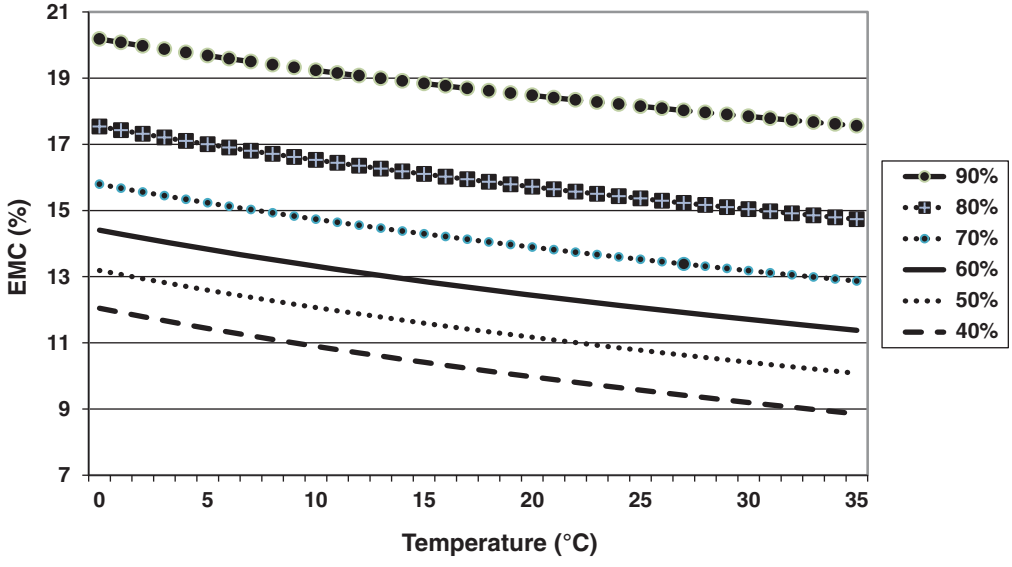

Figure 6.1 Equilibrium moisture content relationship for shelled corn at various temperatures and relative humidities.

RH play a significant role in the design and operation of drying and storage processes (Maier, 2004). As maize is dried, aerated, and/or left in storage undisturbed for longer periods, its moisture content changes until it is in equilibrium with the surrounding temperature and RH of the storage environment (Figure 6.1). If the air temperature increases at a constant RH, then the grain EMC will be reduced (Maier, 2004). If the air RH increases at constant temperature, then the grain EMC will increase. For example, maize grain stored at 15% wet basis and 20°C (68°F) has an equilibrium RH (ERH) of ~75%. If excessive aeration reduced the interstitial RH to ~50% at 20°C (68°F), then the grain moisture content will decline to ~11% wet basis. Safe storage of maize grain depends largely on its moisture content. Keeping stored maize in equilibrium with 70% or lower intergranular RH prevents molding because fungal spores can germinate and grow above that humidity level. The warmer and more humid the conditions, the faster the fungus grows and the spoilage spreads.

Common Fungi and Mycotoxins in Maize

Mycotoxin contamination in maize is a serious problem affecting farmers, grain processors, feed manufacturers, and other end users. The mycotoxins of greatest concern in maize are aflatoxins, deoxynivalenol (also known as vomitoxin), fumonisins, and zearalenone, although these are not the only mycotoxins known to occur in maize. Mycotoxin contamination of maize is greatly influenced by the weather. Aflatoxin is the most studied and important mycotoxin affecting maize globally. Aflatoxins are produced primarily by strains of *Aspergillus flavus* and *Aspergillus parasiticus*. *Aspergillus flavus* grows as a yellow-green mycelium and *A. parasiticus* as a grey-green mycelium, with colonies of both species having a powdery appearance.

Aspergillus contamination occurs most commonly in dry, hot years, especially in the southeastern and southwestern United States. The *Aspergillus* fungi are commonly present in maize subjected to stress from drought, heat, disease, insect or nematode pressure, or nutrient deficiency(ies). Good

agricultural and good storage practices reduce *Aspergillus* growth and aflatoxin accumulation in maize. Aflatoxins are confirmed human carcinogens. Aflatoxin B_1 is the major toxin in the group formed on maize. Aflatoxin-contaminated feed may cause serious health risks in young animals. For example, cows are less susceptible than calves. The FDA has established action levels (Table 6.1) for aflatoxin content in maize depending on the final use of the grain.

Fumonisins are produced by fungi in the genus *Fusarium*. *Fusarium verticilloides* is the most common pathogen of maize and produces fumonisins and symptomatic "starbursts" on the grain.

Fusarium graminearum (also known as *Gibberella zeae*) is responsible for deoxynivalenol in maize and is referred to as *Gibberella* ear rot. Cool, wet weather favors infection by *F. graminearum*. This fungus can be spread by rain splash and air currents to maize silks where they may infect and colonize the developing grain and the cob (Patience and Ensley, 2010). Whitish-red mycelial growth may be seen on heavily infected ears. *Fusarium graminearum* infection and subsequent mycotoxin synthesis in maize is often associated with a delayed harvest during a rainy fall and the consequent storage of the grain at a high moisture content. Some *Fusarium* spp. also may produce deoxynivalenol and (rarely) T-2 toxin on maize under suitable weather conditions (Desjardins, 2006; Leslie and Summerell, 2006).

Zearalenone, also known as F-2, is produced by *Fusarium* species such as *F. graminearum* and *F. culmorum*, which can be found on maize. Zearalenone is heat stable, occurs worldwide, and is commonly reported in the North Central corn belt of the United States. Alternating low and moderate temperatures during storage favors zearalenone production with an optimum of 27°C. Pigs are very sensitive to zearalenone, hence rations exceeding 0.5 μg/g of zearalenone should not be fed to swine.

Blue-eye is a storage disease of maize usually caused by contamination with *Aspergillus glaucus* or *Penicillium oxalicum* (Kurtzman and Ciegler, 1970). The name blue-eye results from the death and discoloration of the maize germ by strains of *Aspergillus* and *Penicillium* that invade during harvest and/or storage. The presence of blue-eye usually indicates that the grain was held too long at high moisture after harvest and before drying. Blue-eye maize may contain mycotoxins, but not all species that produce blue-eye symptoms can produce mycotoxins. Spores of *Penicillium* and *Aspergillus* are present in and around all grain storage facilities. Thus, infection of the grain can occur at any time during the holding, drying, and storage process. The most likely time for infection is during wet holding before drying. Delaying drying of a very high moisture content grain and prolonged wet holding periods favor blue-eye infections.

Harvest Considerations

Harvest should begin as soon as possible after crop maturity and the desired moisture content have been reached. Grain moisture levels at the start of harvest can be as high as 25–28%, provided that the grain can be dried to 15% grain moisture content within a day or two. It is important to check and service all equipment before beginning the harvest. Combine adjustments and operation play a major role in preventing kernel damage and subsequently in the ability of fungi to infect during transport, handling, and storage. It also is important to inspect the field before harvest for fungal infection and, if found, to test samples for mycotoxin content. Infected fields should be harvested as soon as possible and the grain should be dried immediately to prevent further fungal growth and mycotoxin accumulation. It is good practice to maintain 1–2% lower moisture content in maize from infected fields or with high mechanical damage, i.e., 13–14% grain moisture instead of the typical 15%.

Maize Drying

To minimize fungal growth and subsequent mycotoxin synthesis, harvested maize should be dried to less than 18% grain moisture content as soon as possible. Higher moisture contents favor fungal growth in stored maize. If the grain moisture content is <18%, then natural air or low-temperature in-bin (in-silo) drying usually suffices to reduce the grain moisture level to <16%. If the grain moisture is >18%, then higher speed drying is required.

Maize drying can be divided into four categories: (i) low-temperature drying, which uses unheated or slightly heated air; (ii) medium-temperature drying with heated air (<43°C or 110°F); (iii) high-temperature drying with heated air that keeps kernel temperature <82°C (180°F) usually for animal feed; and (iv) a combination of low- and medium-temperature air to optimize capacity and efficiency. There are advantages and disadvantages of different drying systems that can affect the end-use of the grain. For low- to medium-temperature in-bin drying, pre-cleaning the grain before loading and drying yields better results because high concentrations of fines in the center of the grain mass will result in uneven airflow and drying. Maize fines also tend to be wetter than are intact kernels. Fines in uncleaned grain with relatively high moisture may favor fungal growth and self-heating, i.e., hot spots in the stored grain mass, due to improper drying and will increase both drying time and energy consumption. The use of a spreader to distribute fines while filling a bin is less effective than drawing a core after the bin is filled (Maier and Bartosik, 2002).

Mechanical Damage, Broken Kernels, and Foreign Materials

Harvesting, transportation, drying, and handling of maize grain generates broken kernels and other fine materials (Maier, 2004). Foreign material includes material other than grain, e.g., weed seeds, dirt, chaff or hulls, plant pieces, and other grains. A properly serviced and operated combine harvester will pre-clean maize grain quite effectively. Cleaning before drying is not effective due to high moisture in the harvested maize. If maize grain is transferred out of the dryer with a pneumatic conveying system, there is little chance to clean the grain before storage. If the grain is moved out of the dryer into a bucket elevator, then cleaning typically occurs in a gravity screen cleaner instead of at the top of the grain leg. The cleaning of fines and foreign material from stored grain is very important for safe storage, as the broken kernels are three to four times more susceptible to fungal growth and foreign material can act as a potential source of contamination.

Care should also be taken to avoid auger damage. Screw conveyors should be run full and as slowly as possible to minimize damage to the grain. A rotary grain cleaner ahead of the screw conveyor, or a gravity flow cleaner at the top of the elevator leg, should be used to avoid maize kernel damage. Rotating screen cleaners typically are used on farms and, most often in conjunction with in-bin dryers.

Condensation

Condensation of moisture on the underside of bin roofs and along the inside walls is a serious management problem that may lead to fungal growth and self-heating of the grain during storage. Warm grain harvested in the summer or the fall and placed in a bin without aeration results in air currents within the grain mass that move the moisture and may lead to condensation, particularly in the bin headspace beneath the roof and on the grain surface. Without venting, this condensation

Table 6.3 Target maize storage temperatures recommended for the US Corn Belt

Month	Temperature (°C)
September to October	13–18
November	4–10
December to February	1.6–7
April	15

Source: After Buschermohle *et al.* (2005).

creates a wet zone in the top layer of the grain that favors fungal growth and results in a crust forming on the grain surface. Crust formation forecasts potentially severe spoilage. If crusting occurs, the surface must be broken up or, in extreme cases, removed and aeration begun immediately. Moisture movement and condensation can be avoided by forcing low volumes of air, i.e., aeration through the grain mass from the time a bin is filled until a safe storage temperature is reached. Aeration with fans should begin as soon as the outside air temperature is 5°C cooler than that of the grain mass. In late fall and winter, aeration fans should be operated again to reduce the temperature of the grain mass to less than 10°C to reduce fungal metabolic activity, including mycotoxin biosynthesis. The optimum target storage temperature for maize depends on the geographic location and the time of the year (Table 6.3).

The SLAM Strategy

The best management practices for the successful storage of maize can be summarized as SLAM: sanitation, loading, aeration, and monitoring (Mason and Woloshuk, 2010).

Sanitation

Handling equipment should be cleaned before and after harvest and should include combines, trucks, wagons, conveyors, and so on. Sanitation in and around the storage bin is very important to reduce pest migration into the bin. Vegetation around the storage structure should be removed, and a weed-free facility should be maintained as weeds may act as a source of fungal infection. Spills should be avoided as they can attract and shelter pests in the storage areas. Clean storage structures soon after emptying them. For longer term storage, empty storage structures should be treated with an approved insecticide inside and around the outside base before refilling. Stored maize can be protected from storage fungi with propionic acid and other organic acids, although their use is usually not recommended. For example, maize treated with propionic acid can only be used for animal feed and is not permitted in commercial grain channels. Before bin filling, the unloading auger, auger tube opening, and side door openings should be sealed for sanitation and to keep the grain from spilling out of the bin.

Loading

Proper handling and loading of storage structures is important for safe storage. This process includes screening out fines and foreign material, coring, and leveling the grain mass. Cleaning maize grain is critical to remove grain dust and broken kernels that insects and fungi thrive on and to improve

aeration. Minimizing grain transfer operations reduces handling, broken kernels, and fines. Coring will not remove the trash and fines, but repeated unloading helps to distribute this material throughout the bin rather than allowing it to accumulate in the center of the bin. Once the bin is full, the grain surface should be leveled, as peak loading of maize reduces airflow and increases airflow resistance. It may be necessary to operate a fan up to 50% longer to cool an overfilled bin than to cool a similar bin with leveled grain that is filled only to the eaves. Grain peaking also makes it more difficult and more dangerous to monitor the grain during storage. Utilizing a spreader to fill a bin or coring the grain mass also enables more uniform airflow.

Aeration

Aeration is key to maintaining low, uniform grain temperatures and avoiding the localized hotspots that can result from the growth of storage fungi. Grain should be cooled uniformly to as low a temperature as reasonable for the geographic region and the cooling maintained as long as possible during storage. Cooled grain will not kill fungal contaminants, but will slow or halt fungal growth and mycotoxin biosynthesis while also reducing the insect population. Adequate exhaust vents must be installed to avoid condensation on the inside walls and under the roof of the storage bin. Automatic fan controllers should be used to optimize aeration. When not operating, the fans should be sealed to prevent premature warming of the grain mass.

Monitoring

Regular monitoring of stored grain is essential to avoid fungal growth and mycotoxin biosynthesis, usually in hot spots during storage. Monitoring every 3–4 weeks during cold months and every 1–2 weeks during warm months usually suffices. Regular inspection and repair of leaky roofs and other holes is necessary to avoid water infiltration.

CO_2 Monitoring

Traditional methods for detecting spoilage in bins include manual walking, smelling, and sampling the grain and monitoring the temperature. Human sensory detection of fungal spoilage and other quality parameters is not accurate and varies from person to person, and temperature cables will not detect fungal growth several meters from the sensor until the size of the spoiling grain mass is large enough to raise the temperature sufficiently near the cable. Thus, monitoring the condition of hundreds and thousands of tons of grain is a difficult task with only temperature monitoring technology.

These limitations are overcome with CO_2 sensors which detect increases in CO_2 due to fungal respiration and thus help to monitor grain quality. Research in the laboratory and in farm and commercial bins (Maier *et al*., 2010) has clearly shown that CO_2 sensors can be used effectively in the headspace of a grain storage bin to detect increases in CO_2 concentration. Thus, the onset of spoilage due to fungi, mycotoxins, and stored-product insects can be detected more quickly than with more traditional methods such as visual inspections and temperature cables. CO_2 sensors are easy to install, continuously monitor the CO_2 concentration in grain storage structures, and alert operators early about the onset of spoilage conditions. These sensors are inserted through the roof of the grain storage bin and are permanently suspended in the headspace. CO_2 data are automatically recorded and may be sent to a computer server via a wireless telephone network.

If spoilage is detected early as an increase in CO_2 concentration, then the problem can often be corrected by simple management practices such as supplying aeration to cool and dry the grain mass or early unloading of the storage structure. CO_2 detection technology promises to reduce the residue levels of mycotoxins, pesticides, and other foreign material assuring food quality and safety throughout the global grain-based food supply chain.

Treating Mycotoxin-Contaminated Maize Grain

Several methods have been developed to treat mycotoxin-contaminated grain and feed products. However, there are limitations associated with each method. Application of ammonia (ammonification) to contaminated maize, peanut, and cotton seeds and meals is a treatment option used worldwide. Ammonification can reduce fumonisin B_1 levels in cultured and naturally contaminated corn by 30% and 45%, respectively (Norred *et al*., 1991). The use of chlorine dioxide, ClO_2, gas at 500 or 1000 ppm with 24-hour exposure times is effective as a structural fumigant in arresting the growth of some fungi (Wilson *et al*., 2005). Other methods include the use of ozone, O_3 (Kim *et al.*, 1999), and γ-irradiation (Aziz and Moussa, 2002). The addition of binders to contaminated feed diets also has been used to reduce mycotoxin toxicity (Jans *et al*., 2014). These binders bind to mycotoxins and prevent them from being absorbed in the intestinal tract of domesticated animals (Jones, 2008). Some of the potential absorbents include clay, bentonite, montmorillonite, zeolite, phyllosilicates, activated carbon, and synthetic polymers such as cholestyramine and polyvinylpyrrolidone (Jans *et al*., 2014). Fungal growth inhibitors, e.g., propionic acid, food grade phosphates, sulfur dioxide, sodium bicarbonate, sorbic acid usually as potassium sorbate, dillapiol, apiol, and dioctatin-A can also be used to slow fungal metabolism and the mycotoxin contamination of various commodities.

Conclusions

Safe maize storage has become a challenging task with increasing demand for good quality grain to assure food and feed safety. Storage is an important link in the value chain in which the quality and quantity of the stored grain must be preserved through the application of best storage management practices. Despite decades of research, preventing pre- and postharvest fungal infection remains a challenge (Munkvold, 2003, 2014). Regular monitoring and maintenance of proper storage environment conditions remains the best strategy for preventing fungal growth during storage (Northolt and Bullerman, 1982). Proactive monitoring coupled with proper aeration can effectively maintain the quality and quantity of on-farm stored grain (Arthur *et al*., 1998; Ileleji *et al*., 2007; Maier *et al*., 1996; Thompson, 1972). Farmers need to avoid factors that cause crop stress, e.g., insect damage, bird damage, drought stress and early harvest, and kernel damage during plant growth, harvesting, transporting, drying, and storage. Field application of fungicides may reduce fungal infection, thus reducing mycotoxin contamination potential, but is often not economically feasible. Segregating damaged grain from intact grain and controlling insects during storage also reduces fungal infection. To stop fungal growth and avoid mycotoxin accumulation, grain should be dried to less than 0.7 a_w, i.e., usually to no more than 14% and preferably below 13%. Grain dried below 14% moisture content can resist further fungal growth and mycotoxin production; however, the fungi and mycotoxins already present will not be eliminated.

Preventing fungal infection is the best management practice and is strongly preferred to treating moldy grain. It is extremely difficult to destroy mycotoxins in grain and grain-based products. Mycotoxin contamination of grain is a complex situation affecting producers, grain elevator managers, food and feed processors, and consumers. Efforts are underway to find reliable, cost-effective, safe

treatment techniques for limiting mycotoxins entering the food chain, but a number of challenges remain. Most mycotoxin-contaminated grain detoxification methods are either too expensive or leave residues in the commodities that limit their end-use. Diagnosis of mycotoxin problems can be both difficult and challenging, as many mycotoxins induce nonspecific, wide-ranging symptoms in animals and since mycotoxins usually are not uniformly distributed throughout the grain mass. Sanitation, loading, aeration, and monitoring (SLAM) are critically important best management practices for stored maize. CO_2-sensing technology is a promising technology for the early detection of spoilage due to fungal growth. Fungi and stored-product insects also can be detected by more traditional methods such as visual inspections and temperature cables. A preventive fungal management strategy involving integrated pest management (IPM) tactics during maize production and SLAM-based storage practices could save grain producers and the grain handling and processing industry in the United States and elsewhere millions of dollars while assuring food and feed safety throughout the corn supply chain.

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7 Good Food-Processing Techniques: Stability of Mycotoxins in Processed Maize-Based Foods

Lloyd B. Bullerman and Andreia Bianchini

Abstract

Good postharvest and processing techniques and strategies to control mycotoxins begin with harvesting at grain moisture levels low enough to prevent fungal growth, or drying to such levels. Adequate sampling and validated, officially approved, methods of analysis of maize samples should then be used to monitor fungal growth and sporulation and the mycotoxin content of incoming and stored grain. Most of these processes can affect mycotoxin contamination, with those that utilize the highest temperatures having the greatest effects. Good processing techniques may reduce mycotoxin concentration, but mycotoxins are never completely destroyed during processing and can contaminate finished processed foods and feeds.

Keywords: aflatoxins; brewing; cleaning; deoxynivalenol; extrusion processing; fumonisins; milling; ochratoxin A; sorting; thermal processing; zearalenone

Introduction

Maize is susceptible to contamination by fungi both in the field and in storage. Some of these fungi are capable of producing toxic metabolites collectively referred to as mycotoxins. Mycotoxins are secondary fungal metabolites, not part of the primary fungal metabolic growth processes. These metabolites are difficult to classify, due to their diverse chemical structures and biosynthetic origins, their myriad biological effects, and their production by a wide number of diverse fungal species (Bennett and Klich, 2003). Mycotoxins are relatively stable compounds that resist destruction by many food processes and then contaminate finished processed foods.

Mycotoxins have a wide range of potential toxic effects, including carcinogenicity and immunotoxicity, in humans and animals. They also are stable in food and feed processes, so it is imperative that maize and maize-based foods be protected from fungal growth and mycotoxin production postharvest, and during storage and distribution. Thus, hazards associated with postharvest development of mycotoxins must be managed and controlled if the grain is to be used for food or feed. The main postharvest procedures for preventing growth of fungi and the accumulation of mycotoxins and their possible entrance into foods and feeds are good quality control, proper storage, and processing.

A safety management program for preventing mycotoxins from entering the food and feed supply begins with adequate monitoring of incoming and stored products. Adequate sampling plans for obtaining representative samples coupled with analyses that use officially approved and validated

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analytical procedures are essential first steps. Detection and quantification of mycotoxins in the maize product are necessary to prevent mycotoxins from entering the food/feed supply chain and to detect and prevent fungal growth and mycotoxin accumulation in stored maize.

Postharvest strategies for preventing mycotoxin contamination in stored maize begin at harvest. Maize should be harvested, if possible, when the moisture content of the grain is low enough to prevent fungal growth and mycotoxin biosynthesis. If the moisture content of the maize at harvest is not low enough for safe storage, then it must be dried either naturally, with ambient air forced through the storage bin, or artificially in a heated air dryer. Maize should be kept at a moisture content $<14\%$ to be safely stored. Following drying, the moisture content must be monitored and maintained. Aeration with ambient air can be used to maintain moisture in a safe range and prevent heating of the grain due to fungal respiration. Fungal growth in storage often begins with a xerophilic species. Fungal respiration causes a rise in moisture content and heats the maize, which in turn enables the growth of spoilage and toxigenic fungi and possible mycotoxin production. Control of insect and rodent activity in stored grain also is essential to prevent damage and to maintain the moisture and temperature control necessary to prevent the growth of toxigenic fungi.

Removal of broken and damaged maize kernels, fine materials, and dust also helps prevent fungal growth. These materials are ideal substrates for initiating fungal growth because they provide readily available nutrients. Other potential methods for preventing fungal growth in stored maize use antifungal chemicals, e.g., propionic and acetic acids, or biological agents with antifungal activity, e.g., lactic acid and propionic acid bacteria.

Mycotoxins

Numerous mycotoxins pose food safety concerns (Table 7.1). Of these, aflatoxins, ochratoxins, fumonisins, deoxynivalenol, and zearalenone are considered the most important, because of their economic and toxicologic importance and the frequency at which they occur (Chełkowski, 1998; Desjardins, 2006). T-2 toxin is sometimes included in this list because of its high toxicity, while moniliformin is sometimes included because of its potential co-occurrence with fumonisin.

Aflatoxins (Figure 7.1) are produced by *Aspergillus flavus* and related species (Bennett and Klich, 2003). They are acutely toxic, can cause chronic toxicity and immune suppression, and are potent hepatocarcinogens. In addition to maize, aflatoxins may be found in cottonseed, peanuts, tree nuts, and other cereal grains (Bennett and Klich, 2003; Egal *et al.*, 2005).

Ochratoxin A (Figure 7.1) is produced by *Aspergillus ochraceus*, *Aspergillus carbonarius*, and *Penicillium verrucosum* (Bayman *et al.*, 2002; Frisvad and Lund, 1993; Pitt, 1987). It is found primarily in wheat and barley grown in northern climates, e.g., Canada and Northern Europe, where

Table 7.1 Mycotoxins of greatest concern in maize and the fungi that produce them

Mycotoxin	Fungal species
Aflatoxins	*Aspergillus flavus*, *A. parasiticus*, *A. nomius*
Ochratoxin	*Aspergillus ochraceus*, *A. niger*, *Penicillium verrucosum*
Fumonisins	*Fusarium verticillioides*, *F. proliferatum*, *F. subglutinans*
Moniliformin	*Fusarium proliferatum*, *F. subglutinans*, *F. thapsinum*
Deoxynivalenol (DON, vomitoxin)	*Fusarium graminearum*, *F. culmorum*, *F. crookwellense*
Zearalenone	*Fusarium graminearum*, *F. culmorum*, *F. crookwellense*
T-2 toxin	*Fusarium poae*, *F. sporotrichioides*, *F. langsethiae*

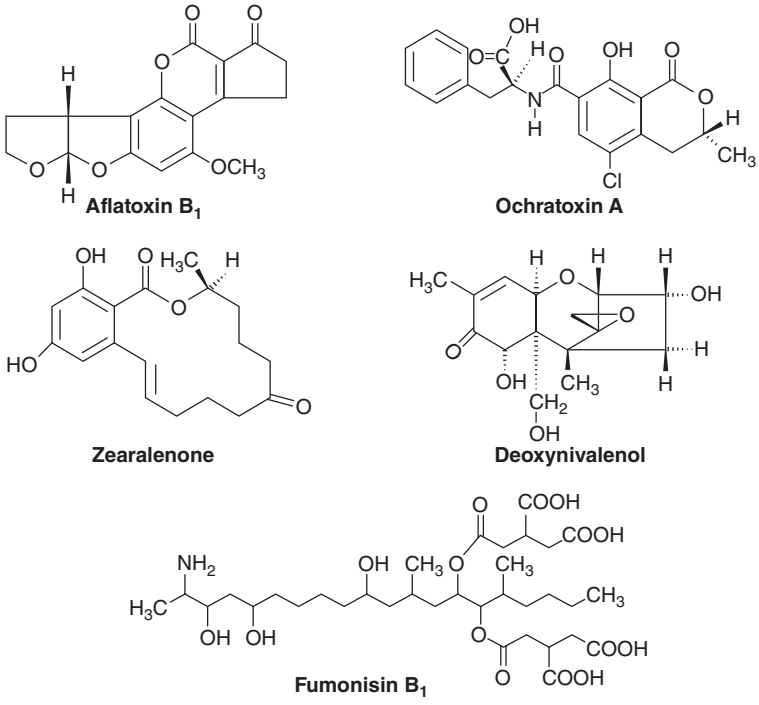

Figure 7.1 Chemical structures of the most important mycotoxins.

it is produced by *P. verrucosum*. Ochratoxin A can also be found in green coffee beans, cocoa beans, raisins, and wine, where it is produced by several species of *Aspergillus* (Scudamore, 2005). It also may occur in maize, but is less common there than in the other commodities. Ochratoxin A has nephrotoxic, teratogenic, and carcinogenic effects in many species (Bennett and Klich, 2003; Kittane *et al.*, 1984).

Fumonisins, deoxynivalenol, and zearalenone (Figure 7.1) are toxins produced by various *Fusarium* species. Fumonisins are produced primarily by *Fusarium verticillioides* and *Fusarium proliferatum* and are common in maize (Rheeder *et al.*, 2002). These *Fusarium* species commonly occur worldwide on maize intended for human and animal consumption (Leslie and Summerell, 2006). *Fusarium verticillioides* is a soil-borne plant pathogen that can cause symptomless infections of maize plants and invade the grain. All of the kernels in a lot of shelled maize may be infected with a strain of *Fusarium*, but lack visible fungal growth or deterioration (Desjardins, 2006; Leslie and Summerell, 2006). These characteristics mean that good quality, food-grade maize can be contaminated with fumonisins without any visible signs of fungal growth. Fumonisin B_1, the most common form of fumonisin, occurs in finished processed maize-based foods, such as maize meal and maize flour.

Fumonisins interfere with sphingolipid metabolism and cause leukoencephalomalacia in horses (Marasas *et al.*, 1988) and rabbits (Bucci *et al.*, 1996), and pulmonary edema in swine (Harrison *et al.*, 1990; Haschek *et al.*, 2001). They are suspected as a causal agent of human esophageal cancer in South Africa and China (Chu and Li, 1994; Rheeder *et al.*, 1992) and to be responsible for some cases of neural tube defects in humans in the United States (Desjardins, 2006; Hendricks, 1999; Marasas *et al.*, 2004). Moniliformin is produced by *F. proliferatum* and *F. subglutinans* and a number of related *Fusarium* species (Fotso *et al.*, 2002) and may co-occur with fumonisin in maize.

Deoxynivalenol, also known as DON and as vomitoxin, is produced most prominently by *Fusarium graminearum*, *Fusarium culmorum*, and *Fusarium crookwellense. Fusarium graminearum* is a pathogen of wheat, barley, and maize, causing *Fusarium* head blight in wheat and barley and ear rots in maize, which may lead to the contamination of these crops with deoxynivalenol. Deoxynivalenol also is found in rye, oats, and rice (Schollenberger *et al.*, 2006; Scott, 1997). Deoxynivalenol causes vomiting and feed refusal in animals, especially swine, and causes gastroenteritis with vomiting in humans (Lun *et al.*, 1985; Luo, 1994). It also adversely affects immune system function (Pestka and Smolinski, 2005).

T-2 toxin and deoxynivalenol are Type A and Type B trichothecenes, respectively, and differ in the presence of a keto group at the C-8 position of the molecule. T-2 is much more toxic than deoxynivalenol and is on the select list of microbial toxins in the United States, but occurs much less frequently than does deoxynivalenol. T-2 causes alimentary toxic aleukia, which killed thousands of people in 1994–1945 in Russia (Desjardins, 2006; Marasas *et al.*, 1984).

Zearalenone, another mycotoxin produced by *Fusarium* species, is not a true toxin. Instead, it has estrogenic activity that affects the reproductive system of mammals, especially swine, and is classified as an endocrine disrupter (Friend *et al.*, 1990). Zearalenone is produced by *F. graminearum* and *F. culmorum* and has been found primarily in high-moisture maize, but may also occur in wheat, sorghum, barley, and processed foods (Mirocha *et al.*, 1977; Schollenberger *et al.*, 2006; Leslie, 2014).

Effects of Food Processing

There are published studies on the effects of processing on mycotoxins in commodities other than maize, which are included in this discussion so that the effects of all processes on mycotoxins are considered and since effects on maize are usually similar to those observed for other commodities. Food processes that affect mycotoxin contamination include sorting, trimming, cleaning, milling, brewing, cooking, baking, frying, roasting, canning, flaking, alkaline cooking, nixtamalization (tortilla processing), and extrusion.

Sorting, Trimming, and Cleaning

Sorting and trimming may lower mycotoxin concentrations by the removal of the toxins, but they do not destroy mycotoxins. Cleaning grain may remove kernels with extensive fungal growth, broken kernels, and fine materials, and thereby reduce mycotoxin concentrations. Fumonisin concentrations in maize can be reduced by 29–69% just with cleaning (Sydenham *et al.*, 1994), and this process is as effective in less developed countries as it is in developed countries (Afolabi *et al.*, 2006). Cleaning also can be used to remove scab-infested wheat and barley kernels and thereby reduce deoxynivalenol concentrations. Cleaning wheat reduced deoxynivalenol levels by 5.5–19% (Abbas *et al.*, 1985), but by only 2–3% for ochratoxin A (Scudamore *et al.*, 2003). For aflatoxins, removing the fungal-damaged kernel, seed, or nut intact from the commodity may reduce toxin levels by 40–80% (Park, 2002). Although, sorting, trimming, and cleaning reduce mycotoxin concentrations, these operations usually do not completely remove all of the contamination, especially because their efficacy is highly dependent on the initial condition of the grain. In developed countries the culled grain is often used for nonfood/feed products, e.g., fermentation to biofuels, or is destroyed. In less developed countries, however, where food security usually is a major concern, the contaminated grain may be retained for on-farm consumption or be milled so that the contamination is no longer visibly evident with the resulting flour then introduced into the effectively unregulated human food chain.

Milling

No step/operation in the milling process destroys mycotoxins; however, the contamination may be redistributed and concentrated in certain mill fractions. During dry milling, mycotoxins tend to be concentrated in bran and germ fractions (Abbas *et al.*, 1985; Brera *et al.*, 2004; Katta *et al.*, 1997; Park, 2002; Scudamore *et al.*, 2003). These maize fractions are used for animal feed and/or oil extraction. The maize fractions used for food production, including flaking grits and flour, usually have the lowest levels of mycotoxin contamination. At the end of the dry milling process of wheat, barley, and other cereals, deoxynivalenol, zearalenone, aflatoxins, and ochratoxin A were concentrated in fractions of the commodity that are the least likely to be used for human food production, i.e., the germ and the bran (Abbas *et al.*, 1985; Alldrick, 1996; Brera *et al.*, 2006; Chełkowski *et al.*, 1981; Lancova *et al.*, 2008; Park, 2002; Scott *et al.*, 1984; Scudamore *et al.*, 2003).

In the wet milling of maize, mycotoxins may be dissolved in the steep water or be distributed among the byproducts of the process, but are not destroyed. By the end of the wet milling process aflatoxin, zearalenone, and fumonisins are found in the steep water, gluten fiber, and germ, while the starch tends to be relatively uncontaminated (Bennett *et al.*, 1996; Lauren and Ringrose, 1997; Park 2002; Ryu *et al.*, 2002). Fumonisin, e.g., can be found dissolved in the steep or process water and distributed in the gluten, fiber, and germ fractions, with no detectable toxin in the starch (Bennett *et al.*, 1996).

Brewing

Aflatoxin B_1, ochratoxin A, zearalenone, deoxynivalenol, and fumonisins B_1 and B_2 may be transferred from contaminated grain into beer during the brewing process (Scott, 1996). The source of these toxins could be the malted grain or adjuncts, including maize adjuncts. In the brewing industry, maize, in the form of grits or syrup, rice grits, unmalted barley, wheat starch, or sorghum grits may be used as adjuncts to provide fermentable carbohydrates for the yeast. The patterns for the loss of aflatoxin B_1 and ochratoxin A during the brewing process are similar (Chu *et al.*, 1975). Both mycotoxins are relatively stable in the mash cooking step (boiling temperatures applied), but more sensitive to mash malting (protein hydrolysis; 12–27% toxin reduction), wort boiling (20–30% toxin reduction), and final fermentation (20–30% toxin reduction). If ochratoxin A, fumonisin B_1, or fumonisin B_2 is added to the wort prior to fermentation, then 2–13% of the ochratoxin A, 3–28% of the fumonisin B_1, and 9–17% of fumonisin B_2 are lost during the fermentation (Scott *et al.*, 1995).

Thermal Processing

Thermal processing involves the application of heat to a product to cook and preserve it. These processes include ordinary cooking, frying, baking, roasting, and canning. Extrusion, which also is a thermal process, is considered separately in the "Extrusion Processing" section below. Studies of the stability of mycotoxins during various methods of thermal processing have been reported for several mycotoxins (Jackson *et al.*, 1996a, 1996b; Pineda-Valdes and Bullerman, 2000; Ryu *et al.*, 2003).

In the preparation of muffins from maize meal contaminated with aflatoxins, 87% of the initial aflatoxin B_1 in the meal was retained in the baked muffins (Stoloff and Trucksess, 1981). Cooking rice contaminated with aflatoxin B_1 resulted in a loss of 34% of the toxin, and even larger reductions (78–88%) if the grain was cooked under pressure (Park and Kim, 2006; Park *et al.*, 2005). Boiling

maize grits reduced aflatoxin levels by 28%, while frying the boiled grits resulted in a total reduction of 34–53% of the toxin (Stoloff and Trucksess, 1981).

Roasting pistachio nuts at different temperatures (90°C, 120°C, and 150°C) for different periods of time (30, 60, and 120 minutes) reduced their aflatoxin content by 17–63%, with the amount of reduction both time and temperature dependent (Yazdanpanah *et al.*, 2005). The reduction of aflatoxin contamination during coffee bean roasting also depends on temperature and the type of roasting, with toxin reductions of 42–56% reported (Soliman, 2002).

Production of tortillas by the traditional method (alkaline cooking and steeping of the maize) decreases aflatoxin levels by about half (46–52%); however, acidification of the final products can enable much of the original toxin to reform (Price and Jorgensen, 2006; Torres *et al.*, 2001). When tortillas were processed into tortilla chips and corn chips, the initial aflatoxin contamination was reduced by ~84% in the tortilla chips and 79% in the corn chips (Torres *et al.*, 2001).

The concentration of ochratoxin A is not reduced during bread baking (Scudamore *et al.*, 2003), but when biscuits are baked about two-thirds of the toxin is destroyed or immobilized. Cooking beans in water under pressure reduces ochratoxin A concentrations by up to 84% (Milanez and Leitão, 1996). Autoclaving oatmeal with 50% water reduces ochratoxin A concentrations by 74%, while autoclaving dry oatmeal or rice reduces ochratoxin A content by 86–88% (Trenk *et al.*, 1971).

Roasting coffee reduces ochratoxin A concentrations by 13–93% (Nehad *et al.*, 2005). Brewing coffee also reduces ochratoxin A contamination, with the extent of the reduction dependent upon the brewing process. Regular brewing can reduce the initial levels of ochratoxin A by 72%, the traditional oriental brewing method (Turkish coffee) reduces toxin levels by 88%, and typical espresso coffee brewing eliminates >90% of the ochratoxin A (Nehad *et al.*, 2005).

Deoxynivalenol levels were not reduced when Egyptian flat bread was baked (El-Banna *et al.*, 1983), but baking regular bread, cookies, and biscuits reduced deoxynivalenol levels by 24–71% in bread and by 35% in cookies and biscuits (El-Banna *et al.*, 1983; Scott, 1984). Canning baby food and dog food did not reduce deoxynivalenol levels, but canning cream-style maize reduced (12%) deoxynivalenol levels slightly (Wolf-Hall *et al.*, 1999). When tortillas were made from naturally contaminated and spiked maize, deoxynivalenol levels were reduced by 71–82% (Abbas *et al.*, 1988).

Fumonisin B_1 is a fairly heat-stable compound. If *F. verticillioides* culture material is boiled in water for 30 minutes and then dried at 60°C for 24 hours, then there is no detectable loss of fumonisin B_1 (Alberts *et al.*, 1990). However, as with deoxynivalenol, fumonisin levels decreased whenever whole kernel maize or cream-style maize was canned and when baked in maize bread (Castelo *et al.*, 1998b). No significant loss of fumonisins occurred when naturally or artificially contaminated (5 μg/g fumonisin B_1) maize–muffin mix was baked. In contrast, roasting naturally or artificially contaminated (5 μg/g fumonisin B_1) maize meal samples at 218°C for 15 minutes resulted in an almost complete loss of fumonisins (Castelo *et al.*, 1998b). In another study (Jackson *et al.*, 1997), baking maize muffins at 175°C and 200°C reduced fumonisin levels by 16% and 28%, respectively. At both temperatures, toxin losses were greater at the surface than at the core of the muffins. Frying maize masa at 140–170°C for 0–6 minutes did not reduce the fumonisin levels, but frying tortilla chips at 190°C for 15 minutes reduced the fumonisin levels by 67% (Jackson *et al.*, 1997).

The rate and extent of fumonisin B_1 decomposition in aqueous buffered systems increased with processing temperature (Jackson *et al.*, 1996a, 1996b). Overall, fumonisin B_1 was least stable at pH 4, followed by pHs 10 and 7, respectively. At temperatures >175°C, more than 90% of fumonisin B_1 was lost after processing for 60 minutes, regardless of pH. Similar results were found for fumonisin B_2. Thus, both compounds were heat stable in aqueous environments. Fumonisin molecules lose the tricarballylic acid side groups, but the carbon backbone of the molecule remains intact (Jackson

et al., 1996a, 1996b). In aqueous solutions heated to 100–150°C containing fumonisin B_1 and maize starch, zein, or glucose as individual matrix components, the greatest losses of fumonisin B_1 occurred in solutions containing glucose (Hlywka, 1997).

N-(Carboxymethyl)fumonisin B_1 is a stable reaction product formed when fumonisin B_1 is heated in the presence of reducing sugars (Howard *et al.*, 1998). The reaction probably proceeds through a common Maillard (nonenzymatic browning) reaction between fumonisin B_1 (an aliphatic primary amine) and a reducing sugar, in a manner similar to the reaction of amino acids with reducing sugars (Murphy *et al.*, 1996). The first reaction product of fumonisin B_1 and D-glucose is *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (Polling *et al.*, 2002), which, following the general Maillard reaction, is converted to NCM-FB_1 (Howard *et al.*, 1998; Lu *et al.*, 2002).

Maize Flake Process

The effect of the maize flake process on aflatoxin and fumonisins has been studied (Castelo, 1999; de Girolamo *et al.*, 2001; Meister, 2001). For aflatoxin, cooking maize grits with and without sugars reduced aflatoxin levels by 64–67%, while toasting the flakes with and without sugar reduced aflatoxin by 78–85% (Castelo, 1999). Ochratoxin A also was reduced during the processing of breakfast cereals, e.g., maize flaking (Aish *et al.*, 2004), as were the levels of fumonisin B_1 and B_2 (de Girolamo *et al.*, 2001). Total fumonisins were reduced by 60–70% across the entire process, with only 30% of the losses attributable to the extrusion step, where the material was subjected to 70–170°C for 2–5 minutes. In another study of fumonisin stability (Meister, 2001), extrusion cooking and gelatinization reduced fumonisin levels by 45–70%, cooking the grits for flaking reduced fumonisin contamination by 35–80%, and roasting the flakes reduced the fumonisin content by 65–94%.

Maize flake processing without sugar reduced fumonisin B_1 levels by 54% and 49%, following cooking and toasting, respectively, whereas processing in the presence of glucose reduced fumonisin B_1 levels by 86–89% (Castelo, 1999). Losses of fumonisin B_1 in the presence of sucrose, maltose, and high fructose maize syrup were similar to the reductions that occurred without sugar. Caution must be exercised when interpreting these data, however, since "hidden" fumonisins, i.e., fumonisins bound to proteins, occur in commercial corn flake samples (Kim *et al.*, 2003), which suggests that the apparent reductions in fumonisins may not be due to their destruction.

Extrusion Processing

Extrusion processing is extremely versatile and used extensively in the production of breakfast cereals, snack foods, and textured foods. During extrusion cooking very high temperatures can be reached. Extruders may be thought of as high-temperature, short-time chemical and bioreactors. During processing through an extruder, a dough-like mixture is forced through a stationary metal tube, or barrel, by a rotating screw shaft. Heat is added in the form of steam, by the mechanical energy of the turning screw, and by the friction of the barrel, and very high temperatures (>150°C) can be reached. Very high pressures (10–20 bar) and severe shear forces, due to the use of screws for changing flight depth and/or screw pitch, also are generated and contribute to the chemical reactions and molecular modifications of compounds and to completing the cooking process in a short time. Extruders may have a single screw or a double (twin) screw.

Mycotoxin concentrations usually decrease during extrusion processing, with the degree of reduction in the finished product dependent on some or all of extruder temperature, screw speed,

residence time in the extruder, and moisture content of the extrusion mixture. Extrusion temperature and screw speed, which affect residence time, seem to have the greatest impact on mycotoxin concentrations. Reductions of 46–76% in fumonisin levels occurred when contaminated corn grits were extruded at 160–200°C with screw speeds of 120–160 rpm (Katta *et al.*, 1999).

The effect of extrusion on aflatoxin content is influenced by the presence of additives, moisture content, and temperature (Cheftel, 1989). Extrusion alone can reduce aflatoxin content by 50–80% (Hameed, 1993). If ammonia is added, either as a hydroxide (0.7 and 1.0%) or as a bicarbonate (0.4%), then the aflatoxin content is reduced by ≥95%. Results with peanut meal were similar to those with maize meal, with aflatoxin levels reduced by 23–66% in the absence of ammonia and by 87% in the presence of 2.0–2.5% ammonium hydroxide (Cheftel, 1989).

Ochratoxin A contamination of wheat can also be reduced during extrusion processing. Screw speed has little effect on ochratoxin A levels, but increasing residence time, temperature, or moisture, all reduces ochratoxin A contamination (Scudamore *et al.*, 2004). When the moisture content was 30%, at 116–120°C, ochratoxin A was reduced by 12%, and at 133–136°C it was reduced by 24%. When the moisture content was 17.5% at 157–164°C, ochratoxin A levels were reduced by 13%, while at 191–196°C the reduction was 31%. Ochratoxin A losses up to 40% occurred with increased residence times, when lower mass flow rates were applied, due to the longer time the product spent in the extruder.

Temperature and screw type, but not the moisture content of maize grits, affected the reduction of zearalenone contamination by extrusion cooking. The reduction in zearalenone contamination was significantly higher when a mixing screw (66–83% reduction) was used than when a non-mixing screw (65–77% reduction) was used. The reduction of zearalenone contamination at 120°C and 140°C was 73–83%, while at 160°C it was only 66–77% (Ryu *et al.*, 1999). This result is counterintuitive since greater loss of zearalenone was expected to occur at the higher temperature.

Extrusion cooking of maize flour contaminated with deoxynivalenol reduced toxin levels under all conditions studied by >95% (Cazzaniga *et al.*, 2001). These conditions included moisture contents of 15% and 30%, temperatures of 150°C and 180°C, and sodium metabisulphite concentrations of 0% and 1%.

The effect of extrusion cooking on fumonisin stability has been studied more extensively than for most other mycotoxins. More fumonisin B_1 is lost in extrusion cooking with a mixing screw than with a non-mixing screw (Castelo *et al.*, 1998a). With a corotating twin-screw extruder (Katta *et al.*, 1999), fumonisin B_1 levels were reduced by 34–95% in maize grits with the losses increasing as the temperature increased and the screw speed decreased.

The inclusion of sugars in the dough also alters the stability of fumonisins during extrusion processing (Castelo *et al.*, 2001). Fumonisin levels were reduced in extruded maize grits that contained 2.5% or 5.0% glucose, fructose, or sucrose. Significant reductions in fumonisin B_1 levels occurred in all treatments. The toxin losses with glucose were greater (45–67%) than those with either fructose (32–52% reduction) or sucrose (26–43% reduction). Both screw speed and glucose concentration affect the amount of fumonisin in the extruded grits, with up to a 93% reduction in fumonisin levels occurring at lower screw speeds and higher glucose concentrations. At 10% glucose in maize grits and extrusion at 160°C and 60 rpm screw speed in a single screw extruder, fumonisins were reduced by 21–37% in the absence of sugar, or 77–87% when glucose was present (Bullerman *et al.*, 2008). The main fumonisin B_1 derivative formed in the presence of glucose is *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1.

The presence of glucose in the extrusion process also affected the toxicity of the extruded product. Male Sprague-Dawley rats fed maize grits extruded with glucose had less severe kidney lesions and no changes in kidney weight, while those fed the extruded product without glucose had more severe kidney lesions and altered kidney weights (Voss *et al.*, 2008). In a more efficient twin-screw

extruder, maize grits were fermented with *F. verticillioides* to attain fumonisin concentrations of ~10 and 50 μg/g and then extruded in the presence or absence of 10% glucose. As in the previous study, extrusion with glucose and a twin-screw configuration reduced the toxicity of the extruded product (Voss *et al.*, 2011).

Conclusions

In this chapter we discussed the effects of good food-processing techniques on the stability and fate of aflatoxins, ochratoxin A, fumonisins, deoxynivalenol, and zearalenone in maize and maize-based products. The most effective strategy to prevent the occurrence of mycotoxins in processed maize products is to prevent the entrance of contaminated maize into the food supply chain. Implementing this strategy requires harvesting when the moisture content is low enough to prevent fungal growth and mycotoxin biosynthesis in stored maize or drying the grain to such a level after harvest (Channaiah and Maier, 2014). Adequate quality control, which includes representative sampling and analyses for mycotoxins, also is needed to monitor incoming and stored grain for mycotoxin contamination.

Processing techniques that alter mycotoxin concentrations include sorting, trimming, cleaning, milling, brewing, cooking, baking, frying, roasting, canning, flaking, alkaline cooking, nixtamalization, and extrusion. Sorting, trimming, cleaning, and brewing all lower mycotoxin concentrations, but usually do not remove all of the contamination and may result in a contaminated final product. Of the techniques that employ heat, ordinary cooking and baking have little or no effect on mycotoxin contamination levels, while those that utilize higher temperatures and dry heat, appear to be more effective. Heating mycotoxins in the presence of sugars, especially glucose, increases their reduction. Heating combined with alkaline pH may destroy some mycotoxins, but these effects may be reversible, especially if the treated material is exposed to acidic conditions. Extrusion processing with 10% glucose greatly reduces or eliminates toxicity attributable to fumonisins. Thus, good food-processing techniques can reduce the concentrations of mycotoxins in processed maize-based foods and help lower mycotoxin levels, but they are insufficient to totally eliminate them.

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8 Mycotoxin Reduction in Animal Diets

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Abstract

Mycotoxins are toxic chemicals produced by fungal species that colonize crops in the field or after harvest and pose a potential threat to the health of humans and domesticated animals. Even when recommended "good agricultural practices" are implemented to decrease mycotoxin production during crop growth, harvesting, and storage, the potential for significant contamination still exists. The significance of these unavoidable, naturally occurring toxicants to human and animal health, the increase in mycotoxin regulations, and the global trans-shipment of agricultural commodities all highlight the need for strategies to reduce mycotoxin contamination. These strategies may include any or all of the physical methods, chemical decontamination, adsorbent treatments, and biological detoxification to decrease mycotoxin bioavailability. Available treatments may reduce levels of specific mycotoxins; however, no single method is sufficiently effective against the wide variety of mycotoxins that co-occur in different commodities.

Keywords: activated charcoal; adsorbents; aflatoxins; bentonites; biological detoxification; chickens; glucomannans; hydrated sodium calcium aluminosilicates; ochratoxin A; probiotics; silage; trichothecenes; zearalenone; zeolites

Introduction

Mycotoxin-producing fungi grow on staple raw materials used for animal feeds and human foods. Consequently, mycotoxins are persistent, common, worldwide contaminants of feed and foods that affect the health of both humans and domesticated animals (Bryden, 2012). Extreme vigilance and careful management is vital to ensure that mycotoxin levels are acceptably low. The complexity of mycotoxin regulation and biosynthesis makes it difficult to predict which toxin will be produced, when, and at what level.

Unfortunately it is not possible to prevent mycotoxin production before harvest, in storage, or during processing. Consequently, numerous strategies are evolving to manage and limit mycotoxin contamination, some of which are more practical and effective than others. In general, large-scale, practical, cost-effective methods for complete detoxification of mycotoxin-containing feedstuffs are not available. Substances used to reduce the absorption of mycotoxins by animals should either promote the excretion of mycotoxins in feces, e.g., binders, or modify their mode of action, e.g., biological detoxifiers.

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One of the most promising approaches is to incorporate sorbents that sequester mycotoxins into the diet and reduce their absorption from the gastrointestinal tract, thereby avoiding both the direct toxic effects and the carryover of these fungal metabolites into animal products. Such sorbents should have a high binding capacity and affinity for the mycotoxin and dissociate poorly from the toxin, thereby reducing the toxin's bioavailability. Sorbents that are effective *in vitro* may be neither safe nor efficacious *in vivo*.

Biological detoxification has become an important research area during the last decade and is an emerging area of interest in animal feed production. Isolation and characterization of microorganisms or enzymes that chemically alter mycotoxins could be an important part of decontamination processes occurring in an animal's intestinal tract. If the resulting metabolites are less toxic than their predecessors, then biological decontamination may be a technology of choice, as these processes are specific, efficient, and environmentally friendly.

Independent of product, a primary research objective is to evaluate efficacy when multiple toxins are present, as such conditions more closely approximate reality. Thus, this chapter focuses on the main binders used for mycotoxin adsorption, their *in vitro* efficacy, and their *in vivo* applications in various animals. We also evaluate biological detoxification as a means of detoxifying mycotoxins that cannot be removed with inorganic or organic sorbents.

Adsorption of Mycotoxins

Practical, economical deactivation procedures are needed in response to the ever-increasing number of reports of the presence of mycotoxins in foods and feeds. The high costs and limitations of physical and chemical treatments of contaminated feeds have prompted a search for other methods to remedy mycotoxin contamination. The most widely evaluated methods involve additions to the diet of nutritionally inert sorbents that bind tightly to mycotoxins in the gastrointestinal tract and reduce toxin availability.

Use of Inorganic Adsorbents

Amongst clay and zeolitic minerals, hydrated sodium calcium aluminosilicates (HSCAS) and similar bentonite/montmorillonite clays are the best adsorbents for aflatoxins (Grant and Phillips, 1998; Phillips *et al.*, 1988; Ramos and Hernandez, 1996; Vekiru *et al.*, 2007). When mixed into feed, these clays markedly reduce aflatoxin uptake and its distribution to target organs, thus avoiding aflatoxin-related diseases and the carryover of aflatoxins into animal products such as milk (Phillips *et al.*, 1990, 1991). These binders adsorb organic substances on an external surface or within their interlaminar spaces or through interaction with or substitution of exchange cations present at these sites. Clay and zeolitic minerals, however, comprise a diverse family of aluminosilicates, which are not all equally effective as mycotoxin sorbents. Effective sorbents have been identified for aflatoxins, but these sorbents have little, i.e., zearalenone, ochratoxin A, and fumonisins, or no, i.e., trichothecenes, ability to bind other toxins under field conditions (Friend *et al.*, 1984; Huff *et al.*, 1992; Kubena *et al.*, 1990; Ramos *et al.*, 1996).

Hydrated Sodium Calcium Aluminosilicate

HSCAS, as used by Phillips' research group, refers to a specific calcium montmorillonite, i.e., phyllosilicate clay, that is an effective enterosorbent of aflatoxins. Its chemical composition and

its mechanism of aflatoxin sorption at interlayer surfaces have been well described. The stability of the aflatoxin–HSCAS complexes may explain the *in vivo* effectiveness of the adsorbent in preventing the toxic effects of aflatoxins (Pettersson, 2004). HSCAS diminishes the effects of aflatoxins in a variety of young animals including rodents, chicks, broilers, turkey poults, ducklings, lambs, pigs, minks and trouts. Levels of aflatoxin M_1 in milk from lactating dairy cattle and goats decreased in the presence of HSCAS (Richard and Payne, 2003). At 0.5% HSCAS in the diet, total aflatoxins ($M_1 + B_1 + B_2$) in liver, kidney, muscle, and fat were significantly reduced (Beaver *et al.*, 1990).

HSCAS has limited effects on zearalenone and ochratoxin A contamination and is ineffective against trichothecenes (Huwig *et al.*, 2001; Pettersson, 2004; Richard and Payne, 2003; Santin *et al.*, 2002). These interactions result from the HSCAS–mycotoxin-binding mechanism. Aflatoxins have a β-keto-lactone or bilactone system, which other mycotoxins lack. This structure is essential for chemisorption to HSCAS.

Ochratoxin A in the diet reduces productivity indexes, e.g., heavier kidneys and enlarged livers, and the addition of HSCAS to the diet does not improve these parameters (Santin *et al.*, 2002). HSCAS in the diet does not significantly alter the hyperestrogenic effects of zearalenone (Bursian *et al.*, 1992). Increased gestation length, decreased litter size, and increased kit mortality of mink were some of the effects reported. Adding HSCAS at either 0.5% or 1.0% to the diet did not alter the average daily gain of pigs exposed to deoxynivalenol (Richard and Payne, 2003).

Bentonites (Montmorillonites)

Including HSCAS, bentonites are clays that result from the decomposition of volcanic ash, which consists primarily of phyllosilicate minerals belonging to the smectite group. In 70% of the cases the smectite is montmorillonite. Promising *in vitro* results were obtained with bentonite adsorption of aflatoxins, fumonisins, and ergot alkaloids, i.e., ergin, ergotamine, and ergovaline. Bentonites have high aflatoxin adsorption, capacity, and affinity (Vekiru *et al.*, 2007) and could adsorb up to 90% of the fumonisins present in a complex medium, e.g., gastrointestinal fluid (Richard and Payne, 2003).

Inclusion of 0.5% sodium or calcium bentonite restored growth performance and serum profiles of growing pigs fed diets containing 800 ppb of aflatoxin B_1 (Schell *et al.*, 1993b). Bentonites can also effectively reduce the toxicity of aflatoxins in broiler chicks, poultry, and pigs; improve growth performance, average daily weight gain, average daily feed intake, and feed conversion ratio; and reduce the negative immunosuppressive effect (Rosa *et al.*, 2001; Shi *et al.*, 2006; Yu *et al.*, 2008). The addition of montmorillonite to aflatoxin-contaminated diets diminished the adverse effects on relative organ weights; hematological, serum, and liver biochemical values; and enzymatic activities associated with aflatoxicosis (Shi *et al.*, 2006).

Reduction of the carryover of aflatoxins into milk varied when bentonites were included in the diet of dairy cattle (Veldman, 1992). Thus, these sorbents may not suffice to meet the low EU tolerance limits for aflatoxins, even though a significant reduction of aflatoxin M_1 levels can occur (Pietri *et al.*, 2009) when a blend of bentonites is fed. Rats fed a diet contaminated with T-2 toxin that contained bentonites had general improvements in performance parameters and excreted more toxin (Carson and Smith, 1983). However, these results required bentonite in the diet at 10–20 times the level needed for aflatoxin control.

Bentonites have little or no effect on zearalenone contamination, although they may be useful if fungal growth results in nonspecific unpalatability. Bentonites are ineffective when feed refusal results from direct effects of the toxin on appetite, as occurs with trichothecenes such as nivalenol (Guerre, 2000; Williams *et al.*, 1994).

Zeolites

Zeolites are aluminosilicate clays with similarities to both molecular sieves and ion-exchange resins. These clays can differentiate molecules by size, shape, and charge. Depending on the dosage, physical structure, type of zeolite, and concentration of aflatoxin in the contaminated feed, the ability of zeolites to reduce aflatoxin toxicity can differ widely. Zeolites normally have less capacity than HSCAS or other bentonites to adsorb aflatoxins *in vitro*, but have some *in vivo* efficacy. Natural zeolite (1% in the diet) given to broilers consuming 2.5 ppm aflatoxin B_1 alleviates growth depression and reduces the increase in liver lipids (Scheideler, 1993). Several studies with the natural zeolite, clinoptilolite, confirmed improved growth performance, decline of liver and kidney lesions, reduction of heterophilia and lymphopenia, and improvements to humoral immunity (Oguz and Kurtoglu, 2000; Ortatatli and Oguz, 2001; Ortatalti *et al.*, 2005).

Other studies, however, report that zeolites were ineffective adsorbents and organ lesions resulted (Harvey *et al.*, 1993; Mayura *et al.*, 1998). In one study, incorporation of a synthetic anion-exchange zeolite into rat diets at 5% completely eliminated the deleterious effects of zearalenone on body weight, feed consumption, and feed efficiency (Smith, 1980). In another study, the use of a synthetic cation-exchange zeolite did not protect rats against zearalenone toxicosis, and piglets fed diets containing zearalenone and the zeolite had higher uterine weights than did piglets fed diets with just zearalenone (Coenen and Boyens, 2001; Guerre, 2000). These observations support the hypothesis that anion-exchange zeolites can bind zearalenone, which is anionic at intestinal pH, and can be used to treat zearalenone toxicosis by reducing the absorption of the toxin and increasing its excretion.

Other Clays

To some extent other clays, e.g., diatomaceous earth also have been evaluated for their ability to bind mycotoxins both *in vitro* and *in vivo*. Some commercial products reduced aflatoxin toxicity in broilers (Denli *et al.*, 2009) and ochratoxin A toxicity in hens, leading to an increase in egg albumen, height, redness of the egg yolk, and serum calcium concentration (Denli *et al.*, 2008).

Nutrient Interactions in Animals

Potential adsorbent activities have resulted in many unanswered questions regarding adsorbent effects on the utilization of nutrients such as carbohydrates, proteins, vitamins, and minerals. At 0.5% in the diet, HSCAS did not impair the utilization of either phytate or inorganic phosphorous (Phillips, 1999). At 0.5% and 1.0% in the diet, HSCAS did not impair the utilization of riboflavin, vitamin A, or manganese, although there was a slight, but significant, reduction in zinc utilization at the 1% clay level (Chung and Baker, 1990). The effects on mineral metabolism are more pronounced when aflatoxin-contaminated maize is fed to pigs (Schell *et al.*, 1993a). Feeding bentonite with aflatoxin-contaminated maize partially restores performance and liver function without greatly influencing mineral metabolism (Schell *et al.*, 1993a). Zeolites had a significant negative effect on vitamin and mineral uptake and on their distribution in the body of sows (Papaioannou *et al.*, 2002). Thus, an important factor in evaluating potential mycotoxin-binding agents is the adsorbent's affinity for vitamins, minerals, and other nutrients (Huwig *et al.*, 2001).

Organic Adsorbents

Substances investigated as potential organic mycotoxin-binding agents include activated charcoal, alfalfa, canola oil, bleaching clays, and organic polymers, e.g., cholestyramine, polyvinylpolypyrrolidone (PVPP), yeast cell walls and components thereof, and bacterial cells.

Activated Charcoal

One of the most effective, nontoxic, nonspecific binding sorbents is activated charcoal. It has a high surface-to-mass ratio (500–3500 m^2/g) and contains binding agents formed by pyrolysis of various organic compounds. All activated charcoals are not the same, as some efficiently adsorb most mycotoxins in an aqueous solution, whereas others have little, if any, adsorptive capacity for mycotoxins (Kolosova and Stroka, 2012). Activated charcoal has been evaluated for its ability to adsorb mycotoxins both *in vitro* and *in vivo*. It adsorbs zearalenone, deoxynivalenol, and nivalenol *in vitro* in a gastrointestinal model and aflatoxins, ochratoxin A, and fumonisins *in vivo* (Kolosova and Stroka, 2012; Pettersson, 2004; Richard and Payne, 2003).

In vitro, different activated charcoals adsorb aflatoxins at different pHs (Huwig *et al.*, 2001). *In vivo* the conversion of aflatoxin B_1 to aflatoxin M_1 decreased in Friesian cows by 41–74% when some granulated activated carbon was included in the diet at 2% (Richard and Payne, 2003). Chicken body weight and feed intake increased when activated charcoal was added to aflatoxin-contaminated diets (Dalvi and Ademoyero, 1984; Edrington *et al.*, 1997). In *in vitro* tests with 1% activated charcoal in the diet, ochratoxin A was completely adsorbed from aqueous solutions with a pH between 3 and 8 (Harvey *et al.*, 1991; Plank *et al.*, 1990).

In trials with superactivated charcoal, which differs from activated charcoal in its reduced particle size and therefore larger surface area, a median effective oral dose of 0.175 g superactivated charcoal per kg body weight prevented deaths in rats given a lethal oral dose of 8 mg T-2 toxin per kg body weight. Survival times and survival rates of rats given T-2 toxin increased if fed 1 g superactivated charcoal per kg body weight (Galey *et al.*, 1987). In mice, activated charcoal administered orally at 7 g/kg body weight either immediately or 1 hour after toxin exposure resulted in survival rates of 100% and 75%, respectively (Fricke and Jorge, 1990). These results are consistent with those of Bratich *et al.* (1990) who observed no lesions in female rats fed 25 mg/kg T-2 toxin and 936 mg/kg charcoal.

No adsorbent materials other than activated carbon bound deoxynivalenol and nivalenol (Binder, 2007). Inclusion of 2% activated carbon in the diet reduced *in vitro* deoxynivalenol adsorption from 51% to 28% and nivalenol adsorption from 21% to 12% (Avantaggiato *et al.*, 2004). The binding activity of activated carbon was higher for zearalenone than it was for these trichothecenes (Avantaggiato *et al.*, 2004). *In vitro*, activated carbon effectively reduced fumonisin levels, but these results were not confirmed in *in vivo* tests (Binder, 2007).

Activated charcoal binds many materials in a nonspecific manner, including important nutrients. For example, activated charcoal adsorbed 100% of the aflatoxin B_1 present, but also adsorbed most of the vitamin B_{12} (99%) and biotin (78%), which are not stored in animal tissues in appreciable amounts and must be regularly added to their diets. Thus, designing diets that are safe and contain activated charcoal and adequate nutrients is difficult (Vekiru *et al.*, 2007).

Cholestyramine

This anion-exchange resin is used pharmaceutically to decrease cholesterol. It can efficiently bind zearalenone, ochratoxin A, and fumonisins *in vitro* and *in vivo*, which is unusual since few adsorbent materials simultaneously bind multiple mycotoxins (Binder, 2007; Pettersson, 2004; Richard and Payne, 2003).

In *in vitro* tests, cholestyramine is the best adsorbent for zearalenone followed by crospovidone, montmorillonite, bentonite, sepiolite, and magnesium trisilicate (Ramos *et al.*, 1996). Cholestyramine–zearalenone complexes form rapidly, i.e., within 1 minute, under gastrointestinal conditions, are stable for 24 hours, and their stability is influenced by neither pH nor temperature (Ramos and Hernandez, 1996). In mice and rats fed diets containing different amounts of

cholestyramine, zearalenone adsorption was reduced 19–52% with the estrogenic effects, toxin urinary excretion, and renal and hepatic residues reduced as well (Avantaggiato *et al.*, 2003; Guerre, 2000; Richard and Payne, 2003; Underhill *et al.*, 1995). In rats, cholestyramine increased ochratoxin A excretion (Madhyastha *et al.*, 1992) and reduced ochratoxin A adsorption (Kerkadi *et al.*, 1998). It also inhibited the enterohepatic circulation of ochratoxin A in mice (Roth *et al.*, 1988). Of several adsorbents evaluated for their *in vitro* capacity to adsorb fumonisin B_1, cholestyramine was the best (Avantaggiato *et al.*, 2005; Solfrizzo *et al.*, 2001).

Polyvinylpolypyrrolidone

Adsorption of mycotoxins by PVPP is based on the hydration hull formed by the adsorbent. Polar particles are attracted to the hull and bound by the adsorbent. PVPP reduces aflatoxin toxicity by decreasing its adsorption in the gastrointestinal tract. In broiler chickens fed a diet containing 2.5 ppm aflatoxin and 0.3% PVPP, the impact of aflatoxins was significantly decreased (Kiran *et al.*, 1998). *In vitro*, 0.3 mg/g of zearalenone was adsorbed by PVPP, but *in vivo* tests of this adsorptive ability are lacking. PVPP did not reduce deoxynivalenol toxicity to pigs when fed as part of a deoxynivalenol-contaminated feed (Friend *et al.*, 1984; Pettersson, 2004).

Yeast Cell Walls and Components Thereof

Yeast cell walls, particularly those of *Saccharomyces cerevisiae*, are an environmentally friendly alternative to other adsorbents, because they are easily biodegraded (Yiannikouris *et al.*, 2005). The structure of these cell walls and the nature of the polysaccharides they contain, i.e., glucan and mannan, have been studied extensively. However, the role of these polysaccharides in the adsorption of mycotoxins has not been widely studied, and the available data are not consistent.

Yeast cell walls may form complexes with dietary toxins, thereby limiting toxin absorption in the digestive tract. The ability of yeast cell walls to bind toxins is increased by their large surface area. The β-D-glucan fraction of the yeast cell wall is directly involved in mycotoxin binding (Yiannikouris *et al.*, 2004, 2006), with the structural organization of the β-D-glucans modulating the binding strength (Jouany, 2007). Yeast glucomannan binds various mycotoxins *in vitro* and/or *in vivo* (Meissonnier *et al.*, 2009; Pettersson, 2004; Stanley *et al.*, 2004). Live yeast culture preparations can reduce aflatoxin-associated problems in several animal species, and supplementing feed with modified glucomannan at 1 kg/t of feed prevents T-2 toxin absorption (Girish and Devegowda, 2006). In other trials, feed supplemented with yeast glucomannan neither prevented nor ameliorated the oxidative damage caused by aflatoxins in broilers (Çinar *et al.*, 2008). Similarly, inclusion of nondigestible yeast oligosaccharides in aflatoxin-contaminated feed did not significantly alter aflatoxin M_1 levels in milk from lactating Holstein cows (Stroud, 2006).

The ability of yeast glucomannans to simultaneously bind multiple mycotoxins is particularly important. *In vitro* binding of aflatoxins, ochratoxin A, and T-2 toxin by a commercial esterified glucomannan was reduced when multiple toxins were present (Raju and Devegowda, 2002). Cumulative mycotoxin binding depended upon the mycotoxins present. Esterified glucomannan (0.05–0.1%) in chicken feed reduced or eliminated many of the combined toxic effects on performance, serum biochemistry, and hematology of aflatoxins, ochratoxin A, and T-2 toxin (Aravind *et al.*, 2003; Raju and Devegowda, 2000). Based on a pig trial, diets supplemented with glucomannan had protective effects against aflatoxin B_1 and T-2 toxin immunotoxicity (Meissonnier *et al.*, 2009). However, a diet fed to minks that contained 0.2% of another glucan polymer product did not alleviate the toxic effects associated with fumonisin B_1, ochratoxin A, moniliformin, and zearalenone (Bursian *et al.*, 2004).

Adsorption by Bacteria

Most studies of bacteria as adsorbents are based on *in vitro* tests. Some dairy strains of lactic acid bacteria and bifidobacteria can effectively bind aflatoxins, probably due primarily to their cell wall peptidoglycans and polysaccharides. In particular, some strains of *Lactobacillus amylovorus* and *L. rhamnosus* removed more than 50% of the aflatoxin B_1 from a sample (Peltonen *et al.*, 2001).

Probiotics, e.g., *L. rhamnosus* strain GG and a mixture of *L. rhamnosus* LC705 and *Propionibacterium freudenreichii* ssp. *shermanii* JS (LC705+JS), bound aflatoxin B_1 *in vitro* and under physiological conditions in the gut (Gratz, 2007). Six dairy strains of lactic acid bacteria reduced aflatoxin M_1 content in liquid media (Pierides *et al.*, 2000). This binding ability provides approaches for decontaminating foods and feeds and for reducing the bioavailability of aflatoxins in the diet, if these bacteria function as absorbents in the gastrointestinal tract.

Similarly, when zearalenone and its zearalenol derivative at 2 mg/ml were incubated with two strains of *L. rhamnosus*, ~55% of the toxins were bound immediately upon mixing with the bacteria (El-Nezami *et al.*, 2002b). The mechanism linking these toxins to the surface of bacteria did not require metabolic activity by living cells. Five *Propionibacterium*, three *Lactobacillus*, and two *Bifidobacterium* strains decrease toxin concentration *in vitro* after incubation with both viable and nonviable cells. The propionibacteria usually were the most efficient (El-Nezami *et al.*, 2002a). Strains of *Lactobacillus* and *Propionibacterium* also removed the trichothecenes deoxynivalenol, 3-acetyl-deoxynivalenol, nivalenol, fusarenone X, diacetoxyscirpenol, and T-2 toxin from liquid media (El-Nezami *et al.*, 2002a).

Probiotic lactic acid bacteria bind aflatoxin B_1 under *in vivo* conditions (El-Nezami *et al.*, 2000). *L. rhamnosus* strain GG reduced aflatoxin B_1 uptake by intestinal tissue by 74% during the first 60 minutes of exposure. Rats fed this *Lactobacillus* strain increased fecal excretion of aflatoxin B_1 and had reduced aflatoxin B_1-associated growth faltering and liver injury (Gratz *et al.*, 2006).

Other Organic Adsorbents

Other organic adsorbents, e.g., humic acid, divinylbenzene-styrene, chlorophyllin, alfalfa, and fibers, also have been studied. Oxihumate, i.e., pure, high-quality humic acid that differs slightly chemically from humic acids obtained from other sources, was highly effective in the amelioration of aflatoxicosis in broiler chickens fed aflatoxin-contaminated diets (van Rensburg *et al.*, 2006). Divinylbenzene-styrene reduced the adsorption and toxic effects of zearalenone (Guerre, 2000) and T-2 toxin in rats (Carson and Smith, 1983) when anion-exchange but not cation-exchange resins were used. The anion-exchange resin prevents zearalenone toxicosis by binding the toxin in the digestive tract and preventing absorption (Smith, 1980). Chlorophyllin is a potent dose-responsive inhibitor of aflatoxin I, DNA adduction, and hepatocarcinogenesis in rainbow trout (Breinholt *et al.*, 1995). Bleaching clays used to process canola oil lessened the effects of T-2 toxin (Richard and Payne, 2003).

Alfalfa and oat fibers reduced the estrogenic effects of zearalenone on rats (Jouany, 2007). Including alfalfa in the diet of rats and swine reduced the inhibitory effects of zearalenone on growth and feed consumption, minimized zearalenone-induced liver enlargement, increased hepatic 3α-hydroxysteroid dehydrogenase (3α-HSD) activity, reduced residual zearalenone in the liver, and decreased uterine enlargement (James and Smith, 1982). Thus, this feedstuff may be of use for treating zearalenone toxicosis in livestock. Micronized wheat fibers decreased the level of ochratoxin A in plasma, kidney, and liver of piglets fed a naturally contaminated diet and prevented the usual increase in kidney and liver weights associated with ochratoxin A-contaminated feeds (Aoudia *et al.*, 2009).

Biological Detoxification

Enzymatic or microbial degradation of mycotoxins, i.e., biotransformation, leading to less toxic or nontoxic metabolites has been studied for more than 40 years as a method for decontaminating crops. Microorganisms, including yeasts, filamentous fungi, and bacteria, have been screened for their ability to modify or inactivate various mycotoxins, but only a few microorganisms with mycotoxin-degrading ability have been found (Karlovsky, 1999). The first was *Flavobacterium aurantiacum*, which can detoxify aflatoxins (Ciegler *et al.*, 1966). The degradation products produced by this bacterium have been extensively characterized (Bata and Lásztity, 1999). Various yeast strains can partially degrade ochratoxin A, nivalenol, deoxynivalenol, zearalenone, and fumonisins (Styriak *et al.*, 2001).

Ochratoxin A is rapidly degraded by microorganisms in the rumen to ochratoxin α and phenylalanine (Hult *et al.*, 1976; Kiessling *et al.*, 1984) and by the aerobic bacterium *Phenylobacterium immobile* (Wegst and Lingens, 1983). *Acinetobacter calcoaceticus* can degrade ochratoxin A in an ethanol-containing medium (Hwang and Draughon, 1994). Various strains of *Lactobacillus*, *Bacillus,* and *Saccharomyces* could degrade up to 94% of the ochratoxin A present under *in vitro* conditions (Böhm *et al.*, 2000), but were much less effective in the degradation of trichothecenes. A *Trichosporon* yeast, *T. mycotoxinivorans*, isolated from a termite hindgut deactivated both ochratoxin A and zearalenone in animal feeds (Molnar *et al.*, 2004; Schatzmayr *et al.*, 2006). This yeast detoxifies ochratoxin A by cleaving the phenylalanine moiety from the isocoumarin derivative ochratoxin α. This metabolite is probably nontoxic, i.e., at least 500× less toxic than the parent compound (Bruinink *et al.*, 1998; Schatzmayr *et al.*, 2003). In feeding trials, inclusion of *T. mycotoxinivorans* in the diet blocked ochratoxin A-induced immune suppression in broiler chicks (Binder, 2007; Politis *et al.*, 2005). In further feeding trials with broiler chicks and a commercial toxin deactivator that contained *T. mycotoxinivorans*, the feed conversion ratio improved and there were less pronounced histological changes in kidneys, liver, bursa, and spleen in animals fed the toxin deactivator (Hanif *et al.*, 2008).

Zearalenone is not acutely toxic, but mimics the reproductive hormone estrogen and may cause fertility problems. Reduction of zearalenone to α- and β-zearalenols occurs in rumen fluid and in many mixed and pure cultures of bacteria, yeast, and filamentous fungi. This transformation, however, is not a detoxification as the zearalenols still have significant estrogenic activity. *Thamnidium elegans*, *T. mycotoxinivorans*, *Mucor baineri*, *Rhizopus* sp., *Streptomyces rimosus*, *Cunninghamella baineri*, and *Gliocladium roseum*, amongst others, can biotransform zearalenone to non-estrogenic compounds (El-Sharkawy and Abul-Haji, 1987, 1988; Kamimura, 1986; Schatzmayr *et al.*, 2003). The strain of *G. roseum* detoxified zearalenone by opening the ring followed by decarboxylation with yields of 80–90% (El-Sharkawy and Abul-Haji, 1988).

Numerous studies have been made of the biotransformation of trichothecenes, whose toxicity is usually attributed to their 12,13-epoxide ring. Reductive de-epoxidation by ruminal and intestinal microbes of pigs, hens, and rats (King *et al.*, 1984; Kollarczik *et al.*, 1994; Swanson *et al.*, 1987, 1988; Yoshizawa *et al.*, 1983) or by a strain of *Eubacterium* isolated from bovine rumen (Schatzmayr *et al.*, 2006) reduces toxicity. This *Eubacterium* strain, BBSH 797, is the first bacterial strain to be cultured, produced, and stabilized for use as a feed additive to reduce the effects of trichothecenes. The nontoxic metabolites produced when BBSH 797 biotransforms type A and type B trichothecenes indicate that deacetylation and de-epoxidation occur simultaneously when the bacterium is added to the culture medium (Fuchs *et al.*, 2002). In piglets fed 2 mg deoxynivalenol per kg and chickens fed 10.5 mg deoxynivalenol per kg, including the additive in the diet reduced mortality and increased weight gain (Binder *et al.*, 2001; Plank *et al.*, 2009). Feed additives containing BBSH 797 reduced

the adverse effects of 2 ppm T-2 toxin (Diaz *et al.*, 2005) and 1 ppm 4,15-diacetoxiscirpenol (Diaz, 2002) on feed intake and body weight of broiler chickens.

Other investigations of microbial biotransformation of trichothecenes have focused on aerobic soil bacteria. *Curtobacterium* sp. strain 114–2 can deacetylate T-2 toxin to the slightly less toxic metabolites HT-2 toxin and T-2 triol (Ueno *et al.*, 1983). A soil bacterium belonging to the *Agrobacterium–Rhizobium* group can biotransform deoxynivalenol to the less-toxic 3-keto-deoxynivalenol (Shima *et al.*, 1997). In one case, soil was incubated with deoxynivalenol and the toxin concentration decreased significantly, but a pure culture of the causal organism could not be obtained (He *et al.*, 1992).

Enzymes capable of degrading fumonisins have been isolated from a filamentous saprophytic fungus growing on maize (Blackwell *et al.*, 1999; Duvick, 2001). Some bacteria, including some in animal gastrointestinal tracts, also can metabolize fumonisins. One potentially useful strain of the Sphingomonadaceae degrades fumonisins by cleaving off the tricarballylic acid side chains and then catabolizing the rest of the molecule to nontoxic products (Hartinger and Moll, 2011).

Biological detoxification also has been tested in feeds contaminated with multiple mycotoxins. A mycotoxin-degrading enzyme completely or partially relieved multi-organ toxicity in pigs due to a combination of deoxynivalenol and zearalenone (Yeong-Hsiang *et al.*, 2006).

Biotechnology contributions to animal feed formulation and production are becoming increasingly important. Microbiological products, both enzymes and intact microorganisms, used in feed have increased dramatically and are prime examples of the need for new approaches to animal production. Isolation and characterization of microorganisms or enzymes that can biotransform mycotoxins could have major practical impacts by enabling specific decontamination processes to occur within an animal's intestinal tract. Such biological decontamination could become a technology of choice, as enzymatic reactions offer a specific, efficient, and environmentally friendly means of detoxification.

Ensiling mycotoxin-contaminated crops could potentially eliminate or reduce mycotoxin contamination, but normal ensiling has only rarely been evaluated for its mycotoxin control potential. In one study (Rotter *et al.*, 1990), ensiling ochratoxin-contaminated barley reduced toxin levels by approximately 68%. However, in feeding studies with chickens there was no improvement in performance or mortality relative to non-ensiled diets. Yeasts in grass silage degraded patulin in silage inoculated with a *Paecilomyces* strain that induces patulin production (Dutton *et al.*, 1984). Both bacteria and yeasts from maize silage can degrade fumonisins (Camilo *et al.*, 2000). Stimulation of mycotoxin degradation by naturally occurring microorganisms in silage or the addition of yeasts or bacteria with known mycotoxin biotransformation capabilities to silage could be a practical method for reducing the impact of mycotoxins in some crops.

Conclusions

Prevention and reduction of mycotoxin contamination during crop and feed production has become important. Good agricultural practices and HACCP in production processes are requirements, and the need for feed additives to prevent absorption by and toxic effects of mycotoxins on farm animals has increased significantly. Since feedstuffs are commonly contaminated with more than one mycotoxin, a good mycotoxin inactivator must be effective against multiple mycotoxins, should be incorporated in small amounts into complete diets, should have a high binding capacity for mycotoxins, and should be free of impurities and odors.

Both organic and inorganic binders are available to help manage mycotoxin problems safely, economically, and easily. Adsorbents that act in the animal's intestinal tract complement the physical and chemical methods commonly used to decontaminate raw materials. Adsorbents in the same category, e.g., clays, may have different physical and chemical properties and dramatically different efficacies. To assure efficacy and safety, all additives should be carefully tested before coming to the market, with *in vitro* tests used primarily for their scientific development and improvement. Moreover, critical parameters must be evaluated, including biochemistry, gross pathology, histopathology, and immunology.

Adsorption currently is not a viable control option for trichothecenes, zearalenone, or ochratoxins, but inactivation by biotransformation is a promising strategy for their detoxification. Indeed, biotransformation has the most potential of the emerging processes for mycotoxin deactivation and is in need of further investigation.

There are many factors that influence and complicate setting acceptable levels of mycotoxin contamination in different countries. Such regulations exist worldwide and the European Union has legislated maximum levels for some mycotoxins in foodstuffs. Most of the feed production industry has implemented production standards, which may be stricter than the legal requirements. The HACCP analysis of production practices required for compliance with EU Feed Hygiene legislation and the quality systems it triggers, e.g., FAMI-QS, are important requirements for operators in the European feed industry.

Mycotoxin contamination, however, still occurs despite strenuous efforts to prevent it. Therefore, there is an obvious need for mycotoxin-inactivating products to complement these efforts in the pursuit of HACCP goals and standards. Mycotoxin-inactivating products may reduce or close safety gaps that remain in spite of the current maximum limits, preventative actions, and analytical control.

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9 Physical and Chemical Methods for Mycotoxin Decontamination in Maize

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Abstract

Mycotoxins are fungal secondary metabolites associated with toxic effects in humans or domesticated animals. Mycotoxin contamination of foods and feeds is a worldwide problem. A number of strategies have been developed to prevent the growth of mycotoxigenic fungi and to decontaminate and/or detoxify mycotoxin-contaminated food and animal feed, including (i) prevention of mycotoxin contamination; (ii) detoxification of mycotoxins present in food and feed; and (iii) inhibition of mycotoxin absorption in the gastrointestinal tract. We focus on the physical and chemical methods relative to point (ii) in the maize grain chain with an emphasis on efficacy and safety. Only a few of these methods are in practical use, probably due to difficulties in complying with FAO requirements. There is no single method that can simultaneously remove all of the mycotoxins known to co-occur in maize.

Keywords: aflatoxins; ammoniation; cleaning; extrusion; fumonisins; irradiation; milling; nixtamalization; ochratoxins; ozonation; sodium bisulfate; sorting, trichothecenes; zearalenone

Introduction

Consumption of mycotoxin-contaminated food or feed may result in cancer, acute mortality, reproductive disorders, or growth impairment in humans and/or domesticated animals (Oswald and Comera, 1998). The Food and Agricultural Organization (FAO) of the United Nations estimates that up to 25% of the world's agricultural commodities are contaminated with one or more mycotoxins. Economic losses due to mycotoxin contamination are estimated as billions of US dollars annually worldwide (Kabak *et al.*, 2006).

Strategies that rely on the prevention of fungal infection to reduce fungal and mycotoxin contamination must be done at an integrative level along the entire food production chain. There are three possible intervention steps: (i) prior to fungal infestation; (ii) during fungal invasion of plant material; and (iii) when an agricultural product is identified as significantly contaminated. This hazard analysis is similar to the HACCP food safety management system.

Control efforts must focus on the two first steps since, once mycotoxins are present, it is very difficult to eliminate them. Unfortunately, the prevention of mycotoxin contamination prior to harvest or during postharvest and storage is not always possible. So, decontamination should occur before such materials are used for food and feed purposes.

Mycotoxin Reduction in Grain Chains, First Edition. Edited by John F. Leslie and Antonio F. Logrieco.

Guidelines for evaluating mycotoxin detoxification and decontamination procedures have been established by FAO. The process should (i) inactivate, destroy, or remove the mycotoxin; (ii) not result in the deposition of toxic substances, metabolites, or by-products in the food or feed; (iii) retain nutrient value and feed acceptability of the product or commodity; (iv) not significantly alter the product's technological properties; and if possible, (v) destroy fungal spores. The process(es) should be readily available, easily utilized, and inexpensive (Richard and Payne, 2003). We evaluate physical and chemical methods that meet most of these requirements and that can be used to reduce levels of aflatoxins, fumonisins, trichothecenes, zearalenone, and ochratoxins, in maize. We organize the chapter by method type, and within each type provide a brief description for each of the toxins for which it may be used, and include a table (Table 9.1) to provide a convenient check sheet.

Table 9.1 Toxicological evaluation of effective processes for maize decontamination

Processing method	Mycotoxin	Toxicity following treatment	References
Physical methods			
Extrusion	Fumonisins	Feeding trial (*rats*)—decreased kidney lesions	Bullerman and Bianchini (2007)
	Deoxynivalenol	Bioassay (*MTT assay*)—loss of toxicity on CHO-K1 (Chinese hamster ovary) cells	Cetin and Bullerman (2006)
	Zearalenone	Bioassay (*MTT assay*)—loss of biological activity on MCF-7 (human breast cancer) cells	Cetin and Bullerman (2005)
Chemical methods			
Ammoniation	Aflatoxins	Bioassay (*Ames assay*)—reduced mutagenicity	Park (1993)
		Feeding trial (*rats*, *ducks*, *pigs*, *chickens*, *and trout*)—reduced toxicity	Park *et al.* (1988)
	Fumonisins	Feeding trial (*rats*)—reduced weight gain; increased serum enzyme levels; increased the number of histopathological lesions	Norred *et al.* (1991)
Ozonation	Aflatoxins	Bioassay (*Hydra attenuata assay*)—no toxicity	McKenzie *et al.* (1997)
		Feeding trial (*turkeys*)—increased body weight gain; no liver discoloration; restoration of the biochemical parameters	McKenzie *et al.* (1998)
	Fumonisins	Bioassay (*Hydra attenuata assay*)—toxicity; elevated Sa/So ratio	McKenzie *et al.* (1997)
	Ochratoxin A	Bioassay (*Hydra attenuata assay*)—no toxicity	McKenzie *et al.* (1997)
	Zearalenone	Bioassay (*mouse uterine weight assay*)—uterine weights unaffected	Lemke *et al.* (1999)
Nixtamalization	Aflatoxins	Bioassay (*Ames assay*)—higher mutagenic activity for extracts from acidified tortillas	Price and Jorgensen (1985)
	Fumonisins	Feeding trial (*rats*)—hepatotoxic and nephrotoxic	Voss *et al.* (1996)
Modified nixtamalization	Fumonisins	Bioassay (*Ames assay*)—no mutagenicity	Park *et al.* (1996)
		Bioassay (*brine shrimp assay*)—reduced toxicity	
Sodium bisulfite	Deoxynivalenol	Feeding trial (*pigs*)—increased feed intake and body weight gain; no emesis	Young *et al.* (1987)
Reducing sugars	Fumonisins	Feeding trial (*pigs*)—increased body weight gain and feed intake; restoration of the biochemical parameters; no liver or kidney lesions; decreased Sa/So ratio	Fernandez-Surumay *et al.* (2005)
Citric acid	Aflatoxins	Bioassay (*Ames assay*)—reduced mutagenic activity	Mendez-Albores *et al.* (2005)
		Feeding trial (*ducks*)—increased body weight gain; restoration of the biochemical parameters; reduced severity of liver lesions	Mendez-Albores *et al.* (2007)

Physical Methods—Removal of Contaminated Materials

Sorting

Aflatoxins. Most mycotoxin contamination occurs on relatively few seeds, so separating damaged and discolored seeds can reduce toxin contamination (Afolabi *et al.*, 2006; Kabak *et al.*, 2006). Sorting maize based on fluorescence following illumination with UV light ($\lambda = 365$ nm) is common for the reduction of aflatoxin contamination. The bright greenish yellow (BGY) fluorescence observed results from a peroxidase transformation product of kojic acid, a metabolite of *Aspergillus flavus* and other fungi. This test, also known as the black light test, is quick and easy, but both false positives (BGY fluorescence and no toxin detected) and false negatives (toxin detected and no BGY fluorescence) may result (Bothast and Hesseltine, 1975). Thus, this test has now largely been abandoned.

More recently, another sorting method based on the preferential invasion of the oil-rich germ by mycotoxin-producing molds was proposed. This method uses the free fatty acid content of the grain as an index of incipient grain deterioration. By using a pair of filters, A750 and A1200, kernels with $>$100 ppb aflatoxin contamination can be distinguished from kernels with no detectable aflatoxin with $>$98% accuracy. In high-speed sorting of maize kernels with this technique, $\sim$5% of the grain was removed along with $\sim$82% of the aflatoxin from samples contaminated with $>$10 ppb aflatoxin (Pearson *et al.*, 2004).

Fumonisins. When a high-speed dual-wavelength sorter was used to sort fumonisin-contaminated maize, fumonisin levels were reduced by an average of 88%, including samples with low levels of fumonisin (Pearson *et al.*, 2004).

Sieving-Cleaning

Cleaning grain reduces mycotoxin contamination by removing kernels with extensive fungal growth, broken kernels, and fine materials such as dirt and debris.

Fumonisins. When maize screenings were sieved into eight different particle sizes, the screenings and broken kernels usually contained $\sim 10\times$ higher levels of fumonisins than did intact kernels (Murphy *et al.*, 1993). The "fines" particles that pass through a 3 mm mesh screen usually constitute 5–20% of a sample by mass. Removing the "fines" fraction reduced the total fumonisin level by 26–69% (Sydenham *et al.*, 1994).

Deoxynivalenol and zearalenone. Removing screenings and broken kernels from maize reduced deoxynivalenol and zearalenone contamination by 73% and 79%, respectively (Trenholm *et al.*, 1991), but up to 69% of the total weight of the maize was removed as well.

Removal of Contaminated Maize: Flotation and Density Segregation

Fungal-damaged, mycotoxin-contaminated kernels have different physical properties than non-damaged kernels and can be separated by density segregation or by fractionation on specific gravity tables. Since many fungi cause this type of damage, these techniques are sensitive to the degree of fungal contamination rather than the toxin(s) present.

Aflatoxins. Removing maize kernels that float in water reduced aflatoxin levels by 60% (Huff, 1980). Increasing the specific gravity of the solution by adding 30% sucrose, so more kernels float, not only reduced the aflatoxin content by 87%, but also removed 50% of the kernels (Huff, 1980). If

a saturated sodium chloride solution is used, then ~3% of the kernels are removed, but this fraction contains ~74% of the total aflatoxin in the sample (Huff and Hagler, 1985).

Deoxynivalenol. Removing maize kernels that float in an aqueous 30% sucrose solution removed >50% of the deoxynivalenol in the sample (Huff and Hagler, 1985).

Fumonisins. Removing maize kernels that float in a saturated salt solution removed >85% of the fumonisins present (Shetty and Bhat, 1999).

Washing with a Sodium Carbonate Solution

Washing methods are used to remove mycotoxins with some water solubility from the outer surface of grains. Sodium carbonate, usually at 0.1 M, is an inexpensive, widely available, nontoxic chemical that forms an aqueous alkaline solution in water. In a feeding trial, soaking the grain in a sodium carbonate solution reduced the toxicity of *Fusarium*-contaminated maize fed to pigs, as measured by increases in feed consumption and weight gain (Rotter *et al.*, 1995).

Deoxynivalenol. Soaking maize contaminated with deoxynivalenol at 25 mg/kg in a 0.1 M sodium carbonate solution for 24–72 hours removed 69–95% of the toxin (Trenholm *et al.*, 1992). The amount of toxin removed increased with soaking time.

Zearalenone. Zearalenone is a weak phenolic acid whose solubility is greatly increased in a sodium carbonate solution. The available data on the effectiveness of sodium carbonate solutions for reducing zearalenone contamination are inconsistent, with some reporting no detectable removal of zearalenone (Bennett *et al.*, 1980), and others reporting the removal of up to 87% of the zearalenone present (Trenholm *et al.*, 1992).

If either a washing or a buoyancy technique is used to reduce toxin contamination, then the grain must be dried (again) before it can be stored.

Dehulling

Dehulling removes the outer layers of grain by abrasion. Manual dehulling was required before grinding. Now, manual dehulling has generally been replaced by mechanical disc dehullers at commercial and semi-commercial scales. The efficacy of this procedure in removing mycotoxins depends on the extent of fungal penetration into the kernel.

Aflatoxins. The outer layers of maize kernels are the most susceptible to fungal attack and aflatoxin accumulation. Physical dehulling of maize reduces aflatoxin contamination by up to 92% (Siwela *et al.*, 2005). A similar reduction in aflatoxin contamination of naturally contaminated maize occurs when maize is processed to "muthokoi," a traditional dehulled maize dish in Kenya (Mutungi *et al.*, 2008).

Milling

There are two major milling processes for maize—dry and wet—with wet milling the major process used. In wet milling, the grain is resolved into different fractions. Thus, identifying the fractions that retain or accumulate mycotoxins is important so that they can be diverted to lower risk uses or decontaminated. During dry milling, mycotoxins usually are concentrated in the germ and bran fractions, while in the wet milling, the mycotoxins may be retained in the steep water or distributed among the by-products of the process (Bullerman and Bianchini, 2007, 2014).

Aflatoxin. Wet milling of aflatoxin-contaminated maize samples distributes 40% of the toxin to the steepwater solubles, 30–38% to the fiber, 6–10% to the germ, and 1% to the starch (Bennett and Anderson, 1978). Thus, most of the aflatoxin ultimately accumulates in the animal feed fractions, which then must be decontaminated by other processes, e.g., ammoniation (Bennett and Anderson, 1978).

Ochratoxin A. Following dry milling, ochratoxin A is concentrated in cleanings, bran, and other fractions derived from the seed coat (Scudamore, 2005).

Fumonisins. In wet milling, the water-soluble fumonisins are usually dissolved in the steep water or distributed to the gluten, fiber, and germ fractions, leaving no detectable toxin in the starch (Bennett and Richard, 1996). In dry milling, fumonisins are usually are concentrated in the bran and germ fractions. These fractions are commonly used as animal feed or for oil extraction, respectively, and the grits are relatively free of contamination (Bullerman and Bianchini, 2007).

T-2 toxin. Based on a laboratory simulation of wet milling, T-2 toxin is distributed to the steep and process water—66%, the germ—30%, and the starch—4% (Collins and Rosen, 1981).

Zearalenone. Following dry milling, zearalenone is usually concentrated in the germ and bran fractions, and the starch fraction is essentially free of the toxin (Bennett *et al.*, 1976).

Steeping

The first step in wet milling of maize, steeping, involves soaking the maize in water containing SO_2 to facilitate germ separation.

Ochratoxin A. This toxin remains in the steeped maize and does not move into the steep water (Wood, 1982).

Fumonisins. Steeping in a 0.2% SO_2 solution may reduce the fumonisin content of naturally contaminated maize (Pujol *et al.*, 1999), as the highly polar fumonisins migrate from the kernels to the steeping water.

Physical Methods—Mycotoxin Inactivation

Most mycotoxins are heat stable in the temperature range used for conventional food/feed processing, 80–121°C, so little or no toxin degradation occurs under normal boiling, frying, or pasteurization conditions. Extrusion cooking can be used to reduce mycotoxin contamination. The efficacy of the reduction depends mainly on the extruder temperature and transit time, with the highest reductions in mycotoxin concentrations usually occurring at temperatures $\geq$160°C and when the time of transit is increased (Bullerman and Bianchini, 2014). Flaking, roasting, toasting, and canning of the grain or flour also can be used to reduce mycotoxin contamination.

Aflatoxins. Extrusion alone can reduce aflatoxin content in naturally contaminated maize by 50–80% and with the addition of ammonia, either as hydroxide (0.7 and 1.0%) or as bicarbonate (0.4%), by >95% (Bullerman and Bianchini, 2007). Roasting also is effective in the detoxification of aflatoxin-contaminated maize and is more efficient when it is combined with ammonia treatment (Conway *et al.*, 1978).

Fumonisins. Extrusion can reduce fumonisin levels in maize and maize grits by 34–95% depending on the extrusion parameters (Bullerman and Bianchini, 2007). The addition of sugars to the mixture to be extruded can decrease fumonisin stability during the extrusion of maize grits (Bullerman and Bianchini, 2007). Both screw speed and glucose concentration affect the extent of fumonisin B_1 reduction in extruded grits, with the highest reductions, up to 93%, observed at low screw speeds and

high glucose concentrations. Extrusion may modify the mycotoxin's chemical structure or enable interactions with the matrix that are neither detectable nor quantifiable by either HPLC or ELISA methods focused on the toxin. For example, Bullerman and Bianchini (2007) found that fumonisin B_1 extruded with 10% glucose formed *N*-(1-deoxy-D-fructos-1-y1) fumonisin B_1, a compound that is less toxic to rat kidney than is fumonisin B_1 (Table 9.1).

Similarly, roasting maize meal and maize flake processing in the presence of glucose resulted in >85% reduction in fumonisin B_1 content (Bullerman and Bianchini, 2007). Reduction of fumonisins by 60% and 100% was also reported after heating dry maize meal at 190°C or 220°C, respectively (Scott and Lawrence, 1994).

Deoxynivalenol. Deoxynivalenol contamination in maize is reduced by >95% under virtually all extrusion conditions tested—15–30% moisture content, 150–180°C, and ± sodium metabisulfite (Bullerman and Bianchini, 2007). The resulting degradation products are less toxic than deoxynivalenol in an *in vitro* cellular toxicity assay (Cetin and Bullerman, 2006; Table 9.1).

Zearalenone. Zearalenone content of maize grits also is reduced by extrusion from 60% to 83%, depending on the cooking temperature (Bullerman and Bianchini, 2007). As with deoxynivalenol, the degradation products have less *in vitro* toxicity than the original toxin (Cetin and Bullerman, 2005; Table 9.1).

Irradiation

Ionizing radiation, i.e., solar radiation, γ-irradiation, and microwaves, can be used to eliminate pathogenic microorganisms in foods. Solar irradiation is a particularly attractive, inexpensive option in many tropical areas, although γ-irradiation is the most widely evaluated technique.

Aflatoxins. Solar irradiation may be more effective than either γ-irradiation or microwave heating, with >60% of the aflatoxins present degraded after 30 hours of exposure to sunlight (Herzallah *et al.*, 2008). The reduction in aflatoxin contamination depends on the length of exposure to the sunlight.

A γ-radiation dose of 20 kGy to yellow maize suffices to destroy 40–100% of the aflatoxin present (Aziz and Youssef, 2002; Herzallah *et al.*, 2008).

Ochratoxin A. Published results on the stability of ochratoxin A to ionizing radiation are mixed. Pure ochratoxin is stable to 75 kGy of γ-irradiation (Paster *et al.*, 1985), but 20 kGy of γ-irradiation suffices to detoxify most of the ochratoxin A in yellow maize samples (Aziz and Youssef, 2002).

Fumonisins. A dose of 15 kGy of γ-radiation sterilizes maize flour and decreases fumonisin content by ∼20%. Following the radiation treatment, the remaining fumonisins are stable in the irradiated maize flour for at least 6 months at 25°C or at least 4 weeks at 40°C (Visconti *et al.*, 1996).

Deoxynivalenol. Other ionizing radiations, e.g., microwaves and convection heat, lower the amount of this toxin persisting in naturally contaminated maize (Young, 1986).

Zearalenone. This toxin is destroyed in yellow maize at a dose of 20 kGy (Aziz and Youssef, 2002).

Physical Methods—Conclusions

Some physical methods were developed specifically for mycotoxin decontamination, e.g., sorting, cleaning, and washing, whereas others were developed for other purposes, e.g., milling, irradiation, and extrusion, and then adapted to reduce mycotoxin contamination. The first set of techniques

focuses on removing mycotoxins from maize, while the second set focuses on either destroying the toxins or sequestering them in a disposable or low-value material. These techniques are generally efficient and meet most of the FAO guidelines, i.e., inexpensive, simple, no production of toxic metabolites, and no change in the nutritional value or the properties of the raw materials. Nonetheless, there are problems with the application of these techniques on a commercial scale which are as follows:

- Sieving and cleaning can reduce the total mycotoxin content significantly, but may simultaneously remove a large portion of the grain.
- Following flotation and washing, the cost of drying the grain is significant. Thus, these techniques are limited to use "just prior" to a manufacturing process that requires the grain to be wetted or tempered, e.g., wet milling or alkaline processing of maize.
- All of the treatments require time, complicate the process, and increase the cost of processing the grain.
- The contaminated waste, e.g., broken kernels, residues, and dust, resulting from the decontamination procedures often are highly contaminated and must be destroyed rather than being used as animal feed.

Processes in the second category, e.g., milling, thermal treatments, and irradiation, commonly are used in the commercial production of human food and animal feeds. Problems encountered with the milling processes include the differential toxicity of the fractions resulting from grain separation. For example, mycotoxins usually are concentrated in the bran and germ fractions that are used in animal feed, and these fractions must be decontaminated before use. Heat treatments may result in the formation of unknown biologically active mycotoxin degradation products or the reversible binding of the toxin to sugar or proteins in the food/feed matrix (Humpf and Voss, 2004). Chemical studies and appropriate bioassays are needed to identify and characterize these "hidden" or "masked" mycotoxins. Finally, the blending of contaminated grain with batches of good quality material is now prohibited in the European Union (Verstraete, 2008), in spite of its effectiveness.

Chemical Methods

Ammoniation

Treatment of maize meal and other products with ammonia in the gaseous phase, or with substances capable of releasing it, effectively detoxifies them. The efficacy of mycotoxin detoxification with ammonia is positively correlated with the quantity of ammonia used, the reaction time, and the temperature and pressure levels.

Aflatoxins. Chemical inactivation of aflatoxin by ammoniation was first reported for cottonseed and peanut meal. Currently, it is the method of choice for the detoxification of aflatoxin-contaminated agricultural commodities and feeds in many locations. At least five types of ammoniation processes have been described or patented, but the two considered the most practical are a high-temperature/high-pressure process used by commercial treatment plants and an atmospheric pressure/ambient temperature process for *in situ* farm usage. Both procedures reduce mycotoxin contamination by up to 90%.

Aflatoxin B_1 degradation by ammonia proceeds through hydrolysis of the lactone ring, followed by decarboxylation to aflatoxin D_1 and loss of the cyclopentone ring yielding a compound with a molecular weight of 206 Da. To be effective, the ammoniation must last long enough to ensure the

hydrolysis of the lactone ring, as the process is reversible up to that point. Addition of formaldehyde reduces the amount of ammonia required for success and eases the irreversible breaking of the lactone ring. The metabolites resulting following ammoniation are less toxic than aflatoxin B_1, as demonstrated in bioassays, in many species and in dairy cattle (Hoogenboom *et al.*, 2001; Park, 1993; Park *et al.*, 1988; Table 9.1).

Fumonisin B_1. Up to 70% of fumonisin B_1 in grain may be degraded by ammoniation at high pressure/ambient temperature or at atmospheric pressure/high temperature (Park *et al.*, 1992), but this process did not reduce the toxicity of the treated material (Table 9.1).

Deoxynivalenol and zearalenone. Ammoniation of maize also reduces deoxynivalenol (Young, 1986) and zearalenone (Chełkowski *et al.*, 1981) levels, but toxicity studies with the decontaminated material have not been reported.

Ozonation

Ozone is a powerful oxidizing agent capable of reacting with numerous chemical groups, though it has an affinity for carbon–carbon double bonds. Ozone decomposes to form oxygen and is classified as a nonpersistent chemical. It is currently used as an industrial disinfectant and has been evaluated for its potential to reduce mycotoxins in agricultural products.

Aflatoxins. Ozone can potentially degrade aflatoxins, as it reacts with the 8,9-double bond of the furan ring through an electrophilic attack. McKenzie *et al.* (1997) developed a continuous source of ozone gas through electrolysis. Total degradation of aflatoxin B_1 in solution occurred within 15 seconds when using 20 wt% ozone. The same process also was used to decontaminate bulk maize contaminated with high levels of aflatoxin B_1. In another study, aflatoxins were reduced by 95% in maize treated with 14 wt% ozone for 92 hours at a flow rate of 200 mg/min (McKenzie *et al.*, 1998). Turkeys fed ozone-treated contaminated maize did not show signs of aflatoxicosis (Table 9.1). One risk of the ozonation process is the formation of fat-soluble reaction products with low mutagenic potential (Prudente and King, 2002).

Fumonisins. In aqueous solutions of fumonisin B_1 treated with 10 wt% ozone gas for 15 seconds, fumonisin B_1 was converted to 3-keto-fumonisin B_1 (McKenzie *et al.*, 1997). Unfortunately, 3-keto-fumonisin B_1 is nearly as toxic as fumonisin B_1 (Table 9.1), so further degradation of the ozonated product is needed.

Trichothecenes. Moisture appears to be essential in the reaction between deoxynivalenol and ozone. Moist ozone (1.1 mol%) in air resulted in a 90% reduction of deoxynivalenol in maize contaminated with deoxynivalenol at 1000 ppm after 1 hour, while dry ozone resulted in only a 70% reduction (Young, 1986). There also are differences between wheat and maize, as ground maize is more amenable to treatment than whole wheat kernels (Young *et al.*, 2006). Aqueous ozone can degrade several trichothecenes probably through the addition of two oxygen atoms to the C9—10 double bond (Young *et al.*, 2006). The toxicity of the breakdown products and the efficiency of the process remain to be evaluated.

Zearalenone. Ozone rapidly degrades zearalenone in water (Lemke *et al.*, 1999) and prevents its estrogenic effects (Table 9.1). In an *in vitro* study, zearalenone was undetectable after 15 seconds of ozone treatment in an aqueous solution, with no new products observed after the treatment (McKenzie *et al.*, 1997).

Ochratoxin A. Treatment of an aqueous solution of ochratoxin A with 10 wt% ozone for 15 seconds reduced the mycotoxin level to undetectable levels, and a bioassay revealed a decrease in the toxicity (McKenzie *et al.*, 1997; Table 9.1).

The commercial Oxygreen® process claims to reduce mycotoxin levels in commodities to levels beneath regulatory limits. This procedure reduced ochratoxin A contamination in grain by 94%. The toxicity of this process was evaluated in a 4-week study of rats fed treated wheat, with the treated wheat considered safe for consumers (Gaou *et al.*, 2005).

Nixtamalization

Nixtamalization, a traditional Mexican process for making tortillas, consists of cooking maize grain in excess water and lime (calcium hydroxide, $Ca(OH)_2$), at temperatures near boiling, followed by steeping.

Aflatoxins. Nixtamalization reduces aflatoxin content by 90–95%, but this reduction can be reversed by an acid treatment. Presumably the alkali opens the lactone ring and the acidic conditions result in its reformation (Price and Jorgensen, 1985). Acidification of aflatoxin extracts, to a pH similar to that in the stomach, enabled aflatoxin reformation (Table 9.1).

Fumonisins. Under alkaline conditions, fumonisins in contaminated maize are converted to hydrolyzed fumonisins. Nixtamalization of maize reduced the fumonisin B_1 levels in tortillas by 50–82%, with most of the fumonisin B_1 and hydrolyzed fumonisin B_1 found in the steeping and washing water (Dombrink-Kurtzman *et al.*, 2000; Palencia *et al.*, 2003). However, when fed to rats, nixtamalized maize that contains hydrolyzed fumonisin B_1 is still toxic (Voss *et al.*, 1996; Table 9.1). Although this toxicity was originally attributed to the hydrolyzed fumonisins, further work (Voss *et al.*, 2009) suggests that the observed toxicity was mediated by residual or "hidden" fumonisin B_1 (matrix bound forms not detected by HPLC) rather than hydrolyzed fumonisin B_1. Further decontamination and detoxification of fumonisin B_1-contaminated maize can occur if the nixtamalization procedure is modified. Other treatments of fumonisin B_1-contaminated maize that are similar to nixtamalization reduce the toxicity of contaminated feed (Table 9.1).

Zearalenone and deoxynivalenol. Boiling contaminated maize in lime water removed 72–82% of deoxynivalenol, 100% of 15-acetyl-deoxynivalenol, and 59–100% of zearalenone (Abbas *et al.*, 1988). No data on the safety of the treated feed, originally contaminated with either deoxynivalenol or zearalenone, has been reported.

H_2O_2/$NaHCO_3$

The addition of oxidizing agents, e.g., H_2O_2, can increase the effectiveness of nixtamalization. A combination of heat treatment with H_2O_2/$NaHCO_3$ and $Ca(OH)_2$ completely eliminated fumonisin B_1 contamination (100 ppm in maize) and reduced toxicity (Park *et al.*, 1996; Table 9.1). Treatment with H_2O_2/$NaHCO_3$ alone can efficiently degrade aflatoxins, fumonisins (Lopez-Garcia *et al.*, 1999), and zearalenone (Abd-Alla, 1997) in contaminated maize and is simple enough to be integrated into industrial maize-processing procedures.

Sodium Bisulfite

Sodium bisulfite is a common food additive.

Aflatoxins. When sodium bisulfite is used at 0.5% and 2% to treat maize, it reduces aflatoxin B_1 levels by 80% and 90%, respectively (Doyle *et al.*, 1982). The main product of the reaction is a

sulfonate, aflatoxin B_1 sulfonate, which results from the addition of bisulfite to the furan ring present in aflatoxin B_1 and G_1, but not in aflatoxin B_2 and G_2. Although this process is less efficient than ammoniation (Piva *et al.*, 1995), it overcomes some of the typical disadvantages of ammoniation, e.g., less hazardous to handle, no off colors in the treated feed, no impact on nutrient value, and cost.

Deoxynivalenol. Sodium bisulfite also reduces deoxynivalenol content in maize by 85–95% (Young *et al.*, 1987) with the greatest reductions occurring when the contaminated maize is autoclaved for 1 hour at 121°C in the presence of 8.3% aqueous sodium bisulfite. Deoxynivalenol-sulfonate, the by-product obtained after treatment, when administered orally to swine did not cause severe emesis (Table 9.1). Deoxynivalenol-sulfonate is stable in acid, but may be hydrolyzed to deoxynivalenol under alkaline conditions.

Reducing Sugars

Fumonisins. The primary amine group in fumonisin B_1 is responsible for its toxicity (Fernandez-Surumay *et al.*, 2005). Transformation of fumonisin B_1 into a fumonisin B_1-reducing sugar adduct through a nonenzymatic browning reaction (65°C for 48 hours) significantly reduces the toxicity of the resulting material on rodents and swine (Fernandez-Surumay *et al.*, 2005; Lu *et al.*, 1997; Table 9.1). Fumonisin B_1-glucose might be a detoxification strategy when widespread fumonisin contamination occurs (Fernandez-Surumay *et al.*, 2005).

Citric Acid

Aflatoxins. Up to 96% of aflatoxin B_1 (93 ppb) in contaminated maize was degraded when treated with an aqueous citric acid solution (Mendez-Albores *et al.*, 2005), with a concomitant decrease in toxicity (Table 9.1). Citric acid treatment is probably less effective for whole grain than for ground maize, since any toxin inside the whole kernels is less likely to be exposed to the treatment than toxin in the ground maize particles. Toxicity of aflatoxin-contaminated feed was reduced in ducks when aqueous citric acid was added to the feed (Mendez-Albores *et al.*, 2007; Table 9.1).

Other Chemical Treatments

Aflatoxin B_1. Butylated hydroxytoluene (BHT) treatment reduced hepatocellular necrosis, biliary hyperplasia, and elevated serum enzymes commonly associated with aflatoxin B_1 intoxication in turkeys. The reduction in pathological symptomatology is consistent with a physiologic protective effect (Coulombe *et al.*, 2005). These results are also consistent with dietary BHT's ability to reduce aflatoxin B_1 bioavailability, aflatoxin B_1–DNA adduct formation in the liver, and aflatoxin B_1 residues in tissues (Guarisco *et al.*, 2008).

Deoxynivalenol. Deoxynivalenol is completely destroyed in maize contaminated with 1000 ppm of deoxynivalenol treated with 30% chlorine (v/v) for 30 minutes (Young, 1986). This treatment might be too drastic for grain destined for human consumption, but might be useable for treating animal feeds.

T-2 and HT-2 toxins. Calcium hydroxide monomethylamine can be used to treat maize contaminated with these toxins (Bauer, 1994).

Chemical Methods—Conclusions

Although chemical detoxification methods could greatly reduce mycotoxin contamination, they do not now satisfy all of the FAO requirements, thus limiting their widespread use. Critical points remaining include the following:

- Identification of mycotoxin degradation/transformation products and suitable methods for their detection. For example, problems with "hidden" mycotoxins, such as those that survive the nixtamalization of fumonisin B_1-contaminated feed (Voss *et al.*, 2009).
- Bioassay and toxicological studies in target species assessing the safety of the treatment and evaluating the fate of processed contaminated food/feed after ingestion. For example, reversion of a mycotoxin to its native toxic form may occur, as happens for aflatoxin following nixtamalization of aflatoxin-contaminated feed (Price and Jorgensen, 1985).
- Assessment of processing impact on nutrient content or on the fate of nutrients post-processing. For example, reduction in the content of some amino acids, e.g., cysteine, methionine, and lysine, and an increase in total nitrogen and nonprotein nitrogen are among the effects attributed to ammoniation (Piva *et al.*, 1995).
- Handling the chemicals involved in detoxification may put workers' health at risk. At this time chemical treatment of commodities destined for human food is not allowed within the European Community.

Finally, any practical procedure must act efficiently and simultaneously on several types of mycotoxins without leaving residual toxicity. Based on the criteria and results summarized above, ozonation and modified nixtamalization are currently the best candidate processes.

Conclusions

Detoxification efforts for mycotoxins have focused primarily on aflatoxins, with numerous strategies to decontaminate aflatoxin-contaminated materials both reported and reviewed. In comparison, the available information on methods to detoxify other mycotoxins is limited. Contamination by multiple mycotoxins is common in commodities. As the impact of these toxins is recognized, successfully removing them from the food/feed supply is becoming a larger and more visible area of research. The effectiveness of a mycotoxin detoxification method depends on the nature of the food/feed, environmental conditions, e.g., moisture content and temperature, the type and concentration of the mycotoxin, and the extent of binding between a mycotoxin and other sample components. A number of processes are effective for one or a few compounds, but no single physical or chemical method can simultaneously remove all of the mycotoxins that might be present in a food or a feed.

Other approaches based on mycotoxin adsorption and biotransformation, which act in the gastrointestinal tract of animals, have been reported (Jans *et al.*, 2014). Thus, when prevention of mycotoxin contamination prior to harvest or during postharvest and storage is not possible, an integrated, multipronged approach will be the best strategy, with a physical and/or chemical method used to lower the mycotoxin content of the raw material followed by adsorption and/or biotransformation protocols to reduce the bioavailability of mycotoxins during digestion.

To conclude, mycotoxin contamination of food/feed remains a global problem. The decontaminated or detoxified crops usually are considered low quality and sell for less than uncontaminated, untreated material. This price differential limits the practical use of the available decontamination

and detoxification methods, which cost money and result in a product whose current primary use is as animal feed.

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10 Aflatoxin B_1 Chemoprevention Strategies in Countries with Frequent Exposure to Mycotoxins

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Abstract

Aflatoxin, and in particular aflatoxin B_1, is a major contaminant of maize and peanuts produced by several species of the fungus *Aspergillus*. More than 90% of people living in sub-Saharan Africa and portions of Asia are chronically exposed to aflatoxins at high levels. At the highest levels, aflatoxins can be toxic to humans and domesticated animals, although such incidents of aflatoxicosis are not common. At somewhat lower levels aflatoxins are a primary cause of liver cancer, especially when combined with infection by the hepatitis B virus, leading to tens to hundreds of thousands of cases of liver cancer annually. At still lower levels, aflatoxins have a deleterious effect on both the immune systems and the growth and development of children. Reliable biomarkers in humans enable the assessment of aflatoxin exposure in diverse populations. Oltipraz, sulforaphane, and green tea polyphenols all can alter aflatoxin B_1 metabolism after it has been taken up from the diet. Uptake inhibitors such as NovaSil® clay, chlorophyllin, and probiotics may bind aflatoxin and prevent uptake of the toxin from the gut. Clinical trials for all of these treatments have provided "proof of concept" confirmation of effectiveness, but none have been tested at a population level. The need to make consistent day-to-day changes in diets and the costs to both educate the populace and provide a sufficient supply of the necessary control items will complement widespread testing and implementation of any of these potential control measures. Long-term success with efforts to reduce smoking suggests that such changes are possible, but success in making these changes should not be expected to occur quickly, easily, or cheaply.

Keywords: aflatoxins; biomarkers; cancer; children; China; chlorophyllin; clinical trials; growth faltering; hepatitis B; humans; immune system suppression; liver; maize; oltipraz; peanuts; polyphenols; pregnancy; probiotics; sub-Saharan Africa

Introduction

Fungal growth and subsequent contamination of foodstuffs with fungal toxins, also known as mycotoxins, is frequent in all regions of the world (Miller, 1995), with up to 25% of crops intended for human consumption contaminated (CAST, 2003). Mycotoxins present both economic and health risks, though the major burdens are restricted to less developed regions of the world. The mycotoxins of major concern for human health are produced by three main genera of fungi: *Aspergillus*—which produces aflatoxins and ochratoxin A, *Fusarium*—which produces fumonisins, zearalenone, and

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trichothecenes, and *Penicillium*—which produces ochratoxin A. In this review I focus on the frequently occurring hepatotoxic and carcinogenic aflatoxins. Fungal growth and aflatoxin contamination can occur during crop growth, harvesting, or storage. Aflatoxin is commonly found on crops at the time of harvest and increases significantly during prolonged storage (CAST, 2003; Miller, 1995). Temperature and humidity are important factors affecting fungal growth and aflatoxin production; however, the risk of human exposure also is strongly related to the regulatory infrastructure and the financial capacity to enforce suitable monitoring and remove contaminated crops. Indeed the ability of the wealthy countries to monitor and regulate mycotoxin levels in imported cereals weakens trade opportunities for some of the poorer regions of the world, and the poorer members of populations that produce contaminated crops are increasingly obliged to consume the most heavily contaminated or poorest quality of their own crops.

A number of intervention activities to restrict exposure to aflatoxins, including breeding resistance, preharvest, harvest, and postharvest activities, are possible and are discussed elsewhere in this volume (Bullerman and Bianchini, 2014; Channaiah and Maier, 2014; Duvellier *et al.*, 2014; Grenier *et al.*, 2014; Jacobsen, 2014; Jans *et al.*, 2014; Lanubile *et al.*, 2014; Magan *et al.*, 2014; Mesterházy, 2014a, b; Munkvold, 2014; Raiola and Ritieni, 2014). This review focuses on chemoprevention approaches, with agents that limit the formation of the reactive metabolites of aflatoxin or the bioavailability of the toxin. Other approaches will not be discussed further here, although it is important to emphasize two points. None of the individual intervention approaches are likely to completely restrict exposure, and thus combinations of intervention strategies are required. Second, all of the approaches will not be applicable in all settings, and it is important that appropriate, sustainable combinations of interventions are sought that reflect the needs and capacity of the groups at risk.

This review provides a brief overview of the toxicity of aflatoxins, aflatoxin metabolism, and the biomarkers used to assess aflatoxin exposure. The latter are particularly important in terms of understanding the efficacy of the chemoprevention approaches. However, the main focus is on the success of animal studies that informed the development of clinical trials and how the data from such trials need to be taken forward. An obvious, but critical, difference between animal trials and clinical trials needs to be emphasized. Animal trials are conducted under controlled conditions—strain, diet, activity, toxin dose, and intervention dose—but the same control is not possible in a clinical trial. Of the many differences, physiological, genetic, etc., the heterogeneous distribution of aflatoxins even in small rural village settings in which there is only modest dietary variation between households deserves special mention. Thus, aflatoxin exposure surveys even in geographically similar and adjacent regions often reveal large variations despite the apparent similarities of a common diet. Thus, it is not trivial to design and implement successful chemoprevention clinical trials that modify metabolism and or bioavailability, which makes the successes even more noteworthy. For more in-depth exploration of the mechanistic aspects of aflatoxin metabolism and toxicity, a number of reviews are available (Groopman *et al.*, 2005; Kensler *et al.*, 2011; Turner *et al.*, 2012; Wild and Turner, 2001, 2002).

Aflatoxins

Aflatoxins are a family of highly toxic, carcinogenic secondary metabolites produced by several species of *Aspergillus*. As many as 4.5 billion people live in regions where aflatoxin exposure can occur in food (Williams *et al.*, 2004). In developing regions of the world, aflatoxins are a frequent contaminant of maize and peanuts consumed by local populations (CAST, 2003; Miller, 1995). The most frequently occurring and the most carcinogenic is aflatoxin B_1, although aflatoxins B_2, G_1, and

Table 10.1 Aflatoxin–albumin adduct level and frequency in selected geographic regions of the world

Country	*N*	% Pos	AF-alb (pg/mg) mean	Range	Age group	Reference
Benin and Togo	479	99	33	(nd–1064)	Child	Gong *et al.* (2002)
Benin[a]	200	98	37	(nd–688)	Child	Gong *et al.* (2004)
	200	99	39	(nd–744)		
	200	100	88	(5–1568)		
Brazil	50	62	15*	(nd–57)	Adult	Scussel *et al.* (2006)
China – Quangxi	143	69	39	(nd–437)	Adult	Wild *et al.* (1992)
– Shangdong	69	0				
– Qidong	362	95	ns	(1–10)**	Adult	Kensler *et al.* (1998)
Egypt	46	67	6*	(nd–33)	Adult	Turner *et al.* (2008)
	19	0			Adult	Wild *et al.* (1992)
France/Poland	74	0			Mixed	Wild *et al.* (1992)
Ghana	785	>75	11	(nd–269)	Adult	Shuaib *et al.* (2010)
	151	100	2		Adult	Wang *et al.* (2008)
Guinea	124	96	9	(nd–262)	Child	Turner *et al.* (2005a)
	300[b]	67	5	(5, 6)	Adult	Turner *et al.* (2005b)
	300[b]	88	15	(10, 25)	Adult	
	300[b]	98	19	(17, 21)	Adult	
Kenya	155	65	43	(nd–580)	Mixed	Wild *et al.* (1992)
Nepal	46	15	9	(nd–18)	Adult	Wild *et al.* (1992)
Taiwan – Case	230	94	57	(54)***	Adult	Wu *et al.* (2009)
– Cont	1052		60	(60)	Adult	
Taiwan	264[c]	ns	22	(5–356)***	Adult	Ahsan *et al.* (2001)
	264[c]	ns	14	(5–205)	Adult	
Thailand – V	60	62	12	(nd–47)	Adult	Vinitketkumnuen
– NV	100	22	7	(nd–50)	Adult	*et al.* (1997)
	160	11	12	(nd–50)	Adults	Wild *et al.* (1992)
The Gambia	119	100	40	(5–261)	Preg	Turner *et al.* (2007)
	99	49	10	(5–190)	Cord	
	118	11	9	(5–30)	16 weeks	
	466	93	24	(nd–456)	Child	Turner *et al.* (2003)
	444	100	41	(3–459)	Child	Turner *et al.* (2000)
– R	272	100	62	(57, 67)	Mixed	Wild *et al.* (2000)
– PU	84	100	39	(31, 49)	Mixed	
	391	83	57	(nd–720)	Child	Allen *et al.* (1992)

Thailand: V, vegetarian; NV, not vegetarian. The Gambia: R, rural; PU, peri-urban (mean and 95% confidence interval). Taiwan case control – mean and standard deviation provided. The absolute mean levels and ranges in all studies cannot be directly compared as different methodologies are used that have not been cross-referenced. The major observations are the frequencies of detection and the large range of adduct levels.

[a] 200 children measured at three time points within an 8-month period.

[b] 300 adults measured at three time points within a 6-month period (mean and 95% confidence interval).

[c] 264 adults measured at two time points separated by on average more than 1.5 years.

*Mean of positives only; **units are pmol/mg; ***units are fmol/mg.

G_2 are also potent toxins; however, this review will focus only on aflatoxin B_1. The use of validated aflatoxin exposure biomarkers in population surveys suggests that >90% of people living in some regions of Asia and sub-Saharan Africa are frequently exposed to aflatoxin at high levels (Table 10.1). Individuals who live in countries such as Thailand, Brazil, and Egypt have more moderate aflatoxin exposure, but sufficient to be a health concern, while those living in European/Northern American regions typically have low and infrequent exposure to aflatoxins.

The liver is the major, but not exclusive, target for aflatoxin toxicity. Acute aflatoxicosis leads to liver damage and at high doses can be fatal. In the last few decades, a number of incidents have been reported both in the technical literature and in the popular press, with the most recent major outbreaks occurring in Kenya. In one incident alone more than 100 individuals (39% of the affected study population) consumed fatal quantities of aflatoxin (Azziz-Baumgartner *et al.*, 2005). Chronic aflatoxin exposure also significantly contributes to the global burden of liver cancer incidence and deaths (IARC, 1993), particularly in populations with chronic infection with hepatitis B virus. Aflatoxin-related deaths due to liver cancer are estimated to be in the tens to hundreds of thousands annually (Liu and Wu, 2010). In animals, aflatoxins also retard growth and suppress the immune system (Bondy and Pestka, 2000; Pier *et al.*, 1977). There is increasing evidence that aflatoxins have a deleterious effect on both the immune system and the growth of infants and children (reviewed by Gong *et al.*, 2008; Turner *et al.*, 2012). Thus the true global health burden of aflatoxins is yet to be determined. The studies on growth retardation include both cross-sectional and longitudinal surveys of young children (Gong *et al.*, 2002, 2004; Turner *et al.*, 2003) and cross-sectional and longitudinal studies of maternal exposure during pregnancy and the effects on birth weight and longitudinal growth during the first year (Shuaib *et al.*, 2010; Turner *et al.*, 2007). The number of children at risk of immune toxicity (Turner *et al.*, 2003) or growth faltering likely dwarfs that of those affected by either acute poisoning or cancer. Clearly there is a strong justification and need for intervention.

Chemoprevention

No single approach suffices to completely prevent the contamination of cereals with mycotoxins. Thus, one important strategy is to accept that the diet will be contaminated and develop interventions that modulate toxicity by either altering the metabolism of the toxin or reducing the adsorption of the toxin in the GI tract. Collectively these strategies are known as chemoprevention techniques. Assessing the modulation of aflatoxin metabolism requires exposure biomarkers to monitor the process(es) and a detailed understanding of the pathways involved in both the phase 1 (activation/detoxification) and phase 2 (detoxification) processes. Modulation of uptake requires the same exposure biomarkers and an understanding of agents that specifically bind aflatoxins and can shuttle them through the GI tract.

These studies include both animal and clinical trials and it is worth reemphasizing a perhaps obvious, but critical, difference between them. The animals are in a very controlled situation—diet, activity, toxin dose, and intervention dose—but the same is not true for a clinical trial. A key difference is that aflatoxin contamination is extremely heterogeneous, even within a small rural village setting in which there is little dietary variation between households. Thus, the level of any given aflatoxin biomarker within a study population can vary 100–1000× and also may vary with the temporal variations in exposure that occur during the time course of the intervention. The range of the variation in biomarker levels within the control population makes it considerably more difficult to detect a significant intervention effect in a real-life setting than in the more tightly controlled animal studies.

Aflatoxin B_1 Metabolism

Aflatoxin B_1 toxicity occurs following activation to one of two reactive epoxides, aflatoxin *exo*-8,9-epoxide (AF-EXO) and aflatoxin *endo*-8,9-epoxide (AF-ENO), by cytochrome P450s 1A2, 3A4, 3A5, and in infants 3A7 (Guengerich *et al.*, 1998). AF-ENO is toxic but nonmutagenic, while AF-EXO is both toxic and carcinogenic; both epoxides form covalent adducts with and disrupt

the function of cellular macromolecules. Binding of AF-EXO to the N-7 moiety of guanine results in a pre-mutagenic lesion (Raney *et al.*, 1993), and, following depurination, aflatoxin-N7-guanine (AF-N7-Gua) is excreted in urine. This analyte is a validated exposure biomarker for aflatoxin ingestion (Groopman *et al.*, 1993). Aflatoxin M_1 is a hydroxy metabolite of aflatoxin B_1 that is less carcinogenic than its parent, and thus frequently regarded as part of a detoxification process, but has similar general toxicity to aflatoxin B_1 (Wild and Turner, 2001, 2002). Aflatoxin M_1 also is a validated exposure biomarker for aflatoxin (Groopman *et al.*, 1993). CYP1A2 produces more AF-ENO than it does AF-EXO, in addition to aflatoxin M_1, while CYP3A4 and 3A5 predominantly generate AF-EXO. CYP3A4 also produces aflatoxin Q_1 (Gallagher *et al.*, 1996; Ueng *et al.*, 1995). Aflatoxin metabolism occurs predominantly in the liver, and CYP3A4 is the dominant liver P450. CYP3A7 is the fetal form of CYP3A4 and may be responsible for the aflatoxin adducts observed in cord blood samples from pregnant women (Turner *et al.*, 2007). Aflatoxin–albumin (AF-alb) adducts also are found in aflatoxin-exposed animals and humans and serve as another validated exposure biomarker for aflatoxin (Wild *et al.*, 1990, 1992).

AF-EXO and AF-ENO are detoxified through conjugation with glutathione via a family of glutathione *S*-transferases (Guengerich *et al.*, 1998; Raney *et al.*, 1992), and eventually excreted as aflatoxin–mercapturic acid (AF-ma). Nonenzymic hydrolysis of both epoxides to aflatoxin B_1-8,9-dihydrodiol also occurs, though a putative enzymic role for epoxide hydrolase in this step remains unsettled. The aflatoxin B_1-8,9-dihydrodiol ring can open to form a dialdehyde which is toxic, but not mutagenic. The dialdehyde can be reduced via aflatoxin aldehyde reductase (AFAR) to the less toxic dialcohol (Bodreddigari *et al.*, 2008; Guengerich *et al.*, 2001; Johnson *et al.*, 2008), although the overall toxicological contribution provided by AFAR has not yet been determined. Thus, there are numerous sites (Figure 10.1) at which chemoprevention approaches could target aflatoxin metabolism.

The use of reliable analytical tools for the exposure biomarkers also is critical. There have been advances in LC/MS methodology for assessing the concentration of AF-alb (Jiang *et al*., 2005; McCoy *et al.*, 2005; Scholl *et al.*, 2006a), AF-N7-Gua (Egner *et al.*, 2006), aflatoxins M_1 and P_1 (Everley *et al.*, 2007), and aflatoxin B_1 dialdehyde metabolites (Johnson *et al.*, 2008). In addition, for the AF-alb measure, there is a strong correlation between a well-established ELISA method, a HPLC method, and a new LC/MS method (McCoy *et al.*, 2008; Scholl *et al.*, 2006b). There also are HPLC methods for the other aflatoxin metabolites. Thus, both relatively "low-tech" and "high-tech" tools can support exposure assessment within chemoprevention clinical trials.

This review focuses on those compounds that have been the most extensively studied, i.e., those for which clinical trials have been initiated, or those thought to be key novel developments.

Oltipraz

One of the key determinants of aflatoxin-induced toxicity is the relationship between activation (to the reactive epoxides) and detoxification via the glutathione *S*-transferase (GST) family of enzymes. Induction of phase 2 metabolism involving glutathione S transferases has been a key research activity in this area. There are many compounds, e.g., ethoxyquin, butylated hydroxyanisole, butylated hydroxytoluene, oltipraz, and indole-3-carbinol, whose chemoprotection against aflatoxin involves induction of glutathione S transferase in animal models (Hayes *et al.*, 1996). These compounds also induce AFAR. Chemically induced increased activity of these detoxification enzymes in rats has been suggested as an explanation for their role in the reduction of aflatoxin-driven hepatocarcinogenesis (Hayes *et al.*, 1996). For example, 1,2-dithiole-3-thiones, e.g., oltipraz, lead to the accumulation

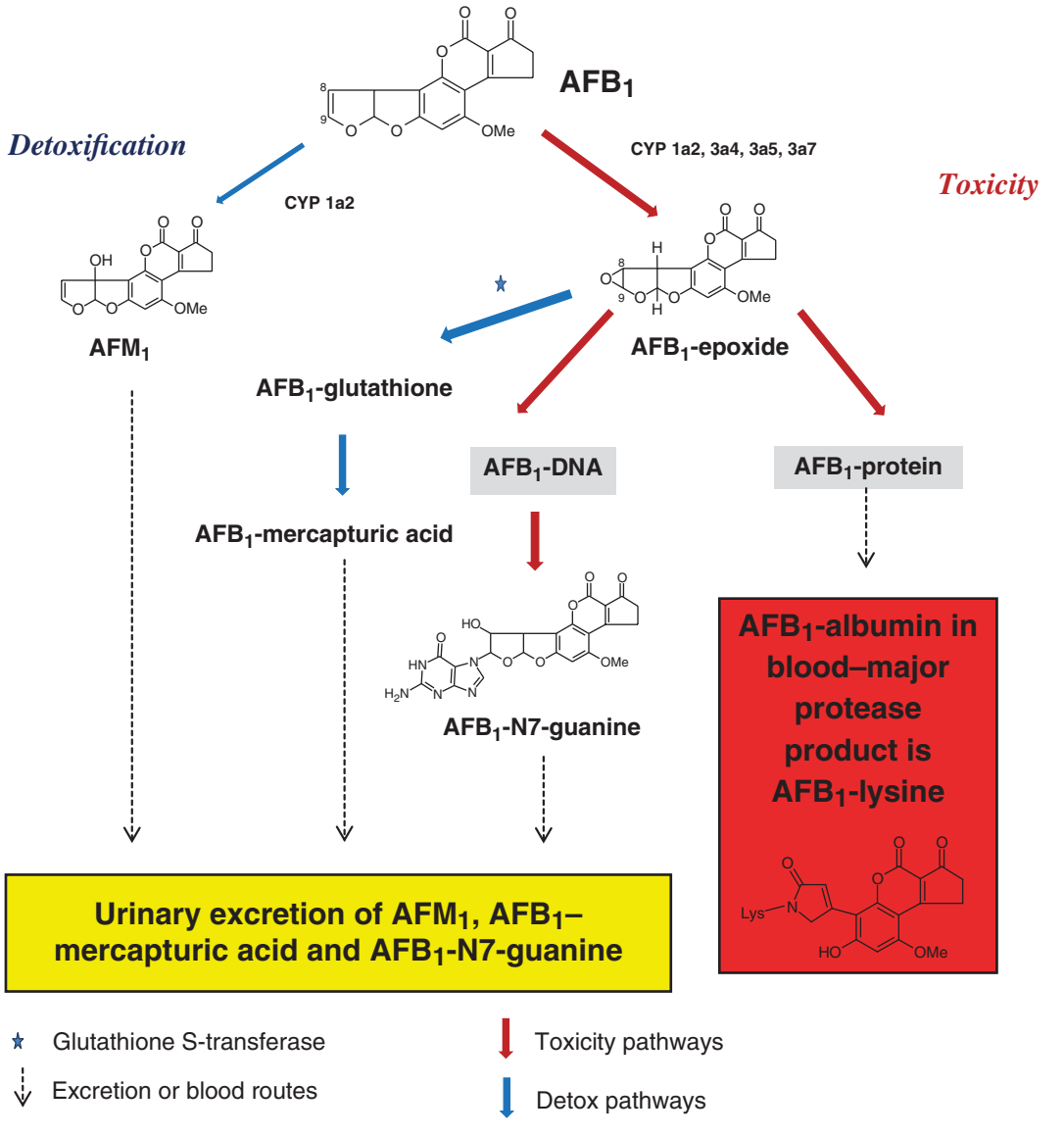

Figure 10.1 Simplified overview of important steps and key metabolites in the established metabolic pathways for aflatoxin B_1 (AFB_1) and biomarkers used to assess the efficacy of aflatoxin intervention studies [modified from Turner *et al.*, 2012]. This set of metabolites and enzymes excludes many other pathways and is simplified here to focus on those important in understanding chemoprevention. AFM_1, aflatoxin M_1. (For a color version, see the color plate section.)

of the transcription factor Nrf2 and its enhanced binding to the antioxidant response element in the nucleus, which activates transcription of genes involved in carcinogen detoxification (Yates and Kensler, 2007).

The understanding of oltipraz in animal models, and its subsequent inclusion in clinical trials, provides one of the most important demonstrations of "proof of concept" for mycotoxin interventions. Oltipraz had many advantages for development as one of the leading chemopreventative

agents in the 1990s. Most prominently, it was already a well-documented compound from animal studies and clinical trials with it as an antischistosomal agent in the 1980s (Archer, 1985), thus safety issues had already been addressed. Second, its mechanism of action included induction of glutathione S transferases, at relatively low doses. Oltipraz effectively blocks aflatoxin adduct formation in rodents, tree shrews, and a nonhuman primate, the marmoset (Bammier *et al.*, 2000; Li *et al.*, 2000; Roebuck *et al.*, 1991), and hepatocarcinogenesis in rodents (Bolton *et al.*, 1993). While the induction of glutathione S transferases is regarded as the dominant effect (Primiano *et al.*, 1995), CYP1A2 and CYP3A4 inhibition also occurs in isolated hepatocytes (Langouet *et al.*, 1995) and CYP1A2 is inhibited in rodents (Scholl *et al.*, 1996). A trial in F344 rats utilizing AF-alb and liver cancer incidence and severity as end points was conducted, in part, to assess the utility of AF-alb as a biomonitor for this chemoprevention approach. Both the AF-alb adduct concentration and the incidence of hepatocarcinogenesis were reduced in animals receiving oltipraz at either of two dosing regimens (Kensler *et al.*, 1997). Thus, oltipraz should restrict both the mutagenic and toxigenic effects of aflatoxin.

The successful demonstration of oltipraz as a chemoprevention drug in animals led to its use in a randomized, placebo-controlled, double-blind intervention clinical trial in 1995 in Qidong, People's Republic of China (Zhang *et al.*, 1997). In this region aflatoxin exposure occurs frequently and at high levels, there is a high incidence of liver cancer, and the tumors from these cancer cases frequently contain a "fingerprint mutation" in *p53* that is strongly suggestive of a causative role for aflatoxin in the carcinogenesis (Kensler *et al.*, 2011). Participants ($n = 234$) were placed randomly into three groups: one receiving a placebo, a second receiving 125 mg of oltipraz daily, and a third receiving 500 mg of oltipraz weekly. Aflatoxin exposure was that which occurred naturally in a given participant's diet. The intervention lasted for 8 weeks, with an additional 8-week follow-up. Numbness, tingling, or pain in the finger tips was reported by 18% and 14% of the treatment groups, respectively. The efficacy of the intervention was assessed by using several measures of aflatoxin in body fluids. There were no consistent changes in serum AF-alb levels in the group receiving the placebo over the 16-week observation period, nor was there any apparent effect observed in the group that received 125 mg of oltipraz daily. During the first month there was no detectable response to treatment for individuals receiving 500 mg of oltipraz, but a significant ($p = 0.001$) reduction in AF-alb level was detected in the second and third months (Kensler *et al.*, 1998), with the third month being a period in which no oltipraz was supplied. AF-alb provides an integrated measure of aflatoxin intake over a period of many weeks. Thus, the apparent delayed response and the continued response into the period in which oltipraz was withdrawn probably reflects the half-life of this adduct, estimated at about 20 days.

Urinary aflatoxin M_1 was measured at the 5-week time point, 2 days after the weekly 500 mg oltipraz dose was administered and within 24 hours of administration of the daily 125 mg oltipraz dose. The urinary aflatoxin M_1 concentration was reduced in both intervention groups relative to the placebo group, although this reduction was statistically significant only for the group receiving the larger weekly dose of oltipraz (Wang *et al.*, 1999). At this dose a 20× higher serum oltipraz maximum concentration occurred compared to the lower daily dose regime (Gupta *et al.*, 1995), which suggests that the lower dose did not provide a sufficient systemic concentration of oltipraz to increase CYP1A2 to a point where significant differences in aflatoxin M_1 are detectable. One concern is whether a 500 mg weekly dose provides only transient protection shortly after the time of dosing.

Urinary AF-ma has not been validated as a biomarker of aflatoxin bioavailability, but high urinary concentrations probably reflect increased detoxification via GST induction, and therefore protection. Urinary AF-ma was higher in both intervention groups, but the increase was statistically significant

only for the lower daily dose of oltipraz relative to the placebo control group (Wang *et al.*, 1999). Thus, the lower daily dose, which did not significantly alter CYP1A2 expression, appeared to modify GST activity.

The modulation of the AF-alb concentration was consistent with either a reduction in CYP levels or an increase in GST levels. The change in the urinary aflatoxin M_1 level was consistent with decreased CYP1A2 activity, while higher urinary AF-ma was consistent with increased GST activity. Together, these data strongly support the hypothesis that an effective reduction in aflatoxin *endo*- and *exo*-epoxides available for toxicity occurred in response to the oltipraz intervention. This series of studies on oltipraz clearly demonstrates the need to link mechanistic approaches through animal models to carefully designed clinical trials to demonstrate efficacy of a chemopreventative approach. Perhaps the only missing piece of the puzzle, and a recurring issue in this research field, is how to implement a public health program based on such results in a sustainable and affordable manner, within any of the high-risk and predominantly poor populations. Given the potential difficulties with a long-term drug delivery program for chemoprevention, a number of alternative approaches that use more natural products also have been investigated.

Sulforaphane

Consumption of cruciferous vegetables, e.g., broccoli, is associated with a reduced incidence of cancers of the gastrointestinal tract and other sites (IARC, 2004). Broccoli, along with numerous other cruciferous vegetables, is rich in glucosinolates that can induce phase 2 detoxification enzymes (Fahey and Talalay, 1999; Kensler *et al.*, 2000). The major inducer results from the breakdown of the glucosinolate, glucoraphanin, to an isothiocyanate, sulforaphane. The glucosinolate is metabolized by the enzyme myrosinase, which is present in the broccoli. Chewing allows the enzyme and the substrate to mix to form the active sulforaphane. Gut microbes also can convert the glucosinolate to sulforaphane in the absence of exogenous enzyme.

A randomized, placebo-controlled trial again in Qidong, China, was used to assess the efficacy of chemoprevention in response to an infusion prepared from broccoli sprouts (Kensler *et al.*, 2004). The trial consisted of 200 adults who consumed a hot infusion that contained either 400 mmol of glucoraphanin (intervention) or <3 mmol of glucoraphanin (control). Due to denaturing of the enzyme in the preparation of the "broccoli tea," this approach relies on the gut microbes to generate the sulforaphane. The broccoli tea was consumed for 14 days during a period in which other cruciferous vegetables were excluded from the diet. The mean concentrations of urinary AF-N7-Gua were not statistically significantly different when comparing the intervention and the control group. However, within the intervention group a urinary measure of isothiocyanate bioavailability did vary, $\sim 40\times$, a reflection of individual differences in conversion by gut microbiota. Thus, in this study, unlike the oltipraz trial, the concentration of the active component was not controlled, and the study appeared to have been unsuccessful. When data for isothiocyanate bioavailability and urinary AF-N7-Gua for individuals within the intervention group were evaluated, a significant inverse relationship was observed. Thus, the approach is heavily dependent upon the availability of the active component and the mechanism of delivery. This trial provided a clear demonstration of the potential effectiveness of the proposed intervention and indicated the need to better understand the role and influences of gut microbes on human health. The inclusion of broccoli, or similar cruciferous vegetables, in the diet may prove protective in populations for which these foods are readily available.

Green Tea Polyphenols

Another interesting chemoprevention uses green tea polyphenols. Green tea polyphenols can modulate aflatoxin B_1 metabolism, reduce the aflatoxin–DNA adduct burden, and reduce aflatoxin-induced hepatocarcinogenesis in animals (Wang *et al.*, 1989). A randomized, double-blind, placebo-controlled clinical trial was conducted to determine the efficacy of this approach, in volunteers from Southern Guangxi, China (Tang *et al.*, 2008). The study was conducted over 3 months and involved 120 individuals to whom green tea polyphenols were supplied in tablet form at either 500 or 1000 mg daily or a placebo control. Blood and 24-hour urine samples were collected at baseline, after 1 month, and after 3 months. Modest, albeit significant, reductions occurred in mean AF-alb concentration, although urinary aflatoxin M_1 levels were unchanged. Of particular note were significant increases in urinary AF-ma and the AF-ma/aflatoxin M_1 ratio in both intervention groups. These data strongly support a role for green tea polyphenols in modulating aflatoxin B_1 detoxification in phase 2 metabolism.

One interesting aspect of the green tea polyphenol clinical trial was the use of ratios of aflatoxin metabolites. Aflatoxin contamination of dietary staples generally is heterogeneous, and biomarker levels can vary considerably within study populations, despite only minor differences in dietary content, an observation that was a major driver in the search for validated biomarkers to assess exposure. Whenever chemoprevention alters biomarker metabolism, the changes in ratios of the biomarkers within an individual across the time of the intervention may ultimately be more informative than any single biomarker measure. Further development and understanding of these ratios is an important tool in future intervention approaches.

Other Compounds

A novel approach for aflatoxin chemoprevention involves a group of compounds based on pentacyclic oleanane triterpenoids. These include 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO), methyl CDDO (CDDO-Me), imidazolide CDDO (CDDO-Im), and a synthetic acetylenic tricyclic bis-(cyano enone) known as TBE-31 (Liby *et al.*, 2008; Yates *et al.*, 2006). The potency of TBE-31 is similar to or better than that of CDDO-Im for inhibiting hepatic tumorigenesis induced by aflatoxin B_1, and this chemoprevention is dependent on phase 2 enzyme induction. Impressively, both CDDO-Im and TBE-31 are 100× more potent than oltipraz and clinical trials of these compounds are eagerly awaited.

Other dietary components that can alter aflatoxin metabolism include coumarin (Kelly *et al.*, 2000), coffee diterpines (Cavin *et al.*, 2001), and bioflavanoids from kola seeds (Farombi *et al.*, 2005). It will be important to link detailed mechanistic information on chemoprevention at the chemical level with natural dietary components that either contain, or can be modified to contain, therapeutic levels of relevant compounds or their precursors. Such foods need to be acceptable dietary components, inexpensive, and frequently available to at-risk populations.

Uptake Inhibitors

This chemoprevention approach assumes that exposure to aflatoxin is unavoidable, as also is done in the other chemoprevention studies, but rather than change the consumer's metabolism they alter the

bioavailability of the toxin. There are three main classes of uptake inhibitors, all with demonstrated efficacy to restrict systemic exposure to aflatoxin B_1. Uptake inhibitors function predominantly by shuttling the toxin through the intestinal tract so that it can be eliminated in the feces, chemically unmodified.

NovaSil® Clay

Phillips and colleagues have championed the use of clay sorbents that are added to the diet to reduce aflatoxin exposure (Phillips *et al.*, 2006, 2008). NovaSil® clay contains layered particles that can intercalate with aflatoxin B_1, thereby reducing aflatoxicosis in a range of animals when added to aflatoxin-contaminated feed. The intercalation occurs in the aqueous solution of the GI tract and reduces the bioavailability of the toxin. NovaSil® clay appears to be specific for aflatoxins and does not bind several other important mycotoxins tested to date (Phillips *et al.*, 2008), though understanding the extent of the binding specificity of various mycotoxins to NovaSil® clay is still an ongoing activity. In addition, the clay does not appear to bind vitamins or minerals in rodents and has no effect on the growth of swine or rodents. A long-term toxicity trial was conducted in male and female Sprague-Dawley rats. Diets were spiked with 0%, 0.25%, 0.5%, 1.0%, and 2.0% w/w with NovaSil® clay, with consumption *ad libitum* for 6.5 months. No effects on morbidity, mortality, body weight gains, feed conversion ratios, relative organ weights, gross anatomy and histological appearance of major organs, hematology, serum biochemistry parameters, or selected nutrients were observed (Afriyie-Gyawu *et al.*, 2005). These data provided enough confidence in the product to initiate clinical trials in high-risk populations.

Clinical trials. Initially a short-term evaluation was carried out in healthy, US-based individuals, not suspected of being exposed to aflatoxin (Wang *et al.*, 2005). The study recruited 50 adults aged 20–45 years, from Texas Tech University. Sterile NovaSil® clay capsules were provided to give either 1.5 or 3.0 g of clay per day for 2 weeks. Based on a physical examination, serum nutrient markers, biochemistry, and hematology, no significant changes from baseline measures occurred. The success of this trial allowed the development of a larger scale field trial in Ghana, where aflatoxins are frequent dietary contaminants. This larger trial followed 177 individuals selected after a baseline screen for AF-alb (Afriyie-Gyawu *et al.*, 2008a). Similar to the chemoprevention trials in China, this study was double blind and placebo controlled. Individuals were randomly assigned to a high dose (3.0 g/day), low dose (1.5 g/day), or placebo control group, for 3 months. Few side effects were reported, and no effects were associated with the NovaSil® clay consumption. No differences were observed between control and intervention groups for hematology, liver or kidney function, serum biochemistry, or serum nutrients (Afriyie-Gyawu *et al.*, 2008b).

Finally, serum AF-alb and urinary aflatoxin M_1 were measured as markers of the efficacy of the intervention. No differences in AF-alb levels were observed at the 1-month time point; however, by 3 months the level of this biomarker was reduced by ~25% in both intervention groups relative to the placebo control (Wang *et al.*, 2008). One month after completion of the intervention the mean level of AF-alb in both intervention groups was not significantly different from the control group. The 1-month time delay before an effect on adduct level again probably reflects the longer term nature of the albumin biomarker. There were no significant differences in median urinary aflatoxin M_1 at baseline between any of the groups. There were no significant differences between either of the intervention groups and the placebo group at 1 month, nor for the low dose of NovaSil® clay at 3 months; however, there was a significant reduction (58%) in mean urinary aflatoxin M_1 concentration for the high-dose treatment. Overall, the combined biomarker measures indicate the

effectiveness of NovaSil® clay for restricting aflatoxin uptake. Given that the use of clay particles at a low dose is acceptable in many at-risk populations, this approach could be well tolerated. Phillips and colleagues are undertaking extensive surveys within West Africa of naturally occurring clays, such that the method is not continually reliant on a more developed country for the clay, a critical requirement if this approach is to become applied more widely over extended time frames. When monitoring at a local level is economically feasible the use of clay may have particular utility, and if acute poisoning incidence can be predicted through weather/drought monitoring and through advanced prediction of food scarcity, then this material could also provide essential temporary relief in aid programs.

Chlorophyllin

Chlorophyllin is a synthetic derivative of the ubiquitous green plant pigment chlorophyll, which, in addition to possessing antimutagenic and anticarcinogenic properties (Breinholt *et al.*, 1995a, 1995b), has been marketed as a food color, deodorizer, and accelerator for wound healing. Chlorophyllin coadministration with aflatoxin B_1 significantly reduced liver aflatoxin–DNA adduct formation in trout (Dashwood *et al.*, 1991) and liver tumor incidence (Breinholt *et al.*, 1995a). Chlorophyllin forms a strong complex with aflatoxin B_1 in aqueous solution and thus reduces the body burden of the toxin within the GI tract (Breinholt *et al.*, 1999; Dashwood, 1997).

The first clinical trial with chlorophyllin was conducted in Qidong, China, where 180 volunteers were randomly assigned to an intervention group (100 mg chlorophyllin thrice daily for 4 months) or a placebo control group (Egner *et al.*, 2001). After 3 months, the mean level of urinary AF-N7-Gua was reduced by 55%, indicative of a strong protective effect of chlorophyllin.

While chlorophyllin is a potentially useful chemopreventative agent its wider application has two significant drawbacks. First, it is a commercial product albeit based closely on a natural product, and thus its distribution is complicated and potentially not affordable. Second, there are concerns over the long-term potential for chlorophyllin to enhance the carcinogenicity of other known carcinogens (Nelson, 1992; Xu *et al.*, 2001). By contrast, chlorophyll is naturally occurring and has no known toxicity at dietary levels. Dietary levels equivalent to that for chlorophyllin are possible with moderate to high consumption of green leafy vegetables (Simonich *et al.*, 2007). In rodents, chlorophyll reduced hepatic aflatoxin–DNA adduct level, AF-alb, and urinary AF-N7-Gua (Simonich *et al.*, 2007). The mechanism for chlorophyll activity is consistent with a role as an uptake inhibitor (analogous to chlorophyllin), rather than as a modifier of metabolism. In a longer term trial, chlorophyll reduced the volume percent of liver occupied by glutathione S transferase placental form-positive foci in the liver of aflatoxin-treated animals, indicating that a reduction in both aflatoxin biomarkers and early markers of carcinogenesis occurred (Simonich *et al.*, 2007).

In the first study of its kind, seven volunteers received 1 mg of ^{14}C-labeled aflatoxin B_1, and the resulting aflatoxin–albumin adduct levels were analyzed by using an extremely sensitive carbon dating procedure that allows human dosing studies to be "safely" conducted (Turteltaub and Dingley, 1998). AF-alb concentrations were similar to those in rats receiving an equivalent dose per kg body weight (Cupid *et al.*, 2004) and was more than 5× below the limit of detection of standard ELISA or RIA methodologies. A separate toxico-kinetic study with four volunteers sampled bio-fluids at multiple time points and found that chlorophyll limited aflatoxin bioavailability (Jubert *et al.*, 2009). Again these authors used a low-level radioactive aflatoxin dosing regimen and utilized the high-sensitivity carbon detection method. While still preliminary, these data strongly support the need for clinical trials in naturally affected populations.

Probiotics

Probiotics are nonpathogenic, nontoxic, live microbes used as food supplements that provide a benefit to the host. Strains of *Lactobacillus* and *Bifidobacterium* are amongst the most commonly encountered. A number of bacterial strains have been tested for their ability to bind aflatoxins and other mycotoxins to their surface (El-Nezami *et al.*, 1998, 2002; Gratz *et al.*, 2005; Peltonen *et al.*, 2001; Styriak and Conková, 2002). Among the strains tested, *Lactobacillus rhamnosus* strain GG (GG) and strain LC705 were the most efficient at removing aflatoxin from aqueous medium (El-Nezami *et al.*, 1998; Haskard *et al.*, 2001). Cell wall peptidoglycans were responsible for binding aflatoxins to the surface of GG and LC705 (Haskard *et al.*, 2000, 2001). Aflatoxin binding to probiotics has been demonstrated *ex vivo* in the intestinal lumen of chicks (El-Nezami *et al.*, 2002). Given that the probiotics that can bind aflatoxin are already commercially viable and safe, these observations suggest a potentially useful approach to intervene in exposure.

In *in vitro* studies, GG protects against aflatoxin B_1-induced disruption of the integrity of Caco-2 monolayers (Gratz *et al.*, 2007), while in rats GG reduced AF-alb in aflatoxin B_1-treated animals compared to controls and significantly, though only partially, restored the growth rate of aflatoxin-dosed rats (Gratz *et al.*, 2006). A double-blind, placebo-controlled clinical trial of 90 healthy males from Guangzhou, China, assessed the efficacy of the intervention in a population naturally at risk from aflatoxin exposure through diet (El-Nezami *et al.*, 2006; Mykkanen *et al.*, 2005). Individuals were randomly assigned to either the intervention group (two capsules daily with food containing a 1:1 mixture of the strains *L. rhamnosus* LC705 and *Propionibacterium freudenreichii* subsp. *shermanii*) or a placebo control group (two capsules daily containing cellulose), for 5 weeks. Morning urine samples were collected at baseline, week 3, week 5, and week 10, the latter being 5 weeks post intervention, to measure AF-N7-Gua. Overall the frequency of non-detection increased for the intervention group, albeit at borderline significance ($p = 0.052$); however, 36% and 55% mean reductions in AF-N7-Gua were observed at weeks 3 and 5, respectively. No differences were observed at baseline or 5 weeks after the intervention ceased. Thus, probiotics may be a useful intervention in middle-income populations, with moderate frequencies and levels of aflatoxin exposures, e.g., Thailand, Brazil, and Egypt, where milk products may be used as a vehicle to supply the probiotic. Probiotic strains of *Lactobacillus casei*, *Lactobacillus plantarum*, and *Enterococcus faecium* also may provide protection against aflatoxins (Fazeli *et al.*, 2009; Hernandez-Mendoza *et al.*, 2009; Topcu *et al.*, 2010). The ability of any particular probiotic strain to limit aflatoxin bioavailability probably depends on the contaminated food consumed (Kabak *et al.*, 2009), so multiple probiotic strains may be needed to provide effective protection. The use of probiotics that are capable of detoxifying mycotoxins during food-processing activities may have both local and industrial benefits, but is beyond the scope of this review. Probiotics in the diet also may provide additional benefits to gastro-hepatic health beyond reducing aflatoxin bioavailability. Probiotic use probably is not a suitable approach to mitigate aflatoxin exposure in rural settings in sub-Saharan Africa.

Summary

All of the approaches described in this chapter followed a meticulous, detailed route, and the resulting clinical trials were based on a solid understanding of the mechanism on which the intervention technology is based and have validated end points to assess their efficacy. The breadth of available knowledge on aflatoxins makes this approach possible and provides a model to emulate when

studying other mycotoxins. A number of possible approaches have been outlined, and greater detail is available (Kensler *et al.*, 2011; Phillips *et al.*, 2008). From a cancer standpoint, if the results of these clinical trials were applied more widely then both a reduction in incidence and an age delay in peak incidence are predicted, with the delay measured in decades (Kensler *et al.*, 2004). At this point these trials, while successful, have demonstrated only a "proof of concept." A key unanswered question is how to apply any of these approaches at the population level. Such an extension will require perhaps as large an effort in training and education as has been needed in research to get to this point, but hopefully can be accomplished in a shorter time than the ~50 years since the alarm bells for aflatoxin were first rung.

While "cancer" as a tragic end point is the most clearly defined outcome from chronic aflatoxin exposure, and has been the means by which the vast majority of grant funding has been obtained, the greater morbidity and mortality burden probably results from life-long chronic toxicity and the suspected immune suppression and growth retardation (reviewed by Turner *et al.*, 2012). Such observations in children have only recently been reported, although they have been observed in other animals for many decades. It is important for international organizations and funding bodies to recognize the need to extend these modest observational studies, with various intervention trials that focus on reducing early life exposure. Education is a significant component of this activity. In addition to chemoprevention in the young, the simple extension of the breast-feeding period may have other important health benefits. For those at greatest risk of aflatoxin exposure, region-appropriate, dietary-driven, sustainable chemoprevention combined with primary preventions, e.g., improved postharvest storage strategies (Turner *et al.*, 2005b) and education, comprises an important set of tools to reduce the burden of acute and chronic aflatoxin toxicity.

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Part II
The Wheat Grain Chain

11 Identification of *Fusarium* spp. and *Penicillium verrucosum* in the Wheat Grain Chain

Antonio Moretti, Cees Waalwijk, and Rolf Geisen

Abstract

Ochratoxin, produced primarily by *Penicillium verrucosum*, and deoxynivalenol and zearalenone, produced primarily by *Fusarium graminearum* are the primary mycotoxin contaminants of wheat. Multiple PCR-based tests are available to identify the trichothecenes produced by various *Fusarium* species that can occur on wheat. Usually these assays are based on sequences from the trichothecene gene cluster, but they may not be derived from the critical genes in the biosynthetic pathway that are responsible for the predicted metabolite. Multiplex and individual real-time PCR assays have been developed to both identify and quantify the *Fusarium* species present within a wheat sample. Other detection technologies, including microarrays, denaturing gradient gel electrophoresis, and surface plasmon resonance biosensors have been developed but not used as extensively as the PCR-based assays. The efforts with *Penicillium* are not as extensive as those made with *Fusarium*. PCR-based assays are available, with some targeted to the polyketide synthase that is critical for ochratoxin production. Polyketide synthase gene expression precedes the detection of toxin by 36–48 hours. This gene expression is sensitive to both temperature and moisture conditions. A better understanding of the genomics of these fungi will likely increase the number, accuracy, and sensitivity of the assays for these mycotoxins.

Keywords: citrinin; deoxynivalenol; environmental conditions; Fusarium head blight; grain storage; microarrays; ochratoxin; PCR assays; polyketide synthase; preservatives; trichothecenes; zearalenone

Introduction

Mycotoxins are secondary metabolites produced by several species of fungi during colonization and growth on plants. The occurrence of mycotoxins in commodities represents a major threat to humans and animals. Their formation in the infected crops and persistence in food and feed is associated with different toxicities including mutagenicity and estrogenic, gastrointestinal, urogenital, vascular, kidney, and nervous disorders as well as the induction of cancer. Some mycotoxins also are immunocompromising and can thus reduce resistance to infectious disease agents (Desjardins, 2006). Moreover, significant economic losses are associated with the impact of mycotoxins on human health, animal productivity, and both domestic and international trade. It is estimated that 25% of the world food production, including many basic foods, is affected by mycotoxin-producing fungi, while cereals appear to be contaminated at higher levels (CAST, 2003). Wheat is

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a crop that can be commonly contaminated by three of the most important mycotoxin-producing fungi: *Aspergillus ochraceus*, *Penicillium verrucosum*, and *Fusarium graminearum* (Marasas *et al.*, 2008). Therefore, three of the most important mycotoxins occurring in agricultural products, ochratoxin (produced on wheat mainly by *A. ochraceus* and *P. verrucosum*) and deoxynivalenol (DON) and zearalenone (ZEA), both produced on wheat mainly by *F. graminearum*, can be common contaminants of wheat (Miller, 1995). The detection and control of each toxigenic fungal species *in planta* is key to reduce fungal contamination and in turn to prevent mycotoxins from entering human food and animal feed.

The Problem of *Fusarium* and Its Toxins in Wheat: *Fusarium* Head Blight (FHB)

Fusarium head blight of wheat is a significant threat to wheat production worldwide. The main causal agent of *Fusarium* head blight worldwide is *F. graminearum* (teleomorph, *Gibberella zeae*) that has recently been described as a species complex and is undergoing a major taxonomic reassessment. However, many other *Fusarium* species also have been associated with the disease, e.g., *F. avenaceum*, *F. culmorum*, and *F. poae*, and to a lesser extent, *F. acuminatum*, *F. cerealis* (syn. *F. crookwellense*), *F. chlamydosporum*, *F. equiseti*, *F. langsethiae*, *F. sporotrichioides*, and *F. tricinctum*. In addition to causing significant yield losses, these *Fusarium* species can produce various mycotoxins that subsequently appear in the grain (Logrieco and Moretti, 2008). The toxigenic potential of FHB pathogens varies greatly between species. *Fusarium culmorum*, *F. graminearum*, *F. langsethiae*, *F. poae*, and *F. sporotrichioides* each produces their own spectrum of trichothecenes, while *F. avenaceum* may produce moniliformin, beauvericin, and enniatins (Desjardins, 2006).

The frequency of *Fusarium* head blight species is determined, to a large extent, by climatic factors, particularly temperature and moisture (Xu *et al.*, 2008). Agricultural practices also play an important role in the prevalence of *Fusarium* head blight pathogens. For example, overwintered plant debris favors infection by *F. graminearum* (Bai and Shaner, 2004). Moreover, combinations of these pathogens can occur on wheat heads, which results in multiple mycotoxins contaminating the harvested grain. Wheat plants are most vulnerable to infection during anthesis, and *Fusarium* head blight is most severe when warm, wet conditions prevail during this time. In addition, each *Fusarium* head blight species may differ in its response to the fungicides used to control *Fusarium* head blight (Bai and Shaner, 2004). Therefore, it is vital to determine the exact composition of the *Fusarium* head blight complex for more reliable disease prediction and management and for managing the food risks associated with mycotoxins.

Trichothecenes

These secondary metabolites are produced by multiple *Fusarium* species in the *Fusarium* head blight species complex. They can be divided into two groups according to their chemical structure: type A trichothecenes have a hydrogen or ester side chain at carbon atom 8 (C-8)—T-2 toxin, HT-2 toxin, 4,15-diacetoxyscirpenol, neosolaniol—whereas type B trichothecenes have a ketone at C-8—deoxynivalenol, nivalenol, and their acetylated derivatives. Both type A and type B trichothecenes exhibit acute toxicity, causing vomiting and feed refusal. T-2 and HT-2 toxins are the most poisonous and are produced primarily by *F. sporotrichioides* and *F. langsethiae*, two pathogens of wheat that commonly occur in colder climates. Several groups have investigated the role of trichothecenes in plant diseases by generating trichothecene-nonproducing mutants. Disruption of the *tri5* gene in *F. graminearum* significantly reduced the virulence of the trichothecene-nonproducing mutant strains (Proctor *et al.*, 1995).

Fusarium graminearum and *F. culmorum* produce different trichothecene analogs, in particular deoxynivalenol (DON) and its acetylated derivatives, 3-acetyl-deoxynivalenol (3-ADON) and 15-acetyl-deoxynivalenol (15-ADON), and nivalenol (NIV). Three chemotypes have been described. Strains with the nivalenol chemotype produce nivalenol and 4-acetyl-nivalenol (4A-NIV), strains with the 3-ADON chemotype produce deoxynivalenol and 3-ADON, and strains with the 15-ADON chemotype produce deoxynivalenol and 15-ADON (Desjardins, 2006). Differentiation between chemotypes is important because hydroxylation and acetylation of trichothecenes leads to marked changes in toxicity and biological activity (Kimura *et al.*, 1998). In the ongoing taxonomic re-evaluation of *F. graminearum* there is no clear relationship between chemotypes and phylogenetic species of *F. graminearum* at either the chemical or the molecular level (Ward *et al.*, 2002).

The F. graminearum Species Complex

Based on sequence variation in multiple genes, 13 phylogenetic species have been recognized in *F. graminearum* species complex (FGSC) (Gale *et al.*, 2010; O'Donnell *et al.*, 2000, 2004, 2008; Starkey *et al.*, 2007; Yli-Mattila *et al.*, 2009). Additional studies should provide insight into the practical implications of these divisions in the FGSC with respect to disease management, quarantine regulations, and plant breeding strategies, and increase our understanding of the ecology, epidemiology, and population dynamics of this widespread group of fungi.

PCR Methods to Detect *Fusarium* in Wheat

Detection and control of *Fusarium* species *in planta* is crucial to prevent mycotoxins from entering the human food and animal feed chains. Since different *Fusarium* species have different mycotoxin profiles, an accurate identification of the *Fusarium* species present and their chemotypes is critical to predicting potential toxicological risks. Traditionally, identification of fungal species in infected plants involved isolation and growth of the fungus, followed by identification based upon morphological characteristics, such as shape, size, and presence or absence of macroconidia, microconidia, chlamydospores and colony morphology (Leslie and Summerell, 2006). However, this approach requires considerable expertise, is time consuming, and does not provide early detection. DNA-based methods, developed for rapid detection and identification of mycotoxigenic fungi, can overcome these disadvantages. The availability of rapid, sensitive, and inexpensive diagnostic tools for the detection of the main toxigenic *Fusarium* species, both in the field and after harvest, could enable real-time data monitoring of contamination and a better assessment of food safety.

In *Fusarium*, a number of potentially diagnostic sequence motifs have been identified. Since the early 1990s, assays have been based on anonymous DNA fragments, e.g., RAPD, RFLP, SCAR, amplified fragment length polymorphism (AFLP), and SSR markers (Suga *et al.*, 2004). A number of specific PCR primers for several of the species involved in *Fusarium* Head Blight, e.g., *F. culmorum*, *F. graminearum* (Nicholson *et al.*, 1998; Schilling *et al.*, 1996), *F. poae* (Parry and Nicholson, 1996), and *F. langsethiae* and *F. sporotrichioides* (Wilson *et al.*, 2004) are all available.

PCR Detection with Conserved Genes

Species-specific PCR methodologies based on evolutionarily conserved genes have their roots in taxonomic and phylogenetic studies. These methods were first designed to evaluate sequence

polymorphisms between species within the same genus and for subsequent studies of their phylogenetic relationships (Taylor *et al.*, 2000).

Ribosomal genes. The majority of DNA sequence data used for fungal taxonomy are derived from the nuclear ribosomal genes, represented by the highly conserved 18S, 5.8S, and 28S coding units, for which universal primers are easily developed. These coding regions are separated from each other by two less-conserved internally transcribed spacer (ITS) regions (ITS1 and ITS2), and the linked, repeated modules are separated by the intergenic spacer (IGS) region. The high number of copies of rDNA per cell makes it an attractive target for diagnostics and sensitive detection (Edwards *et al.*, 2002). Multiple primer sets have been developed that allow detection of single or multiple *Fusarium* Head Blight pathogens (Niessen, 2007).

Other conserved genes. More recently, diagnostic primers that target structural genes, e.g., mycotoxin production genes, were designed. Species-specific PCR assays for *Fusarium* also have been designed based on ubiquitous, single-copy gene regions such as the translocation elongation factor 1-α (TEF-1α) gene that contains sufficient sequence polymorphism to resolve multiple species in the *Fusarium* Head Blight complex (Nicolaisen *et al.*, 2009). Specific identification of *F. graminearum* also was demonstrated by using the DNA sequence of the galactose oxidase (Niessen and Vogel, 1997) and the cytosine phosphate synthase (*CTPS2*) genes (Yang *et al.*, 2008).

PCR detection with trichothecene biosynthesis pathway genes. An alternative strategy that deals specifically with mycotoxigenic fungi is to design primers directed toward critical mycotoxin biosynthetic genes (Edwards *et al.*, 2001). This approach also can be used to simultaneously detect the fungi and predict their chemotype (Ward *et al.*, 2002). The trichothecene biosynthetic gene cluster has been well characterized in *F. graminearum* and *F. sporotrichioides*. (Brown *et al.*, 2002, 2004; Lee *et al.,* 2002). In both species, the core cluster consists of 12 genes (*tri3–tri14*) that are responsible for the synthesis of the core trichothecene molecule and several modifications to it. The sequence of *tri5* has been used extensively to develop both group- (Edwards *et al.*, 2001) and species-specific detection assays (Wilson *et al.*, 2004).

Prediction of Chemotypes in F. graminearum and Related Species

PCR assays have been developed that differentiate the three trichothecene chemotypes. For example, *tri7* and *tri13* PCR assays can differentiate the nivalenol and deoxynivalenol chemotypes of *F. cerealis*, *F. culmorum*, and *F. graminearum* (Chandler *et al.*, 2003). In nivalenol-producing strains, *tri13* and *tri7* are intact and functional, but in deoxynivalenol-producing strains *tri13* and often *tri7* have multiple insertions and deletions that render these genes nonfunctional (Lee *et al*., 2001, 2002). Recently this protocol was used by Quarta *et al.* (2006) to develop a multiplex PCR assay, based on *tri3*, *tri5*, and *tri7* sequences, in order to determine whether wheat seeds were infected with 3ADON, 15ADON, or nivalenol-producing strains of *F. culmorum*, *F. graminearum*, and *F. cerealis*. Other multiplex PCRs have been developed for accurate simultaneous identification of nivalenol, 3-ADON, and 15-ADON chemotypes of *F. asiaticum* and *F. graminearum* by means of primer pairs designed for *tri3* and *tri12* (Ward *et al*., 2002), or the *tri6* gene and one pair for the *tri3* gene (Suzuki *et al.*, 2010). The primers designed by Ward *et al.* (2002) have the widest known applicability, as they can be used with all of the phylogenetic species in the *Fusarium graminearum* species complex. The primers of Suzuki *et al*. (2010) have been tested only in two of the *F. graminearum* lineages and those of Lee *et al*. (2001, 2002) and Quarta *et al.* (2006) have been tested even less extensively.

Quantitative PCR: single and multiplex. Real-time PCR (RT-PCR), which measures quantitative amplification of PCR products in real time, can be used to identify the presence and abundance of particular DNA sequences in agro-food samples. It can be used to identify and quantify *Fusarium* species occurring on wheat and their derivative products in a single assay. Furthermore, multiplex RT-PCR reactions allow the specific and sensitive detection, identification, and quantification of different DNA (or RNA) targets in a single reaction. The two main methods used to detect *Fusarium* species in wheat are TaqMan and SYBR Green.

Several species-specific primers, which were first developed several years ago, were used as templates to develop RT-PCR protocols (Waalwijk *et al.*, 2004). By using rDNA primers against 21 *Fusarium* species, Kulik (2008) amplified the targeted *F. tricinctum*, as well as *F. acuminatum* and *F. nurragi*, which limited quantitative detection. Nicolaisen *et al.* (2009) designed species-specific primers based on the TEF-1α sequences of 10 *Fusarium* species: *F. avenaceum*, *F. equiseti*, *F. graminearum*, *F. langsethiae*, *F. poae*, *F. proliferatum*, *F. sporotrichioides*, *F. subglutinans*, *F. tricinctum*, and *F. verticillioides*. However, two species that can often occur on wheat, *F. culmorum* and *F. cerealis*, could not be distinguished when this gene was used to develop specific primers. In this study, quantitative RT-PCR was validated on artificially and naturally infected grain samples. Quantitative methods, based on the *tri5* sequence, also have been developed for the quantification of trichothecene-producing *Fusarium* species. A competitive quantification system was developed by Edwards *et al.* (2001) and a RT-PCR system by Schnerr *et al.* (2001). Using the same gene, Niessen *et al.* (2004) developed a PCR assay for detection and taxonomy of trichothecene-producing species in *Fusarium* section *Sporotrichiella*, while Strausbaugh *et al.* (2005) set up a TaqMan quantitative RT-PCR assay for the quantification of *F. culmorum* in wheat roots.

Finally, with regard to multiplex PCR, several authors have tried to develop PCR assays that can detect multiple FHB species in wheat at the same time. Demeke *et al.* (2005) proposed a multiplex method that could identify the three main species pathogenic on wheat in Canada: *F. culmorum*, *F. graminearum*, and *F. sporotrichioides*. Brandfass and Karlovsky (2006) described a duplex PCR for simultaneous detection of *F. culmorum* and *F. graminearum* in plant material. Yli-Mattila *et al.* (2008) developed specific TEF-1α primers, as well as TaqMan probes, for a multiplex quantitative PCR of *Fusarium* species from cereal grains (*F. poae*, *F. graminearum*, and *F. langsethiae*/*F. sporotrichioides*). Moreover, a duplex PCR detection was developed for simultaneous detection of *F. poae* and *F. sporotrichioides* (Kulik, 2008). Finally, another multiplex PCR has been developed for accurate simultaneous identification of the nivalenol, 3-ADON, and 15-ADON chemotypes of *F. asiaticum* and *F. graminearum* by means of three pairs of primers designed for the *tri6* gene and one pair for the *tri3* gene (Suzuki *et al.*, 2010).

New Detection Platforms

In general mycotoxin biosynthetic genes are not expressed constitutively (Peplow *et al.*, 2003); However, their expression can be detected before mycotoxins are detected by chemical analytical methods (Mayer *et al.*, 2003). Hence, expression of these genes can be used as early indicators of mycotoxin biosynthesis even though there are only some of the genes in a mycotoxin biosynthetic pathway whose activation is directly coupled to mycotoxin biosynthesis. Focusing on these key genes detects fungal strains that potentially can produce toxins, but may give false positives if nonfunctional alleles are present in other genes in the pathway and the strains cannot produce the predicted toxins. Detection of multiple species that produce the same mycotoxin, rather than a

specific species, has been attempted with primers developed from conserved sequences of toxin cluster genes (Waalwijk *et al.*, 2008).

Microarrays

Microarray technology is a rapid and cost-effective method that has potential for parallel analysis of a high number of target organisms. DNA-microarrays use ordered sets of DNA molecules of known sequence and specificity, fixed to a solid surface. These arrays accelerate the hybridization process of labeled biological samples with a large collection of immobilized probes, thereby enabling the screening of several sequences in a single experiment. The results can be used to detect, at the single-nucleotide level, differences in genes of interest.

A number of microarrays have been made to evaluate various sets of *Fusarium* species. Nicolaisen *et al.* (2005) made an oligonucleotide array based on ribosomal ITS sequences for the detection and differentiation of trichothecene-producing and trichothecene-nonproducing *Fusarium* species occurring in small grain cereals. Kristensen *et al.* (2007a) developed a DNA microarray for fast and easy detection and identification of 14 trichothecene- and moniliformin-producing *Fusarium* species. Schmidt-Heydt and Geisen (2007) developed a microarray that includes most relevant mycotoxin biosynthesis genes, including those for type A and B trichothecenes. For trichothecene-producing *Fusarium* species, genes from the biosynthetic gene cluster specific for *F. sporotrichioides* (type A) and *F. graminearum* (type B) have been included. More recently, Lezar and Barros (2010) developed a microarray chip for the identification of several potential mycotoxigenic species of *Aspergillus*, *Fusarium*, and *Penicillium*. One set of probes was based on ITS and on TEF-1α regions, while a second set targeted biosynthetic genes of the most important mycotoxins, i.e., aflatoxins, deoxynivalenol, fumonisins, nivalenol, and trichothecenes. This diagnostic microarray can resolve 32 different species and predict their potential to produce mycotoxins.

The recent availability of complete genome sequences of several filamentous fungi now provides the opportunity for large-scale functional analysis, including genome-wide expression analysis. The design and validation of an Affymetrix GeneChip microarray based on the entire genome of *F. graminearum* were reported by Güldener *et al.* (2006). This GeneChip can be used to detect fungal genes *in planta* with surprising sensitivity, even without enriching for fungal transcripts.

DGGE-ARMS

Denaturing gradient gel electrophoresis (DGGE) is one of the most commonly used of the culture-independent fingerprinting techniques. Briefly, amplified DNA fragments of the same length but of different sequences are separated on a denaturing electrophoresis gel, according to their melting temperature. This technique has been extensively used for characterization and identification of members of bacterial communities from natural environment or food matrices (Ercolini, 2004), but has not been used extensively with fungal communities. Mach *et al.* (2004) focused on the β-tubulin gene and developed a DGGE protocol that discriminated *F. kyushuense*, *F. robustum*, *F. langsethiae*, *F. poae*, *F. sambucinum*, *F. sporotrichioides*, and *F. tumidum*, from different infected cereal crops, including wheat. These authors proposed an amplification refractory mutation system (ARMS)-PCR that used different primers for different regions of the β-tubulin sequence and produced three different patterns that could distinguish *F. langsethiae*, *F. sporotrichioides*, and *F. kyushuense*/*poae*.

Single-Nucleotide Polymorphism

A single-nucleotide polymorphism (SNP) is a DNA sequence variant that occurs when a single nucleotide in the genome (or other shared sequence) differs between the strains being compared. Kristensen *et al.* (2007b) developed a SNP assay that simultaneously detected 16 trichothecene- and moniliformin-producing *Fusarium* species—*F. avenaceum*, *F. cerealis*, *F. culmorum*, *F. equiseti*, *F. flocciferum*, *F. graminearum*, *F. kyushuense*, *F. langsethiae*, *F. lunulosporum*, *F. poae*, *F. pseudograminearum*, *F. sambucinum*, *F. sporotrichioides*, *F. torulosum*, *F. tricinctum*, and *F. venenatum*. Primers were based on variation in TEF-1α sequences, and the SNP assay was validated in cereal samples. The authors proposed a *Fusarium* chip prototype for those 16 species (Kristensen *et al.*, 2007b).

Loop-Mediated Isothermal Amplification Assay

Loop-mediated isothermal amplification (LAMP) of DNA is a quick, simple, and relatively cheap method for the specific detection of genomic DNA. It consists of a set of primers that hybridize specifically to different regions of a target gene (Notomi *et al.*, 2000). A LAMP assay has been developed to detect and identify *F. graminearum* (Niessen and Vogel, 2010). The assay was based on the *gaoA*, galactose oxidase gene and the amplification product was detected indirectly *in situ* by using calcein fluorescence as a marker without electrophoretic analysis. Among the 132 fungal species tested, only *F. graminearum* was recognized. The method was validated on wheat, providing a promising tool for the *in situ* detection and identification of this species.

Surface Plasmon Resonance Biosensors

Different molecular approaches based on surface plasmon resonance (SPR) have been taken to detect toxigenic *Fusarium* spp. on cereals. Kai *et al.* (2000) showed that DNA-based biosensors are valid, rapid tools for detecting microorganisms. SPR biosensors can be rapid, label-free, selective tools to detect PCR products (Sawata *et al.*, 1999; Giakoumaki *et al.*, 2003). These biosensors measure refractive index changes that occur on a metal film providing a signal that is positively correlated with the mass density changes on the metal surface (Jönsson *et al.*, 1991). By immobilizing a biological recognition molecule (ligand) on the sensing surface, SPR biosensors provide a tool for real-time biospecific interaction analysis. The choice of a specific single-stranded DNA ligand enables the rapid detection of the target DNA by hybridization with higher selectivity than possible with a gel electrophoresis analysis. This technique was used to design a SPR sensor to detect *F. culmorum* on wheat (Zezza *et al.*, 2006). Further studies of this type are in progress and should enable the detection of additional toxigenic *Fusarium* species.

Other Novel Technologies

Other molecular methods have become important for detecting or characterizing fungal species in medical applications, but their use in food microbiology is only at its early stages. The use of integrated platforms that combine detection, identification, typing, and quantification of the fungi present would facilitate the control of mycotoxin-producing fungi in food commodities. With

the sequencing of the entire genome of several mycotoxigenic fungi, identifying usable species-specific sequences should be easier. Various platforms are now available for "next generation" high-throughput DNA sequencing, of which the nanopore-patented technology (Illumina Solexa 1G Genome Analyzer) and pyrosequencing (Roche 454 GS-FLX platform) are the most prominent. PCR-Luminex, a novel system developed for high-throughput analysis of SNPs, was used successfully to identify *F. asiaticum* (syn. *F. graminearum* lineage 6), *F. culmorum*, and *F. avenaceum* on wheat by coupling oligonucleotide probes with fluorescent beads (Ishii *et al.*, 2008). Ward *et al.* (2008) used a Luminex system with allele-specific primer extension (ASPE) probes to identify a shift in trichothecene chemotypes of *F. graminearum* colonizing wheat in Canada.

Conclusions and Future Perspectives

Due to increasing concerns regarding food safety, contamination of wheat with *Fusarium* mycotoxins and related fungal species has become a main topic. *Fusarium* Head Blight, the main disease caused by *Fusarium* species on wheat, is caused by members of a diverse species complex. The mycotoxin contamination profile and related toxicological risk can vary dramatically with the *Fusarium* species present. Therefore, strategies to control the disease must successfully counter a range of *Fusarium* species, each with unique ecological, epidemiological, and population characters. Hence, the combination of accuracy, reliability, and rapidity in the diagnosis of *Fusarium* species in wheat is a major challenge for scientists working in the field. The diagnostic methods described above include a large number of species-specific DNA-based assays that can be used to detect mycotoxigenic *Fusarium* species, also enabling a direct detection from field samples without isolating or purifying fungal cultures. Phylogenetic databases could help resolve taxonomic issues when combined with information on epidemiology and the host crop. Species-specific sequence differences are more readily identified as the complete genome sequence of *F. graminearum* is now available and DNA arrays using these sequences have been developed (Kristensen *et al.*, 2007a; Schmidt-Heydt and Geisen, 2007).

Whenever DNA-based assays are used to estimate the risk of contamination, then fungi that are present but not synthesizing mycotoxins also must be considered. Another approach to detecting *Fusarium* species occurring on wheat include microarrays with probes for biosynthetic genes involved in the trichothecene pathway. This approach enables detection of the main toxigenic *Fusarium* species directly from field samples when implemented with DNA biochips and also permits evaluation of mycotoxin-related gene expression. Specific expression profiles for the entire mycotoxin biosynthetic pathway can be generated with RNA microarrays making the prediction of wheat contamination more accurate. This kind of microarray also can be used to study the effects of various physiological and environmental parameters on mycotoxin gene expression. Thus, it will be possible to use a PCR assay to diagnose the presence of *Fusarium* species and to determine if the relevant mycotoxin genes are expressed in field samples at levels that pose a real toxicological risk.

The Problem of *Penicillium* and Its Toxins in Wheat

Less work has been done with *Penicillium* spp. than with *Fusarium* spp., even though members of this genus are solely responsible for the production of ochratoxin A in cereals. The genus *Penicillium* contains mainly saprophytic species that colonize plant materials after harvest. Many species of *Penicillium* are quite resistant to dry conditions, which makes them ideally suited as

contaminants of stored cereals. In contrast to the plant pathogenic *Fusarium* spp., *Penicillium* spp. are regarded primarily as storage fungi that grow on the stored cereal biomass, which can no longer react to the fungal attack by activating various defense mechanisms. *Fusarium* spp. can grow on stored cereals, but as pathogenic fungi they are adapted to living plants and usually infect plants during growth in the field, as described earlier. Thus, isolates of *Fusarium* spp. that occur on stored cereals usually do not compete with the *Penicillium* spp. This difference in adaptation is further enhanced by differences in sensitivity to low relative humidity. *Eupenicillium*, the teleomorph of *Penicillium*, is particularly well suited to xerophilic conditions and is a spoilage fungus of dry cereal products. In this respect, *Penicillium* spp. are more competitive on stored cereals than other fungi.

Several different *Penicillium* spp. may occur in stored cereals; however, by far the most attention is focused on *P. verrucosum* as members of this species can synthesize both ochratoxin A and citrinin (Larsen *et al.*, 2001). Both ochratoxin A and citrinin are polyketide mycotoxins that are nephrotoxic in humans. Production of these mycotoxins depends on the culture conditions with either high levels of ochratoxin or high levels of citrinin being produced by the fungus. One hypothesis for this production pattern is that the biosynthetic pathways of both mycotoxins compete for a common metabolic intermediate, e.g., acetyl-CoA (Larsen *et al.*, 2001). However, we (Geisen *et al.*, unpublished) identified signal transduction cascades that activate either the ochratoxin A or the citrinin biosynthetic pathways, depending upon environmental conditions.

Both ochratoxin A and citrinin often can be found in cereals; however, it is not clear if they act additively or synergistically (Braunberg *et al.*, 1994; Vrabcheva *et al.*, 2000) with respect to toxicology. Ochratoxin A is regarded as the more important toxin since it is a type 2 carcinogen (Bayman and Baker, 2006). The presence of *P. verrucosum* above a certain contamination threshold indicates that ochratoxin A also is present (Lund and Frisvad, 2003). These authors found that *P. verrucosum* was the only potential ochratoxin A-producing fungus in their sample of nearly 70 species, indicating that ochratoxin A-producing *Aspergillus* spp. do not play a role in the ochratoxin A contamination of wheat. They (Lund and Frisvad, 2003) also analyzed the percentage of infected kernels from their samples containing ochratoxin A by growth on DRYES agar. During this analysis individual wheat kernels were placed on agar media and the percentage of kernels from which *P. verrucosum* grew was used as a measure of infection. From these experiments Lund and Frisvad (2003) concluded that an infection rate $>7\%$, i.e., *P. verrucosum* grew from $>7\%$ of the kernels, indicates that ochratoxin A will be present. These results reaffirm that *P. verrucosum* is adapted to this environment and that a dry storage environment can support ochratoxin A biosynthesis.

The lack of a role in wheat for *Aspergillus* spp. that synthesize ochratoxin A is consistent with the results of Cabanas *et al.* (2008). These authors isolated 105 strains of *Aspergillus* spp. from commercial wheat flours available in the Spanish market. None of these *Aspergillus* strains produced ochratoxin A. However, from the same flour samples the authors did recover *P. verrucosum* strains that could produce both ochratoxin A and citrinin. These results were confirmed in a second study (Bragulat *et al.*, 2008). In both studies, which covered only the Spanish market, only a few isolates of *P. verrucosum* were recovered. These results are consistent with existing anecdotal reports that *P. verrucosum* is better adapted to a more moderate climate and that this adaptation is why *P. verrucosum* poses a problem in mid- to high-latitude European countries.

Even though *P. verrucosum* is a moderately drought-resistant species, drying cereal grain to a relative humidity of $\leq$14–15% prevents the growth of this fungus and ochratoxin A biosynthesis. Lindblad *et al.* (2004) described a mathematical model of ochratoxin A biosynthesis by *P. verrucosum* in wheat. In this model the colony forming units (cfus) and the water activity (a_w) are correlated with the probability of ochratoxin A being present in the sample. They concluded that the

probability of ochratoxin A being present increases as a_w and cfus of *P. verrucosum* increase. This result emphasizes the importance of controlling the inoculum of *P. verrucosum* present in a cereal sample to reducing the amount of ochratoxin A produced. *Penicillium verrucosum* occurs ubiquitously in cereals, especially wheat, and preventing contamination of wheat samples by *P. verrucosum* seems all but impossible. The number of cfus of *P. verrucosum* is a determinant of cereal quality, with high-quality cereals usually having fewer cfus of *P. verrucosum* than do low-quality cereals.

Controlling the amount of *P. verrucosum* in wheat is important for quality assurance within the food chain. The early detection and quantification of *P. verrucosum* can be accomplished by various molecular approaches, e.g., PCR (qualitative), RT-PCR (quantitative), or microarrays. Moreover, by targeting RNA instead of DNA the activation of important genes, e.g., the genes responsible for ochratoxin A biosynthesis, can be followed directly. These data provide information about the conditions within a food system that result in ochratoxin A biosynthesis. Moreover, these molecular approaches can be used for scientific purposes to study growth, physiology, and the regulation of ochratoxin A biosynthesis directly in the food system. Therefore, monitoring *P. verrucosum* in wheat by molecular methods suffices for evaluating the risk of ochratoxin A contamination.

PCR Methods to Detect *P. verrucosum* in Wheat

A prerequisite for the development of a specific PCR reaction is the availability of a suitable specific DNA sequence. For *Penicillium* spp. the ITS regions are not suitable because the variation in these regions is too limited, although there is a PCR detection system based on this region for *P. carneum* and *P. roqueforti*, two spoilage organisms of the bread industry (Pedersen *et al.*, 1997). A specific PCR method for demethylation inhibitor (DMI)-resistant strains of *P. digitatum*, a postharvest contaminant of citrus fruits, also has been described (Hamamoto *et al.*, 2001). This system is based on a target region that precedes *pdcyp*51, which encodes the target enzyme for DMIs. Several fungicides, e.g., thiobendazol, act as DMIs. There is a PCR system for detecting another postharvest decay fungus of fruit, especially apples, caused by *P. expansum* (Marek *et al.*, 2003) that is based on the polygalacturonase gene. A LAMP system has been developed for the pathogenic fungus *Penicillium marneffei* (Sun *et al.*, 2010) that should be adaptable for *Penicillium* spp. that contaminate food, e.g., *P. verrucosum.*

Heterologous PCR primers for fungal polyketide synthase (*pks*) genes have been used to PCR amplify a 616 bp fragment of the chromosomal DNA of *P. verrucosum*. This fragment has high homology to fungal *pks* genes and its transcription coincides with the biosynthesis of ochratoxin A, suggesting that the cloned DNA fragment is part of the ochratoxin A polyketide synthase. By using primers internal to the 616 bp fragment, a PCR reaction specific for *P. verrucosum* was developed that had no cross-reactivity with any of the tested fungi, including ochratoxin A-producing species of *Aspergillus* (Schmidt-Heydt *et al.*, 2008a). However, there was a clear positive reaction with *Penicillium nordicum*, the other species of *Penicillium* known to produce ochratoxin A. *Penicillium nordicum* is well adapted to protein- and NaCl-rich foods, however, and is only rarely found on cereals. Thus, this cross-reactivity does not pose a problem for the use of this method to identify *P. verrucosum* in wheat.

The PCR method was used to detect *P. verrucosum* in wheat samples with different levels of contamination of the fungus. All of the samples with $\geq 10^3$ cfu/g gave positive results, although the intensity of the amplified band varied from intense to weak depending on the quality of the isolated DNA. At this level of contamination with *P. verrucosum*, the probability of a sample containing ≥ 5 μg/kg of ochratoxin A, the EU statutory limit, is $\geq 7\%$ (Lindblad *et al.*, 2004).

Another PCR method for *P. verrucosum* has also been described by Dao *et al.* (2005), who identified a primer pair based on the polyketide synthase gene of *A. ochraceus* that also amplifies a DNA fragment from *P. verrucosum*. However, no experiments testing this PCR under practical conditions have been reported.

AFLP fingerprints were used by Frisvad *et al.* (2005) to analyze 321 strains of *P. verrucosum* from cereals (oats, wheat, and barley) from Denmark, the United Kingdom, and Sweden. Of these, 236 strains produced ochratoxin A under the conditions used and 190 of these strains were analyzed by AFLP. One hundred and thirty-eight strains had unique AFLP patterns and could not be grouped, whereas 52 isolates could be placed in small groups containing 2–4 isolates each. Thus, *P. verrucosum* is genetically a very heterogeneous species. It is not known if the ochratoxin A-negative strains described by Frisvad *et al.*'s (2005) study would react with the PCR systems described here. No PCR system targeted to the citrinin biosynthesis genes of *P. verrucosum* has been described yet.

RT-PCR Methods for Quantification and Monitoring of *P. verrucosum* in Wheat

Targeting DNA by RT-PCR

A conventional PCR system, as described above, is a very useful first step for detecting the presence of potential ochratoxin A-producing *Penicillium* strains. However, this assay delivers only a "yes" or "no" answer regarding the presence of an ochratoxigenic *P. verrucosum* strain and no further conclusions can be drawn. More detailed data require more sophisticated analyses, e.g., from a RT-PCR or a reverse transcriptase RT-PCR. For *P. verrucosum*, a RT-PCR system has been developed that targets an internal fragment of the ochratoxin A polyketide synthase. This system has been used to follow the growth kinetics of *P. verrucosum* in a wheat system. The wheat was inoculated with 10^6 spores/g and then stored at a relative humidity $>15\%$ with samples taken for 42 days and the cfus quantified by conventional plate counts and by RT-PCR (Schmidt-Heydt *et al.*, 2008a).

In the first 10 days, the number of cfus dropped to 10^3 cfu/g, and no RT-PCR signal was detected. These data suggest that the adaptation of *P. verrucosum* from lab media to the new habitat is difficult since more than 99% of the population apparently died. Once adapted however, the fungus begins to grow until it reaches $\sim 10^9$ cfu/g. After the fungus begins to grow in the wheat, its presence was quantifiable with RT-PCR. The RT-PCR values increase in parallel with the cfu values obtained from plate counts. Thus, it is possible to accurately quantify *P. verrucosum* in wheat by using RT-PCR. However, it is not clear why *P. verrucosum* could not be detected in the RT-PCR assays during the lag phase of the first 10 days. One explanation could be that an actively growing fungal population is necessary to isolate DNA and that the DNA is not easily released from the spores present in a nongrowing lag phase culture.

Detection and quantification of DNA by PCR or RT-PCR gives information about the presence and the amount of *P. verrucosum* in a wheat sample, but provides no information about the physiological activity, e.g., ochratoxin A production. Such measures are at least partly possible by using reverse transcriptase PCR or reverse transcriptase RT-PCR, as the mRNA transcripts can be quantified. Transcription of the ochratoxin A biosynthesis genes must precede ochratoxin A biosynthesis, so *in situ* monitoring of gene activation provides an early alert for ochratoxin A biosynthesis. If the conditions that regulate ochratoxin gene activation *in situ* are known, and if the time between gene activation and the first analytical detection of the toxin is long enough, then proactive measures might be applied to reduce or prevent measurable ochratoxin A synthesis. These measures could

include changes in storage conditions such as relative humidity or temperature. Before this approach can be used, knowledge of the relationship between gene expression and toxin biosynthesis must be known.

The expression of some genes, here termed key genes, is tightly coupled to toxin biosynthesis, e.g., the *afl*D gene in the aflatoxin biosynthesis pathway (Scherm *et al.*, 2005), the *TRI*5 gene of *Fusarium* spp. (Doohan *et al.*, 1999), and the *otapks*PV gene of either *P. verrucosum* or *P. nordicum* (Geisen *et al.*, 2004). Such studies have been made of *P. verrucosum* in a wheat storage system. Wheat was inoculated with *P. verrucosum* and stored at ambient temperature and a relative humidity >15%. Samples were taken at various times up to 58 days and the amount of ochratoxin A produced was determined by HPLC, whereas the expression of the *otapks*PV gene was determined by RT-PCR (Schmidt-Heydt *et al.*, 2008a).

In *in vitro* assays, *otapks*PV gene expression precedes the presence of analytically detectable amount of ochratoxin by 36–48 hours (Geisen *et al.*, 2004). Under *in situ* conditions, i.e., directly in the wheat, the conditions are quite different in that an increase in expression occurs several days before high amounts of ochratoxin A (above the statutory limits) accumulate (Schmidt-Heydt *et al.*, 2008a). This time window of several days should enable the application of preventive measures if transcription is monitored regularly.

Based on the expression profile of the ochratoxin A biosynthesis genes under various combinations of water activity and temperature, there are two expression peaks that correspond to ochratoxin A biosynthesis. The larger expression peak occurs near the growth optimum, i.e., cultures maintained under conditions near those that give optimal growth produce high amounts of ochratoxin A. The smaller peak occurs when the conditions are marginal for growth, i.e., when either temperature, water activity, or their combination is unfavorable for the fungus or when growth is retarded due to an abiotic stress (Schmidt-Heydt *et al.*, 2008b). Thus ochratoxin A production is expected whenever wheat is stored at temperatures that are low enough to slow, but not stop fungal growth.

This hypothesis was tested in an experiment in which inoculated wheat was stored in silos at ambient temperatures at a relative humidity >15% for several months (Schmidt-Heydt *et al.*, 2007b). During that time the temperature dropped from 20°C, at which moderate ochratoxin A gene expression occurred, to 12°C in the early winter, and then to 5°C during late winter. *otapks*PV transcription was an order of magnitude higher in November, when the median temperature was 12°C, than it was in any other month between September and February (Table 11.1). Ochratoxin A production also was highest in November, but only by a factor of 1.3. In general, expression levels and toxin production were not tightly correlated ($r = 0.734$, $p \leq 0.097$).

Table 11.1 *otapks*PV expression and ochratoxin A production during storage of wheat in a silo under ambient conditions (Schmidt-Heydt *et al.*, 2007a)

Month	Mean temperature (°C)	*otapks*PV expression (copy number)	Ochratoxin A (μg/kg)
September	21	118	313
October	21	235	311
November	11	2260	437
December	9	193	350
January	6	370	241
February	6	197	206

These results reemphasize the importance of abiotic stresses for the induction of the ochratoxin A biosynthesis genes and how managing these stresses can improve food safety. Storage conditions often are optimized to minimize energy usage, e.g., relative humidity and temperature are set at the growth/no growth boundary for the fungus. The data outlined above suggest that these conditions are not conducive to good grain quality as they are well suited for toxin production.

Suboptimal concentrations of preservatives (in wheat products) or fungicides also may retard, but not stop, the growth of *P. verrucosum* and provide favorable conditions for mycotoxin biosynthesis. For example, suboptimal amounts of the preservatives potassium sorbate or calcium propionate can result in drastic increases in *otapks*PV gene expression and corresponding increases in ochratoxin A biosynthesis (Schmidt-Heydt *et al.*, 2007b). As with temperature or relative humidity, if the parameter is shifted past the marginal value, then ochratoxin A biosynthesis ceases. The fungicide carbendazim does not completely inhibit the growth of *A. carbonarius*, which can produce ochratoxin A, and at some levels the presence of this fungicide may increase the amount of ochratoxin A produced (Medina *et al.*, 2007). Suboptimal amounts of some fungicides also activate the trichothecene biosynthesis genes (Ochiai *et al.*, 2007).

Similar work has not been conducted with strains that synthesize citrinin, although a *pks* gene from the citrinin biosynthesis pathway of *Monascus purpureus* has been identified (Shimizu *et al.*, 2005). Disruption of this gene confirmed its involvement in the citrinin biosynthetic pathway (Shimizu *et al.*, 2007).

The Application of Novel Platforms

In addition to technologies such as RT-PCR, which can be used simultaneously with at most one or a few genes, high-throughput platforms such as the open array Biotrove system or microarrays can also be used for detection and monitoring purposes. MycoChip, a flexible microarray, carries a subarray containing the ochratoxin A biosynthetic genes (Schmidt-Heydt and Geisen, 2007). This subarray can be used for identification purposes, since only ochratoxin A-producing strains of *Penicillium* should hybridize with the ochratoxin biosynthetic gene oligonucleotides in the array and should result in a characteristic pattern. However, this array was developed for expression analysis to determine the influence of food-relevant parameters on the expression of the ochratoxin A biosynthesis genes. In the storage experiment described earlier, in which RT-PCR was used to measure gene expression in wheat and in which the production of ochratoxin A could be predicted several days in advance, similar conclusions could be drawn following analysis with the microarray.

Both the microarray and the RT-PCR detected, roughly at the same time (day 25), the activation of *otapks*PV gene expression, although the microarray also detected the activation of the rest of the ochratoxin A biosynthesis cluster and associated genes as well.

The microarray was used to study the impact of food-relevant parameters, e.g., temperature and water activity, on the activation of ochratoxin A biosynthetic genes of *P. verrucosum* and resulted in a clear expression profile (Figure 11.1; Schmidt-Heydt *et al.*, 2008b). There was a clear, major peak near the growth optimum of the fungus, i.e., at an a_w of 0.98 and a temperature of 25°C. At moderate conditions, i.e., a_w of 0.95 and a temperature of 20°C, both moderate gene expression occurred and moderate levels of toxin were produced. However, if the conditions lead to stress and result in reduced growth, e.g., an a_w of 0.93 and a temperature of 15°C, then the activation of the ochratoxin A biosynthetic genes increases to a minor optimum that was paralleled by an increase in ochratoxin A biosynthesis. Initially, these experiments were designed to find combinations of parameters that could be used to control ochratoxin A biosynthesis at moderate conditions. However,

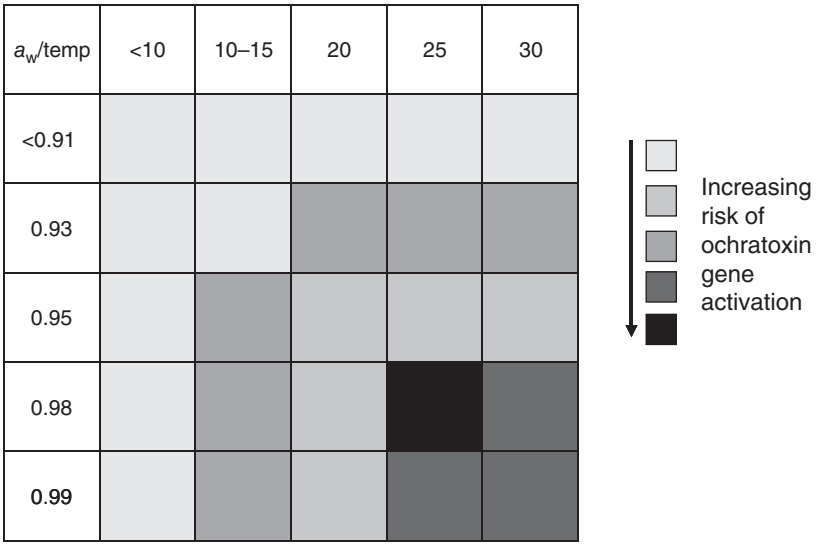

Figure 11.1 Risk map of ochratoxin biosynthesis by *Penicillium* based on expression data.

after extensive expression analysis, it became clear that both *P. verrucosum* and *P. nordicum* can produce ochratoxin A, under nearly all conditions where growth is possible as long as the substrate on which the fungus is growing supports ochratoxin A biosynthesis.

Outlook

Molecular detection methods for most wheat-relevant mycotoxin-producing fungi are available and are in at least experimental use. These methods provide information on a range of characters that would otherwise be unavailable. With the advent of open multi-platforms, e.g., microarrays and Biotrove, questions about gene regulation *in situ* or changes in population due to climatic and other changes can be treated at a high-throughput or genome-wide level. The rapid progress in genomics will almost certainly lead to new technologies that can be used to better understand toxin biosynthesis and its control.

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12 Analytical Methods for Mycotoxins in the Wheat Chain

John Gilbert and Michelangelo Pascale

Abstract

This chapter reviews state-of-the-art analytical methods for the determination of major mycotoxins occurring in wheat and wheat-based products. Screening (commercially available) methods, official methods, and methods that have undergone inter-laboratory validation and research methods for the determination of ochratoxin A, deoxynivalenol, nivalenol, zearalenone and T-2 and HT-2 toxins in the wheat chain are critically assessed. A brief overview is given of emerging technologies, including tandem mass spectrometry, fluorescence polarization immunoassays, immunochromatographic tests, infrared spectroscopy, and biosensors based on surface plasmon resonance and screen-printed electrodes.

Keywords: aptamers; biosensors; deoxynivalenol; ELISA; fluorescence polarization; immune assays; infrared spectroscopy; mass spectrometry; nivalenol; ochratoxin A; official methods; rapid screening; T-2 toxin; zearalenone

Introduction

In Europe, the requirement to determine ochratoxin A, deoxynivalenol, zearalenone, and T-2/HT-2 toxins in the wheat chain in grain, animal feed, and finished products is driven by a regulatory framework with various limits for mycotoxin/commodity combinations. Testing occurs along the food chain and the method(s) selected depends on the requirements for accuracy and speed and constraints such as cost and availability of laboratory facilities.

Screening methods offer speed and the convenience of on-site testing, whereas for enforcement purposes fully validated instrumental methods are required, as unequivocal identification and accurate quantification must be demonstrated. Methodological innovation is occurring rapidly in mycotoxin testing, through the development of novel diagnostic tests and the application of more sophisticated instrumental methods. For a novel diagnostic test to move from research to everyday use, commercial exploitation is necessary. Routine application of more sophisticated instrumental methods, e.g., liquid chromatography–tandem mass spectrometry (LC-MS/MS), requires demonstration of their cost-effectiveness and robustness.

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Screening Methods for Mycotoxins in the Wheat Chain

Screening methods are generally rapid and easy to use, do not require skilled/trained operators, and are versatile enough for field use, i.e., not requiring a laboratory or sophisticated instrumentation (Schneider *et al.*, 2004). Screening techniques have a long history in mycotoxin analysis. In the 1970s a mini-column screening method for detecting aflatoxin was based on the adsorption of aflatoxin onto a small diameter glass column packed either with selected chromatographic adsorbents or with other inorganic adsorbing materials. The extract was passed through the column, the column was washed, and then examined for a tight fluorescent band when illuminated with UV light. These columns were inexpensive, but were severely lacking in specificity. Screening for mycotoxins was revolutionized with the introduction of antibody technology. At least nine companies now offer commercially various products for screening ochratoxin A, deoxynivalenol, zearalenone, and T-2/HT-2 toxins in cereals (Table 12.1). These test kits, which are available as lateral flow devices, test cards, dipsticks, or fluorescence polarization (FP) are quick and easy to use and suitable for on-site testing.

Enzyme-linked immunoassay (ELISA) kits require more skill than the tests mentioned above. ELISA kits may give a semiquantitative measurement, but require either an ELISA plate reader or a reader sold by the kit manufacturer. ELISA tests, especially in the 96-well format, are most cost-effective when screening a large number of samples.

Dipstick and Lateral Flow Devices

The simplest dipstick devices are single-step assays based on a competitive immunoassay. The device is dipped into diluted sample extract, which is drawn up through a reagent zone that contains antibodies specific for the mycotoxin conjugated to colored particles (often colloidal gold). If the mycotoxin is present, it is captured by the particle–antibody complex. The mycotoxin–antibody–particle complex permeates onto a membrane containing a zone of mycotoxin conjugated to a protein carrier. This zone captures any uncomplexed mycotoxin antibody, allowing the particles to concentrate and form a visible line. As the level of mycotoxin in the sample increases, free mycotoxin complexes with the antibody–gold particles reducing the amount of antibody-gold captured in the test zone. Therefore, as the concentration of mycotoxin increases, the test line density decreases. The membrane also contains a control zone where an immune-complex present in the reagent zone is captured by an antibody, forming a visible line. The control line is always visible, regardless of the level of mycotoxin, and demonstrates that the strip is functioning properly. After being dipped for 5 minutes in the sample extract, the strip is removed and read directly either visually, to give a "Yes" or "No" above a predetermined limit, or placed in a simple reader to give a quantitative result.

Lateral flow devices (Anfossi *et al.*, 2013; Krska and Molinelli, 2009) function in a similar manner but operate horizontally, with the sample extract drawn from the spotting zone through the device under capillary action. Lateral flow devices range from free-standing strips to strips presented in a plastic housing. Dipsticks or lateral flow devices are available commercially for ochratoxin A, deoxynivalenol, zearalenone, and combined T-2 and HT-2 toxins. For deoxynivalenol, there are nine different lateral flow type products on the market (Table 12.1). These devices have limits of detection (LODs) from 200 to 1000 μg/kg which meets the EU requirement for monitoring deoxynivalenol in unprocessed wheat (limits of 1250 and 1750 μg/kg for common wheat and durum wheat, respectively), wheat flour, bran and germ (750 μg/kg) and bread, pastries, biscuits, and breakfast cereals (500 μg/kg), but they are not sensitive enough to monitor deoxynivalenol at the 200 μg/kg limit for wheat-based foods for infants and young children (European Commission, 2007).

Enzyme-Linked Immunoassay Screening

Direct competitive ELISA test kits in microtiter well format can provide quantitative measures of concentrations from sub-μg/kg to mg/kg. The concentration range and LODs can be predetermined by juggling the dilutions of the sample extract to ensure analyte concentrations fall within the calibration range of the kit.

In these ELISA tests, the antibody conjugate is bound to the well of the microtiter plate and free toxin in the sample and controls compete with enzyme-labeled toxin (conjugate) for the antibody-binding sites. After a wash step, the substrate reacts with the bound enzyme conjugate to produce blue color. A microwell reader is used to measure optical density. Control optical densities form a standard curve against which sample optical densities are plotted to estimate toxin concentration. Generally a weighed sample is extracted by blending or shaking with methanol/water or water. In a mixing well, 100 μl of conjugate solution is added followed by 100 μl of sample extract and then 100 μl of the mixture is transferred to the antibody wells and allowed to incubate for 10 minutes. The liquid is tipped out of the antibody wells which are then washed thoroughly with deionized water, and the residual water tapped out onto a paper towel. About 100 μL of substrate is added to all of the antibody wells and the incubation is continued for another 10 minutes. Finally, 100 μL of colored stop reagent is added to the antibody wells and the results are read by a microwell reader with a 650 nm filter. ELISA test kits are available for ochratoxin A, deoxynivalenol, zearalenone, and T-2 toxin or combined T-2 and HT-2 toxins from at least three different manufacturers covering the full range of concentrations required for screening for compliance with EU regulatory limits (Table 12.1).

Validation of Screening Assays

Studies comparing the results obtained for the same samples analyzed with a screening technique and with HPLC methods are common. For example, De Saeger *et al.* (2002) conducted an inter-laboratory (10 laboratories) validation of membrane-based flow-through enzyme immunoassays for ochratoxin A and T-2 toxin in cereals. For wheat, barley, maize, and rye samples containing ochratoxin A at levels from 1 to 320 μg/kg, the false-positive rate was 2% and the false-negative rate was 4%. For wheat samples containing T-2 toxin at levels from <20 up to 800 μg/kg, the kits had a false-positive rate of 6% and a false-negative rate of 3% (after exclusion of one outlier test kit). This publication is one of the few to report the critical parameters of false-positive and false-negative rates. This information needs to be more widely available for commercial test kits.

Some commercial test kits have been evaluated by AOAC International and can display the certification mark of "Performance Tested Methods℠." To obtain certification, the manufacturer submits a dossier to AOAC International describing the claimed performance of the method. For screening tests, the entire response range is evaluated by testing a series of test samples and standards consisting of negative test samples, test samples at the specification level (as stated in the kit insert), test samples bracketing the specification level, and strong positive test samples. There also should be 5–10 replicate test samples at each concentration. A response curve of % positive (or negative) against concentration is then constructed.

Accuracy is determined as recovery and should be estimated by comparison of results with an existing method. For qualitative methods, precision cannot be expressed as a standard deviation or coefficient of variation, but instead is expressed as true and false/positive (or negative) rates. These rates are determined at multiple concentrations including the specification level. Comparison with an existing method, when applicable, is strongly recommended (preferably a validated or collaboratively studied method).

Table 12.1 Commercially available screening kits for mycotoxins in the wheat chain

Toxin	Brand name	Company	Principle	Assay time (minutes)	LOD (μg/kg)
Ochratoxin A	OCHRACARD	R-Biopharm	IAC cleanup; apply card; visual, if no color sample >limit	30	Yes/no[a]
	RIDASCREEN® OTA 30/15	R-Biopharm	Competitive ELISA plate reader	45	1.25
	RIDASCREEN® FAST OTA	R-Biopharm	Competitive ELISA plate reader	15	5
	OchraTest™	VICAM	IAC cleanup, elute, fluorometer	10	0.1
	AgraQuant® OTA	Romer Labs	Competitive ELISA plate reader	15	2
	Veratox® for OTA	Neogen Corp	Competitive ELISA plate reader	20	1
	Reveal® Q+ for OTA	Neogen Corp	Lateral flow – reader	9	2
	ROSA® OTA	Charm	Lateral flow—quantitative	10	1
	MycoPlate OTA	AOKIN	Competitive ELISA plate reader	?[b]	?[b]
	QuickTox™ OTA	EnviroLogix	Lateral flow—reader	10	1.5
Deoxynivalenol	RIDA QUICK® DON	R-Biopharm	Lateral flow device—visual/reader	5	500
	RIDASCREEN® DON	R-Biopharm	Competitive ELISA plate reader	45	18.5
	RIDASCREEN® FAST DON	R-Biopharm	Competitive ELISA plate reader	8	200
	RIDASCREEN® FAST DON SC	R-Biopharm	Competitive ELISA plate reader	8	74
	DONCheck™	VICAM	Dipstick test strip—visual	3	1000
	DON-V	VICAM	Dipstick test strip—reader	5	200
	AgraQuant® DON ELISA	Romer Labs	Competitive ELISA plate reader	20	200
	AgraStrip® DON	Romer Labs	Lateral flow—reader	4	500
	Agri-Screen® for DON	Neogen Corp	Competitive ELISA plate reader	10	1000
	Reveal® Q+ for DON	Neogen Corp	Lateral flow—reader	3	300
	Veratox® for DON 2/3	Neogen Corp	Competitive ELISA plate reader	5	100
	Veratox® for DON 5/5	Neogen Corp	Competitive ELISA plate reader	10	100
	Veratox® for DON HS	Neogen Corp	Competitive ELISA plate reader	20	5
	QuickTox™ DON	EnviroLogix	Lateral flow—reader	10	200
	ROSA® DON P/N	Charm	Lateral flow—visual	3	500
	ROSA® DON	Charm	Lateral flow—reader	10	?[b]
	ROSA FAST5 DON	Charm	Lateral flow—reader	5	?[b]
	DON FPA qualitative	Diachemix	Fluorescence polarization	5	1000
	DON FPA quantitative	Diachemix	Fluorescence polarization	15	170

Table 12.1 (*Continued*)

Toxin	Brand name	Company	Principle	Assay time (minutes)	LOD (μg/kg)
Deoxynivalenol	Mycontrol DON	AOKIN	SPE cleanup, fluorescence polarization	6	50
	MycoPlate DON	AOKIN	Competitive ELISA plate reader	?[b]	?[b]
	DONsensor	UNISENSOR	Dipstick strip test—reader	5	200
Zearalenone	RIDASCREEN® ZON	R-Biopharm	Competitive ELISA plate reader	150	1.75
	RIDASCREEN® FAST ZON	R-Biopharm	Competitive ELISA plate reader	15	17
	RIDASCREEN® FAST ZON SC	R-Biopharm	Competitive ELISA plate reader	15	5
	ZearalaTest™	VICAM	IAC cleanup, elute, fluorometer	15	100
	AgraQuant® ELISA ZON	Romer Labs	Competitive ELISA plate reader	15	10
	Veratox® for ZON	Neogen Corp	Competitive ELISA plate reader	10	5
	Reveal® Q+ for ZON	Neogen Corp	Lateral flow—reader	6	50
	ROSA® ZON	Charm	Lateral flow—reader	10	25
	MycoPlate ZON	AOKIN	Competitive ELISA plate reader	?[b]	?[b]
	Mycontrol ZON	AOKIN	SPE cleanup, fluorescence polarization	8	25
T-2	RIDASCREEN® T2	R-Biopharm	Competitive ELISA plate reader	90	5
	RIDASCREEN® FAST T2	R-Biopharm	Competitive ELISA plate reader	15	20
	AgraQuant® ELISA T2	Romer Labs	Competitive ELISA plate reader	15	35
T-2+HT-2	ROSA® T2-HT2	Charm	Lateral flow—reader	10	10
	Reveal® Q+ for T2/HT2	Neogen Corp	Lateral flow—reader	6	50
	Veratox® for T2/HT2	Neogen Corp	Competitive ELISA plate reader	10	25
Deoxynivalenol Zearalenone T-2 + HT-2	4-Mycosensor	UNISENSOR	Dipstick strip test—reader		1400 (deoxynivalenol) 80 (zearalenone) 400 (T-2/HT-2)

[a]LOD depends on the dilution used.
[b]Not reported by the manufacturer.

Cross-reactivity is the study of the test kit response to nontarget analytes in the sample matrices. Stability studies of the test kit components derive from ruggedness testing and might include sensitivity to factors such as time, temperature, and freeze/thaw cycles.

The dossier setting out method performance is assessed by AOAC-appointed experts and, if they are satisfied, then evaluated by an independent testing laboratory. Three of the test kits listed in Table 12.1 currently claim to be AOAC Performance Tested Methods℠—Ridascreen® Fast DON,

VERATOX® for DON 2/3, and the AgraQuant® DON ELISA test kit. The manufacturers of these kits paid an initial fee to AOAC for the initial assessment and an annual renewal fee to continue to claim the status of an AOAC Performance Tested Method℠.

Some test kits receive endorsement of their performance by organizations such as the USDA Grain Inspection Packers and Stockyards Administration (GIPSA), which performs an assessment similar to that of AOAC International. Again the test kit manufacturer submits a data package supporting the performance claims, and GIPSA staff review the data. If the data support the claims, GIPSA staff then conduct an in-house validation of the rapid test and if the manufacturer's claims are verified, a Certificate of Performance is issued. There are 25 GIPSA Performance Verified Rapid Tests for the analysis of ochratoxin A, deoxynivalenol, zearalenone, aflatoxins, and fumonisins in the wheat chain listed on the GIPSA website (http://www.gipsa.usda.gov/fgis/tech-servsup/metheqp/GIPSA_Approved_Mycotoxin_Rapid_Test_Kits.pdf).

While both AOAC International and GIPSA play important roles in verifying performance claims, there are no requirements for kit manufacturers to provide access to the performance data, e.g., false-positive and false-negative rates. The manufacturers use the endorsement for marketing purposes, but promote only those aspects of kit performance that provide a marketing advantage, e.g., speed and sensitivity. Unlike instrumental methods, for which a criteria-based approach is used to establish minimum performance of official methods, there are no criteria in the screening area other than European Directive 96/23/EC which stipulates only that screening methods with a false compliant rate of $<5\%$ (β-error) at the level of interest shall be used (European Commission, 2002).

Official Methods for Mycotoxins in the Wheat Chain

Official methods have been validated in an inter-laboratory collaborative study and are used by official control laboratories or, in the event of a dispute, are used as referee methods. There are many validated methods, but only those that have been critically assessed, recommended, or endorsed by an organization with some "official" standing become known as official methods. What constitutes official standing is debatable, but clearly organizations such as AOAC International, IUPAC, ISO, and Comité Européen de Normalisation (CEN) are recognized international bodies with remits that include the standardization of methods. These bodies do little to initiate collaborative studies themselves and, to a large extent, fund their activities by "selling" adopted methods to end users.

AOAC International

AOAC International requires payment from any organization requesting adoption of their validated method as an AOAC Official Method. The method together with the inter-laboratory validation data are scrutinized by an expert committee of volunteers before the method is adopted as a First Action AOAC Official Method. After a period of time, during which AOAC gets feedback on the method performance, if nothing adverse emerges, the method is adopted as a Final Action. From 1973 to 2000, eight AOAC Official Methods were adopted for wheat and wheat products for mycotoxins contaminated with ochratoxin A, deoxynivalenol, and zearalenone (Table 12.2). Many of these methods are dated, and there are no AOAC Official Methods that use immunoaffinity column (IAC) cleanup for either deoxynivalenol or zearalenone and no AOAC Official Methods at all available for either T-2 or HT-2 toxins in cereals.

Table 12.2 AOAC International Official Methods for mycotoxins in the cereal chain

Mycotoxin	Matrix	Method principle[a]	Date[b]	AOAC ref
Ochratoxin A	Barley	Column cleanup and TLC	1973	973.37
	Barley	IAC cleanup and HPLC (Fl)	2000	2000.03
	Maize and Barley	Liq/liq extr. + SPE and HPLC (Fl)	1991	991.44
Deoxynivalenol	Wheat	Charcoal/alumina and TLC	1986	986.17
	Wheat	Silica gel cleanup and GC	1986	986.18
α-Zearalenol and zearalenone	Maize	Liq/liq partition and HPLC (Fl)	1985	985.18
Zearalenone	Maize	Column cleanup and TLC	1976	976.22
	Maize, wheat, and feed	ELISA	1994	994.01

[a]TLC, thin layer chromatography; HPLC, high performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; IAC, immunoaffinity column; SPE, solid phase extraction; (Fl), fluorescence detection; GC, gas chromatography.
[b]Date method was adopted as a First Action.

Comité Européen de Normalisation

In contrast to AOAC International, CEN, the European body responsible for standardization, does not charge for adoption of proposed methods. CEN's role is to standardize methods to meet the needs of the European Commission for enforcement purposes and, therefore, it requires a mandate from the commission to incorporate new methods in its work program. CEN has a committee structure consisting of representatives of 30 national standardization bodies and 16 additional affiliate members. Technical Committee 275 (TC275) deals with horizontal methods of analysis for food and has a number of working groups of which WG5 is responsible for methods for determining biotoxins (mycotoxins and phycotoxins) in food.

The European Commission uses a criteria-based approach (Table 12.3; Commission Regulation 401/2006; European Commission, 2006) to determine if a method is fit for purpose, in terms of

Table 12.3 Minimum performance criteria of methods to be used for food control purposes in the EU for mycotoxin in wheat and wheat-based products (taken from EC Regulation 401/2006)

Mycotoxin	Commodity	Limit (μg/kg)	RSD_r (%)	RSD_R (%)	Recovery (%)
Ochratoxin A	Cereal infant foods	0.5	≤40	≤60	50–120
	Unprocessed cereals	5.0	≤20	≤30	70–110
	Processed cereals	3.0	≤20	≤30	70–110
Deoxynivalenol	Cereal infant foods	20	≤20	≤40	60–110
	Bread, pastries, biscuits, etc.	500	≤20	≤40	60–110
	Wheat flour, bran, germ	750	≤20	≤40	70–110
	Unprocessed wheat	1250	≤20	≤40	70–110
	Unprocessed durum wheat	1750	≤20	≤40	70–110
Zearalenone	Cereal infant foods	20	≤40	≤50	60–120
	Bread, pastries, biscuits, etc.	50	≤40	≤50	60–120
	Wheat—flour, bran, and germ	75	≤25	≤40	70–120
	Unprocessed wheat	100	≤25	≤40	70–120
T-2	Unprocessed wheat and products	50–250[a]	≤40	≤60	60–130
	Unprocessed wheat and products	>250[a]	≤30	≤50	60–130
HT-2	Unprocessed wheat and products	100–200[a]	≤40	≤60	60–130
	Unprocessed wheat and products	>200[a]	≤30	≤50	60–130

[a]Limits for the sum of T-2 + HT-2 toxin in cereals still to be stipulated. Proposed EU limits are 25–200 μg/kg for unprocessed cereals and cereal products, 1000 μg/kg for unprocessed oats, and 50–200 μg/kg for oat products.
The limit is the EU regulatory limit as stipulated in Commission Regulation No. 1126/2007.

critical performance parameters. There are small differences between the European Commission method criteria and those originally published by CEN, but the latter are being revised and eventually are becoming more closely aligned to one another. CEN has two official HPLC methods for ochratoxin A in cereals, one with a silica gel cleanup (European Committee for Standardization, 1998a) and a second with a bicarbonate cleanup (European Committee for Standardization, 1998b). A CEN method that includes IAC cleanup has been adopted for ochratoxin A in barley (European Committee for Standardization, 2009) and should perform similarly for wheat products. A similar CEN standard is available for ochratoxin A in cereal-based foods for infants and young children that uses IAC cleanup together with HPLC and fluorescence detection (European Committee for Standardization, 2010a). Moreover, two HPLC methods based on IAC cleanup and fluorescence or UV detection have been adopted as European standards for the determination of zearalenone and deoxynivalenol in cereals (grain and flour), cereal products, and cereal-based foods for infants and young children (European Committee for Standardization, 2010b, 2010c).

Inter-laboratory Validated Methods for Determining Mycotoxins in the Wheat Chain

In recent years, most of the validated methods for routine assessment of regulated mycotoxins are based on IAC cleanup (Senyuva and Gilbert, 2010). Numerous commercially available IACs for cleanup of ochratoxin A, deoxynivalenol, zearalenone, and T-2/HT-2 toxins are validated for a variety of matrices, including wheat and wheat products. Performance of the main commercial columns has been compared (Senyuva and Gilbert, 2010). For ochratoxin A, only R-Biopharm Rhone, Ltd. indicates that their columns cross-react with ochratoxin B and ochratoxin C, although the other products probably behave similarly. This cross-reaction is not a problem as the three ochratoxins are separated by HPLC. Similarly, the DONPREP® column for deoxynivalenol cross-reacts with 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol, which is not a problem as the three compounds are separated by HPLC or GC. One manufacturer sells a column that is specific for T-2 toxin, while another cross-reacts with HT-2 toxin. Similar cross-reactivity occurs with zearalenone columns that cross-react with α-zearalenol, β-zearalenol, zearalanone, α-zearalanol, and β-zearalanol (Erbs *et al.*, 2007), although this cross-reactivity is not generally acknowledged by the manufacturers.

There is a validated IAC HPLC method for ochratoxin A in barley (Entwisle *et al.*, 2000) with an average recovery of 93% and a relative standard deviation for between-laboratory reproducibility (RSD_R) of 12–17% which was adopted as the CEN standard. For ochratoxin A in wheat, a UK collaborative study compared two different brands of IACs. For the Ochratest™ column, recoveries averaged 91% with a RSD_R between 20% and 32%, whereas with the OCHRAPREP® columns recoveries averaged 93% with a RSD_R between 14% and 20% (Scudamore and MacDonald, 1998). There also is a validated HPLC method for ochratoxin A in cereal-based infant foods (Burdaspal *et al.*, 2001) that uses IAC cleanup (fluorescence HPLC) and has an LOD below that of the regulatory limit of 0.5 μg/kg. Unfortunately the RSD_R can be high, i.e., between 29% and 63%, meaning that the method does not meet the maximum criteria for an RSD_R of 60% at the regulated level (European Commission, 2006).

For deoxynivalenol, a validated HPLC method uses low-wavelength UV detection and IAC cleanup for wheat flour and wheat-based breakfast cereal (MacDonald *et al.*, 2005a). For deoxynivalenol concentrations between 200 and 2000 μg/kg, recoveries averaged 78–87% and RSD_Rs ranged from 11% to 26%. This method had acceptable precision since its HorRat values were <1.3 (Table 12.2). This method (MacDonald *et al.*, 2005a) also was subjected to an inter-laboratory study

(Neumann *et al.*, 2009) for soft wheat at levels from 200 to 2050 μg/kg. Recoveries ranged from 66% to 98% (average 85%) and RSD_Rs ranged from 21% to 33%, which was slightly poorer than the previous study but still satisfied the method performance requirements. An inter-laboratory study (Aoyama *et al.*, 2012) involving 11 laboratories in Japan evaluated deoxynivalenol and nivalenol in wheat by LC-UV and LC-MS/MS methods. Recoveries at spiking levels of 100, 500, and 1000 μg/kg ranged from 90% to 110% for deoxynivalenol and from 76% to 83% for nivalenol. RSD_Rs were 7.2–25% for deoxynivalenol and 7.0–31% for nivalenol with HorRat values between 0.4 and 1.4. Thus, both LC-UV and LC-MS/MS methods are suitable as official methods.

Inter-laboratory validation (MacDonald *et al.*, 2005b) of an IAC method for zearalenone in wheat flour uses HPLC and fluorescence detection and gave average recoveries of 91–115% and RSD_Rs between 16% and 38%. This method had acceptable precision as its HorRat values were <1.7 (Table 12.2). A similar IAC method was validated for baby food and animal feed (Arranz *et al.*, 2007), with an average recovery of 92% and RSD_Rs between 8.2% and 13%. This method was also assessed for cereal grains and feed ingredients (Campbell and Armstrong, 2007) with slightly improved performance characteristics.

Commercial IACs are now available for T-2 and HT-2 toxins but the lack of a chromophore makes detection by HPLC problematic. Pre-column fluorescence derivatization with 1-anthroylnitrile (1-AN) prior to HPLC with fluorescence detection has been proposed (Pascale *et al.*, 2003). Blank samples contain multiple peaks, but T-2 toxin elutes in a clean area of the chromatogram and a LOD of 5 μg/kg resulted with a recovery of 80–99%. There has been no inter-laboratory validation of an IAC method for T-2 toxin that uses the fluorescence HPLC approach, underivatized detection with LC-MS, or detection of a derivative with GC-MS.

Research Methods for Mycotoxins in the Wheat Chain

The need for more sensitive methods for use in risk assessment studies has increased the demand for new methods, including those that can detect multiple mycotoxins, for rapid screening of materials in the food and feed chain. Also needed are methods to detect known mycotoxins in new food matrices, to identify new mycotoxins, and to identify masked related metabolites in food commodities. These new methods have both benefits and limitations when used to analyze mycotoxins in the wheat chain (Table 12.4).

Chromatographic Methods Including Liquid Chromatography-Mass Spectrometry (LC-MS)

Gas-chromatographic (GC) methods in combination with electron capture (ECD) or mass spectrometric (MS) detection are routinely used for quantitative determination of type-A and type-B trichothecenes in cereals (including wheat) and derived products. Comparative inter-laboratory studies of GC-ECD and GC-MS methods for deoxynivalenol, nivalenol, T-2 toxin, and HT-2 toxin (Krska *et al.*, 2001, 2005) identified analytical problems including nonlinearity of calibration curves, overestimation of toxins due to matrix effects, poor repeatability, and memory effects from previous sample injections. The most recent developments in GC methodology applicable to the wheat chain include the determination of type-A and type-B trichothecenes in wheat grain without sample cleanup by using two-dimensional GC time-of-flight mass spectrometry (Jelen and Wasowicz, 2008). A recent comparison of chromatographic methods for deoxynivalenol in wheat found that GC-MS with ^{13}C-deoxynivalenol as an internal standard (IS) produced results comparable to a

Table 12.4 Benefits and limitations of research methods for analysis of mycotoxins in the wheat chain

Method[a]	Benefits	Limitations	Mycotoxins	References
GC	Simultaneous analysis of mycotoxins Good sensitivity Can be automated (autosampler) Provides confirmation (MS detector)	Derivatization required Matrix interference problems Nonlinear calibration curve Drifting response Carryover effects from previous sample Variation in reproducibility and repeatability	Type-A and type-B trichothecenes	Krska *et al.* (2001) Jelen and Wasowicz (2008) Neuhof *et al.* (2009)
HPLC	Good sensitivity and selectivity Good repeatability Short analysis times Can be automated (autosampler)	Derivatization required for type-A trichothecenes Cleanup required	Deoxynivalenol Ochratoxin A T-2 and HT-2 toxins Zearalenone	Pascale and Visconti (2008)
LC-MS	Simultaneous analysis of mycotoxins and/or masked mycotoxins High sensitivity (LC-MS/MS) Provides confirmation Can be automated (autosampler) No derivatization required	Matrix-assisted calibration curve (for quantitative analysis) or use of internal standards is not always available/possible Very expensive equipment Specialist expertise required	Aflatoxins Enniatins Ergot alkaloids Fumonisins Moniliformin Ochratoxin A Type-A and type-B trichothecenes, masked deoxynivalenol Zearalenone, its derivatives, and masked zearalenone	Sulyok *et al.* (2006) Krska and Molinelli (2007) Lattanzio *et al.* (2008) Berthiller *et al.* (2009) Vendl *et al.* (2009) Zachariasova *et al.* (2010) Vaclavik *et al.* (2010) Kadota *et al.* (2011) Lattanzio *et al.* (2011) Nakagawa *et al.* (2011) de Boevre *et al.* (2012) Lattanzio *et al.* (2012b) Tang *et al.* (2013) Veprikova *et al.* (2012)

FP immunoassay	Rapid High sample throughput Can be automated Good sensitivity No cleanup required Cost effective Easy to perform	Synthesis of specific tracer Matrix effects Sensitive to organic solvent	Deoxynivalenol	Lippolis *et al.* (2006) Lippolis *et al.* (2011) Bondarenko and Eremin (2012)
Biosensor	Suitable for screening Possibility of multi-analyte detection Inexpensive analysis Sensor regeneration (reusability)	Labeling may be needed Matrix interference problems Cleanup may be needed Lack of validated methods Low sensitivity	Deoxynivalenol Nivalenol Ochratoxin A T-2 and HT-2 toxins	Prieto-Simon *et al.* (2007) Maragos (2009a) Ricci *et al.* (2009) Meneely *et al.* (2010) Dorokhin *et al.* (2011) Maragos (2011) Li *et al.* (2012) Meneely *et al.* (2012)
Immunochromatography test	Simple and fast (5–10 minutes) Visual assessment No cleanup required Can be used *in situ* Inexpensive equipment	Qualitative or semiquantitative (cutoff level) Possible false-positive/-negative results Cross-reactivity with related mycotoxins Matrix interference problems Lack of validated methods	Deoxynivalenol Ochratoxin A T-2 toxin Zearalenone	Krska and Molinelli (2009) Lai *et al.* (2009) Anfossi *et al.* (2011) Huang *et al.* (2012) Lattanzio *et al.* (2012b) Liu *et al.* (2012a)
IR/UV-visible spectroscopy	High sample throughput No sample preparation required Nondestructive analysis Inexpensive analysis	Semiquantitative (cutoff levels) Applicable at high contamination levels Large calibration sets required Lack of appropriate calibration materials	Deoxynivalenol	de Girolamo *et al.* (2009) Siuda *et al.* (2008) Peiris *et al.* (2010) Dvoracek *et al.* (2012)

[a]GC, gas chromatography; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography/mass spectrometry; FP, fluorescence polarization; IR, infrared; UV, ultraviolet.

^{13}C-IS-LC-MS/MS method with similar sensitivity, whereas ECD detection was slightly less sensitive (Neuhof *et al.*, 2009).

IAC cleanup and HPLC with fluorescence detection (FD) or UV-diode array detection are currently used for quantitative analysis of deoxynivalenol, ochratoxin A, and zearalenone in cereals and derived products (Pascale and Visconti, 2008). The possibility of using HPLC-FD for type-A trichothecene, i.e., T-2/HT-2 toxins, determination depends on the availability of suitable derivatizing reagents. 1-AN, 1-naphthoyl chloride (1-NC), 2-naphthoyl chloride (2-NC), and pyrene-1-carbonyl cyanide (PCC) are all efficient fluorescent-labeling reagents for T-2/HT-2 under mild conditions. A derivatizing reaction with 1-AN was used to develop a sensitive, reproducible, and accurate method for the simultaneous determination of T-2/HT-2 in raw cereals (including wheat) and processed products by HPLC-FD after IAC cleanup (Pascale *et al.*, 2008a; Visconti *et al.*, 2005). A rapid ultra-performance liquid chromatographic (UPLC®) method with IAC cleanup of extracts for the simultaneous determination of T-2 and HT-2 toxins in wheat and oats avoids the pre-column derivatization step. Toxins were separated and quantified by UPLC® with photodiode array (PDA) detector in less than 5 minutes with an 8 μg/kg detection limit for both toxins, similar to those reported for HPLC methods that use fluorescence detection. There is good correlation between results from the UPLC® method or a reliable HPLC method with fluorescence detection and derivatization with 1-AN (Pascale *et al.*, 2012).

A phosphate buffer extraction procedure containing maize esterase activity that hydrolyzes T-2 to HT-2 can be used for quantitative determination of the sum of T-2/HT-2 in wheat, maize, and oats by LC-MS/MS (Lattanzio *et al.*, 2009a). This procedure uses no organic solvents and could enable better antibody-based methods for detecting these toxins, as the compatibility of the sample extract with the antibody is a common limiting factor.

LC-MS has been used for many years to confirm mycotoxin presence and identification. LC-MS/MS is the most promising technique for simultaneously screening, identifying, and measuring a large number of mycotoxins. The first validated method determined 39 mycotoxins in wheat (and maize) with a single extraction step followed by LC-ESI-MS/MS (Sulyok *et al.*, 2006). The 39 analytes included type-A and type-B trichothecenes, zearalenone and derivatives, fumonisins, enniatins, ergot alkaloids, ochratoxin A, aflatoxins, and moniliformin. Extracts were injected without any cleanup after dilution. Ion suppression effects due to co-eluting matrix components were negligible for wheat, but signal suppression resulting in systematic errors occurred in maize. Quantitative measurement of mycotoxins by LC-MS is often unsatisfactory due to matrix effects and ion suppression. Matrix-matched calibration, use of isotope-labeled standards, and extract dilution all have been proposed to overcome these matrix effects (Krska and Molinelli, 2007). External matrix calibration, which is extremely time consuming, is needed for each commodity analyzed. Commercially available (^{13}C) labeled mycotoxins, which can be used as internal standards, have made this technique more manageable. Sensitive, robust LC-MS/MS methods that use reversed phase solid phase extraction (SPE) Oasis® HLB columns or Myco6in1™ IACs for extract cleanup can simultaneously determine aflatoxins B_1, B_2, G_1, and G_2, ochratoxin A, nivalenol, deoxynivalenol, zearalenone, and T-2 and HT-2 toxins in a number of cereals, including wheat, barley, and maize, and in cereal-based foods including durum wheat flours and rye- and wheat-based crisp bread with detection limits in the μg/kg range (Lattanzio *et al.*, 2008, 2011; Tang *et al.*, 2013).

LC-MS/MS is a powerful technique for the determination of masked mycotoxins, e.g., deoxynivalenol-3-glucoside or zearalenone-4-glucoside, in wheat (Berthiller *et al.*, 2009). Masked mycotoxins are mycotoxins conjugated to more polar substances that are not detected by routine analytical methods for mycotoxins, which can result in significant underestimation of the toxic potential of a food/feed sample. A LC-MS/MS method for the simultaneous determination of

deoxynivalenol, zearalenone, and their major masked metabolites in cereal-based food matrices, including pasta, was used to determine their levels in approximately 100 different cereal-based food samples (Vendl *et al.*, 2009). From 2010 to 2012 many papers were published on the development of LC-MS/MS methods for the simultaneous determination of trichothecenes and for identification and characterization of masked *Fusarium* mycotoxins, e.g., T-2 and HT-2 or fusarenone glycosyl derivatives, in wheat. Different sample preparation procedures, ionization techniques, and mass detectors, including high-resolution mass spectrometry (HRMS), were used (de Boevre *et al.*, 2012; Kadota *et al.*, 2011; Lattanzio *et al.*, 2012a; Nakagawa *et al.*, 2011; Vaclavik *et al.*, 2010; Veprikova *et al.*, 2012; Zachariasova *et al.*, 2010).

Emerging Technologies

Many rapid methods based on fluorescence polarization (FP) immunoassays, immunochromatographic tests, immunosensors, or infrared (IR) spectroscopy have been evaluated for mycotoxin analyses (Lattanzio *et al.*, 2009b; Maragos and Busman, 2010; Prieto-Simon *et al.*, 2007). Due to their low costs, rapid completion, and ease of use, these methods have great potential for the determination of mycotoxins in food matrices.

FP immunoassays measure interactions between a fluorescently labeled antigen (tracer) and a specific antibody. A rapid FP immunoassay for deoxynivalenol, optimized for common wheat, durum wheat, semolina, and pasta (Lippolis *et al.*, 2006), performed as well or better than a validated HPLC/immunoaffinity method. Average recoveries from spiked samples were >98%, with RSDs <5%, with a detection limit of 80 μg/kg for all matrices. The overall time for deoxynivalenol analysis in wheat-based products, including sample preparation and FP immunoassay measurement, was 10 minutes. A fully automated FP prototype was developed by combining an FP reader with an autosampler and analyzing the data with a PC (Pascale *et al.*, 2008a). This automated FP immunoassay system can screen deoxynivalenol at levels that occur naturally in wheat and wheat-based products. An FP immunoassay has been developed for the simultaneous determination of T-2 and HT-2 toxins in naturally contaminated wheat. The most sensitive antibody was specific for HT-2 and 100% cross-reactive with T-2 toxin. The average recovery from spiked wheat samples was 96% with RSDs lower than 8% and a detection limit of 8 μg/kg for the combined toxins (Lippolis *et al.*, 2011). The concept of determining the total content of T-2 and HT-2 in cereal samples is consistent with EU legislation. FP immunoassays also can be used to determine zearalenone and ochratoxin A levels in grains, but with a detection limit of 10 μg/kg for ochratoxin A that is too high for routine screening (Bondarenko and Eremin, 2012).

Immunochemical biosensors that use surface plasmon resonance (SPR), biolayer interferometry, or screen-printed carbon electrodes may be used to detect mycotoxins in cereals (Li *et al.*, 2012; Maragos 2009a, 2011; Prieto-Simon *et al.*, 2007). Competitive SPR-based immunoassays are available for determining deoxynivalenol in wheat and wheat-based breakfast cereals with or without extract cleanup. The results obtained with the biosensor were similar to those obtained with GC-MS, HPLC-UV, and HPLC-MS/MS (Meneely *et al.*, 2010; Schnerr *et al.*, 2002; Tudos *et al.*, 2003). Rapid SPR-based multiplex immunoassays are available for the simultaneous determination of the sum of T-2 and HT-2 toxins and deoxynivalenol in wheat, breakfast cereals, and maize-based baby food (Meneely *et al.*, 2012), for deoxynivalenol and nivalenol in wheat (Kadota *et al.*, 2010), and for zearalenone and deoxynivalenol in wheat and maize (Dorokhin *et al.*, 2011). The results obtained from the SPR-based immunoassays and those from the confirmatory mass spectrometry methods were highly correlated.

Competitive electrochemical ELISAs based on disposable screen-printed carbon electrodes are available for quantitative determination of ochratoxin A in wheat. Results from screen-printed carbon electrodes and HPLC/IAC cleanup methods for naturally contaminated wheat samples were similar (Alarcón *et al.*, 2006). A 96-well electrochemical plate equipped with screen-printed electrodes coupled with microwave hydrolysis can detect deoxynivalenol and nivalenol in wheat after SPE cleanup, but the detection limit (1100 μg/kg) is too high for it to be used for analyzing samples intended for human consumption. This method also cannot distinguish between deoxynivalenol and nivalenol (Ricci *et al.*, 2009). Gold nanoparticles coupled to the antigen increase the sensitivity of a nanostructured electrochemical immunosensor that uses polyclonal antibodies and disposable screen-printed electrodes to determine ochratoxin A in wheat. These immunosensors can measure ochratoxin A in wheat samples with a linear range of 0.4– 7.2 μg/kg, a LOD of 0.2 μg/kg, and recoveries from 104% to 107% (Bonel *et al.*, 2010). Colloidal gold also can amplify signals in a biolayer interferometry-based immunosensor for the rapid determination of deoxynivalenol in wheat, with a detection limit of 90 μg/kg (Maragos, 2012).

A fluorescence-based array biosensor, i.e., the NRL-array biosensor, was developed to simultaneously detect ochratoxin A and deoxynivalenol in cereal samples, including wheat. This technique needs no sample pretreatment, which reduces cost, but the detection limits for ochratoxin A in wheat and barley were high, suggesting that further assessments of matrix effects or pretreatment of sample extracts still are needed (Prieto-Simon *et al.*, 2007).

Sensitive, quantitative lateral flow tests have been developed for measuring deoxynivalenol in matrices of the durum wheat chain (durum wheat, semolina, and pasta) and zearalenone in wheat, maize, and cereal-based baby food. Results from the lateral flow tests were consistent with LC-MS results, so the test is a reliable rapid screen for wheat samples that satisfies the regulatory requirements for deoxynivalenol and zearalenone (Liu et al., 2012a, 2012b).

Immunochromatographic tests for the semiquantitative determination of T-2 toxin and deoxynivalenol in wheat have been combined as a qualitative lateral flow one-step test for the simultaneous detection of deoxynivalenol and zearalenone (Huang *et al.*, 2012; Krska and Molinelli, 2009). A colloidal gold strip for the rapid detection of ochratoxin A in wheat and maize with a mimotope peptide has low sensitivity (Lai *et al.*, 2009). A sensitive, quantitative lateral flow immunoassay for detecting ochratoxin A in common wheat, durum wheat, and maize has a detection limit of 1.5 μg/kg. Results from this test were correlated with those from the reference method (Anfossi *et al.*, 2011). A multiplex dipstick immunoassay based method also has been validated for the simultaneous determination of zearalenone, T-2/HT-2 toxins, deoxynivalenol, and fumonisins in wheat, oats, and maize. The optimized immunoassay could detect these mycotoxins at 280, 400, 1400, and 3200 μg/kg, respectively, for zearalenone, T-2/HT-2 toxins, deoxynivalenol, and fumonisins in maize, and 80, 400, and 1400 μg/kg, respectively, for zearalenone, T-2/HT-2 toxins, and deoxynivalenol in wheat and oats, in less than 30 minutes (Lattanzio *et al.*, 2012b).

Infrared spectroscopy is an important emerging technique for detecting mycotoxins in grains because it requires minimal sample preparation and can be used for screening. Near-infrared (NIR) spectroscopy combined with principal component analysis (PCA) has been used for rapid screening of deoxynivalenol in wheat (Dowell *et al.*, 1999; Pettersson and Aberg, 2003). NIR transmittance is used to determine deoxynivalenol levels in whole wheat kernel samples at levels >500 μg/kg, whereas NIR reflectance is used to detect scab and to estimate deoxynivalenol and ergosterol contents of single wheat kernels. Fourier transform near-infrared (FT-NIR) spectroscopy also can be used to determine the deoxynivalenol content of durum and common wheat. A semiquantitative model was developed to discriminate between blank and naturally contaminated wheat samples at 300 μg/kg (de Girolamo *et al.*, 2009). FT-NIR spectroscopy has been used to estimate deoxynivalenol content

in intact wheat kernels, providing a useful tool for breeders in artificially inoculated experiments for selection of wheat cultivars resistant to *Fusarium* (Dvoracek *et al.*, 2012; Peiris *et al.*, 2010). A similar approach uses diffuse reflectance UV-vis spectroscopy to evaluate deoxynivalenol content in scab-damaged wheat (Siuda *et al.*, 2008). The amplitude of the acoustic signal that penetrates the sample is strongly correlated with deoxynivalenol, zearalenone, and T-2/HT-2 toxin levels in the kernels (Juodeikiene *et al.*, 2011). Advantages of these spectroscopic methods include (i) the ease of operation, (ii) the speed of the analysis, and (iii) their nondestructive nature, leaving the sample intact. Major limitations to these methods include (i) high matrix dependence, (ii) mandatory uniform particle size distribution in the ground material, (iii) large calibration sets, and (iv) availability of calibration materials (Lattanzio *et al.*, 2009b).

Demand is increasing for new and inexpensive materials for mycotoxin analyses that mimic the functionality of antibodies, e.g., molecularly imprinted polymers (MIPs) or aptamers, for use as SPE adsorbents or recognition elements in sensing systems. MIPs are cross-linked polymers that are thermally, photochemically, or electrochemically synthesized by the reaction of a monomer and a cross-linker in the presence of an analyte, e.g., a mycotoxin, serving as the template (Appell *et al.*, 2008; Maragos 2009b; Yu and Lai, 2010). After polymerization, the analyte is removed, leaving specific recognition sites inside the polymer. MIPs are inexpensive, easily prepared, chemically stable, and have a long shelf life. For wheat, MIPS in several forms are available: (i) a molecularly imprinted solid phase extraction-pulsed elution (MISPE-PE) method (Zhou *et al.*, 2004), (ii) an automated MISPE coupled with an online fluorescence detector (Vidal *et al.*, 2012), and (iii) a molecularly imprinted polypyrrole film synthesized on the sensor of a miniaturized SPR device (Yu and Lai, 2005). MIPs also have been used to clean up zearalenone and α-zearalenol in cereals, including wheat, and in feed sample extracts (Maragos, 2009b). Itaconic acid polymers synthesized without the template, i.e., deoxynivalenol, were effective adsorbents for SPE cleanup and preconcentration of deoxynivalenol from pasta extracts prior to the HPLC analysis (Pascale *et al.*, 2008b).

Aptamers are single-stranded DNA or RNA oligonucleotides selected by an *in vitro* process (SELEX – systematic evolution of ligands by exponential) that bind specifically to their target. Aptamers, like antibodies, have numerous potential applications including biosensors, affinity chromatography, and lateral flow devices. An electrochemical competitive aptamer-based biosensor for ochratoxin A has been used for the analysis of certified and spiked wheat samples (Bonel *et al.*, 2011). A DNA aptamer-based SPE column with high affinity and specificity for ochratoxin A also was developed for the cleanup of wheat extracts before HPLC analysis. HPLC analyses of naturally contaminated wheat samples cleaned up with this column or with IACs gave similar results (de Girolamo *et al.*, 2011).

Conclusions and/or Future Perspectives

Several chromatographic methods for mycotoxin determination in the wheat chain have been developed/validated, but there are few official methods based on HPLC-FD or UV and IAC cleanup available for the determination of the major mycotoxins occurring in wheat and derived products. Methods for deoxynivalenol and zearalenone in cereals, cereal products, and cereal-based foods for infants and young children that were adopted as European standards by CEN and a CEN method that uses IAC to determine ochratoxin A in barley should both perform similarly for wheat products.

Many commercial screening methods are available and some have been marked "Performance Tested Methods^{SM}" by AOAC International or have been verified by GIPSA. Methods that can

simultaneously detect multiple mycotoxins and more rapid and reliable assays remain highly desired. HPLC coupled with mass spectrometry detection is the most promising technique for the simultaneous determination and identification of a large number of mycotoxins, including masked mycotoxins. Other novel technologies, including FP immunoassays, immunochromatographic tests, IR spectroscopy, and biosensors may be useful as rapid, low-cost tests of mycotoxins in the wheat chain. The performance of the LC-MS/MS methods and of the novel technologies remains to be validated in inter-laboratory comparison studies, especially for levels close to legal limits.

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13 Breeding for Resistance to *Fusarium* Head Blight in Wheat

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Abstract

Resistance to *Fusarium* head blight appears to be long lasting, race and species nonspecific, and durable. The first moderately resistant varieties are on the market, and resistance is clearly the most powerful means to limit contamination with deoxynivalenol. However, combining high levels of resistance with appropriate agronomic characters remains problematic and progress is slow. A better understanding of the genetic background and the identification of resistance genes, not just quantitative trait loci, are needed to move breeding projects forward. Pathogen biology and population structure also need to be better understood. In some cases, better methodology and a better understanding of the entire disease process may be needed and our breeding philosophy may need to be updated.

Keywords: aggressiveness; alien species; deoxynivalenol; doubled haploids; exotic resistance; environmental conditions; fungicides; inoculums; phylogenetic species; populations; QTLs; transgenics

Introduction

The working procedure for identifying emerging food safety issues in Europe (Marvin *et al.*, 2009) does not include the word prevention for deoxynivalenol contamination, although the deoxynivalenol forecasting system, which is based on weather variables, is mentioned (Schaafsma and Hooker, 2007; de Wolf and Paul, 2014). The real prevention of deoxynivalenol contamination occurs in the field, not later, and this toxin would not be a problem if cultivars with sufficient resistance were available. Thus, the real risk is the susceptible variety, and not the meteorology, and the real prevention is resistant cultivars. The wheat–*Fusarium* system is a good example for other toxic plant pathogen–host plant systems.

Fusarium head blight of wheat has been reviewed several times during the past few decades (Dill-Macky, 2003; Gilbert and Tekauz, 2000; Mesterházy, 1995, 2003; Miedaner, 1997; Parry *et al.*, 1995; Snijders, 1994, 2004; Sutton, 1982). In the United States, the disease was not significant for decades, but in the 1990s the disease reemerged and devastated millions of acres of wheat and barley (McMullen *et al.*, 1997). These epidemics have continued since then, with only the regions affected differing. This disaster increased research efforts to control this disease with numerous papers published. One goal was to determine the role of breeding in decreasing mycotoxin contamination. Thus, the central focus of this chapter is to evaluate how breeding can contribute to this goal, how

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the breeding process should be modified to reach the goal, and which methodical approaches seem to be most suitable.

Toxigenic fungi do not cause significant epidemics in cereals every year, so efficient selection based on natural infection severity is not possible. Thus, susceptible and highly susceptible cultivars are planted widely on a global basis. Even in regions with a high probability for an epidemic, the severity of natural epidemics is usually low and there is not enough selection pressure to enable an effective breeding program. Consequently, nurseries with artificial inoculation are used to ensure the necessary selection pressure is present for successful breeding.

Breeding becomes more important as there are now three European regulations for contamination with *Fusarium* toxins. European Commission Regulation (Anonymous, 2005), EC 856/2005 (Anonymous, 2006), which came into force on July 1, 2006, was modified by EC 1126/2007 (Anonymous, 2007) and sets limits for contamination of component materials for human foods and food products. For animal feeding, Commission Regulation EC 576/2006 (Anonymous, 2007) gives suggested values. These regulations increase the need for healthy grain for food and feed purposes. The consumers of organic food think that they consume the healthiest food, as their food is free from mineral fertilizers, pesticides, and other materials forbidden in organic production. Yet they also are the group most exposed to mycotoxin contamination as the cultivars planted are usually susceptible and no effective fungicides are allowed to be used. Trade also is severely affected as Canada and the United States use these arguments to retain export markets by selling better quality grain.

In epidemic years, 5–10 mg/kg of deoxynivalenol occurs regularly in cereal commodities at harvest, and in some cases far higher levels are observed. Deoxynivalenol has a LD_{50} value toward mice of 47 mg/kg, nivalenol 4.8 mg/kg, and aflatoxin 7–8 mg/kg. These toxins are resistant to degradation during baking and cooking. Their degradation through physical processes takes years, but can be speeded up significantly by soil microbes although only a small fraction usually is degraded in a short time (Snijders, 1994). Fungicides effective against *Fusarium* have LD_{50} values of 5000 mg/kg for tebuconazole and 6300 mg/kg for prothioconazole, which are 100–1000 times less toxic than the fungal toxins produced in their absence. Without fungicide applications, consumers are exposed to mycotoxins and health risks in epidemic years as grain from susceptible varieties will be contaminated with fungal toxins. Fungicides are far less toxic than are the *Fusarium* toxins, but the general public is more fearful of fungicides than it is of the much more highly toxic mycotoxins. Additionally, fungicides usually are degraded to traces by harvest, while mycotoxins linger much longer.

Mycotoxins and Their Origin

Since the detection of aflatoxin B_1 in the 1960s hundreds of mycotoxins have been detected, described, and analyzed (Bartók *et al.*, 2006; D'Mello and McDonald, 1997; Marasas *et al.*, 1988) and their epidemiology studied (Logrieco *et al.*, 2003). There are numerous articles regarding the occurrence and concentration of different mycotoxins in various products and commodities. As the effects of mycotoxins on humans and animals became better known and detection of toxins simpler, governmental regulations were developed and implemented to reduce the exposure of humans and domesticated animals to these compounds (Verstraete, 2008).

Toxigenic fungi generally are considered a storage problem, with the exception of *Fusarium*, *Cladosporium*, *Alternaria*, and occasionally *Aspergillus* (Christensen and Kaufmann, 1969). This perception focused control efforts on toxin destruction or neutralization in stored commodities, and left breeding for disease resistance in the field as at best a secondary idea, and largely ignored the

connections between toxin resistance and disease resistance. In the last 10–20 years, however, disease resistance and toxin resistance have been joined and may be amongst the most important characters being selected, e.g., *Fusarium* head blight resistance and reduced accumulation of trichothecene toxins by *Fusarium* spp. in wheat and other small grains such as barley, rye, oats, and triticale. The most important *Fusarium* spp. associated with *Fusarium* head blight in northern and central Europe are *F. graminearum*, *F. avenaceum*, *F. culmorum*, *F. poae,* and *F. equiseti*, and the most important toxins are deoxynivalenol, nivalenol, zearalenone, moniliformin, and diacetoxyscirpenol. In southern Europe, *F. graminearum* usually dominates and *F. culmorum* and *F. avenaceum* are less common, but the toxin profile is almost exactly the same (Bottalico and Perrone, 2002). Interestingly *F. sporotrichioides* occurs at only trace levels among the fungal isolates cultured, but T-2 toxin, which this fungus is known to produce, is regularly isolated, usually at low levels, in Hungary (Varga *et al*., 2004)

In Europe, the most important toxin on wheat is deoxynivalenol, which is produced by most isolates of *F. graminearum* and *F. culmorum* (Tóth *et al.*, 2004, 2005). Breeding, fungicides, and agronomic practices are now viewed as important partners in controlling both plant disease and toxin production (Lehoczki-Krsjak *et al.*, 2010). These efforts are also critical for grain storage, since the quality of commodities infected in the field never improves while it is stored.

The Wheat/*Fusarium* Breeding System

There are several important conditions for successful breeding. First, without significant differences in resistance, no method will yield good results. Next, reliable inoculation methods are essential, so that differences in resistance can be replicated and consistently observed. The method used depends on the purpose of the evaluation. For example, large-scale screening requires high throughput but less precision, while scientific research requires greater precision but usually smaller throughput. Third, the nature of resistance and its variation need to be known. Resistance may be complex, and the different components of the resistance may need to be assessed in different ways. Fourth, sources of resistance must be identified. Only then does the mode of inheritance become important and is there an opportunity to systematically incorporate the resistance into a commercial germplasm. Finally, basic information on the population structure of the pathogen is needed to delineate the geographic area and conditions under which the resistance is expected to be useful.

Pathogen Population, Composition and Its Significance

Many *Fusarium* spp. have been identified from wheat (Table 13.1; Leslie and Summerell, 2006). In general, multiple *Fusarium* spp. infect wheat, with the most common species present varying by region and to some extent by year. In most regions *F. graminearum* is the most important species, although in cooler regions *F. culmorum* and *F. avenaceum* become more common (Liddell, 2003). *F. graminearum* and *F. culmorum* also are the most pathogenic species. Members of the other species are less pathogenic, but under favorable conditions they too may cause disease and produce toxins. There is some confusion regarding the taxonomic status *F. graminearum* at this time as O'Donnell and colleagues (O'Donnell *et al.*, 2004, 2008; Sarver *et al.*, 2011; Starkey *et al.*, 2007; Yli-Mattila *et al.*, 2009) have proposed splitting *F. graminearum* into at least 16 different phylogenetic species (genetic lineages) even though the data suggest that all of the strains belong to a single biological species (Leslie and Bowden, 2008).

Table 13.1 *Fusarium* spp. recovered from wheat in various countries

Country	*Fusarium* species	Reference
Hungary	*F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. sporotrichioides*, and *F. poae*	Mesterházy (1984)
Belgium	*F. graminearum*, *F. culmorum*, *F. avenaceum*, and *F. poae*	Isebaert *et al.* (2009)
Poland	*F. graminearum*, *F. culmorum*, and *F. crookwellense* (= *F. cerealis*)	Stepien *et al.* (2008)
Germany	*F. poae*, *F. avenaceum*, *F. culmorum*, and *F. graminearum*	Sommer *et al.* (2008)
Lithuania	*F. avenaceum*, *F. sporotrichioides*, *F. graminearum*, and *F. culmorum*	Mankeviciene *et al.* (2008)
Japan	*F. graminearum*, *F. culmorum*, and *F. avenaceum*	Chung *et al.* (2008)
Kenya	*F. poae*, *F. graminearum*, *F. equiseti*, and *F. avenaceum*	Muthomi *et al.* (2008)
Canada	*F. poae*, *F. graminearum*, *F. culmorum*, and *F. avenaceum*	Gordon (1952)
	F. graminearum	Clear *et al.* (2005)

For breeding, the critical point is whether resistance to one of these phylogenetic species results in resistance to all of them. The genes responsible for toxin production do not have the same evolutionary history as those used to discriminate the various phylogenetic species/lineages. Thus, the reported correlations between phylogenetic species/lineage are empirical and may not be consistent. In Canada, fungal strains producing 3-acetyl deoxynivalenol (3ADON) and 15-acetyl deoxynivalenol (15ADON) are both found in *F. graminearum* sensu stricto (lineage 7), but the relative frequencies of these two strain types vary by location and over time, with the proportion of 15ADON-producing strains increasing 14× within a few years (Ward *et al.*, 2008). Here the relationship between resistance and toxin production needs further clarification. A third issue is the possibility of selecting more and more aggressive pathogens as more and more resistant cultivars are planted (Voss *et al.*, 2010). The resistance to the different phylogenetic species/lineages did not differ, so from the point of breeding it has no significance (Tóth *et al*., 2008).

Specialized vs. Nonspecific Resistance

The first report of races in *F. graminearum* is attributed to Tu (1930). This report was interpreted for years to support the occurrence of races in *F. graminearum*, although the data presented by Tu (1930) must be misinterpreted to reach this conclusion. The origin of the misinterpretation was that the only criterion for the presence of the races was that significant fungal isolate–host plant interactions occurred, but these interactions were not consistent over time. Thus, stability is an important component of the resistance character. For a race of rust, every experiment with different near-isogenic lines gives the same results. Here, however, the case is different, as this consistency need not exist for *Fusarium* spp. Within *F. culmorum* (Mesterházy, 1983, 1995; Mesterházy *et al.*, 1999), the overall situation is similar and there is no race specificity. Additionally, these studies have shown that the correlations between strains of *F. culmorum* and *F. graminearum* are as strong as those amongst the *F. culmorum* or the *F. graminearum* strains.

The observation that resistance to *F. culmorum* and *F. graminearum* was correlated led to further tests of cross-resistance to other *Fusarium* spp. (Mesterházy, 2002). The presence of common resistance enables the selection for resistance to the entire group of *Fusarium* species even while practically selecting for resistance to only a single species, usually, *F. graminearum*. The common resistance also means that wheat genotypes selected for resistance to *F. graminearum* usually retain their resistance phenotype even when cultivated at locations or in years in which *F. culmorum* or other *Fusarium* spp. are the primary *Fusarium* head blight pathogens. Studies of the phylogenetic

species/lineages within *F. graminearum* also have found that this common resistance applies to all of them (Tóth *et al.*, 2008), and that these groups do not pose any new threats to the disease resistance breeding process. This result was consistent with the finding that the resistance to different *Fusarium* spp. is found in the same host genomic background and that resistance can be mapped in quantitative trait locus (QTL) analyses (Mesterházy *et al*., 2007). This common resistance response simplifies the selection and screening processes and enables the development and utilization of wheat varieties in regions with differing *Fusarium* species composition.

Inoculum Use

Dill-Macky (2003) analyzed methods of inoculum production, but several approaches to the use of inocula do not have solid scientific support. Many researchers use mixtures of isolates to more broadly test genetic differences within *Fusarium* populations even though there is no known specialization within a species or between the species associated with *Fusarium* head blight. We have tested a number of mixtures (Mesterházy, 1977) and the aggressiveness of a mixture was always lower than the arithmetic mean of the mixture's components. This result is consistent with those of Miedaner *et al.* (2004b), who reported that the aggressiveness toward and toxin contamination of plants treated with a mixture of strains were less than when the strains were applied individually. This finding helps to explain the results we observed nearly 20 years ago (Figure 13.1). These data contain highly significant differences in aggressiveness between different isolates. Thus, the amount of resistance cannot be determined for any of the fungal isolates or mixtures. Instead, resistance rankings can be determined only when differences in severity are above a minimum threshold.

More data are needed to determine reliable mean reaction values, but how these data should be collected is not settled. Breeders normally use a ring test at multiple locations that are cultivated under primarily artificial conditions. This process works well for advanced materials, e.g., the US

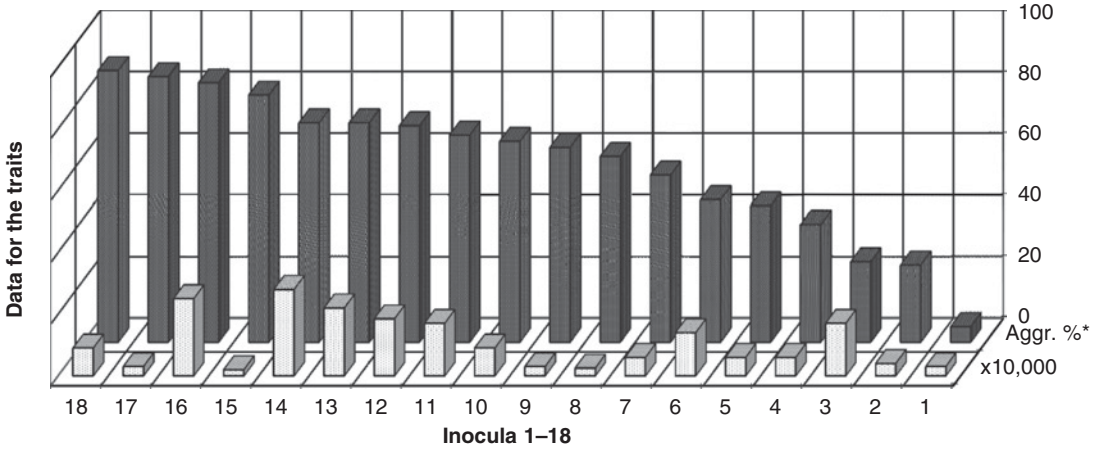

Figure 13.1 Aggressiveness (diseased germs %) and conidium concentration ($\times 10^4$) of inocula of *F. culmorum* isolate 12551. *Aggressiveness: percentage of healthy germs across two cultivars, five recording times (2–6 days after inoculation), and four concentrations (original and diluted 1:1, 1:2, and 1:4 with distilled water; Mesterházy, 1985).

Wheat and Barley Scab Initiative's SWWFHB and NWWFHB in the United States or at CIMMYT, with sets of lines that contain a relatively small number of entries, ~30–50. However, in a breeding program this process is impossible. We now evaluate with parallel isolates (four or more) by inoculating 15–20 heads in one group, with their means giving more precise information about the level of resistance. This technique has much smaller environmental interaction components than are found in multilocation tests. For genotyping, e.g., this protocol works very well.

Another common misperception is that conidial concentration is correlated with aggressiveness and that appropriate adjustments of conidial concentrations can create a homogeneous basis for the comparison of the aggressiveness of different isolates. Unfortunately, aggressiveness and conidial production capability are not interchangeable (Mesterházy, 1981). Some isolates can produce numerous conidia but have low aggressiveness and vice versa. Inocula also may respond differently to dilution. For example, for some inocula, a 1:20 dilution results in no change in aggressiveness, while in another case a 1:1 dilution can reduce aggressiveness by 50% or more (Mesterházy, 1977).

Aggressiveness may not be a consistent phenotype. In the ANOVA tables of Mesterházy (1983, 1995), Mesterházy *et al.* (1999, 2005), or Tóth *et al.* (2008), the isolate × year interaction is highly significant, which indicates large changes from year to year. The source of this variation is not completely environmental, as inocula produced from the same test tube may differ widely in aggressiveness. In 2008 we made 18 inocula from the same test tube of *F. culmorum* strain 12551. A Petri dish test was used to check aggressiveness (Mesterházy, 1985), and the suspensions were made by a bubble breeding method (Mesterházy, 1983). The correlation between conidial concentration and aggressiveness was $r = 0.31$, which was nonsignificant, and the aggressiveness of the inocula ranged from 5% to 90% (Figure 13.1). Each inoculum also reacted differently to dilution. As there is no consistency in aggressiveness, each inoculum must be controlled individually for aggressiveness, and conidial concentration does not seem to be an appropriate approach. This problem could affect the aggressiveness tests for a number of isolates. To give the data some genetic meaning, several inocula of the same isolate should be tested; otherwise the ranking of isolates can be misleading. An important practical implication is that changing the inoculum during a test can lead to significant mistakes. Thus, each unit of inoculum should be large enough that it can be used for an entire test.

What is Measured in a Resistance Test?

Schroeder and Christensen (1963) described two types of resistance. Type I resistance is resistance against the initial infection. Although there are a number of definitions, in practice type I resistance is identified as the reaction to the spray inoculation method. Type II resistance measures the spread of infection from one floret inoculated in the middle of the head. Type II resistance is relatively easy to work with as the tests can be made under controlled conditions and a limited number of plants can provide good results. There also are excellent resistance sources, e.g., Sumai 3, that have this type of resistance (*3BS* or the *fhb1* QTL). Marker-assisted selection was developed and the future seemed to be bright. In Europe several variations of the spray inoculation method have been used, whereas point inoculation is used only for experimental purposes and not as part of a breeding program. However, this QTL explains only about 30% of the observed resistance (Brown-Gudeira *et al.*, 2008; Mesterházy *et al.*, 2007). In another test, Mesterházy *et al.* (2007) found that spray inoculation can be used to assess both type I and type II resistance and that for selection purposes the spraying methods assess a much wider range of resistance than do the point inoculation methods. The difference between the results of the spray and the point inoculation methods could define type I resistance.

Spraying methods also are suitable for testing all types of resistance, such as resistance to kernel infection, or the tolerance described by Mesterházy (1995) and Mesterházy *et al.* (1999). Lemmens (personal communication, 2007) termed such resistance "overall resistance" and concluded that the spraying method was the best to use when considering all of the resistance factors. Most breeding programs focus on visual head symptoms. This focus is good initially, but plants that appear resistant should be checked for the percentage of *Fusarium* diseased kernels (FDK) produced. About 30–40% of the low symptom severity plants may have a high percentage of *Fusarium* diseased kernels. This correlation is now being studied much more intensively. The evaluation of *Fusarium* head blight severity often ends about 3–4 weeks after flowering, even though it might still be 3–5 weeks until harvest. During this time the disease may progress further, especially if the weather is warm and humid. In early generations of germplasm development this approach is suitable, but later in the process toxin contamination also must be evaluated.

Plant height (Mesterházy, 1987; Parry *et al.*, 1995), the compactness of the heads (Steiner *et al.*, 2004), and the presence of awns (Mesterházy, 1987) all significantly influence disease reactions. In the maize grain spawn inoculation method, where wheat is sown after maize and there is a large amount of plant debris on the soil surface, or when the entire nursery is sprayed several times, or when seed is sown in wet fields, the morphological traits are important, therefore the background noise to measure genetic resistance is large. If additional mist irrigation is provided, then the materials inoculated early receive much more humidity than do those inoculated later. The length of the wet period also strongly influences disease development and severity. Another problem is that the weather is neither constant during the 2-week inoculation period nor consistent from year to year. This variation creates major problems when comparing data from different inoculation times. In a variety resistance test, where each ripening group has its own controls, the problem can be managed. However, in mapping populations such problems can result in the assignment of incorrect phenotypes. Correctly phenotyping plants is one reason why the point inoculation technique is used widely, even though it assesses only a relatively small part of the resistance phenotype.

Thus, different methodological approaches preferentially measure different parts of the complex we call *Fusarium* head blight resistance. Methods that use maize seed spawn or maize stalk residue or that spray the entire field with mist irrigation may result in selection for "pseudo"-resistance that results from differences in plant height, loose and awnless heads, and other morphological traits. Under severe epidemic conditions the available resistance traits do not suffice to protect the crop from major losses. I think that research efforts should be concentrated on resistance traits that function under natural conditions and, preferably, have a known physiological basis. Traits identified in plants following artificial inoculation also must be expressed under field conditions and resist the disease during natural epidemics. There are relatively few data that compare artificial and natural infections. There was a severe *Fusarium* head blight epidemic in Hungary in 1985. The correlation between natural incidence (infected heads/m^2) and artificial inoculation (inoculation of groups of heads which were then covered with a polyethylene bag for 24 hours) was $r = 0.30$ when all genotypes were considered (Mesterházy, 1987). When the genotypes at ~90 cm were compared (awned and awnless combined) the linear correlation improved to $r = 0.67$ ($p = 0.001$). If only the awnless genotypes were considered, then the correlation improved to $r = 0.76$ ($p = 0.001$). The corresponding correlations when the data were modeled with polynomial functions were $r = 0.72$ and $r = 0.89$, respectively ($p = 0.001$). This correlation is consistent with that of Tomasović *et al.* (2010) who found a correlation between natural and artificial inoculation results, $r = 0.78$ and $r = 0.82$, indicating that artificial inoculation tests have forecasting power to predict field behavior of the given genotypes.

Repeatability of Results

Initially, Scott (1927) reported that differences in resistance were not very large. Most of the local materials were from native wheat populations and most were highly susceptible to *Fusarium* head blight (Brown-Gudeira *et al.*, 2008; Mesterházy, 2003). Snijders (1990a, 1990b) evaluated numerous European wheat genotypes, and many landraces, varieties, and lines were found with considerable resistance. In his review, Snijders (2004) stressed the role of high resistance in controlling mycotoxin contamination. As the severity of infection varied dramatically and clear, consistent differences were not usually reported, the general experience was that the degree of resistance of these more resistant materials depended upon epidemic conditions. This widespread opinion meant that effective breeding for *Fusarium* head blight resistance was not possible and relatively little effort was made to systematically improve the resistance of commercial lines. However, not all of the data were consistent with this opinion as moderate correlations, up to $r = 0.67$, occurred between years (Hanson *et al.*, 1950), and these correlations could be improved even further when more sophisticated statistical procedures were used for the analysis (Mesterházy, 1995, 1997). Others (Nishio *et al.*, 2004; Tomasović *et al.*, 2010) also identified significant correlations ($r > 0.72$) between years. If the weather within a year changes during inoculation time and thereafter or if the years being compared have different weather conditions, then the correlations between years may be lower than expected (Lehoczki-Krsjak *et al.*, 2009).

Sumai 3, registered in 1973 from China, and Nobeoka Bozu, an old landrace from Japan, were the first wheat accessions highly resistant to *Fusarium* head blight. Sumai 3 in particular has been widely used to improve resistance, but success has been moderate. Resistance levels range from highly susceptible to highly resistant. Although varieties without symptoms at high epidemic pressure have not yet been found, the difference between 1–2% and 100% severity of *Fusarium* diseased kernels is very large and can serve as a breeding target. In this highly variable material it is possible to study the relationship between disease resistance and deoxynivalenol contamination. Identifying traits associated with deoxynivalenol contamination is important because the presence of deoxynivalenol in what otherwise appears to be high-quality grain can limit the uses for the grain and greatly reduce its market value.

Progress in QTL Research and Resistance Sources

To date, no genes have been identified or cloned that confer resistance to *Fusarium* head blight. QTLs identify chromosomal regions associated with a given trait. The first QTL for *Fusarium* head blight resistance was reported in 1997 (Ban, 1997a), while a more recent review (Buerstmayr *et al.*, 2009) lists 52 QTLs that affect this trait. In general, if more than five QTLs can be identified, then the trait is polygenic. In fine mapping, the position of the gene(s) underlying the QTLs on a chromosome can be localized precisely. QTLs can be divided into two major groups, *i.e.*, *3BS* (*Fhb1*), for type 2 resistance and *5AS* for Type 1 resistance, which may explain 40–50% of the observed variation. These loci are validated, i.e., they are confirmed to work in different backgrounds, and marker-assisted selection protocols are then developed. However, Sumai 3 contains more QTLs than those that localize to the 3BS or 5AS regions, and *Fusarium* head blight resistance clearly has a polygenic basis (Buerstmayr *et al.*, 1997; Yu, 1982). Most of the resistance studies have been of type II resistance. Type I and other native resistances have been examined much less thoroughly. Additionally, most studies evaluated only *Fusarium* head blight symptoms and not deoxynivalenol accumulation. Thus, papers that link the *Fusarium* head blight QTLs to deoxynivalenol accumulation are relatively few. The

linkage is important as different QTLs might influence *Fusarium* head blight resistance rather than influence toxin accumulation (Draeger *et al.*, 2007; Lu *et al.*, 2011). Most QTLs have small- or medium-level effects and are difficult to validate, which may indicate methodological difficulties or other problems (Jiang *et al.*, 2007; Mesterházy *et al.*, 2006). In spite of considerable progress with QTLs and associated markers, phenotypic selection for disease resistance remains more effective than selecting for QTLs (Wilde *et al.*, 2007). Phenotypic selection also benefits from synergistic effects and the influence of uncharacterized QTLs that cannot yet be accounted for by using solely the known genes and QTLs. The role of QTLs in limiting toxin accumulation remains unknown, and phenotypic selection is the primary breeding tool used to alter this character in a breeding program.

Sources for resistance to *Fusarium* head blight can be divided into three main groups. In the first group are found the exotic, often highly resistant, commonly used spring wheat genotypes, e.g., Sumai 3, Nobeoka Bozu, Shanghai 7-31B, Nyubai, Fan 1, Ning 8343, Ning 7840, and Wangshubai (Nishio *et al.*, 2004). Most of these lines have the *3BS* QTL (*fhb1*) and exhibit some degree of type II resistance. Frontana and Encruzilhada appear to have type I resistance (Steiner *et al.*, 2004). These lines and varieties cannot be used directly in variety breeding, with one to three prebreeding cycles necessary to produce plants with a suitable level of resistance and good agronomic properties.

In the second group are the alien species including several grass species. Sources with higher resistance to *Fusarium* head blight than found in Sumai 3 include the hybrids *Triticum aestivum-Leymus racemosus*, *T. aestivum-Roegneria komoji*, and *T. aestivum-R. ciliaris*. Some alien additions were identified by RFLP (Chen *et al.,* 1997). *Roegneria* was the most resistant genus of Triticeae when 1463 entries from 85 species were tested. In addition to *Roegneria*, the genera *Hystrix*, *Kengyilia*, *Agropyron (Elymus*), and *Haynaldia* also contained accessions that were resistant to *Fusarium* head blight, but whose breeding value is not yet known (Liu and Wang, 1991; Yong-Fang *et al.*, 1997). Some *Thinopyrum intermedium* accessions are highly resistant to *Fusarium* and there was no spread from the inoculation site (Fedak *et al.*, 1997). Japanese entries of *Agropyron humidus*, *A. tsukushiensis*, and *A. racemifer* are more resistant to *Fusarium* head blight than Nobeoka Bozu (Ban, 1997b). Excising useful resistance from these distantly related species is a difficult, time-consuming process.

A third group of resistance sources has until now been rather overlooked. These sources are local resistance sources with resistance levels that usually are lower than those found in the first two groups. These local sources, however, have a big agronomic advantage in that they are mostly well adapted. To date, most of the work has been with the group 1 resistance sources, less with group 2, and relatively little with group 3. There are a number of European resistance sources with unidentified background and considerable resistance to *Fusarium* head blight (Lemmens *et al.*, 1993; Snijders, 1990a), whose potential for incorporation into breeding programs is promising. In our experience, the wheat genotypes available from different breeding programs are significantly different. *Fusarium* head blight resistance commonly is expressed following transgressive segregation or can be traced back to Sumai 3 (Liu and Wang, 1991). Thus, pyramiding different resistances could yield durable, highly resistant genotypes.

Regulation of Toxin Contamination

The physiology of toxin production and contamination influences both the breeding philosophy and the breeding goals. Disease and toxin resistance often are used incorrectly as synonyms (Clements and White, 2004; Menkir *et al.*, 2006; Williams *et al.*, 2003). The data are consistent with hypotheses that both resistance to disease and resistance to toxin contamination exist as distinct entities, probably based on different mechanisms. Disease resistance decreases toxin

contamination indirectly, whereas toxin resistance has a direct effect. The critical question is which mechanism is more important.

- Toxin-producing ability of *F. graminearum* and *F. culmorum* isolates correlates well with their aggressiveness. Understanding the genomic background can help explain the observed results (Brown *et al.*, 2006). The number of identified putative pathogenicity genes in *F. graminearum* is increasing (Dufrense *et al.*, 2008). Deoxynivalenol production is an aggressiveness factor, as isolates that produce higher levels of deoxynivalenol are also more aggressive than those that produce lower levels of deoxynivalenol (Brown *et al.*, 2006; Mesterházy, 2002; Mesterházy *et al.*, 1999, 2005; Miedaner *et al.*, 2004a; Proctor *et al.*, 1997, 2002), and deoxynivalenol has been suggested to be phytotoxic (Hoppe, 1997). The problem is that the aggressiveness of members of natural *Fusarium* populations cannot be regulated. Therefore, while understanding the role played by deoxynivalenol in the disease process answers a number of questions, it does not suggest any means to control either the disease or the accumulation of toxin.
- *Resistance to deoxynivalenol*: A mechanism that decomposes deoxynivalenol much more rapidly in resistant plants than in susceptible ones also decreases toxin contamination by ∼20% (Boutigny *et al*., 2008; Miller, 1994; Miller and Arnison, 1986; Miller *et al.*, 1985). A deoxynivalenol glycoside transferase is known in *Arabidopsis* (Poppenberger *et al.*, 2003), and deoxynivalenol can be detoxified by putative deoxynivalenol-glycosyl transferases in wheat (Lemmens *et al.*, 2005). The amount of the nontoxic product can be measured and correlated with reduced aggressiveness, which results in resistance. This trait is found, to some extent, in *3BS* plants, but in other tests (Mesterházy *et al.*, 2007) the difference between *3BS* and non-*3BS* lines is moderate, perhaps 10%, and this difference in deoxynivalenol detoxification does not seem to be the primary difference that is responsible for the observed differences in resistance to *Fusarium* head blight.
- *Resistance to disease*: Doubled haploid (DH) populations often have higher correlations between AUDPC and DON ($r = 0.77$) than between AUDPC and the relative spike weight ($r = 0.59$; Gosman *et al.*, 2005; Lemmens *et al.*, 2003; Miedaner *et al.*, 2003; Paul *et al*., 2005; Polisenska and Tvaruzek, 2007). My experience is that the relative spike weight does not give results as good as those given by grain yield, as the spike/grain weight ratio is highly variable. In a spike with a large grain ratio, the decrease in head weight is larger than in large robust ears with a smaller grain ratio. Thus, relative spike weight might be usable as a rapid test, but is less suitable for a scientific analysis. I prefer to use visual symptoms and grain mass rather than relative spike weight data.

Deoxynivalenol tolerance assessed by the Petritox method does not correlate with the deoxynivalenol contamination level of the grain and is not a suitable technique for selection purposes in a breeding program (Lemmens *et al.*, 1994). Selection for toxin resistance need not simultaneously select for disease-resistant material as regenerated plants from toxin-selected and control calli progeny of a doubled haploid cross have similar ranges of divergence in *Fusarium* resistance (Ahmed *et al.*, 1992, 1996a, 1996b). Seedling resistance to *Fusarium* and resistance to *Fusarium* head blight are not correlated (Mesterházy, 1987), so seedling and *Fusarium* head blight resistance cannot be used interchangeably. The reports in which seedling resistance was correlated with *Fusarium* head blight resistance appear to be exceptions. A weak correlation has been reported between resistance to *F. avenaceum* and moniliformin concentration; however, both infection severity and moniliformin concentrations were low (Kiecana *et al.*, 2002). In barley, there was a highly significant correlation between AUDPC and deoxynivalenol contamination ($r > 0.90$), although the correlation with the infected grain ratio was lower ($r > 0.60$; Legzdina and Buerstmayr, 2004). Infection was most

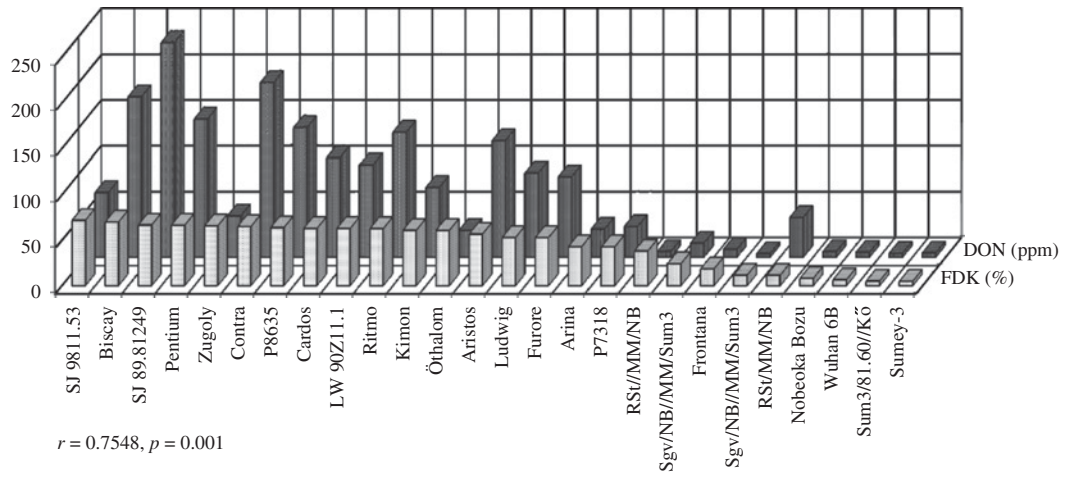

Figure 13.2 Deoxynivalenol (DON) and *Fusarium* diseased kernels (FDK) values of Western European, Hungarian cultivars and highly resistant varieties and lines from the Szeged breeding program. Data are means across four environments and 2 years.

significantly influenced by plant height ($r > 0.80$). The hulless varieties generally had higher correlations than those for the covered varieties. In our tests, data from 20 years support the view that resistance is significantly correlated with toxin accumulation. Highly aggressive fungal isolates that produce hundreds of ppm of deoxynivalenol on grain produce only traces of deoxynivalenol on highly resistant wheat genotypes (Figure 13.2; Mesterházy *et al.*, 2005). Toxin contamination of some Western European wheat genotypes with the same *Fusarium* diseased kernels level can vary by fivefold. Thus, not only is resistance correlated with toxin concentration, but other currently unknown regulators may affect toxin contamination in the late, long vegetation period cultivars.

The relatively strong correlation between *Fusarium* diseased kernels and deoxynivalenol contamination levels ($r = 0.87$; Figure 13.3) strongly supports the hypothesis that increasing disease resistance decreases deoxynivalenol contamination, as do the data from several other studies (Mesterházy, 2002; Mesterházy *et al.*, 1999, 2005; Tóth *et al.*, 2008). However, the observed variation is large and some genotypes have higher or lower deoxynivalenol values than the predicted value from the regression equation. Thus, not only is *Fusarium* head blight resistance important in deoxynivalenol production, but also extremes of disease sensitivity and toxin overproduction. The significance of these extremes had not previously been appreciated and no research has been conducted to discern its genetic basis. However, based on the food safety risks of many cultivars, this trait is important.

The strongest correlation with deoxynivalenol contamination is physiological resistance. The difference between aggressiveness of isolates or inocula is expressed only on susceptible genotypes, and various toxin-degrading or -neutralizing mechanisms in the plant probably play only a minor role.

1. *Weather conditions*: Warm, humid weather has been known to favor *Fusarium* head blight since 1920 (Atanasoff, 1920). The ratio of *Fusarium* diseased kernel to deoxynivalenol contamination depends heavily on the weather. In humid seasons, deoxynivalenol contamination in inoculated tests is much higher, e.g., 500–700 mg/kg, than in a dry season, when deoxynivalenol contamination rarely exceeds 20–30 mg/kg even following artificial inoculation. In an analysis of 10 years of data, the amount of precipitation was the most significant agent influencing deoxynivalenol contamination ($r = 0.96$, $p = 0.1\%$). Heavy rains prior to harvest contribute the most to toxin contamination levels (Mesterházy *et al.*, 2006). Resistant cultivars that also tolerate the late rains while remaining healthy need to be identified and developed.

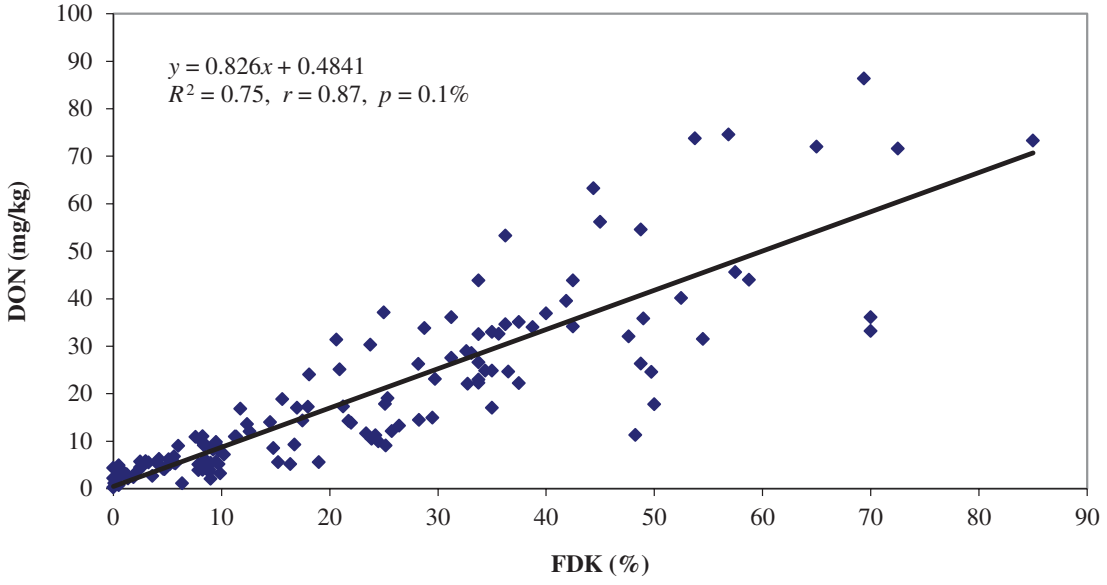

Figure 13.3 Regression between *Fusarium* damaged kernels (FDK) and deoxynivalenol (DON) contamination, 2006, $n = 139$. Data represent means across four isolates with different aggressiveness.

The most powerful toxin-regulating agent appears to be disease resistance, although toxin accumulation also can be modified by additional traits, e.g., toxin-decomposing mechanisms. I do not think such traits should be a focus since even if toxin contamination is decreased, the diseased tissue and quality problems will remain. For many years we have observed that highly resistant genotypes have very low *Fusarium* diseased kernels levels, sometimes zero, and that deoxynivalenol contamination usually is correlated with *Fusarium* diseased kernels levels.

The Transgenic Approach

A transgenic approach might help increase resistance to *Fusarium* head blight (Dahleen *et al.*, 2001). The process is complex, as only a few highly effective QTLs exist, and most have only medium to low phenotypic effects. Normally, if more than five QTLs are identified in a plant, then the trait is treated as a polygenic trait. These studies also promise to contribute to a basic understanding of the resistance background operating in wheat. Glucanases and chitinases surely can play a role in resistance (Kang *et al.*, 2008). MacIntosh *et al.* (2007) tested several transgenic plants with different genes. The best disease- and toxin-reducing effect under both greenhouse and field conditions was found in a line carrying β-1,3-glucanase. Shin *et al.* (2008) found that chitinases enhance *Fusarium* head blight resistance, and that a barley II chitinase gene could confer type II resistance in greenhouse tests. Genes encoding pathogenesis-related (PR) proteins were isolated from a cDNA library of *F. graminearum*-infected wheat spikes of scab-resistant cultivar Sumai 3 and then transformed into a susceptible spring wheat, Bobwhite (Anand *et al.*, 2003, 2004). One line co-expressed a chitinase and β-1,3-glucanase gene combination and had increased type II resistance. Other transgenes that encoded thaumatin-like proteins were ineffective. It has been proposed that genes encoding fungal sex pheromones might suffice to confer *Fusarium* head blight resistance, but

constructs to test this hypothesis have not yet been made. At present there are no transgenic cultivars in commercial production, and I do not think that this technology will be used by breeders within the next few years.

How Does a Breeding System Operate?

In most breeding programs, one to two breeders work with a small staff. They do not have specialists for entomology, phytopathology, and grain quality, but instead share the scoring of these traits amongst members of their group. They rely on access to a university or research institute to provide help with specialized advice and to produce inocula, as necessary.

All generations of plants in the program must be exposed to infection pressure. We also inoculate the F_1 generation. As resistance is additive and transgenic segregation may occur we need to be sure that no resistant plants can be selected from a susceptible F_1. Thus, improved variants will be found and possible escapes can be identified and discarded. The F_2 is sown widely spaced, but no inoculations are made. Of the 200–300 plants per combination, the best 20–30 will be marked, harvested, and threshed separately. The best of the progeny are then sown. We normally test 100–200 new combinations annually. The best ears, usually more than 1000, are sown as ears to rows in an F_3 nursery.

The system needs to be as simple as possible. The part of the nursery for natural selection can be sown after no-till maize, where there is significant stalk residue remaining in the field. Even a 1–2 ha nursery can be treated with head mist irrigation to ensure the necessary infection pressure for selection. An alternative spawn method can be used for smaller fields of several hundred square meters. Finally, an entire nursery can be sprayed by tractor and then mist irrigated. For the last two types, other specialists are needed to produce either a liquid spore suspension or infected grains colonized with *F. graminearum* for ascospore release. In Europe, the nurseries are sown with Wintersteiger or Hege planters that are suitable for ear to row planting.

In the F_3–F_5 nursery one to two rows of each combination, ~10 heads, will be inoculated with an aggressive fungal isolate and the wheat heads bagged for 48 hours. We spray inoculate the first 20 cm of the plot and ensure high humidity with mist irrigation. Both protocols produced good results and enabled effective selection. The spawn method has been more unreliable. During the dry winter of 2011/2012 no perithecia were produced on the maize residue, and this might negatively influence epidemic development. Usually each field is scored two to three times, and rows with high infection severity are discarded. Infected heads from the less heavily infected plots are harvested, threshed, and cleaned. The proportion of *Fusarium* diseased kernels is determined and about half of the lines are discarded. Selected rows are sampled (18 heads) and the remainder of the plot harvested. When the lines are nearly homogenous, they are planted in a 5 m^2 nursery (B lines) to estimate the yield, standability, and other traits. Each of these plots is supported by 12 additional rows to allow further selection. If the results are uneven, but promising, then the material is selected further and replanted in another 5 m^2 nursery for reevaluation. The B lines also are checked. Each row is sown separately and inoculated with an aggressive fungal isolate. The best B lines progress to the four-replicate yield trial (C lines). The C lines are planted in three row plots for inoculation tests with four fungal isolates used in one replicate. So that resistance can be identified more precisely, it is scored as the mean of four epidemic situations. The number of *Fusarium* diseased kernels is determined and the grain from the best lines is analyzed for deoxynivalenol. The best lines go to a multilocation trial, where six-row plots are sown for *Fusarium* head blight tests and four fungal isolates are tested in two replicates. Toxin analysis also is conducted on these materials. The two replicates are pooled

for analysis. As correlations between the number of *Fusarium* diseased kernels and deoxynivalenol accumulation are usually high, in the first cycles of selection toxin analysis may not be necessary.

The variety candidates are tested in a similar manner, but two plots are sown instead of one to obtain more robust results. In all generations, control cultivars with known resistance and susceptibility phenotypes are included to indicate the selection limits. Other traits required as part of the cultivar's development are checked by using an appropriate (standard) protocol.

Summary and Outlook

In addition to exotic resistance sources, considerable resistance to *Fusarium* head blight has been detected in local breeding materials. The significance of the local resistance to the breeding efforts will increase, as these materials are already well adapted. The keys to success are well-planned crosses and regular testing of material in each generation under artificial selection if the natural selection pressure is not sufficiently high. This continuous selection provides the best chance to increase the resistance of new cultivars. The level of resistance needed for effective disease control remains the subject of much debate. I do not think that the Sumai 3 or Nobeoka Bozu resistance will be enough. Experience with resistant and more-resistant genotypes is needed to answer this question. In China, Ma (personal communication, 2012) says that the commercial cultivars are moderately resistant, and that a higher resistance level could not be achieved without altering other essential parameters. I agree that the main problem is to combine *Fusarium* head blight resistance with high yield, good grain quality, and resistance to diseases other than *Fusarium* head blight. I think that there is sufficient existing knowledge to run efficient breeding programs. Significant progress has been made in the United States, Canada, China, and several European countries. It would help to compare existing breeding practices and identify some best practices and novel approaches. Progress in resistance breeding is slower than anticipated. The overestimation of type II resistance surely has played a role. There is a slow transition amongst the breeders to reconsider the role of resistance types. I hope that the progress that occurs will result in more resistant cultivars and that the gaps in scientific knowledge will be filled effectively and efficiently. To speed up the process, existing knowledge should be utilized more efficiently, and, consequently, we need to know more about the genetic background of resistance, and we need to move from QTLs to genes.

In spite of the complications and problems, the *Fusarium* head blight/wheat system is a relatively simple one. There is no race structure in *Fusarium* populations such as found in powdery mildew or rusts, and the resistance obtained to *Fusarium* head blight is effective against all *Fusarium* spp. tested thus far. However, effective breeding under artificial inoculation and selection requires skill and experience. Current knowledge should enable us to breed genotypes with much better resistance than is currently available in the present popular varieties. In each nursery significant native resistance from an unidentified genetic background was detected and is now being used. Efforts to incorporate highly resistant Asiatic resistance sources have been very intensive, but the results have been much less than hoped for. There are at least three reasons for these disappointments. (i) The resistance sources are agronomically of poor quality and require several cycles of prebreeding to develop suitable crossing partners for variety development. (ii) Most selection has been concentrated on the *3BS* (*fhb-1*) QTL, which represents only 30–40% of the available resistance in Sumai 3 accessions. (iii) Although many highly resistant lines are available, some are very similar to Sumai 3. A successful cultivar also needs adequate yield, quality, resistance to other diseases, abiotic stresses, etc. This phase of the breeding process is very important and the most important questions about many of the resistant lines are in this area.

A functional, efficient selection system is essential for a successful breeding program. This methodology has been developed at many breeding institutions. We have summarized several ideas that could be used to make the selection process more effective. Continued exchange of ideas and practices is very important and could lead to more effective breeding and screening methodologies.

Highly significant information has resulted from molecular genetic analyses and studies of the genetics of resistance. However, with a few exceptions, the QTLs identified are weak, numerous, and often not validated. The search for new, effective resistance sources is of global importance as a broader diversity of resistance sources is sorely needed. In addition to resistance sources, cloned genes also are needed. The resistance genes in the *3BS* QTL region should be cloned soon and their function determined. Most of the available data refer to *Fusarium* head blight symptoms, but the effect of this phenotypic selection on *Fusarium* diseased kernels, toxin contamination, and other traits is not clear. Much remains to be done.

The fundamentals that underlie an effective breeding program are in place, which enables the breeding of more resistant cultivars. However, many questions are partially answered, if they are answered at all. Continued intensive research in this area should result in new cultivars with higher resistance coming to the market in the next few years and new scientific results should make the breeding process even more effective. However, without the incorporation of resistance tests against *Fusarium* head blight into the registration process the results of breeding cannot be measured and susceptible cultivars will continue to cover our fields, even though resistance to *Fusarium* head blight is the most important control measure to increase food and feed safety in wheat.

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14 Good Agricultural and Harvest Practices to Reduce Mycotoxin Contamination in Wheat in Temperate Countries

Barry J. Jacobsen

Abstract

Mycotoxin contamination of wheat in the field is most commonly due to growth of fungal pathogens, such as *Fusarium* spp. which cause *Fusarium* head blight or *Claviceps purpurea* which causes ergot. These species usually infect during or shortly after flowering. Several species of *Fusarium*, *Penicillium*, and *Alternaria* may infect grain if the harvest is delayed due to wet conditions, and isolates of *Aspergillus* and *Penicillium* may infect during storage, if there is sufficient moisture to support fungal growth. These fungi produce numerous mycotoxins under field and storage conditions that differ in chemical structure and toxicity. Reduction of mycotoxin contamination in wheat depends upon the integration of management measures from planting through harvest and storage.

Keywords: *Alternaria*; biological control; *Claviceps*; crop rotation; cultivar selection; delayed harvest; drying; *Fusarium*; fungicide; grain moisture; insect control; irrigation; *Penicillium*; residue management; resistance; storage conditions; weed control

Introduction

Mycotoxin contamination of wheat results from fungal pathogens that infect their host during or shortly after flowering, when harvest is delayed due to wet conditions, and in storage. Reduction of mycotoxin contamination in wheat requires the integration of several management measures, including (i) selection of cultivars with resistance to infection and colonization by mycotoxigenic fungi; (ii) planting pathogen-free seed; (iii) crop rotation; (iv) crop residue management; (v) irrigation management; (vi) fungicide application; (vii) timely harvest; (viii) avoiding mechanical damage to kernels during harvest and handling; (ix) weed control; (x) cleaning before binning; and (xi) binning at moisture and temperature levels that restrict the growth of strains of *Aspergillus* and *Penicillium*. Mycotoxins produced in wheat or found in wheat products produced by these fungi include aflatoxins, ochratoxins, sterigmatocystin, penicillic acid, citrinin, alternariol, altenuene, tenuazonic acid, ergot alkaloids, deoxynivalenol (also known as vomitoxin or DON), nivalenol, T-2, HT-2, diacetoxyscirpenol, and zearalenone. Of these, aflatoxin and sterigmatocystin are found only postharvest under improper storage conditions, while the others may occur in the production field, when harvest is delayed or when the grain is stored improperly (Jacobsen, 2010c). Moniliformin

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and the hexadepsipeptides (enniatins and beauvericin) have been identified as mycotoxins of potential importance in Europe (Logrieco and Moretti, 2008). Globally and historically, deoxynivalenol, nivalenol, T-2, HT-2, diacetoxyscirpenol, ergot toxins, ochratoxins, and zearalenone have been the most prominent, so these mycotoxins are the primary focus of this chapter.

Fusarium Head Blight

Fusarium head blight is caused by one or more *Fusarium* species (Table 14.1) depending on location. *Fusarium* species that dominate globally are *F. graminearum*, *F. pseudograminearum*, *F. culmorum*, and *F. avenaceum*. In temperate climates, *F. graminearum* and *F. culmorum* most commonly are involved in *Fusarium* head blight and trichothecene mycotoxin contamination. In addition, *F. acuminatum*, *F. poae*, *F. cerealis* (syn. *F. crookwellense*), *F. equiseti*, *F. langsethiae*, *F. sporotrichioides* and *F. tricinctum* have been associated with *Fusarium* head blight at some locations. In maritime regions of northwestern Europe, *Microdochium nivale* also may be involved in *Fusarium* head blight (Desjardins, 2006; Liddell, 2005; Logrieco and Moretti, 2008; Shaner, 2005).

Not all *Fusarium* species associated with *Fusarium* head blight produce mycotoxins and the literature contains conflicting information (Council for Agricultural Science and Technology, 2003; Desjardins, 2006; Liddell, 2005; Logrieco and Moretti, 2008), thus showing the importance of taxonomic work (Leslie and Summerell, 2006) particularly on reference specimens (Table 14.1).

Isolates of *F. graminearum* have been placed into multiple phylogenetic lineages or phylogenetic species (O'Donnell *et al.*, 2004; Starkey *et al.*, 2007). Lineage 7 (*F. graminearum sensu stricto*) dominates in North America and Argentina while lineage 6 (*F. asiaticum*) dominates in Asia (McMullen *et al.*, 2012; Reynoso *et al.*, 2011; Zhang *et al.*, 2007). Isolates of *F. graminearum* also may be distinguished by the trichothecenes inferred to be produced, i.e., 3-acetyl deoxynivalenol (3-ADON), 15-acetyl deoxynivalenol (15-ADON), and nivalenol (Starkey *et al.*, 2007; Ward *et al.*, 2008). The *15-ADON* genotype dominates in most of the United States and Canada with the *3-ADON* genotype most common in the eastern parts of these countries. The NIV genotype is much less common than the *3-ADON* or *15-ADON* genotypes in North America. Elsewhere, the three genotypes occurred in equal amounts in Belgium in 2007 and 2008 (Audenaert *et al.*, 2009), while

Table 14.1 *Fusarium* species associated with *Fusarium* head blight (scab) of wheat and the mycotoxins they produce in wheat (after Desjardins, 2006; Leslie and Summerell, 2006)

	Mycotoxins produced					
Fusarium species	Deoxynivalenol[a]	Diacetoxyscirpenol	Nivalenol	T-2 Toxin	HT-2 Toxin	Zearalenone
acuminatum				√	√	
avenaceum						
culmorum	√		√			√
equiseti		√	√			√
graminearum	√		√			√
langsethiae		√		√	√	
poae	√		√			
pseudograminearum	√		√			√
sporotrichioides		√		√	√	
tricinctum						

[a] Deoxynivalenol and its acetylated derivatives.

the *15-ADON* genotype was more prevalent than the *3-ADON* genotype in Italy, Turkey, Poland, Argentina, and the United Kingdom (Jennings *et al.*, 2004; Prodi *et al.*, 2009; Reynoso *et al.*, 2011; Yörük and Albayrak, 2012). In China, the *15-ADON* genotype dominates in colder areas, while the *3-ADON* genotype dominates in warmer regions (Ji *et al.*, 2007). While there are claims that strains with the *3-ADON* genotype are more aggressive than those with the *15-ADON* genotype, there is no evidence that there are virulence differences on wheat lines with some resistance to *Fusarium* head blight (McMullen *et al.*, 2012).

There are no documented sources of immunity to *Fusarium* head blight in either hexaploid or tetraploid wheats, but differences in disease susceptibility between cultivars have been recognized for over a century. Multiple components of quantitative resistance have been postulated including (i) flower size (smaller florets are less likely to be infected by ascospores); (ii) duration of flowering (shorter flowering periods provide less time for infection); (iii) resistance to initial infection (type I resistance) and spread within the spike (type II resistance) (Schroeder and Christensen, 1963); (iv) plant height (shorter cultivars are more susceptible); (v) presence of awns (awned genotypes are more susceptible); and (vi) reduced deoxynivalenol accumulation are the most widely accepted resistance mechanisms (Dill-Macky, 2010; Mesterházy, 2005a). Mesterházy (1995) described type III resistance as resistance to kernel infection and type IV resistance as tolerance. Type V resistance is the ability of the host to detoxify toxins or host insensitivity to toxin accumulation (Bai and Shaner, 2004; Miller *et al.*, 1985).

Currently, the Chinese spring wheat, Sumai 3, is recognized as the best source of genetic resistance to *Fusarium* head blight with resistance genes identified on chromosomes 1B, 2A, 5A, 6D, or 7D (Bai *et al.*, 2005). The Sumai 3-derived quantitative trait locus (QTL), *Fhb1*, is now being widely used in breeding programs worldwide (McMullen *et al.*, 2012). QTLs *Qfhs.ndsu-3AS* from *Triticum dicoccoides* and *Qfhs.pur-7EL* from tall wheat grass also are being used in breeding programs (McMullen *et al.*, 2012). Other reported sources of resistance include the cultivars Nobeoka Bozu, Ringo Star, Arina, Shanghai 7-31B, Nyubai, Fan1, Fan 635, Fan 60096, Funo, Fu 5125, Nanda 2419, Taiwanxiaomai, Xiaofan 24, Ning 8343, Ning 7840, Ningmai 7, Chuanmai 25, Longmai 19, Changjang 8809, Xiangmai 1, Xiangmai 10, Pekin 8, Frontana (type I resistance), Senta, Sparta, Branka, Sofia, Xanthos, Sorbas, and Encruzilhada (Bai *et al.*, 2005; Mesterházy, 2005a). The known sources of resistance do not provide immunity, but only reduced infection and spread in the spike (type II resistance) (Bai *et al.*, 2005). Thus, wheat producers where *Fusarium* head blight is a threat must select resistant cultivars, utilize crop rotation and tillage to minimize inoculum, and utilize effective fungicides at heading (Bai *et al.*, 2005; Burrows *et al.*, 2008; Mesterházy, 2014a,b; Willyerd *et al.*, 2012). Suspension of irrigation during flowering also reduces infection (Burrows *et al.*, 2008).

Durum (tetraploid) wheat appears to be more susceptible to *Fusarium* head blight than hexaploid wheat. Sources of resistance in durum wheat currently are limited to the cultivar Divide, breeding lines with Sumai 3 genes, some Tunisian durum lines, Langdon *T. dicoccoides* 3A and 7A substitution lines, and some accessions of their wild relatives including *Triticum turgidum* subsp. *carthlicum* and *T. turgidum* subsp. *dicoccum* (Oliver *et al.*, 2008).

Ergot

Ergot (*Claviceps purpurea*) is a common endemic disease in temperate climates if cool (0–5°C for ~25 days) wet weather occurs during flowering (Kichhoff, 1929). Wheat cultivars vary widely in their susceptibility to ergot and nonimmunity-type resistance is available.

In general, wheat cultivars with shorter flowering times and more closed florets are less susceptible to ergot. In general, shorter periods of susceptibility to colonization following pollination reduce disease incidence and severity. Florets are most susceptible to infection just before anthesis and become resistant to infection, depending on genotype, a few hours to 5 days after pollination. Based on limited data, triticale is more susceptible to ergot than durum wheat, durum wheats are more susceptible than hard wheats, and soft wheats are the least susceptible (Jacobsen, 2010a). Australian cultivars Halbred, Kalkee, and Zenith are amongst the least susceptible cultivars to ergot (Bretag and Merriman, 1980). Ergot resistance is an important factor in cultivar selection in regions where ergot is endemic. For example, severe ergot infection of the hard red spring wheat cultivar, Waldon, resulted in its abandonment in the early 1970s in Minnesota and North Dakota. In some areas, Waldron grain was more than 10% ergot bodies by weight (Jacobsen, unpublished).

Pathogen-Free Seed

The use of pathogen-free seed can reduce the potential for introduction of *Fusarium* head blight and ergot to areas where these diseases are not endemic. There is little evidence that infected seeds play a significant role in *Fusarium* head blight epidemics (Shaner, 2005), although seed treatment with carboxin + thiram reduced the incidence of *Fusarium* head blight by 37% (Teich and Hamilton, 1985). Presumably, triazole seed treatment fungicides could result in similar or better control of *Fusarium* head blight.

Practical control of ergot is based on planting seeds free of sclerotia. Sclerotia can be removed from seed lots by seed-cleaning equipment or by brine flotation (Dewick, 2009). Seed treatment triazole fungicides applied to ergot-infested seed lots reduce the viability of the sclerotia. Not all ergot sclerotia in wheat grain are from wheat infection, as they also may form on grassy weeds. These sclerotia typically are smaller and more slender than the sclerotia produced by wheat ergots, but produce ascospores capable of infecting wheat (Jacobsen, 2010a).

Fungicides

Fungicides applied at full flowering are an important control mechanism for *Fusarium* head blight (McMullen *et al.*, 2012; Mesterházy, 2005b, 2014b). Alone, even the best fungicides are not effective under severe disease pressure, but when fungicides are combined with partially resistant cultivars farmers can obtain acceptable disease control (Burrows *et al.*, 2008; Paul *et al.*, 2007; Willyerd *et al.*, 2012). The best individual fungicides for controlling *Fusarium* head blight have been benomyl, MBC, tebuconazole, metconazole, and prothioconazole, and the combination of tebuconazole and prothioconazole (Jacobsen, 1977; McMullen *et al.*, 2012; Paul *et al.*, 2007). BMBC was commonly used in China before resistance problems became widespread. Other triazoles, benzimidazole or strobilurins (QoI), or combinations of these fungicides generally are less effective, although a combination of pyraclostrobin (a QoI fungicide) and epoxiconazole was effective in China when MBC resistance was present (Chen *et al.*, 2012). The use of QoI fungicides often is accompanied by higher levels of accumulated deoxynivalenol (McMullen *et al.*, 2012). Fungicide efficacy is strongly influenced by application timing (full flower is best) and the coverage of the spike. Fungicides may not control all of the *Fusarium* species involved in *Fusarium* head blight, but they can limit the damage caused by *F. graminearum* and *F. culmorum*.

Crop Rotation and Residue Management

Crop rotation and the appropriate management of crop residue by tillage are critical for the control of *Fusarium* head blight and ergot. *Fusarium* head blight pathogens survive between growing seasons on undecomposed host crop residues and may persist for years if these crop residues remain relatively intact. Residues of wheat, barley, and maize are typical inoculum sources. Major *Fusarium* head blight epidemics in wheat commonly occur following maize (Shaner, 2005). Soybean stubble and sorghum residue in no tillage or minimum tillage production systems also provide inoculum for *Fusarium* head blight epidemics and perithecia can be recovered from numerous species of Poaceae (Campagna *et al.*, 2005; Reis, 1990). Even though the *Fusarium* head blight pathogens are endemic in most wheat-producing areas, crop rotations of one or more years with non-host crops reduce disease incidence (Dill-Macky and Jones, 2000). Tillage, which results in the destruction or burying of infected crop residues, hastens their decay and reduces *Fusarium* head blight incidence since the ascospore and conidial inocula produced on stubble can contribute to epidemics only if they are present above the soil surface where they can be dispersed (Dill-Macky and Jones, 2000). Production of perithecia and ascospores by *F. graminearum* also requires light (Leslie and Summerell, 2006). The increased incidence of *Fusarium* head blight worldwide has been associated with reduced tillage (Dill-Macky, 2010).

Ergot sclerotia present in or on soil from previous hosts, e.g., indigenous grasses or other cereal crops, or sown with the seed, germinate following cold induction in the spring. Sclerotia can survive in the soil in temperate regions for approximately 1 year. Rotations that include a 1-year absence of a gramineous host markedly reduce the number of viable sclerotia in soil. Tillage that buries sclerotia deeper than 4 cm prevents the aerial dispersal of ascospores. Ergot may be reduced if grassy weeds in wheat production fields are controlled. Burning wheat stubble to reduce the number of viable sclerotia and mowing or chemical treatment of headland, roadway, and ditch bank grasses to prevent heading also reduce ergot in adjacent wheat fields (Jacobsen, 2010a).

Irrigation

When wheat is produced under irrigation, minimizing irrigation during the period from anthesis to 4–5 days later reduces infection by the fungal ascospores that cause either *Fusarium* head blight (Shaner, 2005; Stack, 2005) or ergot (Wood and Coley-Smith, 1982). Ascospores are the primary inoculum for both pathogens, although macroconidia may serve as the primary inoculum for *Fusarium* head blight under some conditions. Both ascospores and conidia are dispersed by wind and rain splash and infect through the floret. For ergot, florets are most susceptible to infection just before anthesis and become resistant to infection, depending on genotype, within a few hours to 5 days after pollination. *Fusarium* head blight infection can occur any time between spike emergence and grain fill. In Montana, where conditions are usually too dry for significant *Fusarium* head blight epidemics, farmers growing irrigated spring wheat reduce *Fusarium* head blight incidence and severity by saturating the soil moisture profile before spike emergence and then avoiding irrigation from spike emergence until 5 days after pollination (Burrows *et al.*, 2008). In nonirrigated areas, growers should select cultivars that flower at different times so that if favorable conditions for infection occur during the critical period for infection, then no more than a portion of their total crop will be severely affected.

Delayed Harvest

Mycotoxin problems associated with weather-delayed harvests include the ochratoxins (A, B, and C) produced primarily by *Penicillium verrucosum* and *Penicillium viridicatum* in temperate climates; penicillic acid and citrinin produced by members of several *Penicillium* species; alternariol, alternariol methyl ester, altenuene, alterntoxin, and tenuazonic acid produced by *Alternaria alternata*, *Alternaria triticina*, and perhaps members of some other *Alternaria* species (Li *et al.*, 2001); and the *Fusarium* mycotoxins deoxynivalenol, nivalenol, diacetoxyscirpenol, and T-2 and HT-2 toxins produced by several *Fusarium* species (Council for Agricultural Science and Technology, 2003; Desjardins, 2006; Jacobsen, 2010b; Leslie and Summerell, 2006; Table 14.1). Several *Penicillium* species and some species of *Fusarium* can grow at temperatures as low as 0–5°C and infection of weather-delayed, lodged, or snow-covered wheat by these fungi is common in temperate climates. These fungi usually require host moisture in equilibrium with relative humidity (RH) of 85–100% (Jacobsen, 2010c).

Storage

If production fields contain numerous weeds or late maturing tillers, then significant storage problems can result due to the growth of members of *Aspergillus* and *Penicillium* species. These fungi are ubiquitous saprophytes and their spores are almost always present. Weed seeds and immature wheat seeds are typically high enough in moisture (70–85% RH equivalent) to allow these fungi to grow. The mycotoxins aflatoxin, sterigmatocystin, penicillic acid, and citrinin often are found in improperly stored wheat. As these fungi grow and respire they produce both heat and moisture that alter the preferred cool, dry storage conditions. These changes enable infection of nearby grain kernels, which had previously been too dry to support fungal growth. Grain readily adsorbs or loses moisture and the moisture levels of individual seeds will be in equilibrium with the moisture available in the air surrounding them. The storability of wheat is directly related to the growth of these fungi (Channaiah and Maier, 2014), which is determined by both temperature and moisture (Jacobsen *et al.*, 2007).

Aflatoxins

Aflatoxins (B_1, B_2, G_1, and G_2) are produced in stored wheat by *Aspergillus flavus* or *Aspergillus parasiticus*. Contamination usually results when these fungi grow in improperly stored grain with infection occurring in storage, and not preharvest as is common in maize, peanuts, or cotton. *Aspergillus flavus* grows in starchy cereals, e.g., wheat, whenever the grain moisture exceeds that in equilibrium with 80–85% RH (~16–18% grain moisture) and temperatures are >10°C (Jacobsen, 2010c). Wheat is not a high-risk crop for aflatoxin contamination, although aflatoxin, primarily aflatoxin B_1, contamination does occur in wheat grain, flour, and pasta.

Sterigmatocystin

Sterigmatocystin is produced by several *Aspergillus* species including *A. versicolor*, *A. fumigatus*, *A. nidulans*, *A. terreus*, *A. sydowii*, members of the *A. glaucus* group with *Eurotium* teleomorphs,

and *Bipolaris sorokiniana*. Sterigmatocystin is the principal mycotoxin produced by *Aspergillus* spp. in stored wheat and other cereals in Canada (Mills, 1990; Scott *et al.*, 1972). The fungi involved are relatively common in stored grain in both temperate and tropical regions and probably will be found in wheat stored at moistures in excess of that in equilibrium with 70–75% RH or ∼14–15% moisture and temperatures >5–10°C.

Ochratoxins

Ochratoxins (A, B, and C) are produced primarily by *P. verrucosum*, *P. viridicatum*, and other *Penicillium* species in temperate regions. In tropical areas *Aspergillus alutaceus* var. *alutaceus* can produce ochratoxins, but this fungus is not common on wheat. Ochratoxin A is the most common, the most studied, and the most toxic of the ochratoxins and may be found in wheat grain, all milled wheat fractions, and bread and pasta products. These fungi grow well under storage conditions in equilibrium with 80–85% RH (∼16–18% grain moisture) and at temperatures as low as 0–5°C. Ochratoxin A contamination by *Penicillium* spp. is common when plants lodge or wet weather delays harvest in temperate climates (Jacobsen, 2010c). Ochratoxin A exposure in Europe is primarily from consumption of contaminated grain and not from other potential sources, e.g., meat, wine, coffee, and chocolate.

Control of storage fungi begins in the field through the establishment of uniform stands, so that late maturing tillers and associated immature kernels are not common, and managing weeds, so that high-moisture weed seeds are not included in the harvested grain. Storage fungi often infect through breaks in the seed coat, so adjusting harvest equipment and augers is crucial to reducing the mechanical damage that can lead to growth of storage fungi and mycotoxin contamination.

Integrated Management to Reduce Losses from Mycotoxigenic Fungi

Integrated management reduces the risk of mycotoxin contamination. Wheat production fields should be selected based on cropping history and tillage to minimize *Fusarium* head blight and ergot. Selection of cultivars with disease resistance and/or differing flowering times can limit losses. The relatively low levels of resistance to *Fusarium* head blight in most cultivars frequently necessitates the use of fungicides applied at flowering to limit losses under epidemic conditions. The use of pathogen-free seed is more important for ergot than for *Fusarium* head blight. Weeds can be hosts for either ergot or *Fusarium* head blight pathogens and can contribute to storage problems, so weed control is critical. For irrigated wheat, avoiding irrigation during heading and flowering can reduce the likelihood of infection by either the ergot or the *Fusarium* head blight pathogens. Avoiding weather delays in harvesting often is beyond the control of the farmer, even though it is one of the most important factors in mycotoxin contamination in temperate climates. Kernels infected by strains of *Aspergillus*, *Penicillium*, and *Fusarium* generally are lighter in weight and friable, so adjusting the combine and pre-binning cleaning to remove light-weight, broken kernels can reduce the level of mycotoxins in the stored grain. As these kernels commonly are contaminated by fungi, blowing them out of the combine into the field effectively provides inoculum for successive crops. Yet, removal of light-weight *Fusarium*-infected kernels can reduce deoxynivalenol concentrations in grain lots by 50% or more (Burrows *et al.*, 2008; Salgado *et al.*, 2011). Cleaning removes ergot sclerotia and high-moisture weed seeds that contribute to fungal growth in storage and subsequent contamination problems. Cleaning also enables better air movement in the bin and helps remove

excess moisture from pockets in the grain mass, thereby limiting fungal metabolism. Finally, proper storage moisture/temperature management and grain storage insect management are essential to limit growth by storage fungi and to minimize mycotoxin contamination.

Future Prospects

Biological control induced systemic resistance and transgenic wheat lines may be used to help manage mycotoxins in wheat. Biological control studies have focused primarily on *Fusarium* head blight control through reducing pathogen inoculum, competition for nutrients, use of antagonism to reduce infection, induction of systemic resistance, or inhibiting mycotoxin synthesis. The fungus, *Clonostachys rosea* strain ACM941, a known mycoparasite, reduced production of perithecia on wheat residues and reduced infection, disease severity, and deoxynivalenol accumulation when sprayed on wheat spikelets at 50% anthesis and again 3–4 days later. Significant disease control was achieved, but tebuconazole sprays were more effective (Xue *et al.*, 2009). This organism may be very useful in programs to reduce *Fusarium* head blight inoculum potential. Several biological control agents have shown promise for reducing fungal infections that lead to *Fusarium* head blight and deoxynivalenol accumulation in grain (da Luz *et al.*, 2005; Jochum *et al.*, 2006; Khan and Doohan, 2009; Palazzini *et al.*, 2007; Schisler *et al.*, 2002a,b, 2006, 2011; Xue *et al.*, 2009). Biological control agents evaluated include yeasts, *Bacillus* sp., *Pseudomonas* sp., *Paenibacillus* sp., *Lysobacter enzymogenes* C3, and *Streptomyces* sp. These organisms exert their effects by competing for nutrients (yeasts), producing antifungal antibiotics (*Bacillus* sp., *Streptomyces*, and *Pseudomonas* sp.), or reducing disease severity and deoxynivalenol accumulation (*Pseudomonas* sp.). Biological controls currently do not provide the level of control provided by fungicides and, like fungicides, will be most effective when used in programs that include resistant varieties, cultural controls, and/or fungicides. Combining biological controls with triazole fungicides can increase the control of *Fusarium* head blight (McMullen *et al.*, 2012). Currently, there are no labeled products available to growers although this situation likely will change in the near future. An advantage for biological controls is that they may be used post-anthesis, when fungicide applications are not allowed due to residue problems.

Microbial or chemical inducers of systemic resistance could improve control of *Fusarium* head blight, particularly in integrated management programs. Induction of resistance by microbes such as *L. enzymogenes* C3 (Jochum *et al.*, 2006) or with chemical inducers, including the sodium salt of salicylic acid, isonicotinic acid, and DL-β-amino-*n*-butyric acid (BABA), reduces *Fusarium* head blight severity (Zhang *et al.*, 2007). Overexpression of the *Arabidopsis NPR-1* gene in wheat reduces *Fusarium* head blight severity (Makandar *et al.*, 2006). *Bacillus mycoides* isolate BmJ, a known activator of this gene (Neher and Jacobsen, 2009), is now marketed by CERTIS USA to induce disease resistance for foliar application in a wide range of crops, including wheat for *Fusarium* crown rot (Moya *et al.*, 2010).

The potential for transgenic wheat to express antifungal proteins, genes involved in plant defense reactions, and genes involved in reduced production, accumulation, or detoxification of *Fusarium* mycotoxins is significant (Han *et al.*, 2012; Karlovsky, 2011; Makandar *et al.*, 2006; Muehlbauer and Bushnell, 2005; Shin *et al.*, 2008; Woriedh *et al.*, 2011). Similar transgenic approaches may be useful for reducing contamination by other mycotoxins; however, these transgenic technologies are not presently available in commercial wheat lines.

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15 Good Management Practices for Minimizing the Risk of *Fusarium* Head Blight and Mycotoxin Contamination in Nontraditional Warmer Wheat-Growing Areas

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Abstract

Wheat is a temperate crop grown during the cooler months in tropical areas of the world. We provide an overview of the regions at risk for *Fusarium* head blight and the accumulation of mycotoxins, particularly deoxynivalenol. In some countries, awareness of the need to mitigate the accumulation of mycotoxins is increasing, but data on actual contamination levels are incomplete. In subsistence farming, where wheat is grown on a small scale, the risk of consuming mycotoxin-contaminated wheat products is generally overlooked. We provide guidelines appropriate for wheat producers in warmer wheat-growing areas.

Keywords: breeding; crop rotation; deoxynivalenol; elevation; fungicides; harvesting; irrigation; prediction models; regulations; resistance; seed treatment; sowing time; subsistence farming; tillage

Introduction

Wheat is the most widely grown and important food crop worldwide (217 million ha; Dixon *et al.*, 2009). Most wheat is cultivated in temperate climates lying between 30°N and 50°N and 25°S and 40°S. Although wheat is a temperate crop, more than 40% of the world's wheat is produced in the tropics and subtropics (Rehm and Espig, 1991), and about 47% is grown in less-developed countries (LDCs). At the global level, China and India are the top two wheat-producing countries. Common wheat, *Triticum aestivum*, is by far the most cultivated species of wheat, with spring bread wheat, which does not require vernalization, accounting for ∼70% of the 116 million ha sown to common wheat in developing countries (Dixon *et al.*, 2009). Durum wheat, *T. turgidum* var. *durum*, is grown mainly in Mediterranean countries and in Ethiopia. In areas with mild winters, e.g., parts of China, India, North Africa, Southern Africa, and South America, spring wheat often is sown in the autumn for growth during the cooler months.

In general, wheat in LDCs is grown in three types of climatic zones. In regions with winter rainfall, wheat is sown in the autumn or early winter during the coolest period of the year. In subtropical areas with summer rainfall, wheat is grown in the dry, cool part of the year, usually with irrigation or, in particularly favorable situations, with residual moisture from the rainy season. In more tropical environments and at lower latitudes, wheat is grown at higher elevations (>1500–1800 masl; Rehm and Espig, 1991). Wheat production has expanded horizontally in recent years, with wheat cultivation spreading into nontraditional areas that formerly were considered unsuitable

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for wheat production. For example, wheat now is grown on a large area (>500,000 ha) during the winter in Bangladesh.

Scab, or *Fusarium* head blight, is a major disease of wheat caused by several *Fusarium* species and *Microdochium nivale*. These fungi are distributed globally and some of them produce mycotoxins, i.e., secondary metabolites that can adversely affect the health of humans and domesticated animals. *Fusarium graminearum* (sexual stage: *Gibberella zeae*) is the most common causal agent of the disease, particularly in warmer wheat-growing areas. Depending on the location, however, a number of toxigenic and nontoxigenic species could be involved. *M. nivale*, *F. avenaceum* (sexual stage: *G. avenacea)*, *F. culmorum*, *F. equiseti*, *F. graminearum*, and *F. sporotrichioides* are common in warm areas and can produce a variety of mycotoxins (Bottalico and Perrone, 2002). Mycotoxins produced by *F. graminearum* include two type B trichothecenes—deoxynivalenol and nivalenol—as well as zearalenone, a pseudo estrogen. These fungi not only cause direct yield losses due to plant disease, but also reduce the quality and market value of the harvested grain due to the mycotoxin threat to the health of humans and domesticated animals.

In LDCs and at lower latitudes, *Fusarium* head blight epidemics are more severe when they occur at temperatures of 25–30°C. As in temperate wheat-growing areas, these epidemics are highly dependent on rainfall patterns and on the availability of free moisture around anthesis. Therefore, even though the fungi (particularly *F. graminearum*) are widely distributed, *Fusarium* head blight epidemics in the major wheat-producing LDCs commonly occur in only a few geographical areas, e.g., China, Brazil, Argentina, Uruguay, Ethiopia, and along the Caspian Sea in Iran, where climatic conditions are particularly favorable for the disease. In South Asia, which cultivates 36 million ha of wheat, *Fusarium* head blight is of very limited importance because wheat is grown in the dry winter season, mostly under gravity-irrigated conditions (Duveiller, 2004).

In addition, *F. graminearum* is present in many tropical regions where commercial wheat production is negligible from a global market perspective, but plays a vital role in food security in the local communities. In these areas, e.g., Burundi, Malawi, parts of Kivu in the Democratic Republic of Congo, parts of Ethiopia, and Vietnam, *Fusarium* head blight often is reported as a biotic constraint when rainfall and humidity are high, although other wheat diseases, e.g., leaf and stem rusts caused by *Puccinia triticina* and *P. graminis*, respectively, and spot blotch (*Cochliobolus sativus*), generally need more attention (Dubin and van Ginkel, 1991). In many LDCs, regulations on mycotoxin content either do not exist or are not enforced. Therefore, many people, especially in rural areas, consume cereals either directly or in an indirect form as meat from animals fed with contaminated grain. In the 1980s, with support from the United Nations Development Program (UNDP), efforts were made to develop wheat production in warmer areas in collaboration with CIMMYT. Following the food crises and the high price of cereals in 2008, the need to satisfy local demand from an ever larger population prompted renewed interest in growing wheat in nontraditional warmer areas. If the trend continues, in view of the absence of in-depth local expertise on food safety, guidelines will be needed to mitigate the effects of *Fusarium* head blight and mycotoxins occurring in tropical environments.

Nontraditional Warmer Growing Areas Where *Fusarium* Head Blight Occurs

Warm humid areas are more favorable for *Fusarium* head blight. Severe *Fusarium* head blight epidemics occur when warm, wet weather coincides with anthesis and the availability of natural inoculum. *F. graminearum* is the *Fusarium* species most often associated with the disease. The fungus may survive in the soil as chlamydospores and is found in many areas, including those where there are no severe *Fusarium* head blight symptoms on potentially susceptible crops. During

long periods of free moisture and when rainfall coincides with flowering and relatively warm day temperatures (24–29°C), the fungus can attack the central axis (rachis) of the spike, resulting in the premature death of the ear. Mycotoxin levels can increase if the harvest is delayed and also may increase during storage if temperature and/or relative humidity is sufficiently high. The mere presence of *Fusarium* head blight-infected grain does not necessarily mean that mycotoxins are present.

Fusarium head blight is very important in some parts of Asia. In China, *Fusarium* head blight could affect up to 7 million ha, and 2.5 million tons of grain could be lost in epidemic years (Gilchrist and Dubin, 2002). Most wheat-growing regions in China need *Fusarium* head blight-resistant wheat. The most severely impacted regions are the central and lower Yangtze valleys, the south China winter wheat zone, and the eastern part of the northeast spring wheat zone. In 2012 the disease was severe in other wheat-growing areas of central and northern China where its incidence usually is low. *Fusarium* head blight is severe on the border of the Caspian Sea in northern Iran. In South Asia, *Fusarium* head blight is a disease of very limited importance (Duveiller, 2004). Other regions in Asia where the disease is a constraint include those where wheat cultivation is relatively small or marginal, as in the hilly regions of Southeast Asia.

In South and Central America, increasing *Fusarium* head blight epidemics are occurring in southern Brazil, Uruguay, and Argentina, where climatic conditions were more favorable for *Fusarium* head blight outbreaks in the late 1990s than they were in the 1970s and 1980s (Fernandes *et al.*, 2007a; Peyrera and Dill-Macky, 2008). The disease also can be severe in Paraguay. In Mexico and Guatemala, *Fusarium* head blight occurs during the rainy season in some hilly areas (Gilchrist and Dubin, 2002). In Mexico, affected areas include the Central High Valley and the Mixteca. The disease occurs in several states, including Edo de Mexico, Hidalgo, and Oaxaca, when wheat is grown during the rainy season (June/July to October/November).

In Africa, the area cultivated to wheat is limited, other than in North Africa, Egypt, Ethiopia, Kenya, Sudan, and South Africa (Tanner and Raemaekers, 2001). In rainy years, *Fusarium* head blight has been reported on wheat in South Africa, Ethiopia, the central African highlands (Burundi and Rwanda), and parts of Tunisia. In South Africa, *Fusarium* head blight also can occurs on the irrigated wheat in the Orange River valley

The results of advanced *Fusarium* head blight research, information, training, and resistant germplasm often reach breeding programs and farmers with insufficient resources and facilities in regions where *Fusarium* head blight incidence is sometimes less visible or overlooked, but where farmers and consumers are vulnerable to grain losses or food safety hazards. With few exceptions, studies of the incidence of *Fusarium* mycotoxins in wheat in subtropical and tropical areas are either nonexistent or insufficiently documented (Desjardins, 2006). Limited information is available for China, Argentina, and Uruguay (Desjardins, 2006; Luo *et al.*, 1992), all of which are large wheat consumers and/or exporters. The mycotoxin requiring priority attention is deoxynivalenol.

Guidelines for Minimizing the Risk of Mycotoxins in Warmer Growing Areas: A Discussion

Deoxynivalenol is the most widely occurring mycotoxin, but it often coexists with other toxins (Table 15.1) or derivatives such as 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, or fusarenone-X. Since strict regulations or recommendations for reducing the amount of mycotoxins, primarily deoxynivalenol, often are lacking or incomplete in more tropical wheat-growing areas, guidelines are needed to help scientists and farmers in these areas mitigate the impact of *Fusarium* head blight on yield and reduce the level of mycotoxin contamination. Many factors of varying importance favor the disease. Mitigating *Fusarium* head blight and improving food safety requires that many needs

Table 15.1 Mycotoxins produced by toxigenic species of *Fusarium* associated with *Fusarium* head blight (Bottalico and Perrone, 2002; Desjardins, 2006; Glenn, 2007; Thrane *et al.*, 2004; Waalwijk *et al.*, 2008)

	Mycotoxin										
Species	Beauvericin	Deoxynivalenol	Diacetoxyscirpenol	Enniatins	Fusarenone X	HT-2 toxin	Moniliformin	Neosolaniol	Nivalenol	T-2 toxin	Zearalenone
F. acuminatum	✗			✗			✗			✗	
F. avenaceum	✗			✗			✗				
F. cerealis (syn. *F. crookwellense*)									✗		✗
F. culmorum		✗							✗		✗
F. equiseti	✗		✗						✗		✗
F. graminearum		✗							✗		✗
F. langsethiae			✗			✗		✗		✗	
F. poae	✗		✗		✗				✗	✗	
F. sporotrichioides	✗		✗			✗	✗	✗		✗	
F. subglutinans	✗						✗				
F. tricinctum				✗			✗				

be addressed to enable effective control of *Fusarium* head blight based on current knowledge. Since it is difficult to monitor and impossible to control these factors, an integrated approach is needed to reduce deoxynivalenol contamination to the lowest possible level.

Best practices for LDCs and tropical regions are fundamentally the same as those for advanced countries in temperate regions (Jacobsen, 2014; Mesterházy, 2014a,b). Our goal is to suggest good agricultural practices for minimizing the occurrence of mycotoxins in wheat grain that takes into account the local climate, local agronomic practices, and economic constraints. These good agricultural practices are the main line of defense against contamination of wheat by *Fusarium* mycotoxins. The implementation of good practices during handling, storage, and processing and the distribution of human food and animal feed also are clearly important, but are secondary to the good agricultural practices (Channaiah and Maier, 2014; Magan *et al.*, 2014; Raiola and Ritieni, 2014).

Below, we discuss sound practices that could form general guidelines for various levels in the wheat production chain in warmer areas, where economic and technical constraints are generally higher than in more advanced agricultural systems. Control of the disease in the field relies upon the use of fungicides, resistant varieties, or agronomic practices that minimize inoculum buildup and spread (Nicholson *et al.*, 2004), but limiting mycotoxin contamination in the grain also depends on postharvest conditions.

Seed Treatment

Bergstrom (1993) speculated that *F. graminearum* survives poorly on seed, and that several months of on-farm storage could improve the germination of lightly infected seed in situations where chemical seed treatment is not available. However, research on spring wheat in Canada suggested that the *F. graminearum* survives very well on seed as it can still be found on infected kernels 24 months after storage (Inch and Gilbert, 2003). Although seed infection might play a less important role in disease transmission than survival on residues, affected seed lots should be cleaned thoroughly to eliminate shriveled grains. This cleaning also reduces the risk of seedling blight at emergence and improves crop establishment.

Zero tolerance should be the general rule for the presence of *F. graminearum* in grain intended for use as commercial seed. Chemical seed treatments, e.g., chlorothalonil and thiabendazole, can be used when available. More recently described active ingredients include the triazoles tebuconazole, triadimenol, and triticonazole and azoxystrobin. Among the phenylpyrroles, fludioxonil also is effective in controlling *F. graminearum* on wheat seed. However, adequate seed treatments will not be available everywhere and, even if successful, will not prevent subsequent *Fusarium* head blight infections from wind-borne ascospores. Even if *Fusarium* species are cosmopolitan and several months of storage could reduce endogenous *Fusarium* contamination, chemical seed treatments should still be applied when germplasm is exchanged to prevent the dissemination of isolates with new mycotoxin production abilities. When it is possible to store seed in cold rooms, the increased survival of *F. graminearum* in seed stored as usually recommended, i.e., between 10°C and 2.5°C, must be considered (Gilbert *et al.*, 1997). Therefore, infected seed stored under these conditions should be treated before planting to ensure good crop stands and to reduce seedling blight in disease-prone environments.

Tillage and Sowing Time

Sometimes the burial of infested crop residue is an option, but neither debris management nor crop rotation will be completely effective if wind-borne ascospores are available as inoculum in

neighboring fields. Conservation agriculture practices that combine zero or minimum tillage with the retention of crop residue are being more widely adopted and are important practices for protecting vulnerable soils as they make cropping systems more cost effective and sustainable. However, crop residues on the soil surface are associated with higher *Fusarium* head blight severity in areas where wheat is grown after maize, which is an alternate host for the fungus (Dill-Macky and Jones, 2000). This problem may occur anywhere in the world where wheat is grown following maize and conservation agriculture practices are followed. As the knowledge base on the long-term impact of conservation agriculture practices is somewhat limited, site-specific considerations might lead to different conclusions.

In preliminary data from a long-term sustainability trial in the semiarid, subtropical highlands of central Mexico, *Fusarium* head blight severity in wheat monoculture under zero tillage with crop residue retention did not increase relative to conventional tillage. Similarly, in the same environment there is no indication of increasing disease severity in wheat grown after maize, suggesting that the improved soil conditions resulting from conservation agriculture may reduce *Fusarium* epidemic development (Troch, 2006). Sowing time manipulation can help reduce spore dissemination when it rains during flowering or when mycotoxins accumulate due to rain during harvest or excessive grain moisture, e.g., West Bengal and Assam in India.

Rotation

Crop rotation is important in reducing the amount of inoculum. When *F. graminearum* causes stalk rot in maize, the fungus infects all parts of the plant during the growing season and then survives on the maize stubble. Thus, it is important to minimize wheat after maize, especially when zero tillage is practiced in *Fusarium* head blight-prone regions. Wheat and barley residues also support *F. graminearum* colonization longer under zero tillage production conditions than under reduced tillage production systems. These residues could, therefore, be major contributors to Fusarium head blight inoculum, as reported in Uruguay (Pereyra and Dill-Macky, 2008). *Fusarium graminearum* also colonizes rice (Salleh *et al.*, 2014), but *Fusarium* head blight does not usually occur on wheat planted after rice in South Asia because the climate is not suitable for ear infection during the wheat-growing season. However, sporadic spikelet infection due to *F. graminearum* has been observed near harvest time in Bangladesh (Duveiller, 2004). As a rule, rotations with a nonhost crop, e.g., potato, alfalfa, soybean, and vegetables, should be considered to minimize the risk of mycotoxins in *Fusarium* head blight-affected areas.

Resistance and Varietal Diversification

Field resistance is the cornerstone of integrated control strategies against *Fusarium* head blight in all environments. The relevance of resistance breeding is particularly acute in more tropical wheat-growing areas, where many farmers rely almost exclusively on improved varieties for disease control, and chemical control, even when available, might not be cost effective. Genotype diversification is paramount. International networking and international screening nurseries play a critical role in the distribution of *Fusarium* head blight-resistant germplasm. Since disease severity varies and depends on climatic conditions, sources of resistance need to be identified and tested in reliable hot-spot locations where *Fusarium* head blight occurs every year.

High-throughput screening for *Fusarium* head blight can be conducted with artificial inoculation in Mexico (Duveiller *et al.*, 2008). In 1989, CIMMYT and China initiated a shuttle-breeding and germplasm exchange program focusing on the integration of the *Fusarium* head blight resistance of Chinese wheats into high-yielding CIMMYT germplasm. Consequently, many Chinese derivatives are included in the international CIMMYT nurseries that are distributed worldwide. Currently, improved resistance to FHB is based primarily on the deployment of the 3BS and 5A QTLs derived from the Chinese wheat Sumai-3 and the Brazilian wheat Frontana (Duveiller *et al.*, 2008). Other accessions highly resistant to *Fusarium* head blight, originating mainly from Chinese and Japanese germplasm, also are known (Yu *et al.*, 2008).

Continuous support is needed to broaden the available genetic base with the help of marker-assisted selection and the accumulation of minor resistance genes. Since lodging leads to humid conditions that favor mycotoxin production in high-yielding areas, lodging resistance is another trait that also could help to limit the impact of infection by *F. graminearum* on deoxynivalenol accumulation.

Chemical Control in the Field

The effect of fungicides on the growth of *Fusarium* species associated with *Fusarium* head blight and on mycotoxin production has been widely studied (Mesterházy, 2014b). Field trials have shown that pathogens causing *Fusarium* head blight are differentially affected by fungicides and that fungicide applications can lead to differential levels of mycotoxin accumulation in the grain. The effectiveness of chemical treatments is related to the complex interactions between the fungicide (active ingredient and concentration), the fungal populations involved in the disease, and the mycotoxin that is accumulating (Simpson *et al.*, 2001). For example, azoxystrobin is best for controlling *M. nivale*, whereas tebuconazole, metconazole, and fluquinconazole provide more effective control of most *Fusarium* species. The application of azoxystrobin in regions where plants are infected with species of both *Fusarium* and *Microdochium* may increase the risk of subsequent grain mycotoxin contamination by reducing the competition resulting from infection with *Microdochium*.

Farmers in warmer wheat-growing areas often do not use fungicide treatments. Active ingredients that are commercially available in Europe and North America are extensively used in parts of Brazil, but they are not found in the marginal wheat-growing areas of most LDCs because they are not cost effective there. In general, information on the effectiveness of compounds in these regions and environments is lacking compared to more advanced countries. Some compounds that are more widely available, e.g., propiconazole, might not suppress *Fusarium* head blight to the extent suggested by studies conducted in more temperate climates.

Access to the most recently released active compounds, alone or in the most effective combination against *Fusarium* head blight, is limited in LDCs and they may not even be registered for use unless the country produces large amounts of wheat. Cost is an obstacle for small farmers, and if fungicides cannot be obtained at what is locally considered a reasonable price, then the fungicide treatment option will not be considered by many farmers. This economic driver must be considered before proposing any fungicide-based treatment, particularly in marginal areas. The economic justification for fungicide treatment might be limited to high-value seed production schemes.

Tebuconazole and metconazole are both among the most effective compounds available against *Fusarium* head blight and deoxynivalenol accumulation in grain (Nicholson *et al.*, 2005). Tebuconazole is becoming more widely available in LDCs. Timely spraying at the full rate is recommended to avoid inducing fungicide resistance.

In Europe, the effectiveness of fungicides such as tebuconazole decreases as the intervals between applications and the amounts of inoculation increase. Similarly, the effectiveness decreases and is lower in susceptible varieties than in varieties with some resistance to the disease (Matthies and Buchenauer, 2000; Mesterházy, 2014b). Thus, when fungicides are available and if it is economical, the decision to use them needs to be made carefully if the available varieties have little or no resistance to *Fusarium* head blight. Mycotoxin accumulation following triazole application has been reported (Audenaert *et al.*, 2010). Hence, even if the most effective fungicides are applied, disease control and reductions in mycotoxin accumulation might not occur (Nicholson *et al.*, 2004). Control of *Fusarium* head blight is unlikely solely from the use of fungicides. Growing resistant varieties in combination with good agricultural and storage practices remain critically important to reduce the risks associated with *Fusarium* head blight.

Harvesting

Timely harvesting is essential in areas where there is a risk of late rainfall, e.g., Assam. Farmers must be prepared to harvest on time and avoid delays as soon as the grain reaches the proper moisture content. Using equipment in good condition protects the grain and helps reduce the number of broken kernels, which results in a higher risk of deoxynivalenol contamination (del Ponte *et al.*, 2007). The growth stage and the time of infection influence *Fusarium* head blight incidence and severity and deoxynivalenol production, with the highest effect on yield and deoxynivalenol accumulation occurring when plants are infected around flowering.

The possibility of late infection and deoxynivalenol contamination also must be considered in grading systems that use the presence of visibly damaged kernels to estimate deoxynivalenol content (del Ponte *et al.*, 2007). When wheat is harvested by combine, as in Brazil, the cleaning mechanism leaves the shrunken, infected kernels on top of the soil with the crop residue. More efficient combine cleaning reduces the percentage of infected seed collected and more infected kernels are deposited on the soil surface (Reis, 1991). The fan speed should be adjusted to eliminate as many lightly *Fusarium* head blight-affected kernels as possible to minimize the risk of deoxynivalenol accumulation and the infection of noncontaminated grain during storage. Seed cleaning machines and gravity tables in seed production processing plants should remove even more of the lightly infected grains (Reis, 1991). Thus, winnowing, as practiced by many small farmers in marginal areas, reduces the risk of mycotoxin contamination and should be a recommended practice.

Storage

Grain and seed should be stored, where possible, in dry, temperature-controlled, aerated conditions, which may be difficult to find in some tropical environments. Grain harvested from lodged fields should be stored separately from grain from fields that were not lodged. Good storage also helps prevent the accumulation of ochratoxin A produced by members of several *Aspergillus* and *Penicillium* species. Insect control reduces both ochratoxin A and deoxynivalenol accumulation during storage. Fungicide, insecticide, or chemical preservatives, e.g., propionic acid, can be useful, but their use may be limited by the intended end use of the product (Anonymous, 2006).

Sampling, Testing and Regulations

Grain sampling and testing are required to assess the amount of mycotoxins, but public data on such contamination are scarce and often incomplete. Efforts to increase the awareness of the public, scientists, and decision-makers of the need to improve food safety should be encouraged in warmer areas and detailed studies should not be limited to the northern hemisphere and temperate regions. FAO (2004) conducted a survey and updated the information on mycotoxin regulations and guidelines worldwide, making toxicological data and data on the occurrence of mycotoxins in different commodities available. As of December 2003, ~100 countries had regulations for one or more type of mycotoxins, which confirms the concerns about food and feed safety in more tropical regions where wheat is cultivated. For example, China and Iran both have extensive, detailed regulations. In South America, information from Argentina, Brazil, and Uruguay suggests that deoxynivalenol accumulates to levels well above recommended levels of tolerance (Desjardins, 2006). In China, where *Fusarium* head blight infection occurs widely, variable, but high, levels of deoxynivalenol have been reported and toxicosis has been observed in years of severe epidemics.

In Nepal, the levels of deoxynivalenol in wheat are low because *Fusarium* head blight usually does not occur as the grain matures during the hot, dry spring, even though very high levels of deoxynivalenol, up to 6.5 μg/g, have been found on maize (Desjardins *et al.*, 2000). Research aimed at minimizing mycotoxin levels in cereals has become a higher priority for grain-producing countries due to the potential implications for trade, but mycotoxin researchers should not ignore the potential risks to the people who consume the products locally either.

There clearly is a need for cheaper and more reliable testing methods for conducting systematic surveys and monitoring mycotoxin levels in LDCs. There are multiple methods available that differ in sensitivity. The accuracy of the tests depends on the sampling procedure adopted and the preparation of the samples for analysis. Sampling procedures have been described by several organizations and institutions, e.g., http://www.gipsa.usda.gov and http://www.scabusa.org. In practice, the enzyme-linked immunosorbent assay (ELISA) technique is one of the most affordable tests for breeding programs and for all researchers in many geographic regions. High-performance liquid chromatography (HPLC) and LC-MS/MS technology are much more expensive and should be reserved for special cases when extremely accurate toxin quantification is needed. Efforts are underway to develop and validate more affordable and rapid tests, e.g., qualitative lateral flow dipstick methods, that could be used on a broader scale and in areas with limited scientific laboratory facilities (Gilbert and Pascale, 2014).

Forecasting

Forecasting systems based on mathematical models (de Wolf and Paul, 2014; Battilani and Logrieco, 2014) are powerful new tools for monitoring and predicting risk in disease-prone areas. Their ability to assess the risk of *Fusarium* head blight occurrence and severity in warmer wheat-growing areas has been validated against observed epidemic data from Rio Grande do Sul in southern Brazil, a region where *Fusarium* is a major constraint. The model accurately predicted the severity of *Fusarium* head blight under distinct environmental conditions (del Ponte *et al.*, 2005; Fernandes *et al.*, 2007a, 2007b). The goal of this system is to deliver real-time accurate forecasts of *Fusarium* head blight epidemics and to provide farmers with guidelines on the need for and timing of fungicide applications. A similar approach should be encouraged in other areas where the disease occurs across large areas, e.g., China. Forecasting *Fusarium* toxin production has been more difficult, but would

help reduce the entry of mycotoxins into the food and feed chains. Climatic-based forecasting models can be used to identify high-risk areas where agricultural practices to limit *Fusarium* head blight should be encouraged. Research is needed on potential applications in other regions to assess the risk faced by subsistence farmers in marginal agricultural areas of small countries where surveys cannot be easily conducted. Fungicide use might not be an option in all of these situations, but the awareness of food safety issues should increase.

Conclusion

Wheat is a temperate crop grown mainly in the cooler season in more tropical environments. The distribution and severity of *Fusarium* head blight in these areas varies greatly, depending on the cropping system. The disease is a global threat to wheat production and causes losses in yield and grain quality. In spite of the increased awareness of the risk of mycotoxin contamination in the wheat chain, regulations and testing are not frequently implemented, even in countries where specific regions often are affected by the disease, e.g., China or southern Brazil. Information is generally lacking for small wheat-producing countries even though the disease occurs and small farmers are at risk. We have summarized the current status of the situation in the tropics on different continents, as well as guidelines for minimizing the risk of mycotoxin contamination in these regions.

Consumers and farmers in LDCs and nontraditional wheat-growing areas should increasingly be able to access and benefit from improved technologies and scientific knowledge of *Fusarium* head blight and mycotoxin contamination. At present, no wheat varieties have been identified that are completely resistant to the disease. Nevertheless, the combination of less susceptible genotypes, good agricultural practices, good harvest practices, and good storage conditions should mitigate the risk of mycotoxin contamination even when adequate fungicide treatments are unavailable. These treatments may be neither affordable nor cost effective for small-scale farmers in the poorest and most marginal areas of the tropics where wheat is grown and *Fusarium* head blight occurs. Thus, breeding efforts to develop high-yielding broadly adapted wheat genotypes with quantitative resistance to *Fusarium* head blight, based on the accumulation of minor genes, need to continue (Mesterházy, 2014a). Public awareness of the risk posed by mycotoxins, especially in more tropical environments, should be increased to inform leaders and decision-makers of the importance of mycotoxin contamination in agricultural products and to increase food safety and the availability of high-quality grain in LDCs. Action plans to address these deficiencies in communication, prevention, and control structures are needed.

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16 Chemical Control of *Fusarium* Head Blight of Wheat

Ákos Mesterházy

Abstract

Even the best fungicides alone are insufficient to control *Fusarium* head blight and prevent the accumulation of deoxynivalenol during most years. In combination with improved application methods, more accurate disease forecasts, newer generation fungicides, and host lines with moderate disease resistance, both *Fusarium* head blight and deoxynivalenol accumulation can be minimized. Even then, the cost of fungicide purchase and application may be greater than the value of the increased grain yield. Controlled experiments with defined inocula and environmental conditions are essential for consistent results, as natural field conditions can vary significantly from year-to-year. Treatment with suboptimal doses of a fungicide or with a non-conventional mixture of fungicides can reduce the effectiveness of mixed products and increase the amount of deoxynivalenol accumulated relative to untreated controls. Pathogen resistance to fungicides used to control *Fusarium* head blight is not currently a major problem. If the probability of a *Fusarium* head blight epidemic is high, then a routine preventive fungicide treatment at flowering is advised to maintain yield and grain quality.

Keywords: application methods; biological control; crop rotation; disease – incidence, resistance, severity, and spread; environment; fungicides; inoculum; yield potential

Introduction

Fungicides and agronomy remain important control methods for *Fusarium* head blight (FHB) as varieties with higher levels of resistance to this disease remain rare in European fields and breeding efforts are taking longer than expected. The last major review of this area (Mesterházy, 2003) and practical applications (McMullen *et al.*, 1997; Wilcoxson, 1996) indicate incomplete control of *Fusarium* head blight by fungicides. Developments such as improved application technology, more efficient fungicides, and more sophisticated agronomic practices combined with advanced forecasting of deoxynivalenol (DON) contamination have helped to minimize the damage caused by *Fusarium* head blight.

Fusarium spp. produce a number of toxins harmful to humans and other animals. Most important wheat production areas have either mandatory or suggested limits for toxins in the grain (FAO, 2004), so successful control of *Fusarium* head blight has become even more important than it was before. Consumers also are better informed and markets need healthy grain, so a *Fusarium*

Mycotoxin Reduction in Grain Chains, First Edition. Edited by John F. Leslie and Antonio F. Logrieco.

head blight epidemic with high infection severity resulting in high levels of toxin contamination has serious economic consequences. Thus, in most major wheat-producing regions worldwide, the prevention of toxin contamination in the field and in marketable grain is of primary importance. As cultivar resistance often is insufficient, chemical protection has a significant role in achieving this goal. Consequently, the effectiveness of *Fusarium* head blight control is measured neither by the yield response, nor by reduced incidence of the disease, but rather by the toxin contamination of the harvested grain. Lower levels of mycotoxin contamination usually are accompanied by higher yields and lower disease incidence, but increases in profit rely on lower toxin concentration levels, since grain with toxin levels above legal limits may not be salable at all.

Decisions to apply fungicides should be part of a crop management system (Aldred and Magan, 2004; Sip *et al.*, 2007; Wilcoxson *et al.*, 1989) that includes variables such as cultivar resistance, other variety traits, cropping history, tillage practices, and many other parameters. Indeed the goal is to develop a variety- and field-specific plant protection system where the control of *Fusarium* head blight has a central role.

In this chapter, I summarize the available information and synthesize some answers. Much of the information is published in extension papers, commercial leaflets, unrefereed research reports, and conference proceedings that contain recommended protocols, but lack either the data or methodology to support the recommendations, although recently more of this information has begun to appear in scientific journals. Thus, the cited literature is more diverse than that normally found in a peer-reviewed publication.

Factors Influencing Fungicide Effect

The effects of fungicides often are not constant. For *Fusarium* head blight, however, the results often are discouraging, variable, and influenced by a number of factors (Table 16.1). Data on modes of action of specific fungicides are not included in this chapter, but can be found in other publications, e.g., Thomson (1994) or Hopkins (1997), or obtained directly from the fungicide's manufacturer.

Inoculum

Although spores of *Fusarium graminearum* can travel hundreds of miles via aerial dispersal (Schmale *et al.*, 2006, 2012), they differ significantly from rusts or powdery mildew, since the local inoculum is thought to be the primary source of inoculum. *Fusarium* head blight pathogens, e.g., *F. graminearum*, *F. culmorum*, and *F. avenaceum*, usually have only one or two vegetative spore cycles per growing season and overwinter in infected debris. These fungi do not produce chlamydospores (Leslie and Summerell, 2006) and do not survive in field soil for lengthy periods of time. Thus, the disease has strong endemic characters and local conditions play an important role in the epidemics. Wheat growing in fields planted with maize in the previous year is more prone to *Fusarium* head blight than wheat following other crops (Table 16.2). The-wheat-after-wheat risk also is supported by data from the United States (Dill-Macky, 2008). Generally, the risk for deoxynivalenol accumulation depends upon the number of *Fusarium* propagules found in the fields, and a good correlation ($R^2 = 0.85$) between the amount of inoculum and amount of maize debris has been reported (Maiorano *et al.*, 2008). The relatively local nature of *Fusarium* head blight epidemics means that the possibilities of inoculum control are much better than they are when the inoculum is from more distant sources (Yuen and Schoneweis, 2007). Reducing inoculum is important since

Table 16.1 Factors involved in the success of fungicide control of *Fusarium* head blight in wheat (after Wale, 1994)

Factor	Description
Inoculum	Accurate identification of disease
	Incidence and/or severity of diseases within crop
	Presence of disease in locality
	Previous crop and residues
Cultivar resistance, host plant traits	Level of resistance
	Developmental growth stage
	Flowering type
	Plant height, presence or absence of awns
	Fungicide receptivity
Climatic factors	External humidity, temperature, and rainfall
	Probability of epidemics
	Canopy microclimate at head level
Yield potential	Canopy structure
	Crop nutrition
	Soil moisture
Fungicide	Protectant/curative activity
	Persistence
	Time from last treatment
	Frequency of resistant fungal strains
Management inputs	Cost of application
	Time of fungicide application
	Available application technology
	Use of forecasting data to determine when to apply
	Fungicide availability
	Competing farm activities

in severe epidemics, the efficacy of the fungicides can decrease (Mesterházy and Bartók, 1996; Mesterházy *et al.*, 2003).

After maize, or other cereals, chemical protection is more likely to be needed because the amount of inoculum probably will be high. The corn rootworm (*Diabrotica virgifera*) also increases the risk of *Fusarium* head blight epidemics in wheat because it forces rotation out of maize, commonly into wheat, leading to the concomitant increase in *Fusarium* head blight that goes with this rotation. Currently, at least 50% of the wheat crop in Hungary follows maize.

Variety Resistance—Host Plant Traits

Resistance differences among European and American wheat cultivars are rather modest (Brown-Gudeira *et al.*, 2008; Griffey, 2005; Wagacha and Muthomi, 2007), so all varieties in commercial

Table 16.2 Risk factors for deoxynivalenol accumulation in wheat (after Obst *et al.*, 1992, 1997, 2002)

Previous crop	Tillage	Relative risk
Non-cereal	–	1
Wheat	Minimum tillage	4
Maize (harvested for grain)	Minimum tillage	17.2
Maize (harvested for grain)	Maize residue plowed in	4–5
Maize (harvested for silage)	Maize residue plowed in	1.5

production are potentially subject to fungicide treatment. Most of the area planted is sown with cultivars with moderate to high susceptibility to *Fusarium* head blight. In a bad epidemic year, virtually every variety would need chemical control, as occurred in Hungary in 2010 when the worst epidemic in the last 40 years occurred. Varieties with the same resistance level may react differently to fungicide treatment, and the genetic contributions to fungicide tolerance are poorly understood, even though they could be important in developing variety-specific fungicide applications. In Hungary, national epidemics reoccur every 4–6 years (Aponyi *et al.*, 1998), and elsewhere these recurrences may be more frequent, but nowhere are these epidemics sufficiently frequent for a resistance breeding program to be able to rely solely on natural inoculum (Yuen and Schoneweis, 2007). Thus, without a reliable nursery in which artificial inoculation and screening for resistance can occur, breeding highly resistant varieties is not possible.

The translocation of a fungicide, or its absence, also is an important host trait. There is no proven fungicide translocation from leaves to heads or from one side of the head to the other. The available data refer only to translocation within a leaf (Mauler-Machnik and Zahn, 1994). Indirect data speak, however, for a low level of translocation. This pattern appears to hold for both the triazoles and the strobilurines.

The speed of flowering also influences the success of fungicide treatments. Cultivars in which there is little or no difference between heading and flowering of main and secondary tillers are the best protected by fungicide treatments. If the timing of the main and secondary tillers is different by a week or more, then there is no optimal application time. Waiting for the late ones allows the early ones to be infected, while early applications leave heads in the boot stage at spraying unprotected and exposed to late infection.

The susceptibility window, i.e., how long a cultivar is susceptible to infection is not well defined, but its length is very important for timing fungicide applications. Usually the susceptibility window is considered to be about a week in length, although in highly resistant genotypes the window is zero, since host resistance provides protection, while for highly susceptible genotypes the susceptibility window can last 10 days or more. We (Mesterházy, unpublished) tested the length of the susceptibility window by treating with fungicide at flowering and then inoculating with the fungus 12 days later (Figure 16.1). There was no difference between the results of the two inoculations, so the

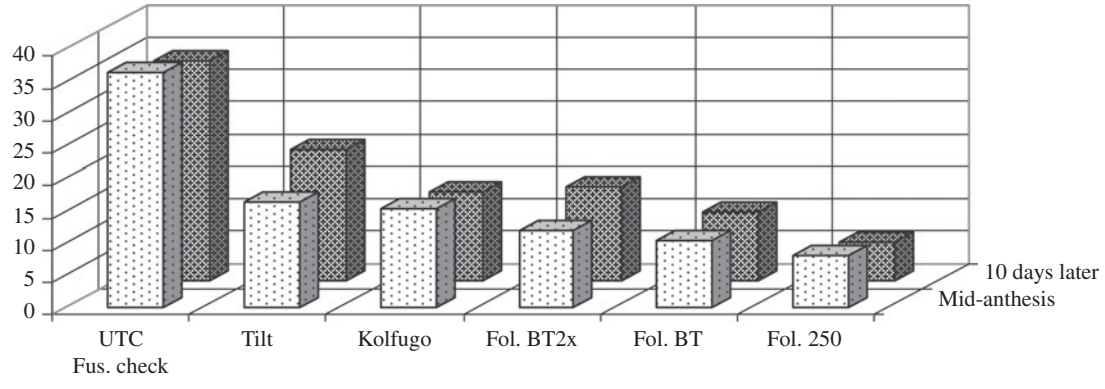

Figure 16.1 *Fusarium*-diseased kernels values from a susceptibility window test in 1992. Inoculation of fungicide-treated plots followed 2 days after fungicide treatment and 10 days later. Fungicide rates: Tilt: 1.0, Kolfugo 1.5, Fol. BT 2.0, Fol. BT 1.0, and Fol. 250 1.0 L/ha. Composition of the fungicides is given in Table 16.3

Table 16.3 Composition of commercially available fungicides tested for the control of *Fusarium* head blight

Commercial fungicide	Active ingredient(s)
Alert	Carbendazim 250 g/L and flusilazole 125 g/L
Amistar	Azoxystrobin 250 g/L
Amistar Xtra	Azoxystrobin 200 g/L and ciproconazole 80 g/L
Artea 330EC	Ciproconazole 80 g/L and propiconazole 250 g/L
Caramba	Metconazole 60 g/L
Eminent 125 SL	Tetraconazole 125 g/L
Falcon 460 EC	Spiroxamine 250 g/L, tebuconazole 167 g/L, and triadimenol 43 g/L
Folicur = F. solo	Tebuconazole 250 g/L
Input	Prothioconazole 250 g/L
Juwel	Epoxiconazole 125 g/L and kresoxim-methyl 125 g/L
Juwel TT	Epoxiconazole 83 g/L, fenpropimorph 317 g/L, and kresoxim-methyl 83 g/L
Kolfugo	Carbendazim 250 g/L
Matador	Tebuconazole 250 g/L and triadimenol 125 g/L
Opus (Tango)	Epoxiconazole 125 g/L
Opus Team (Tango Star)	Epoxiconazole 84 g/L and fenpropimorph 250 g/L
Nativo	Tebuconazol 200 g/L and trifloxystrobin 100 g/L
Prosaro	Prothioconazole 125 g/L and tebuconazole 125 g/L
Prospect	Carbendazim 200 g/L and propiconazole 80 g/L
Sphera	Ciproconazole 88 g/L and trifloxystrobin 187.5 g/L
Sportak 45 EC	Prochloraze 45%
Tilt 250 EC	Propiconazole 250 g/L
Topsin M	Thiophanate-methyl 70%

susceptibility window was at least 10 days long. In other tests there normally was a significant decrease in protection at later inoculations. However, in this trial the weather was dry and warm for the first inoculation and rainy and warm following the second inoculation. We concluded that the protective period for the fungicide must be at least 2–3 weeks to override the susceptibility window and provide protection until early ripening.

There are several characters, e.g., resistance to *Fusarium* head blight, tendency to lodge, presence of awns, and earliness, that affect the need for and efficacy of chemical treatments. In general, chemical applications act synergistically with genetic resistance, thereby reducing *Fusarium* head blight damage more in resistant plants than sensitive ones (Koch *et al.*, 2006; Mesterházy *et al.*, 2003). Taller plants usually have less resistance to lodging. Lodging increases the risk of toxin contamination (Nakajima *et al.*, 2008), since lodged plants do not dry until later in the day, which lengthens the wet period and the exposure to *Fusarium* head blight infection and the time during which toxin synthesis can occur. Lodged materials should be protected chemically to counteract the negative effects of lodging. Awned plants are easier to protect (Mesterházy, unpublished) than those without awns, as their awns catch fungicides and the paleas receive more of the active agent(s), if the chemical application did not completely cover the head. However, plants with awns are more susceptible to *Fusarium* head blight than are otherwise equivalent awnless types (Mesterházy, 1987). Earliness increases fungicide efficacy by reducing the time between flowering and maturity, and thereby increasing the probability of control until near waxy ripening as the efficacy of the long-acting fungicides usually is 3–4 weeks (Mesterházy, unpublished). Additionally, the susceptibility

window also is smaller, which allows better and more efficient protection of the earlier varieties, whether resistant or susceptible, than is possible for a late cultivar.

Climatic Factors

Climatic factors can affect the severity of epidemics and their impact can be estimated with disease-forecasting models (de Wolf and Paul, 2014; Battilani and Logrieco, 2014). Precipitation at flowering is critical: 5 mm or more of precipitation per day significantly enhances the risk of FHB, especially if combined with temperatures of ∼25°C. Heavy rains during and after flowering can initiate severe epidemics such as those in Hungary in 1997–1999, 2006, and 2008 or in the United States and Canada since the 1990s. Forecasting models have been developed in both the United States and Canada. The Canadian model, called "DONcast," is being tested in Europe (Schaafsma and Hooker, 2007) and uses data from satellites to estimate temperature and precipitation. Both the American and the Canadian models used data from Weather Innovations, Ltd. during program development. The American data are still supplied by this firm, but in Europe the system was bought from Geosys, Ltd. by Bayer CropScience. Both models are ∼85% accurate once on-the-ground factors such as resistance level, previous crop, and tillage are taken into consideration.

The models also identify the time at which protection is needed. Bayer, Inc. (Haeuser-Hahn *et al.*, 2008; Suty-Heinze and Dutzmann, 2004) suggests that the presence of tebuconazole or prothioconazole is most important ∼2 days after a rain that could initiate an epidemic. Unfortunately, it may be a week after a heavy rain before the fields are dry enough to be worked. In such cases, the late protection results in only moderate efficacy. Practically, the most efficacious fungicides may be locally unavailable immediately after a heavy rain, with only less effective fungicides available for purchase. Management plans for crops grown in areas at high risk for *Fusarium* head blight routinely include preventative fungicide applications. In other areas, especially if a resistant variety is planted after a low-risk crop, then forecasting models and local weather reports can be used to estimate when and if fungicide applications are efficacious.

Warm rainy conditions during and after flowering may significantly increase the risk of an *Fusarium* head blight epidemic. The length of the rainy period, i.e., the time that the ears are wet, is strongly correlated with deoxynivalenol production. For example, the correlation between the amount of rain and the amount of deoxynivalenol present 3–4 weeks before harvest was $r = 0.96$ (Mesterházy *et al.*, 2006). In dry years, 20–30 mg/kg deoxynivalenol in the untreated control (UTC) is high (no fungicide, only artificial inoculation). In a rainy season, e.g., 2008, the deoxynivalenol contamination in the untreated control reached 300 ppm although the level of *Fusarium*-diseased kernels (FDK) was the same (70–80%) in both years.

Yield Potential

In many wheat-growing areas, yield is generally low, normally <3 t/ha. At present grain and fungicide prices, fungicide applications in these areas are not economically worthwhile. In these areas and in most developing countries, resistant host plants are the only economical solution. Fungicides are commercially useful in high-yielding areas, including most of Europe, China, Egypt, Mexico, Japan, and New Zealand and parts of the United States and Canada. The price of a treatment, approximately the value of 500 kg of grain, should be returned as either a higher yield or a higher price for better quality grain or both.

Fungicides

Disease Assessment in Fungicide Tests

Several methods are used to estimate *Fusarium* head blight (Bekele *et al.*, 1994; Wilcoxson, 1996). The visual estimation of *disease incidence* (proportion of spikes infected) or *disease severity* (proportion of a spike infected) commonly is used in both naturally and artificially inoculated trials. The disease index is the product of the disease incidence and the disease severity. Estimates of fungal mass made with rt-PCR (Nicholson *et al.*, 1997; Simpson *et al.*, 2001) and ergosterol (Varga *et al.*, 2006) correlated well with severity of ear symptoms. Percentage of infected (scabby) kernels is the best measure, but in combine-harvested experiments it may be misleading because it does not include aborted kernels or those lost at harvest. Other traits, e.g., 1000-grain mass or yield reduction, also are commonly used, but their values have the same problem as does *Fusarium*-diseased kernels. This preferential loss of diseased kernels prior to scoring explains why in such tests the correlations between different symptoms are often weak (Wilcoxson, 1996).

Over the last decade the amount of toxin, usually deoxynivalenol, contamination has become one of the most important traits due to the regulatory limits (FAO, 2004) on these compounds, with most articles on fungicides and *Fusarium* head blight now containing toxin data.

There are two general approaches used in field tests of *Fusarium* head blight control by fungicides:

1. Tests with only *natural infection* pressure seldom give acceptable results, as the disease pressure is usually is not high enough for significant infection. These tests do not suffice for routine evaluations of fungicide efficacy. However, they are important when confirming small plot results on a larger scale.
2. There are several artificial inoculation testing methods:
   - Nurseries are sown in several locations and tested over 2–3 years. The whole field may be sprayed with a spore suspension of the pathogen(s). To assure infection, it is advisable to repeat the inoculation the next evening or use mist irrigation to keep the spikes wet (Milus *et al.*, 2001).
   - Artificially infected grains of barley or other cereals are dispersed in the nursery to increase infection by *Fusarium* (Obst *et al.*, 1992). This spawn method has been used for *F. graminearum* and *F. culmorum* in China, Mexico, and the United States. Perithecia of *G. zeae* and sporodochia with macroconidia develop on the infected grain and provide inoculum. This method works better than natural infection and often is combined with mist irrigation as it fails under dry conditions.
   - Greater accuracy can be obtained with a microplot method (Lehoczki-Krsjak *et al.*, 2008, 2010; Mesterházy, 1987, 1995; Mesterházy and Bartók, 1996, 1997; Mesterházy *et al.*, 2003, 2011). *Fusarium* head blight severity is evaluated in the field and *Fusarium*-diseased kernels is evaluated after harvest. As many as 12 different epidemic situations can be studied simultaneously with less uncontrolled environmental variation and at a lesser cost than in a multi-location test series. The grain is threshed first at very low wind speed to retain all of the infected grains, which are very light. This initial cleaning is then followed by an optimal cleaning with optimal wind speed, but with no grain loss.

Toxin analyses may be combined with any of the disease assessment methods. There is no standard for toxin assays, with various rapid tests (usually antibody based), HPLC, GC, or LC-MS/MS and others all being used.

Results of Fungicide Experiments

Numerous fungicides are available commercially (Table 16.3). Older formulations not explicitly described in this chapter are summarized elsewhere (Mesterházy, 2003).

Most fungicides are of medium to low efficacy, providing ~50% control at best (Hershman and Draper, 2004; Hershman *et al.*, 2001; Homdork *et al.*, 2000; Maufras *et al.*, 1994; Mesterházy and Bartók, 1996; Mielke and Weinert, 1996). The French data are the most comprehensive in Europe and perhaps in the world (Maufras *et al.*, 1994). No fungicide was rated in the good to very good category across all cereals when tested for efficacy against stem, leaf, and head diseases, including *Fusarium* head blight. Only metconazole (60 g/L) 1.5 L/ha, bromuconazole 1.25 L/ha, and tebuconazole (250 g/L) 1 L/ha were rated at medium efficacy. Other compounds have lower efficacies even when mixed with carbendazim. In epidemic years, the ratio of visibly scabby grains to all grains was only rarely <5%, the cut off for successful practical control (Wilcoxson, 1996). Nonetheless, in some cases higher reductions in both disease severity and deoxynivalenol levels were observed, e.g., Haidukowski *et al.* (2004), although in other cases no observable reduction occurred in either disease symptoms or DON levels (D'Mello *et al.*, 1998; Gregoire, 2002; Milus and Parsons, 1994).

Prothioconazole is the most recent new fungicide available for controlling *Fusarium* head blight. Like other triazoles, prothioconazole is a demethylation inhibitor, with broad effects against many fungal diseases, including leaf spots, powdery mildew, and rusts (Haeuser-Hahn *et al.*, 2004). Bayer (Suty-Heinze and Dutzmann, 2004) claims that it is efficacious against *Fusarium* head blight on wheat. In initial tests, deoxynivalenol levels were reduced by 58–60% with Prosaro but by only 43–48% with Folicur, with other formulations reducing deoxynivalenol levels by 33–72%, although prothioconazole was usually the most efficacious of the tested fungicides (El-Allaf *et al.*, 2001; Hart *et al.*, 2001; Hershman *et al.*, 2001; McMullen *et al.*, 2001; Milus *et al.*, 2001). Prosaro also was more efficacious than Folicur in reducing *Fusarium* head blight disease severity (Hart *et al.*, 2001; Milus *et al.*, 2001), with reductions of 22–61% depending upon the severity of the epidemic. The percentage reduction in both disease severity and in deoxynivalenol contamination was higher in milder epidemics than in more severe ones. Even with these relatively large reductions, however, fungicides were not sufficient even in a mild epidemic to reduce deoxynivalenol below the regulatory threshold in either the United States (2 mg/kg) or the European Union (1.25 mg/kg). Follow-up tests in subsequent years have confirmed the initial results for both spring and winter wheats (reductions of deoxynivalenol by 32–61%) with large differences noted between locations (Blandino *et al.*, 2006; Hershman and Milus, 2003).

Other fungicides and fungicide test levels also have been evaluated, sometimes with unexpected results. For example, in some cases azoxystrobin can increase deoxynivalenol contamination (Mesterházy *et al.*, 2003; Obst *et al.*, 1997), or reduced or eliminated the effectiveness of other fungicides, e.g., tebuconazole, when applied as a mixed application (Blandino *et al.*, 2006) even though control by tebuconazole can be good (Simpson *et al.*, 2001). Treatment with a triazole fungicide often decreases deoxynivalenol levels, but treatment with a mixture of triazoles and strobilurines can increase deoxynivalenol levels relative to the untreated control (Blandino *et al.*, 2009). Deoxynivalenol levels also may increase when a suboptimal fungicide dose is used to treat the crop (D'Mello *et al.*, 2001). The fungicide JS399-19 is a member of the cyanoacrylate fungicide group (Li *et al.*, 2008) that seems to be more effective than carbendazim but in need of more extensive evaluation.

Microplot tests. The results from the first tests with microplots (Mesterházy and Bartók, 1996, 1997) suggested two advantages for these analyses. First, the efficacy of the treatments was significantly higher than most of those previously reported, with 60–70% reduction in deoxynivalenol

Table 16.4 Fungicides against *Fusarium* head blight in wheat

Fungicides and rates (L/ha)	FHB severity (%)	FDK (%)	DON (μg/g)
Prosaro 1.0	2.4	0.82	8.3
Nativo 1.0	9.4	3.2	23
Prospect 1.5	18	5.7	35
Juwel 1.0	14	5.6	37
Amistar Xtra 1.0	11	5.4	38
Falcon 0.8	14	5.5	41
Tango Star 1.0	16	6.1	44
Artea 0.5	21	7.2	54
Eminent 1.0	23	8.1	56
Untreated control + *Fusarium*	35	11	75
Mean	16	5.8	45
LSD 5%	2.2	3.5	11

Correlations

	Fusarium head blight (%)	*Fusarium* diseased-kernels (%)
Fusarium-diseased kernels (%)	0.9649***	
Deoxynivalenol (μg/g)	0.9547***	0.9898***

***$p = 0.001$

levels, not an exceptional result. Follow-up work was consistent with the initial reports in this area (Lehoczki-Krsjak *et al.*, 2010; Mesterházy *et al.*, 2003), with even the weakest fungicides reducing deoxynivalenol contamination by 30–40% across multiple years. The tebuconazole-containing fungicides were the best, with higher application rates generally giving better control. The second advantage was that the correlation between the ratio of the *Fusarium*-damaged kernels and the level of deoxynivalenol contamination generally was high (Table 16.4).

Influence on Other Diseases and the Greening Syndrome

The fungicides most effective against *Fusarium* head blight also are effective against a broad spectrum of fungal diseases, including rusts and other leaf diseases. The broad spectrum of control means that fungicide treatments often are of value even when an *Fusarium* head blight epidemic does not occur. In some cases a greening effect is observed. Most triazoles have some growth regulatory effects based on their cytokinin-like activity (Brück *et al.*, 1984; Sutton, 1985) and can result in increased yields even in the absence of disease (Fletcher, 2000). Other fungicides such as strobilurines also may increase yields (Grossmann and Retzlaff, 1997). The reason for these increases is not known, but might be attributed to either or both of an extension of the vegetative growth period or a longer period of time in which the genetic potential of the plant can be realized.

Biological Control

This area has attracted a great deal of research attention. Many bacterial antagonists have been tested from genera such as *Bacillus*, *Lysobacter*, and *Pseudomonas* (Yuen and Schoneweis, 2007), with results varying widely. Li *et al.* (2006) found a strain of *Lysobacter enzymogenes* that slightly reduced *Fusarium* head blight severity, but no data on deoxynivalenol were given. In initial greenhouse tests (Zhang *et al.*, 2007) with resistance inducers and *Cryptococcus flavescens* OH 182.9, 50–70%

reduction in disease severity was observed. Khan and Doohan (2009) used *Pseudomonas* strains, but obtained only 12% and 21% reduction in deoxynivalenol on wheat and barley, respectively. Palazzini *et al.* (2007) tested a *Brevibacillus* and a *Streptomyces* strain under greenhouse conditions and obtained deoxynivalenol reductions between 32% and 100%. *Clonostachys rosea* (Xu *et al.*, 2008) gave promising results, but was not as efficacious as tebuconazole. In general, based on a large number of field experiments the best fungicides are superior to the tested biological controls (Canty *et al.*, 2004, 2005, 2006; Edwards, 2004).

Resistance to Fungicides

There are relatively few published reports on this important topic. There is a report of isolates of *F. graminearum* and *F. culmorum* that were resistant to thiophanate-methyl (Ishii *et al.*, 2008) and two reports of *F. sporotrichioides* resistant to carbendazim (D'Mello *et al.*, 1998; Klix *et al.*, 2007). In the presence of carbendazim the amount of T-2 toxin increased significantly. None of the available data predict a significant increase in triazole resistance in *Fusarium* spp. similar to that found for strobilurines in the *Septoria* populations.

Management Inputs

Coverage of Heads by Fungicides

Present technologies result in low, uneven, and inconsistent coverage of heads by fungicides (Hooker and Schaafsma, 2004; McMullen *et al.*, 1999a,b). Penetration of the spray into the heads down to the rachis is as important as the coverage of the outer surface of the head (Ruden *et al.*, 2005). The back of the head seldom receives more than 10% coverage, and the front usually receives no more than ~20% coverage with ground spraying (Halley *et al.*, 1999) and only 1–3% coverage with aerial applications (Hooker and Schaafsma, 2004). Thus, poor coverage may be a major cause of the generally poor efficacy observed in many fungicide trials, since a low level of the active agent on the head may keep effective protection from occurring.

We conducted field application experiments (Lehoczki-Krsjak *et al.*, 2008, 2010) with different nozzle types (Figure 16.2). There are differences among fungicides and among nozzle types. The less effective fungicides are more sensitive to nozzle type than are the most effective fungicides. The correlation between general means of visual symptoms from artificial and natural infection tests (large-scale farm trials with different nozzles) is $r = 0.95$ ($p \leq 0.001$), and for deoxynivalenol contamination $r = 0.84$ ($p \leq 0.001$). Thus, field performance of fungicides can be predicted based on microplot tests. Deoxynivalenol contamination was reduced in Prosaro treatments by 95% with artificial inoculation and 70% with natural deoxynivalenol contamination with ground spraying.

Application Time

For susceptible cultivars planted after maize, preventive fungicide control is suggested at flowering. The current fungicides also protect against other diseases, so even if there is no *Fusarium* head blight epidemic, the control of leaf rust and other diseases will be enhanced and the treatment is economically justified. For other conditions, the fungicide application(s) can be made based on disease forecasts.

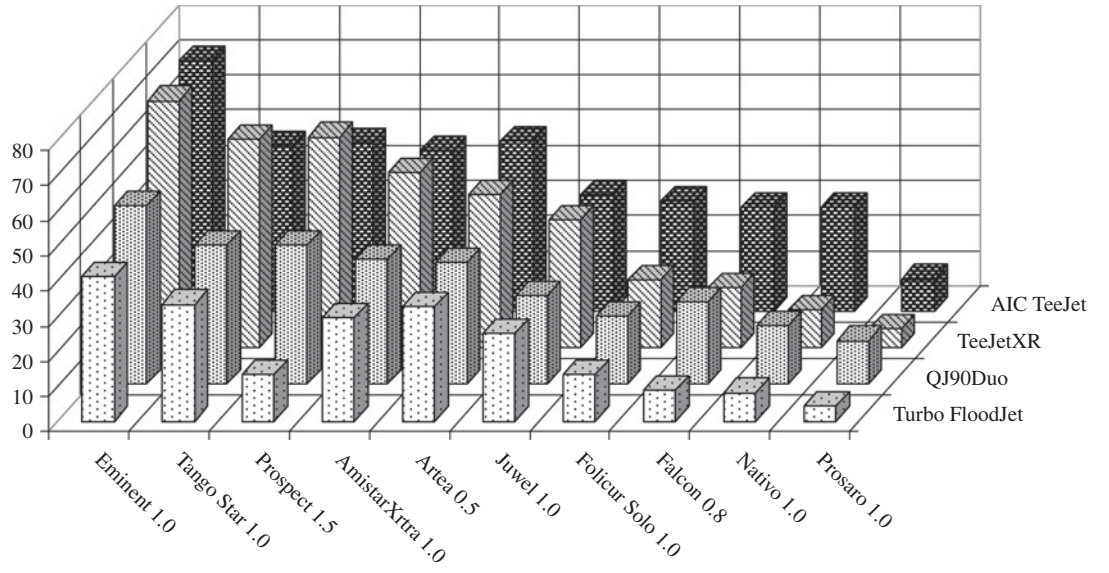

Figure 16.2 Comparison of nozzle types on fungicide protection against *Fusarium* head blight in wheat in 2007 in a large-scale field application, 21 days after application. Data: means across three wheat cultivars and number of infected heads as a percentage of the non-protected control.

Conclusions

Since the first review in 2003, the susceptibility level of the cultivars has not changed much, although new, more resistant cultivars are coming slowly to market. This process is somewhat faster in the United States and Canada than it is in Europe. Thus, long-term chemical control of *Fusarium* head blight remains a necessity. The Chinese found that many very high level resistant lines are not suitable for commercial production, because high *Fusarium* head blight resistance has not generally been combined with other important traits. Consequently, cultivars with medium or slightly better resistance will dominate the market. These cultivars need chemical help in severe epidemic situations, but due to their better resistance should yield more consistent control.

Also since 2003, there is much more data available to confirm that decreased visual symptom severity due to fungicide application is correlated with decreases in the proportion of grain infected, yield loss, and deoxynivalenol contamination. Thus, an effective fungicide not only controls disease symptoms, but also limits toxin contamination.

New fungicides are continually coming to the market. Prothioconazole has the strongest anti-*Fusarium* effect and should be more effective in controlling natural deoxynivalenol contamination. However, prothioconazole's efficacy against brown rust is not outstanding, which has led to its mixture with tebuconazole to provide a product with broad-spectrum control of different diseases. The efficacy of this product is easiest to observe under severe epidemic conditions. Biological control is not yet ready for commercial applications. The development of fungicide-resistant strains has been slow, but several isolates resistant to various fungicides used to control *Fusarium* head blight have been identified. No massive epidemic by fungicide-resistant populations has occurred, suggesting that resistance to fungicides by fungi causing *Fusarium* head blight will not follow the strobilurine-resistant *Septoria tritici* pattern. To minimize the danger posed by resistant strains, a

larger number of stronger fungicides effective against *Fusarium* head blight are needed, so that their use can be rotated and thereby lessen the risk of directional selection within the pathogen population. At this time, prothioconazole, tebuconazole, and metconazole are the best available active agents. I think that the growing market for *Fusarium* head blight fungicides will bring more investment in research and that new fungicides against *Fusarium* head blight will come to market during the next decade.

The identification of poor head coverage as a problem was an important insight. Inadequate spraying technology can ruin *Fusarium* head blight control, even if the best fungicides are used. The microplot method is important as it tests the fungicides at optimal coverage. Farm field applications can be measured for effectiveness against the optimal coverage data obtained from the microplots. Tests with new nozzle types have begun, but much remains to be done. Considering the EU limit for deoxynivalenol is 1.25 mg/kg, 50% efficacy means 2.5 mg/kg in a natural epidemic that could be decreased to 1.25 mg/kg. Epidemics with higher levels of deoxynivalenol contamination cannot now be controlled. If the 90% efficacy observed in the microplots can be achieved in the field, then natural epidemics of up to 10 mg/kg deoxynivalenol probably could be effectively controlled. Epidemics with deoxynivalenol contamination $>$10 mg/kg have little current prospects for control, but such epidemics are relatively rare. Thus, highly susceptible cultivars should probably be taken out of production, as their safe control is not always possible with the existing fungicides and technology (Mielke and Weinert, 1996).

Spraying technology, timing of application, and other management activities remain critical for effective control. Deciding to apply a fungicide goes far beyond the decision to spray. The amount of inoculum, soil cultivation methods, and many traits of the variety influence fungicide efficacy. I think, we should move toward variety- and field-specific technologies as integrated units with the components of the units changing by variety and by field. Even effective fungicides may fail to protect the crop if mistakes are made in application. When the probability of an *Fusarium* head blight epidemic is high, then including a routine preventive fungicide treatment at flowering in the wheat protection management program is advisable.

We are much better positioned to effectively control *Fusarium* head blight in farmers' field than we were in 2003. Many researchers and farmers have contributed to this improvement, and I expect this trend to continue into the future. As more effective methods continue to spread among farmers, the first more resistant cultivars also will appear and will increase even further the safety of our food and feed.

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17 Predicting Mycotoxin Contamination in Wheat

Erick de Wolf and Pierce A. Paul

Abstract

Advanced warning of an elevated risk for mycotoxin contamination in wheat can provide time for the food production system to respond to emerging problems and allow the agricultural industry time to allocate resources to minimize the potential exposure of humans or domesticated animals. During the last decade, at least six models have been proposed for predicting mycotoxins in wheat. All of these models targeted the prediction of deoxynivalenol either indirectly by predicting *Fusarium* head blight incidence or directly by estimating mycotoxin levels. These models share many similarities and represent a conceptual framework indicative of the current understanding of these complex biological systems; however, the development of prediction models is at times limited by the availability of reliable information. As awareness of potential mycotoxin risks increases and quantitative procedures for mycotoxin testing become more affordable, the current *Fusarium* head blight/deoxynivalenol prediction models could be expanded to include additional mycotoxins.

Keywords: computer models; deoxynivalenol; fungicides; *Fusarium* head blight; humidity; surveys; temperature; weather

Introduction

Mycotoxin contamination of wheat is a serious concern throughout the world. During the last decade, several surveys evaluated the incidence and potential risk of mycotoxin contamination in wheat grown for grain (Berthiller *et al.*, 2009; Isebaret *et al.*, 2009; Mashinini and Dutton, 2006; Vogelgsang *et al.*, 2009). These surveys suggest that deoxynivalenol is the most common mycotoxin found in wheat. Most of the deoxynivalenol contamination of wheat is associated with a fungal disease known as *Fusarium* head blight, which is caused by *Fusarium graminearum* and related species. In this chapter we provide a brief overview of international efforts to predict the risk of deoxynivalenol contamination in wheat, review some recent applications of these models as practical decision tools, and discuss some of the challenges related to future model development and use.

Why Try to Predict Mycotoxin Contamination in Wheat?

Mycotoxins are harmful to humans and livestock and once they contaminate grain, there are very few, if any, options for remediation (Grenier *et al*., 2014). Deoxynivalenol, for example, is water

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soluble and heat stable, and as such, can be very persistent during grain processing (Bullerman and Bianchini, 2014; Dexter and Nowicki, 2003), remaining in finished products destined for human and animal consumption. Consequently, most approaches for avoiding deoxynivalenol contamination of grain are preventive and include adjustments to crop rotation or tillage practices (Duveiller *et al.*, 2014; Jacobsen, 2014), fungicide applications (Mesterházy, 2014b), and the selection of varieties to minimize the risk of severe *Fusarium* head blight (Mesterházy, 2014a).

Advanced warning of an elevated risk for mycotoxin contamination in wheat can provide time for the food production system to respond to the emerging problem. Regulatory thresholds for deoxynivalenol in wheat grain range from 0.5 to 5 ppm, depending on the intended use of and market for the grain. For farmers, advance warning of an elevated risk of disease and mycotoxin contamination may provide an opportunity to respond with fungicide applications that can reduce the impact of these problems on their operation (Mesterházy, 2014b). However, the fungicide(s) are most effective when applied at anthesis (opening of the flowers and emergence of anthers), when the host is most susceptible and long before symptoms of *Fusarium* head blight develop or deoxynivalenol accumulates (Mesterházy, 2003). The predictions also may help farmers determine when fungicides are not needed, thereby reducing both production costs and exposure to pesticides. Advance warning of potential mycotoxin contamination also helps the agricultural industry allocate resources for transportation, testing, storage, and cleaning of questionable grain and to minimize exposure of humans or animals to the mycotoxin(s).

Using Visual Estimates of *Fusarium* Head Blight or *Fusarium* Head Blight Prediction Models to Predict Deoxynivalenol Contamination

In general, the same set of environmental-, host-, and pathogen-related factors that favor *Fusarium* head blight development (visual symptom manifestation) also favor deoxynivalenol accumulation (Figure 17.1). These relationships enable the use of models developed to predict epidemics of *Fusarium* head blight also to predict deoxynivalenol accumulation; however, under some conditions, these factors may differentially influence *Fusarium* head blight incidence/severity and deoxynivalenol accumulation (Paul *et al.*, 2005, 2006). For example, on the environment side of the triangle, differences may occur because the temperature and moisture ranges that favor deoxynivalenol production are not exactly the same as those that favor development of *Fusarium* head blight symptoms (Hope *et al.*, 2005; Ramirez *et al.*, 2004) or because weather conditions after anthesis have a greater influence on deoxynivalenol accumulation than they do on *Fusarium* head blight severity (Cowger *et al.*, 2009; Culler *et al.*, 2007; del Ponte *et al.*, 2007; Odenbach, 2009; Yoshida *et al.*, 2007). On the pathogen side of the triangle, differences may be due to differences in toxin-producing abilities and profiles among isolates, species, and populations of fungi that cause *Fusarium* head blight (Gale, 2003). On the host side, varieties with similar levels of resistance to *Fusarium* head blight may have different levels of resistance to deoxynivalenol accumulation (Li, 2009), and weaken the association between *Fusarium* head blight severity and deoxynivalenol accumulation (Paul *et al.*, 2006) and lower the accuracy of estimates of deoxynivalenol accumulation based on disease symptoms.

While *Fusarium* head blight intensity and disease prediction models will continue to play an important role in identifying years and locations with the most serious yield losses and mycotoxin problems, models must now focus directly on deoxynivalenol contamination. This approach bypasses the complex relationships between disease symptoms and mycotoxin levels in the grain and should improve predictions of mycotoxin levels in damaged grain. The accuracy of these models

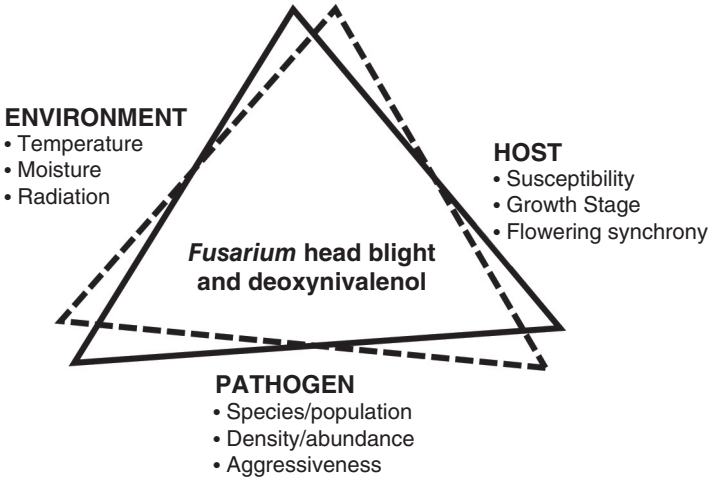

Figure 17.1 Conceptual diagram of the similarities in factors influencing the development of *Fusarium* head blight caused by *Fusarium graminearum* and the resulting contamination of the grain with the mycotoxin deoxynivalenol. Although similar factors result in disease development and mycotoxin accumulation (symbolized by the overlapping triangles), important differences in these complex relationships among host plants, pathogens, and the environment may affect the disease and toxin differentially (offsetting the triangles), making it difficult to reliably predict the risk of deoxynivalenol contamination based on actual estimates of *Fusarium* head blight or with models developed to predict *Fusarium* head blight.

will depend upon the statistical approaches on which they are based and how well the available data represent the full range of factors that influence the final mycotoxin levels.

Comparison of Models Used to Predict *Fusarium* Head Blight and Deoxynivalenol Accumulation

There have been many advances in the development and application of prediction models for mycotoxins in wheat, with at least six models currently available for predicting mycotoxins in wheat (Table 17.1). These models predict deoxynivalenol accumulation either indirectly through the prediction of *Fusarium* head blight incidence and severity or directly by predicting mycotoxin levels. Although the modeling approaches differ, these models have many important similarities. These similarities may result from the available data, but they also suggest a common framework for modeling the biology of these complex systems. For example, most of the current prediction models rely on research that describes the role of temperature and moisture on the reproduction of *Fusarium* species or the infection process (Parry *et al.*, 1995; Shaner, 2003; Xu, 2003). Some of these models also attempt to quantify the impact of weather conditions during grain development. Relatively few models incorporate variables or functions that use information on varietal resistance or cropping practices to help determine the risk of deoxynivalenol accumulation. We now focus on the key components of *Fusarium* biology and how they are represented in the prediction models for deoxynivalenol accumulation.

Various representations of temperature, humidity, and precipitation during anthesis and the early grain-filling stages often are incorporated into prediction models because weather during these times influences reproduction of the fungus, dispersal of spores, and the subsequent infection of the plants (Dufault *et al.*, 2006; Hart *et al.*, 1984; Lacey *et al.*, 1999; Paul *et al.*, 2004; Shaner, 2003; Xu, 2003). For example, a model developed to predict *Fusarium* head blight in the United States uses a variable

Table 17.1 Prediction models recently developed to evaluate the risk of *Fusarium* head blight, caused by *Fusarium graminearum*, or deoxynivalenol

Country[a]	Model description	Reference
Argentina	Predicts *Fusarium* head blight incidence based on rainfall, temperature, and relative humidity during heading	Moschini and Fortugno (1996)
United States	Predicts the risk of an *Fusarium* head blight epidemic based on temperature and relative humidity before and after anthesis	de Wolf *et al.* (2003)
Canada	Predicts deoxynivalenol levels in mature wheat heads based on rainfall and temperature before or after heading	Hooker *et al.* (2002)
Belgium	Assesses the risk of *Fusarium* head blight based on simulated leaf wetness, duration, and temperature	Detrixhe *et al.* (2003)
Italy	Estimates indices of *Fusarium* head blight and toxin risk based on *Fusarium* reproduction, spore dispersal, and infection of wheat as influenced by temperature, relative humidity, and rainfall	Rossi *et al.* (2003)
Brazil	Estimates infection index of *Fusarium* head blight based on wheat growth stage, *Fusarium* inoculum density, and infection frequency as influenced by temperature, rainfall, and relative humidity	del Ponte *et al.* (2007)
Netherlands	Predicts deoxynivalenol based on field location, variety resistance, heading date, fungicide program, temperature, and relative humidity before and after heading	Franz *et al.* (2009)

[a]Country for which the prediction model was originally developed.

that summarizes the hours that the temperature is between 15°C and 30°C and the relative humidity is >90% (de Wolf *et al.*, 2003). This model was later modified to include a broader temperature range (9–30°C) based on research findings that suggested that the fungus could reproduce sexually at cooler temperatures than previously reported (de Wolf *et al.*, 2004; Dufault *et al.*, 2006). Other representations of temperature used to predict deoxynivalenol accumulation include the frequency of days with minimum temperature >10°C and days with maximum temperatures <32°C (Hooker *et al.*, 2002). More complex summaries of temperature also have been proposed. For example, a dynamic simulation model developed by Rossi *et al.* (2003) uses temperature functions derived from experiments done under controlled conditions to simulate fungal sexual reproduction, and infection of wheat to estimate deoxynivalenol accumulation in grain. del Ponte *et al.* (2005) used a similar approach to develop models for use in South America.

Many of the models also contain representations for moisture. Precipitation often is present within the models as the number of days with rainfall greater than some threshold, e.g., >5 mm (Hooker *et al.*, 2002; Moschini *et al.*, 2001), or the duration of rainfall (hours) at critical stages of crop growth (de Wolf *et al.*, 2003). Rainfall by its nature is spatially variable, and as such presents a unique modeling challenge even in regions with relatively dense networks of weather-monitoring equipment. A model developed for use in Belgium, for example, combines ground-based measurements with radar estimates of rainfall to improve the representation of precipitation over large geographic areas (Detrixhe *et al.*, 2003).

Prediction models often quantify available moisture as relative humidity. In many models, relative humidity is a conditional variable for which the amount of time (hours or days) above a predetermined threshold is recorded. Several models consider the duration of time that relative humidity is >90% (de Wolf *et al.*, 2003) or the number of 2-day periods with rain and relative humidity above thresholds that are conducive for the disease (Moschini *et al.*, 2001). As with temperature, the mechanistic approaches to model development used by Rossi *et al.* (2003) and del Ponte *et al.* (2005) allow for more complex representations of atmospheric moisture and their relationship to specific aspects of *Fusarium* biology and the infection cycle.

Application of Prediction Models

The application of *Fusarium* head blight incidence/severity and deoxynivalenol accumulation prediction models as integrated management tools is not as well documented as is the development of the models. The difference between the number of publications addressing model development and the application of the models is well known for many types of pest prediction models or other integrated pest management strategies (de Wolf and Isard, 2007). The reasons for this difference are unclear, but may be attributable to the research interests of the investigators, the complexities of the models, and, in some cases, the proprietary nature of the tools developed for integrated management. Regardless of the rationale, additional effort is clearly needed to improve both the implementation and the documentation of the utility of these tools.

Large-scale deployment of prediction models for *Fusarium* head blight is currently underway in the United States. This application provides daily estimates of disease risk for 30 states where severe outbreaks of *Fusarium* head blight and deoxynivalenol contamination have occurred in recent years (de Wolf and Isard, 2007; de Wolf *et al.*, 2004; McMullen *et al.*, 2012). The predictions are delivered via a web-based prediction tool, the *Fusarium* Head Blight Prediction Center (www.wheatscab.psu.edu). This tool provides information regarding the probability of a severe outbreak of *Fusarium* head blight based on ground-based weather stations and spatially interpolated sources of weather obtained from the National Weather Service in the United States. The weather information is used to produce maps of disease risk (Figure 17.2) with a 5 km spatial resolution throughout the region covered by the prediction system. This prediction effort is the product of a multidisciplinary team of researchers including plant pathologists, meteorologists, geographers, and information technology specialists at Kansas State University, Ohio State University, and Pennsylvania State University.

Models predicting deoxynivalenol accumulation developed by researchers in Canada also have been widely applied as a decision support tool (Pitblado *et al.*, 2007). These models, collectively known as "DONcast," are based on work originally published by Hooker *et al.* (2002) and have been deployed in diverse environments including wheat production regions in North America, South America, and Europe. The prediction tools, managed by Weather Innovations Incorporated (Chatham, ON, Canada), include both map-based regional and site-specific predictions of deoxynivalenol accumulation. The site-specific forecasts improve the model's ability to account for variation resulting from variety susceptibility, crop rotation, and tillage practices and are considered to be more accurate than the regional-scale predictions (Pitblado *et al.*, 2007).

Challenges in Developing Mycotoxin Prediction Models

Many factors influence the severity of *Fusarium* head blight and the amount of deoxynivalenol accumulated. For example, variation in pathogen populations is a potential cause of disparities in the association between *Fusarium* head blight severity and deoxynivalenol accumulation. *Fusarium* head blight is caused by a number of *Fusarium* species, of which *F. graminearum* is the most common. Members of these different pathogen species may be equally capable of causing *Fusarium* head blight, but the type and amount of toxin produced may vary by species and by population (Gale, 2003). Some of this variation could be associated with geographic regions and may result from climatic conditions and cropping practices within the region. It may be possible to address geographic variation in species distributions and pathogen populations by incorporating one or more additional variables describing regional differences into the models. The challenge, however,

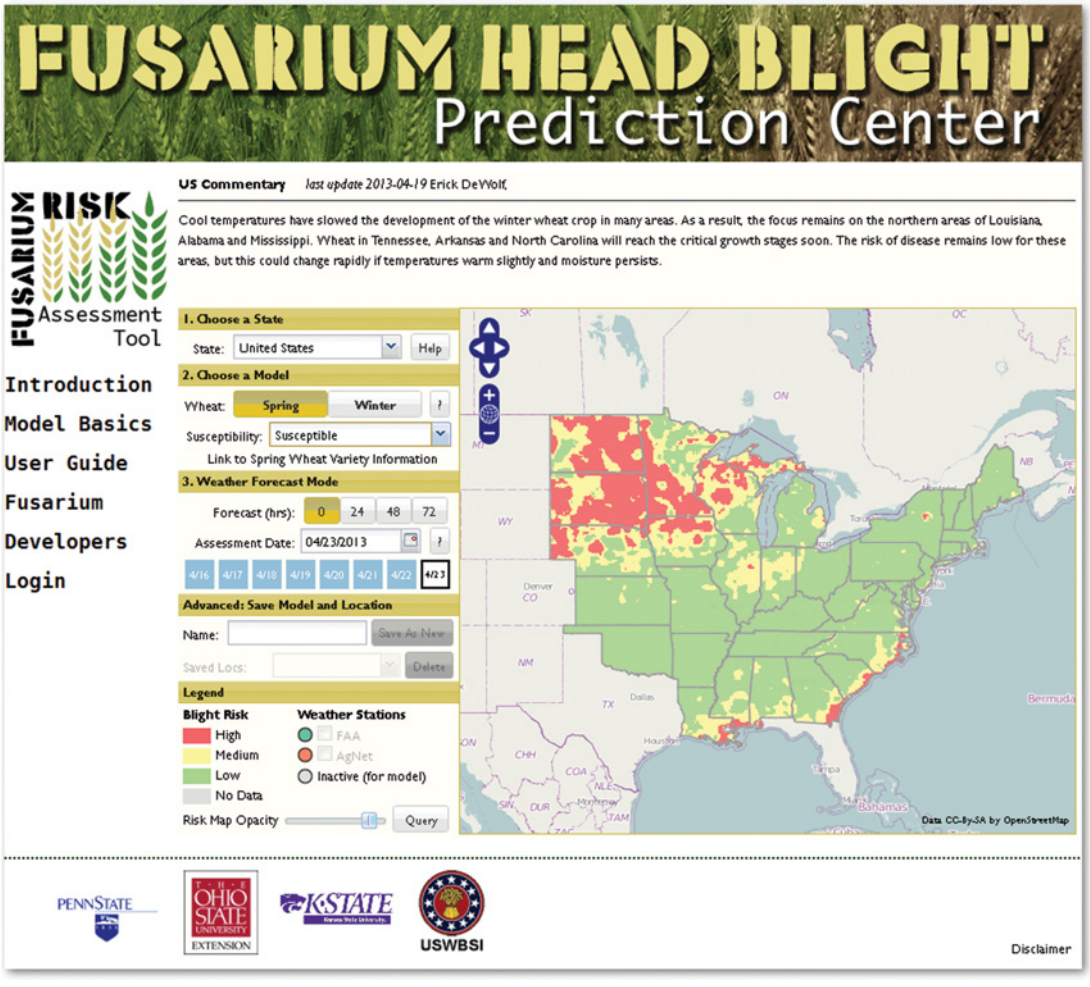

Figure 17.2 Prediction models for *Fusarium* head blight of wheat caused by *Fusarium graminearum* are available for public use via the Internet (www.wheatscab.psu.edu). The user interface displays daily estimates of disease risk for 30 states east of the Rocky Mountains in the United States. The prediction models deployed through the site were developed through the collaboration of researchers at Kansas State University, the Pennsylvania State University, and the Ohio State University. This interface for the model was designed by the Center of Environmental Informatics at the Pennsylvania State University. (For a color version, see the color plate section.)

is to accurately represent the impact of these factors within the models without adding unnecessary complexity. Until these complexities can be addressed more fully, it is likely that differences in pathogen populations may reduce the accuracy of the models in some regions.

Most infection-based disease-forecasting models are developed based on the assumption that inoculum is not limiting, and they focus on weather conditions favorable for infection and subsequent disease development (de Wolf and Isard, 2007). This assumption underlies all *Fusarium* head blight/deoxynivalenol models except that of del Ponte *et al.* (2005). In general, *Fusarium* head blight risk assessment models have not incorporated representations of pathogen population, inoculum

density, and fungal biomass as predictors of disease or toxin accumulation. This omission is because it often is extremely difficult to monitor and quantify these factors in a manner amenable for model implementation. There are functional relationships among *Fusarium* head blight severity, spore density, deoxynivalenol accumulation, and fungal biomass (Li, 2009; Odenbach, 2009; Stein *et al.*, 2009), suggesting that accounting for these relationships could potentially improve the accuracy of *Fusarium* head blight prediction models.

While it is clear that quantifying inoculum density and fungal biomass may not be feasible from a model application standpoint, mathematical models can be used to generate functions of inoculum density within the wheat canopy for *Fusarium* head blight severity/deoxynivalenol accumulation prediction purposes. For example, one set of models estimates the density of *F. graminearum* inoculum on wheat spikes as a function of weather variables (Paul *et al.*, 2007). Similar efforts are needed to formally model fungal biomass of the grain as influenced by host variety and environmental factors. Recent advances in the use of molecular tools to study the epidemiology of *Fusarium* head blight should be invaluable for this purpose (Nicholson *et al.*, 2003). In particular, rt-PCR-based techniques can be used to characterize conditions under which grain colonization and toxin accumulation may occur in the absence of visual symptoms. With further advances in these areas, modules ultimately could be developed and integrated into existing *Fusarium* head blight severity/deoxynivalenol accumulation models to more directly account for pathogen effects, the component of the disease triangle that is missing most often from the current forecasting models.

Challenges in the Application of Mycotoxin Prediction Models

Most disease prediction models are dependent on weather variables. In the application of these models, weather variables often are spatially interpolated to provide estimates of disease severity or mycotoxin contamination risk. Nearly all weather-monitoring networks introduce some uncertainty into model predictions. This uncertainty is compounded further if the mycotoxin prediction models incorporate outputs from meteorological prediction models. Although progress is being made in expanding weather networks and developing technologies for remotely sensing weather, the challenge of identifying consistent, reliable sources of weather information remains and will increase if funding for these weather-monitoring systems is neither stabilized nor increased.

The validation of mycotoxin prediction models within a country, state, or specific wheat production region also is a significant challenge. Plant disease specialists often conduct limited surveys of production fields to monitor symptoms of *Fusarium* head blight during the final stages of grain development. These visual assessments provide useful information, but mycotoxin accumulation within the harvested grain can vary significantly as the toxins usually are found in "hot spots" within the sample and are not uniformly distributed across an entire grain lot. Harvested grain often is tested at grain terminals or other points of delivery for mycotoxin contamination levels. Unfortunately, this information is rarely linked to the grain's specific geographic origin and, at best, provides only an estimate of regional problems.

Enhancing routine mycotoxin surveys to more effectively monitor potential problems, and to collect data that can be used for model validation, is an important goal. Such surveys might include approaches that encourage farmers to provide grain samples from previously identified fields at known geographic locations with well-described cropping practices. Unfortunately, the cost of implementing and sustaining this type of survey may be prohibitive. Sustainability problems for such surveys are magnified by the sporadic nature of the disease outbreaks and associated mycotoxin problems, as a survey could go for several years without detecting a major outbreak of

mycotoxin-related problems. Protocols that involve consistent low-level monitoring of grain until a potential mycotoxin problem is identified may be the most effective. If a potential problem was identified, then more intense sampling would begin to accurately delimit the geographic regions affected. Once validated, mycotoxin prediction models could direct survey efforts and further improve sampling protocol efficiency.

The Future of Modeling Mycotoxins in Wheat

The development of prediction models can be limited by the availability of reliable information. Many of the earliest models for *Fusarium* head blight severity and deoxynivalenol accumulation were based on historical observations of disease and associated weather records (de Wolf *et al.*, 2003; Moschini *et al.*, 2001). In most cases, these historical data sets do not contain quantitative estimates of deoxynivalenol accumulation, making it impossible to directly model mycotoxin accumulation. As awareness of the potential risk posed by deoxynivalenol increases and procedures for quantifying mycotoxin accumulation become more routine and less expensive, the modeling objectives have expanded to incorporate the new information. This cycle of awareness, data collection, and modeling continues today. As this cycle continues, mycotoxin prediction models probably will be expanded to include additional mycotoxins such as nivalenol and glucosylated ("hidden") forms of deoxynivalenol, which also may be present in wheat.

Conclusions

Modeling *Fusarium* head blight occurrence and mycotoxin contamination in wheat is an active area of research in many parts of the world, and there will undoubtedly be more models developed to assess these problems. These models will probably draw on the fundamental concepts common to the current prediction models. New models will be able to incorporate important sources of variation related to varietal resistance, cropping practices, diversity within and between pathogen populations, and the aerial movement of fungus across a production region to increase the accuracy of their predictions. Incorporating this information could further enhance the utility of these models as tools for disease management and help ensure a healthy global food supply.

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18 Good Postharvest Storage Practices for Wheat Grain

Naresh Magan, David Aldred, and Esther S. Baxter

Abstract

The safe storage of wheat grain is determined by the interactions between the moisture content of the grain at harvest, the drying efficiency, and the temperature when the grain is placed into storage. If wheat is dried to 14.5% moisture content, then short- and medium-term storage can be ensured. Poor postharvest management of stored grain resulting from poor silo structural quality, ingress of water, or the failure to exclude insect pests can result in ochratoxin A contamination and, under wetter conditions, increased deoxynivalenol contamination as well. An understanding of the ecology of the *Fusarium* and *Penicillium* species that produce these mycotoxins was combined with environmental controls to develop guidelines for storage conditions and to identify conditions that have lower and higher risks of mycotoxin contamination.

Keywords: deoxynivalenol; drying; HAACP; insects; moisture; ochratoxin A; sampling; silos; temperature; zearalenone

Introduction

Mycotoxigenic spoilage fungi can contaminate cereal commodities both pre- and postharvest, and mycotoxins represent a significant hazard to consumer health. Thus, these compounds are receiving increasing attention from food safety authorities, legislators, and scientists. In the wheat grain chain, the mycotoxins of greatest importance are the trichothecenes, especially deoxynivalenol, which is produced by numerous *Fusarium* species, and ochratoxin A, which is produced by *Penicillium verrucosum*. There also is interest in contamination by zearalenone, which usually is produced by *Fusarium graminearum* or *Fusarium culmorum*, and by T-2 and HT-2 toxins, which usually are produced by *Fusarium langsethiae*, a fungus that is most commonly problematic on oats and rye. Legislative limits for combined T2 + HT-2 toxins are being considered by the European Union and have been finalized in some countries, e.g., Norway (Mylona and Magan, 2011).

In Europe, the management of wheat grain follows a HACCP approach. Much of the current knowledge is based on the identification of the critical control points (CCPs) in the wheat chain, both pre- and postharvest. The European Union has recommendations to prevent contamination of cereals and cereal products with *Fusarium* toxins (Commission Recommendation 2006/583/EC). We focus on the risks that occur between harvest and effective short- and medium-term storage in wheat in relation to deoxynivalenol and ochratoxin A (Magan *et al.*, 2008, 2010; Seitz *et al.*, 1982).

Mycotoxin Reduction in Grain Chains, First Edition. Edited by John F. Leslie and Antonio F. Logrieco.

We considered the ecology of the fungi relative to growth and toxin accumulation, the environmental conditions at harvest, and the management of the drying and storage system. The most critical factors are the original moisture content and temperature at harvest, as they directly influence events that may occur during storage and may result in spoilage, self-heating, and increased mycotoxin contamination. Grain is a living material and its respiration is very low when stored at the proper temperature and moisture content (<14.5%). Increases in moisture content or temperature also increase respiration and the available water, which encourages spoilage. Thus, management of harvesting/drying and the subsequent storage phase is critical whenever effective grain management is required.

Moisture Sorption Curves

The relationship between the measured moisture content and the equilibrium relative humidity (= water activity, a_w) varies (Figure 18.1) with temperature and grain type and is an important variable in determining the range of environmental conditions conducive to fungal growth (Pixton, 1967). If the storage temperature is lowered, then the safe moisture content can be increased slightly, e.g., at 10°C; 0.70 a_w corresponds to ~15% moisture content. The respiration of spoilage and mycotoxigenic fungi is much lower at lower temperatures, which lengthens the safe storage time (Channaiah and Maier, 2014).

Risks of Deoxynivalenol Contamination during Grain Storage

At harvest, wheat grain enters storage containing a variety of potential spoilage and toxigenic organisms. The population on the grain depends largely on the field conditions and the harvesting process, and changes during storage. Sometimes grain is kept for short periods of time on farm in buffer storage before drying. This process can result in conditions conducive to growth of *Fusarium* and possibly increase deoxynivalenol contamination, which usually is produced preharvest. Thus,

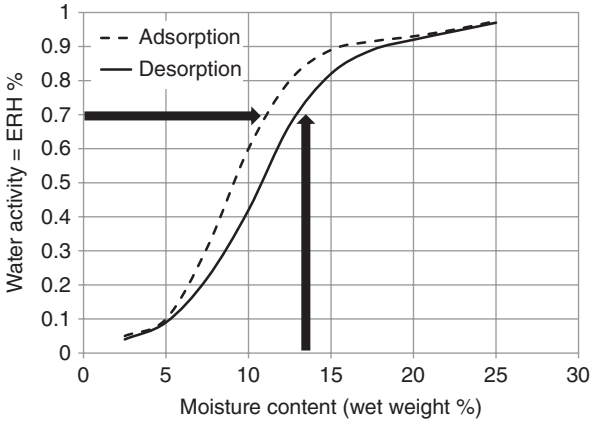

Figure 18.1 Moisture sorption curve for wheat grain relating the adsorption and desorption curves to moisture content (%, wet weight basis). The adsorption lines indicate the effect of wetting grain and the desorption lines indicate the effect of wet grain being dried. The difference is due to the relative binding of water to the grain components during these phases and is called the "hysteresis effect." These curves change with storage temperature. In general, spoilage problems normally would not occur below the conditions indicated by the arrow (~14.5% moisture content = 0.70 a_w).

poor postharvest management can result in rapid quality loss and increase the risk for mycotoxin contamination. This problem is particularly acute in wet harvest years. As both toxins are very heat stable, once they are formed, they are difficult to destroy, even if the fungi involved are no longer viable or growing. Thus, traceability and quality criteria are critical practical measures.

Preharvest assessment of the crop for the presence of diseases, e.g., *Fusarium* head blight, is important. If the disease is present, then separating visibly diseased material from healthy grain may reduce toxin accumulation as deoxynivalenol is more common in the diseased material. Deoxynivalenol usually is a preharvest problem, but improper storage may increase the contamination level. Often shriveled, *Fusarium*-infected grains are lost in the field during the harvesting process, which reduces the apparent contamination. Thus, harvest is the first key CCP in the wheat chain where moisture management becomes the dominant control measure to minimize or avoid increases in deoxynivalenol contamination. Wheat often is harvested at moisture levels >14.5% and usually is traded on a wet weight basis. There are also technological challenges associated with bulk drying and storage of grain, in addition to cases of poor practice and negligence. Thus, there is a significant risk for mycotoxin contamination during grain production, and this risk may increase during postharvest handling and processing.

Information on the ecology of *Fusarium* species involved in *Fusarium* head blight and deoxynivalenol contamination of wheat already is available (Hope and Magan, 2003; Hope *et al.*, 2005; Sanchis and Magan, 2004). The minimum conditions for spore germination, hyphal growth, and deoxynivalenol production also are known (Figure 18.2). The moisture content × temperature profiles for fungal growth are wider than those for deoxynivalenol production. Thus, only under relatively wet conditions, i.e., >20% moisture content, is there a significant risk of deoxynivalenol production.

Moisture management requires prompt, accurate measuring methods and bulk drying, as necessary. Heated air drying is best for drying grain to the target moisture content of 14.5%, if the grain will be used for food or feed, but not for seed. When ambient temperature drying is used, there is a higher risk of mycotoxin contamination because the process depends upon the outside temperature and humidity. The drying front also moves slowly upward through the grain, often overdrying the bottom layers while remoistening the top layers. This moisture distribution is conducive to further toxin biosynthesis in portions of the stored grain. At the margins for growth of *F. graminearum* a dry matter loss of 0.8–0.9% is the threshold. Higher dry matter losses usually lead to deoxynivalenol contamination levels above the EU legislative limits (Magan *et al.*, 2010; Mylona *et al.*, 2012). Insect pests also must be effectively controlled, as they can survive a wider range of humidity conditions and can produce metabolic water, which may result in hot spots in stored grain masses.

Risks from Ochratoxin Contamination of Wheat Grain

The cool damp conditions found in Scandinavia are conducive for contamination by *P. verrucosum* and the production of ochratoxin A. This contamination usually occurs during harvest and storage. Minimizing the delay between harvesting and drying reduces colonization of pockets of wet grain by *P. verrucosum* and subsequent ochratoxin A synthesis. *Penicillium verrucosum* was the main ochratoxin A producer in several national investigations of cereal grains in northern Europe (Magan *et al.*, 2008).

In contrast to the *Fusarium* species, the minimal conditions for growth of and ochratoxin A production by *P. verrucosum* are very similar, and almost any growth has the potential to produce

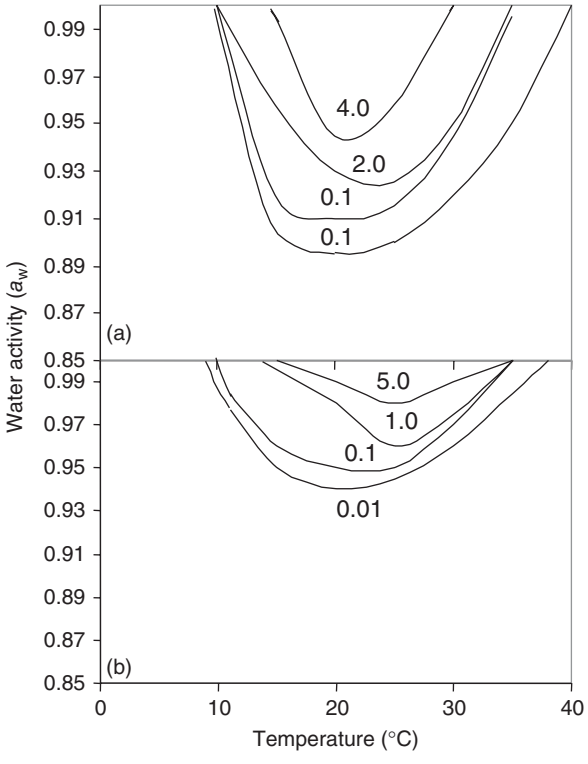

Figure 18.2 Comparison of profiles for (a) growth (mm/day) and (b) deoxynivalenol (μg/g) production by *Fusarium graminearum* on wheat grain (after Magan *et al.*, 2006).

toxin (Figure 18.3; Cairns-Fuller *et al.*, 2005). Previous work by Northolt *et al.* (1979) showed that a moisture content of 16–17% suffices for the growth of *P. verrucosum*, with a 1% higher moisture content sufficient for ochratoxin A production. Thus, an understanding of the ecology of these mycotoxigenic species is required to institute practical control measures.

The on-farm occurrence of ochratoxin A in grain usually can be attributed to insufficient drying or to excessively long pre-drying storage. Studies of the safe storage period for cereal grain are few (Channaiah and Maier, 2014), and those available are based on visible molding (Kreyger, 1972), dry matter loss (Steele *et al.*, 1969; White *et al.*, 1982), or reduced seed germination (Kreyger, 1972; White *et al.*, 1982), all of which are measures of fungal growth and not measures of mycotoxin production. This gap was filled by Mylona *et al.* (2012) who modeled the relationship between dry matter losses and mycotoxin production and found that for deoxynivalenol in wheat any loss >0.9% resulted in contamination exceeding the EU legislative limits. Thus, the margins for error are very small in postharvest storage management.

Grain in storage is an effective thermal insulator. The periphery of the grain mass changes temperature faster than does the less exposed grain in the center of the grain mass, with this tendency exaggerated for grain masses >50 tons, which fail to cool or warm uniformly during seasonal thermal changes (Foster and Tuite, 1992). The resulting temperature gradient causes moisture to move from warmer to colder parts of the grain bulk. Small grains, e.g., wheat, offer

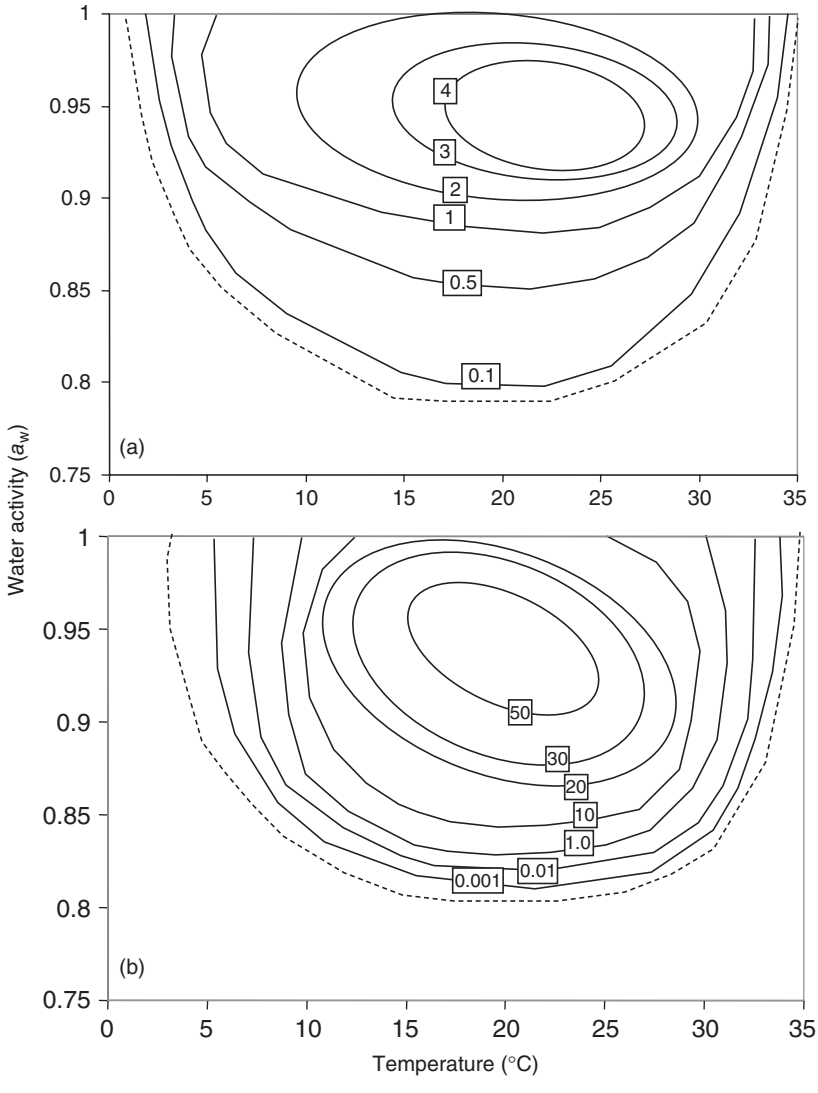

Figure 18.3 Profiles for (a) relative growth rates (mm/day) and (b) ochratoxin A (μg/g) production by *Penicillium verrucosum* on wheat grain. The lines are isopleths at which similar amounts of growth or ochratoxin A production occur (after Cairns-Fuller *et al.*, 2005).

more resistance to air movement within the grain mass, which makes maintaining moisture content at a level safe for storage even more important than it is for larger grains, e.g., maize. Drying and maintaining grain under "safe" storage conditions should reduce or eliminate the risk of ochratoxin A contamination. In practice, however, maintaining these conditions often is difficult, and routine surveys continue to detect contaminated grain and cereal food products, e.g., Wolff (2000).

The growth of *P. verrucosum* and the production of ochratoxin A in wheat grain has been quantified and modeled relative to the maximum permissible European Union levels of 5 μg/kg in

raw cereals (Lindblad *et al.*, 2004). This effort enabled the establishment of practical guides based on the concentration of spores relative to the likelihood of exceeding the established limits and an evaluation of the effect of moisture content and temperature on the length of safe storage, i.e., periods free of ochratoxin A.

In many surveys of stored cereals in Europe, e.g., Puntarić *et al.* (2001) and Wolff (2000), samples sometimes exceeded the 5 μg/kg level. Even if the percentage of samples contaminated at the statutory limits was as low as 3%, it would still represent a large tonnage of grain (∼6 million tons) and a potentially serious economic loss. In monetary terms, the loss would be €800–1000 million, assuming that no alternative use for the grain is available. In addition, the high cost of the monitoring programs, i.e., ∼€0.3 and 100 million for official and internal controls, respectively, that prevent contaminated grain from entering the food chain also should be included.

Sampling Issues

Obtaining a representative sample to assess postharvest mycotoxin contamination is difficult, with the error associated with obtaining a representative sample responsible for 25–60% of the total error (Whitaker, 2004; Whitaker *et al.*, 2000). The European Union has specific sampling requirements for different sized lots of wheat and related commodities. For *Fusarium* toxins in cereals there is a specified European Union sampling plan (Commission Directive 2005/38/EC) to control *Fusarium* in foodstuffs. For individual lots traded as packs, sacks or bags

$$\text{Sampling frequency } (n) = (W_I / W_A) \times (W_L / W_P),$$

where W_I is the weight of the incremental sample (∼100 g); W_L is the weight of the lot; W_A is the weight of the aggregate sample (1–10 kg); and W_P is the weight of an individual container. For larger lots, the number of incremental samples to be taken is determined by the weight (tons) <50, 50–300, 300–1500, and >1500. For samples >50 tons, ∼100 incremental samples are taken and an aggregate sample of ∼10 kg is used.

Sampling is a practical problem for the industry. A common question is whether a regular grid-type sampling approach or a random sampling approach should be used and whether the choice is dependent on the mycotoxin of interest. Some mycotoxins are distributed homogenously throughout a lot, but more commonly the toxins are heterogeneously distributed either in pockets or randomly throughout the sample.

Geostatistics are now being used to analyze *in situ* mycotoxin distribution in three dimensions on almost any scale. Rivas-Casado *et al.* (2009a,b,c) suggested that some mycotoxins, e.g., deoxynivalenol, have a spatial structure in stored grain and that a regular grid may be preferable to a random sampling approach in such cases. Further work is needed to enable accurate representative samples to be acquired from throughout the cereal food chain.

Another area that has received much attention is the development of simple, rapid diagnostics for mycotoxins such as deoxynivalenol and ochratoxin A. These diagnostics are only as effective as the samples they are used on. There are a number of diagnostics kits now available for field use that are based on lateral flow technology and calibrated to test regulatory limits. These tests should be coupled with accurate moisture and temperature sensors to monitor stored grain and to improve the management of the wheat food chain, especially in the postharvest phases.

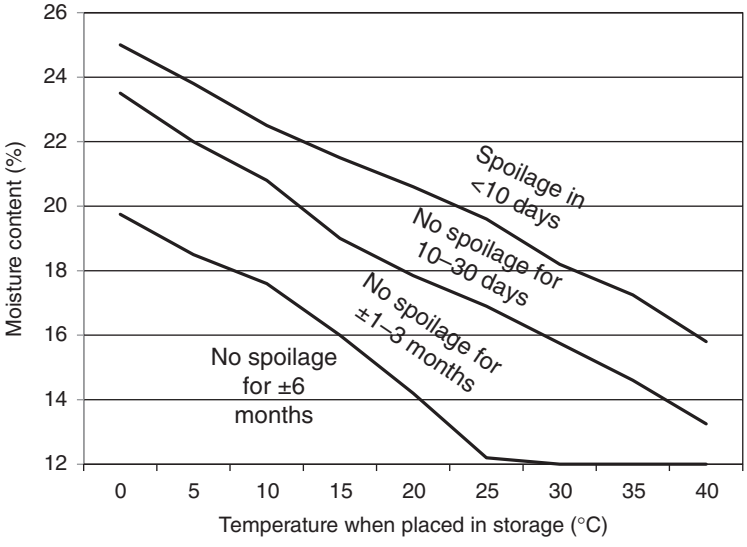

Figure 18.4 The relationship between temperature and moisture content of grain and the length of time for which the grain can be stored.

Discussion and Conclusions

Harvested wheat grain may pass through the hands of a number of "owners" on its way to the primary processor. In the simplest case it is stored on-farm for short periods of time before being moved directly to a processing facility. In other cases the grain may pass through the hands of merchants and/or third-party grain-drying facilities, if it was harvested wet and on-farm drying facilities were not available. In these latter cases, the grain will have been stored at more than one geographic location with transportation steps separating the storage locations. During any of these stages the grain could become susceptible to fungal spoilage if the storage conditions are not strictly controlled. In most cases the key to adequate storage is drying the freshly harvested material to 14–14.5% moisture content and maintaining the grain in this condition. In general, the cooler and drier the grain, the longer it can be safely stored (Figure 18.4; Channaiah and Maier, 2014; Magan and Aldred, 2007; Magan *et al.*, 2010; Wallace *et al.*, 1983).

The most important control measures relevant to storage are:

- Usage of correct combine settings, in particular fan speed, to reduce contamination of harvested grain with *Fusarium*-damaged grain and chaff.
- Harvesting and storing separately weathered or lodged crops, which may have significantly higher levels of deoxynivalenol.
- Cleaning the grain postharvest to reduce the number of *Fusarium*-diseased kernels.
- Taking regular, accurate moisture measurements.
- Promptly and efficiently drying wet grain, and monitoring the holding time/temperature prior to drying as well as the actual drying conditions.
- Rapid drying to 14.5–15% moisture content to reduce risk of postharvest deoxynivalenol biosynthesis.

- Adequate infrastructure, including provision for grain segregation and appropriate transportation.
- Appropriate storage conditions, in terms of moisture and temperature control, and proper maintenance of facilities to prevent pest and water ingress.
- Ability to efficiently identify and reject material below standard in terms of fungal disease and/or mycotoxin levels, e.g., when transferring material to a third party.
- Hygiene to prevent insect contamination or survival in storage, where they can produce metabolic water that results in pockets of spoilage/mycotoxin contamination.
- Reduce insect infestation to reduce grain damage (facilitates fungal infection and mycotoxin production) and to prevent movement of fungal spores carried by insects.
- Management of approved suppliers and specifications for acceptance/rejection.

Based on the available scientific information on the ecology of *F. graminearum* and related *Fusarium* species and deoxynivalenol production, and that of *P. verrucosum* and ochratoxin A production, we generated a simple moisture content/water availability curve. This curve identifies conditions when the contamination risk is high and when it is marginal, i.e., the "zone of uncertainty." Grain lots in the "zone of uncertainty" require more detailed evaluations because of the higher potential contamination with mycotoxins (Figure 18.5).

In summary, there are a number of CCPs that must be managed in the cereal grain chain to ensure that contamination with mycotoxins, such as deoxynivalenol and ochratoxin A, is minimized.

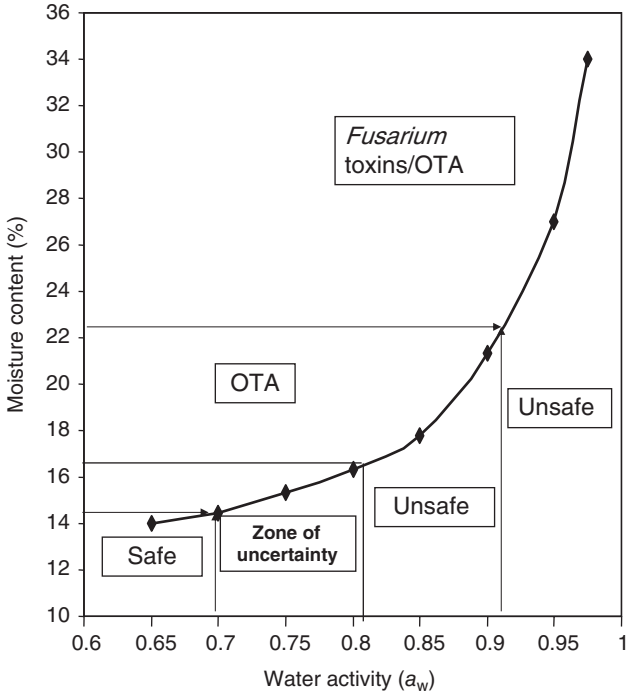

Figure 18.5 Ranges for deoxynivalenol (DON) and ochratoxin A (OTA) contamination and the zone of "uncertainty" where conducive conditions exist for increased contamination by ochratoxin A.

Prevention is better than cure, so it is important to follow good agricultural practices as much as possible in the pre- and postharvest components of the chain to maintain grain quality and reduce the need for intervention in storage. Technologies such as remote real-time sensing of environmental conditions during drying and storage also help minimize the contamination of food and feed grains.

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Part III
Other Grain Chains

19 Good Food and Feed Processing Techniques

Assunta Raiola and Alberto Ritieni

Abstract

The natural occurrence of mycotoxins in foodstuffs intended for human consumption is considered a severe risk to the health of consumers by the European Community and by many other governmental agencies. Many studies have focused on field-level reduction of mycotoxins, particularly aflatoxins, trichothecenes, fumonisins, and ochratoxin A. The purpose of this chapter is to highlight the main industrial detoxification processes of mycotoxins in various foodstuffs, with particular attention to the processes in wheat. After harvest, wheat is subjected to many technological processes in which temperature is the main parameter. Various temperature and moisture conditions are used in food processing and cooking. The main mycotoxins present in wheat are aflatoxin, ochratoxin, deoxynivalenol, and zearalenone. In the literature there also are reports about the presence of fumonisins and moniliformin, but there is little or no data on their detoxification.

Keywords: aflatoxins; cooking; deoxynivalenol; fermentation; fumonisins; industrial detoxification; maize; moniliformin; ochratoxin A; patulin; rice; roasting; temperature; wheat; zearalenone

Introduction

Mycotoxins are secondary metabolites produced by fungi under specific humidity and temperature conditions. The most important mycotoxin-producing fungi belong to the fungal genera *Fusarium*, *Penicillium*, and *Aspergillus*. The United Nations Food and Agricultural Organization (FAO) estimates that up to 25% of the world's grain crops are significantly contaminated by mycotoxins (Dowling, 1997). The most important mycotoxin families are aflatoxins, ochratoxins, fumonisins, trichothecenes, and zearalenone, with less prominent mycotoxins, including patulin, ergot alkaloids, beauvericin, citrinin, and moniliformin, also important in some cases. Mycotoxins are commonly produced and accumulate in the field and during the postharvest portion of the food chain. Postharvest contamination depends on storage conditions, as even excellent quality field material may be contaminated with mycotoxins if it is stored improperly.

The thermal processes used in cooking have significantly increased food safety, in general. Unfortunately, most mycotoxins are resistant to heat treatments of 80–120°C and can persist through the heat treatments commonly associated with most cooking processes. Other factors that influence the stability of mycotoxins include pH, ionic content, water content, temperature, heating time, and

Mycotoxin Reduction in Grain Chains, First Edition. Edited by John F. Leslie and Antonio F. Logrieco.

heating rate. The remainder of this chapter is organized by the mycotoxin of interest, with relevant processing techniques included under more than one mycotoxin heading, if appropriate.

Aflatoxins

Members of the aflatoxin family are very thermostable. The temperature must reach at least 237°C for them to decompose completely although decomposition may begin at temperatures as low as 150°C (Rustom, 1997).

Wheat

Aflatoxin-producing fungi are ubiquitous and may grow in cereals such as wheat. The reduction of aflatoxin B_1 toxicity in wheat is directly proportional to washing time, but the concentration of aflatoxin B_1 is reduced more by heating than by washing. The level of aflatoxin B_1 in dried wheat decreases by 50% and 90%, respectively, following heating at 150°C and 200°C (Hwang and Lee, 2006).

The reduction of aflatoxin B_1 was increased by 8% and 23% following heat treatment of 10% water-added US wheat (soft red/white wheat) and Korean wheat (Anbaekmil) compared to dried US and Korean wheat, respectively. Traditional processing used in Korean foods such as Sujebi (a soup with wheat flakes) and steamed bread caused 71% and 43% decrease in aflatoxin B_1 content (Hwang and Lee, 2006). In the process of baking, a temperature of ~100°C is reached in the center of the bread. During the preparation of whole wheat bread, most of the aflatoxin B_1 that is lost is lost going from meal to dough and not during baking. The relatively low temperature for baking and the low amounts of volatile acids in the bread itself do not suffice to degrade aflatoxin B_1 to any great extent (Reiss, 1978).

Maize

Aflatoxin may be reduced by up to 92% during preparation of the Mexican drink atole by simple heating at 94°C for 10 minutes and then drying at 40°C for 48 hours. During preparation of corn flour (pinole), more aflatoxin is lost during the toasting portion of the process than during the boiling portion (Méndez-Albores *et al.*, 2004). In the process of making corn flakes, aflatoxins are reduced by 64–67% (Lu *et al.*, 1997).

Rice

Fungi that produce aflatoxin also can colonize rice. If rice is cooked with normal or excess water, then up to 89% of the lactone rings in the aflatoxin molecules can be hydrolyzed to the corresponding carboxylic acid. Parboiling of rice occurs when hulled rice is immersed in water at temperatures >58°C, followed by a partial gelatinization of the starch. During the parboiling, the water migrates into the inner layers of the grain that carry water-soluble compounds. In parboiled rice, aflatoxin B_1 contaminated 9% of the samples evaluated at 11–74 ng/g (Dors *et al.*, 2011). From 2.2% to 4.4% of the aflatoxin in a sample may migrate into the grain during parboiling (Coelho *et al.*, 1999). Up to 82% of the aflatoxin in rice naturally contaminated at 17 ng/g could be removed by increasing the

soaking time in the process from 4 to 6 hours (Dors *et al.*, 2009). Aflatoxins in white rice are very stable up to 50 kGy of radiation, and doses in excess of these levels are needed to destroy the toxin (Simionato and de Sylos, 2004).

Nuts

The presence of aflatoxins in matrices such as coffee and peanuts, requires evaluating the roasting process as a part of the potential detoxification process. Roasting pistachios at 90°C, 120°C, and 150°C for 30–60 and 120 minutes, reduced the aflatoxin contamination by 17% to 63% (Yazdanpanah *et al.*, 2005). The presence of NaCl at 50 g/kg during the pistachio roasting process reduces the aflatoxin level by 48% (Ozkarsli, 2003). The available moisture (5.6%) in powdered pine nuts generally is low for the temperature used during the roasting process, and natural aflatoxin contamination of 120–575 ng/g can be reduced by up to 81% during processing. Extrusion of peanuts in the absence and presence of 2–2.5% NH_4OH reduced aflatoxin contamination by 23–66% in the absence of the NH_4OH and by up to 87% when the NH_4OH was present (Cheftel, 1989).

Milk

Pasteurization of milk at 62°C for 30 minutes reduced aflatoxin M_1 contamination by 32%. In a different type of heating, aflatoxin M_1 in milk decreased by 12% and 35% (Ozkarsli, 2003).

Beer

Aflatoxin in beer is sensitive to the steps in the process where malt protein is hydrolyzed (27% reduction), boiled (30% reduction), and fermented (30% reduction). Cooking of the muffin reduces the aflatoxin content by up to 87% (Chu *et al.*, 1975).

Barley

Increasing radiation of barley from 0 to 4 kGy is correlated with a lower level of aflatoxin formation (Chang and Markasis, 1982).

In conclusion, the aflatoxin family of mycotoxins is very stable to heat treatment and technology. The reduction in the level of aflatoxin B_1 is related directly to the temperature or duration of the treatment of the contaminated food. The presence of a basic environment favors increased hydrolysis of aflatoxin, especially if the moisture level of the product is not particularly low.

Ochratoxin A

The ochratoxin family includes numerous molecules with various levels of toxigenicity. The most important member of this family is ochratoxin A, which has a melting point of 169°C. Ochratoxin A's insensitivity to thermal processes enables it to persist through the processing of products such as coffee, cocoa, wine, beer, and cereals.

γ-Radiation reduces ochratoxin A production by *Aspergillus ochraceus* (Refai *et al.*, 1996). In poultry feed, fungal growth ceases at 4 kGy of radiation. Ochratoxin A production ceases at 3 kGy and is reduced from 60 to 1.9 ng/g after exposure to 2 kGy. γ-Radiation in doses of 15 and 20 kGy, respectively, completely destroys ochratoxin A in yellow maize and soybeans. In feed for poultry layers, broiler's feed concentrates, and cottonseed cake, however, the detoxification rates are 40%, 47%, and 36%, respectively, with a radiation dose of 20 kGy (Refai *et al.*, 1996). Pure ochratoxin is stable even to 75 kGy (Paster *et al.*, 1985).

Wheat

Ochratoxin A in wheat at 100°C dry heat is not degraded after 40–160 minutes of treatment, but >50% of the toxin was destroyed after 120 minutes of treatment if wet heat was used instead of dry heat (Boudra *et al.*, 1995). Ochratoxin A is stable during the baking of bread, but during biscuit preparation two-thirds of the ochratoxin A is either destroyed or immobilized (Subirade, 1996).

Coffee

Roasting coffee at 200°C for 10–20 minutes reduces the amount of ochratoxin A present between 12% and 100% (Blanc *et al.*, 1998; Suàrez-Quiroz *et al.*, 2005).

Liquorice

Heat treatment of liquorice at 150°C for 60 minutes (Ariňo *et al.*, 2007) does not reduce the ochratoxin A level.

Common Beans

Pressure cooking common beans, variety Carioca, in water results in >84% loss of ochratoxin A (Milanez and Leitao, 1996).

Rice

In parboiled rice, ochratoxin A contaminated 13% of the samples evaluated at between 13 and 26 ng/g (Dors *et al.*, 2011). Cooking rice in excess water reduces ochratoxin A levels by 86%, whereas normal cooking results in a loss of 83%, and cooking to dryness results in a loss of 86–88% of the ochratoxin A present (Simionato and de Sylos, 2004; Trenk *et al.*, 1971).

Oats

Autoclaving oatmeal with 50% of the normal amount of water reduces the ochratoxin A present by 74%, while autoclaving oatmeal or rice to dryness results in a loss of 86–88% of the ochratoxin A present (Trenk *et al.*, 1971).

Ochratoxin A levels also are reduced during the production of cereals due to extrusion cooking by up to 40% (Castells *et al.*, 2005). The loss of ochratoxin A during extrusion is directly proportional to the temperature and the humidity (Scudamore *et al.*, 2004). When the moisture content is 30% and temperature is between 116 and 120°C the ochratoxin A reduction is 12% while at a temperature of 113–136°C the reduction in ochratoxin A level is 24%. With the moisture level at 17.5% and a temperature of 157–164°C the average loss of ochratoxin A is ∼13%, and increasing the temperature to 191–196°C increases the average loss to ∼31%. Increasing the residence time in the processing equipment also increases the proportion of the toxin that is lost (Scudamore *et al.*, 2004).

The lack of detection of ochratoxin A need not imply a reduction in potential toxicity, since the breakdown by-products can be as toxic as the ochratoxin A itself (Suàrez-Quiroz *et al.*, 2005).

Trichothecenes

The trichothecene mycotoxins produced by *Fusarium* spp., e.g., deoxynivalenol, nivalenol, diacetoxyscirpenol, and T-2 toxin, are commonly found in cereals, e.g., wheat, maize, barley, oats, and rye, which are used as raw materials for food and feed production worldwide. Consumer exposure to trichothecenes results from consuming contaminated food products.

Deoxynivalenol

Deoxynivalenol is stable at 120°C, moderately stable at 180°C, and partially stable at 210°C in a weakly acidic environment, but is unstable under alkaline conditions (WHO, 2001). While deoxynivalenol levels were unaffected by heat treatment of 100–120°C at pHs 4.0 and 7.0, heat treatments of 120°C for 30 minutes or 170°C for 15 minutes led to complete degradation of deoxynivalenol at pH 10 (Wolf and Bullerman, 1998).

Wheat. The conditions used for baking bread and other leavened products, e.g., cakes and biscuits, vary and variables such as fermentation conditions, dough additives, and the length and temperature of the baking process can all affect the amount of deoxynivalenol lost during the process. In some cases deoxynivalenol levels are not decreased by processing, although the toxin may not be evenly distributed into the various fractions following milling (Scott *et al.*, 1984). In other cases, deoxynivalenol may not be detected after baking but an isomer of deoxynivalenol of unknown toxicity may remain (Kushiro, 2008). Widely varying effects of deoxynivalenol on yeast have been reported. Deoxynivalenol levels increased during the production of leavened products, which is attributed to the enzymatic conversion of its precursors (Young *et al.*, 1984). Some studies on the effects of additives in bread on the levels of deoxynivalenol after cooking showed that adding potassium bromate–ascorbic acid or L-ascorbic acid did not reduce deoxynivalenol levels, while the addition of sodium bisulfite, L-cysteine, or ammonium phosphate could reduce deoxynivalenol levels by >40% (Hazel and Patel, 2004). Significant reductions in deoxynivalenol levels occurred during each of the processing steps from uncleaned durum wheat to cooked spaghetti. The average levels of deoxynivalenol remaining were 77% in cleaned wheat, 37% in semolina, 33% in spaghetti, and 20% in cooked spaghetti with respect to uncleaned wheat (Visconti *et al.*, 2004).

Pearling durum wheat reduces the level of deoxynivalenol contamination (Rìos *et al.*, 2009). Approximately 45% of the deoxynivalenol is lost with the first 10% of the tissue removed, with an additional 25% of the deoxynivalenol (total reduction of 70%) removed with the removal of

an additional 35% of the tissue. Thus, the pearling process efficiently reduces deoxynivalenol contamination and may also reduce or prevent recontamination of the starchy endosperm.

In laboratory tests that simulate noodle production from deoxynivalenol-contaminated wheat with extrusion at 42°C and 80–100 bar, followed by drying and boiling for 7 minutes there was an average decrease of 20% in deoxynivalenol concentration (Visconti *et al.*, 2004). Meeting a limit of 0.75 μg/g of deoxynivalenol for the pulp is not guaranteed if the unprocessed wheat is contaminated at 2.0 μg/g. Baking reduces deoxynivalenol by 24–71% in bread and 35% in biscuits (El-Banna *et al.*, 1983).

Loss of >95% of the deoxynivalenol in maize may occur following extrusion at 150–180°C. However, studies describing the fate of trichothecenes during extrusion may reach various conclusions. For example, Wolf-Hall *et al.* (1999) reported stable levels of deoxynivalenol in maize flour and extruded pet food and that the deoxynivalenol is not degraded at the temperatures and pressures used in these processes. During the preparation of tortillas from contaminated maize, 72–88% of the deoxynivalenol is lost.

Soaking wheat in the presence of sodium bisulfite reduces detectable deoxynivalenol through the formation of sulfonate salts. These salts are stable under acidic conditions but may break down resulting in free deoxynivalenol under alkaline conditions. In maize meal, experimentally contaminated with deoxynivalenol at 5 mg/kg, moisture, temperature, and the addition of sodium bisulfite all affect the reduction of deoxynivalenol (Cazzaniga *et al.*, 2001).

Deoxynivalenol levels do not change with treatment at 100–120°C and pH 4 or 7, but treatment at 120°C for 30 minutes or 170°C for 15 minutes results in the complete loss of the toxin at pH 10. The loss of deoxynivalenol from naturally and artificially contaminated Empanada coatings during preparation in corn oil was evaluated (Samar *et al.*, 2007). In this study, flour naturally contaminated with deoxynivalenol (1200 μg/kg) and artificially contaminated fortified flour (260 μg/kg) were used to prepare turnover pie dough covers. Deoxynivalenol reduction was greater in the artificially contaminated samples (>66% at 169°C, 43% at 205°C, and 38% at 243°C), than in the naturally contaminated samples (28% at 169°C, 21% at 205°C, and 20% at 243°C). The thermal decomposition products of deoxynivalenol are unknown, and there are no data on their toxicity, so there is currently no evidence that deoxynivalenol's thermal instability leads to the detoxification of human foods or animal feeds.

Rice. In parboiled rice, deoxynivalenol contaminated 22% of the samples evaluated at between 180 and 400 ng/g (Dors *et al.*, 2011). In a related study (Dors *et al.*, 2009), the shortest soaking time (4 hours) and the lowest level of deoxynivalenol contamination (720 ng/g) led to the highest level of migration of deoxynivalenol into the endosperm starch. A 6-hour soak resulted in the least migration of deoxynivalenol into the endosperm starch.

Zearalenone

Zearalenone is a chemically stable compound with a melting point of 164–165°C. The compound is stable at 120°C for 4 hours, but complete reduction occurs within 30 minutes at 225°C in Teorell and Stenhagen's citrate-phosphate-borate buffer adjusted to a pH of 4.0, 7.0, and 10.0 (Ryu *et al.*, 2003). The extrusion of maize grits reduces zearalenone levels by 77–83% at 120°C, by 74–83% at 140°C, and by 66–77% at 160°C (Ryu *et al.*, 1999). Of 40 samples of parboiled rice, 20% were contaminated with zearalenone at levels between 317 and 396 ng/g (Dors *et al.*, 2011). The best conditions for parboiling rice to reduce the migration of zearalenone into the endosperm include a soaking time of 4 hours (Dors *et al.*, 2009).

Patulin

Patulin is a toxic metabolite produced by several species of *Penicillium*, *Aspergillus*, and *Byssochlamys*. The accumulation of patulin is independent of fungicide applications during prestorage with the temperature prior to refrigeration probably the most important variable in toxin production (Morales et al., 2007a,b). As refrigerated storage in a controlled atmosphere does not suffice to prevent fungal growth and patulin production, additional treatments also have been evaluated, including the application of fungicides. Vapor *t*-2-hexenal, an aromatic compound produced by many fruits and vegetables, at 12.5 μg/L can potentially control blue mold, minimize patulin content, and maintain quality of Golden Delicious apples (Neri *et al.*, 2006).

Immersion of apples in a 3% solution of sodium hypochlorite for 5 minutes at 25°C completely inhibited fungal growth and fruit damage attributed to a number of fungi (Hasan, 2000). In other studies of immersion in acid solutions, immersion in 2–5% acetic acid suffices to inhibit the growth of *Penicillium expansum* and the production of patulin. For industrial applications, guaranteed contact time is important, and apples are either dipped in a 2% solution or sprayed with a 5% solution of acetic acid. In general, a combination of disinfection of fruit with acetic acid before storage and subsequent treatment with low levels of fungicides (Morales *et al.*, 2010) appears to be a promising method for storing large quantities of apples. The amount of patulin in unprocessed fruit stored indoors reaches 90 ng/g after 5 days, 400 ng/g after 15 days, and 2200 ng/g after 33 days. These values decrease to 75, 100, and 695 ng/g, respectively, after washing. Washing with water under high pressure can reduce patulin levels by 10–100% depending on the initial amount of the mycotoxin present. When the apples are heavily contaminated, i.e., 350 μg/L, washing does not reduce the level of patulin to <50 μg/L, regardless of the solution used (Acar *et al.*, 1998). Clean up can reduce the total patulin in apples by 93–99%.

The concentration of patulin in apple juice increases with the decomposition of the apple surface. The levels found in juice products prepared from healthy apples are all <50 ng/g, while juices produced from apples that are >30% decomposed contain patulin at levels that are >50 ng/g. Patulin can be reduced in fruit juice by >98% with granular activated carbon at the cost of lowering color and phenol content (Kadakal and Nas, 2002). Other carbon-based adsorbents also have been studied and can efficiently reduce the patulin levels in apple juice, but these substances, although effective for patulin removal, also result in a loss of flavor (Huebner *et al.*, 2000). Synthetic polymers, including DVB (polyester divinyl benzene), can reduce patulin levels by as much as 45% in fruit juices (de Smet *et al.*, 2011).

Fumonisins

The fumonisin mycotoxins occur worldwide in maize and maize-based products, and a European Commission report on tasks for scientific cooperation contains some occurrence data for fumonisins in wheat and wheat flour from France and Italy (European Commission, 2003).

Fumonisins are stable at 100–120°C. Boiling cultures of *Fusarium verticillioides* for 30 minutes does not reduce the levels of fumonisin B_1 present (Alberts *et al.*, 1990). When processed for 10–60 minutes at 100–235°C, fumonisin B_1 and fumonisin B_2 in aqueous buffers are most stable at pH 7 and progressively less stable at pHs 10 and 4 (Jackson *et al.*, 1996). Fumonisin decomposition begins at 150°C, and at temperatures >175°C, more than 90% of the fumonisin B_1 and the fumonisin B_2 are degraded within 60 minutes, regardless of pH. Furthermore, the fumonisin B_1 hydrolysis products are toxic, although their mode of action is not yet known.

Wheat

A survey of fumonisin contamination in cereals conducted in Spain reported fumonisin B_1 in 8/17 wheat samples in the range of 0.2–8.8 mg/kg (mean 2.9 mg/kg) and FB_2 in 1 sample (0.2 mg/kg) (Castella *et al.*, 1999). Although 19 Italian wheat, 11 durum wheat paste, and 27 wheat semolina samples were not contaminated, 87 of 91 wheat samples from France were reported to contain fumonisins at levels up to 1040 μg/kg. Of the 47 white wheat flour samples analyzed in Italy, five were contaminated at levels <100 μg/kg; of the 214 white wheat flour samples from France, 76 were contaminated, mostly (93%) at levels <100 μg/kg (European Commission, 2003). However, only limited data on the effects of processing on detoxification of fumonisins in wheat are available (Bullerman and Bianchini, 2014).

Maize

Heating wet and dry maize flour at 190°C for 60 minutes reduces fumonisin levels by 60–80%, while almost 100% of the available toxin is degraded after baking at 220°C for 25 minutes (Scott and Lawrence, 1994). Muffins made from maize meal contaminated with 5 mg/kg of fumonisin B_1 and baked at 175°C and 200°C lose 16% and 28% of the fumonisins, respectively, while a tortilla fried at 190°C for 15 minutes lost 67% of the fumonisins initially present (Jackson *et al.*, 1997). If maize flour, either artificially or naturally contaminated with 5 μg/g of fumonisin B_1, was roasted at 218°C for 15 minutes, then no fumonisins could be detected (Castelo *et al.*, 1998).

During the processing and packaging of fumonisin-contaminated products, fumonisins can bind to various components of the matrix and/or react with ingredients such as reducing sugars. For example, if fumonisin B_1 is incubated with D-glucose, then *N*-carboxy-methyl-fumonisin B_1 results. Milling also differentially distributes fumonisins in the resulting products. In particular, fumonisin levels are lowest in the flour, and markedly higher in the bran and germ used for animal feed and/or oil extraction. During wet milling of corn, fumonisins may be dissolved in the process water or distributed among the by-products, but not destroyed. Fumonisins also may be transferred from contaminated grain to beer during the malting process, and then degraded by 3–28% during the fermentation (Scott *et al.*, 1995). Artificially contaminated samples of roasted polenta (5 μg/g of fumonisin B_1) and muffins made with naturally contaminated flour did not lose a significant amount of fumonisins during their preparation (Castelo *et al.*, 1998). In general, fumonisins are more sensitive to dry heat than to moist heat.

The toxin level in cooked products may be underestimated due to the formation of fumonisin derivatives with unknown biological activity, i.e., "masked fumonisins," as in the case of corn flakes (de Girolamo *et al.*, 2001). In this process, fumonisin levels are reduced by 60–70%, with 30% of the losses attributable to extrusion at temperatures between 70°C and 170°C for 2–5 minutes. In other studies (Meister, 2001), extrusion and gelatinization reduce fumonisin levels by 30–55%, while toasting the flakes reduces the contamination by an additional 6–35%. Producing corn flakes without sugar reduces the fumonisin B_1 present by 49%, while production in the presence of glucose results in an 89% reduction. If sugars other than glucose are present, then the reductions are similar to those observed for the process without sugar (Castelo, 1999). When maize is extruded with a twin screw extruder at different temperatures (140°C, 160°C, 180°C, and 200°C) and different screw speeds (40, 80, 120, and 160 rpm), then the loss of fumonisin B_1 increases as temperature goes up and as screw speed goes down (Katta *et al.*, 1999). Pasteurizing milk contaminated with 50 ng/mL fumonisin B_1 and fumonisin B_2 at 62°C for 30 minutes did not significantly reduce the fumonisin contamination (Maragos and Richard, 1994).

Moniliformin

The heat stability of moniliformin is similar to that of other *Fusarium* mycotoxins, e.g., deoxynivalenol and fumonisin B_1. In stability studies, maize and wheat containing moniliformin at a level of 1 μg/g were held at 50°C, 100°C, and 150°C for 0.5–2.0 hours with a concomitant mean reduction in moniliformin of 55% (Scott and Lawrence, 1987). The reduction is correlated with increases in temperature and pH, with a reduction of 99% following heating at 175°C for 60 minutes at pH 10. Complete loss of moniliformin occurs when naturally contaminated maize containing 1.4 μg/g of moniliformin is cooked in an alkaline medium as part of a tortilla preparation process (Pineda-Valdes *et al.*, 2002). The stability of moniliformin is comparable to or greater than that of other *Fusarium* mycotoxins when the raw materials were baked, fried, roasted, or extruded (Pineda-Valdes *et al.*, 2003).

Discussion

Food processing has an impact on mycotoxins in wheat. Cleaning removes broken and moldy grain kernels. The milling processes dilute and more evenly distribute mycotoxins. High-temperature processes result in varying degrees of reduction of mycotoxin levels, but most mycotoxins are moderately stable in most food-processing systems. Aqueous cooking, steeping, roasting at high temperatures (>150°C), and extrusion cooking reduce mycotoxin concentrations. However, in the case of fumonisins, the fate of the toxin in wheat is unclear since the available literature discusses only maize and maize-based products. Fumonisins also may be modified or matrix bound and be non-recoverable for chemical analyses, but retain toxicity. The discrepancies between the findings reported in various cited studies may be due in part to differences in the analytical methods, laboratory equipment and/or experimental conditions employed, the concentration of toxin added, variation in sample source, and/or other environmental factors. However, the amount of reduction is highly dependent on cooking conditions, e.g., temperature, time, water, and pH, as well as the type of mycotoxin.

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20 Mycotoxins in the Sorghum Grain Chain

John F. Leslie

Abstract

Sorghum is a critical cereal crop for domesticated animals and humans in the world's hottest and driest areas. Although not free of mycotoxin contamination, the level of mycotoxin contamination in sorghum usually is much lower than in more widely grown cereals such as maize. The most commonly reported mycotoxins in sorghum are aflatoxins, fumonisins, and zearalenone, with others such as ochratoxin A, cyclopiazonic acid, gliotoxin, and trichothecenes reported much less often. Mycotoxin contamination may occur anywhere that sorghum is grown, but is more common when the grain is harvested and/or stored wet or damaged by insects or harvesting. Sorghum lines are not equally susceptible to mycotoxin contamination, but breeding for resistance to mycotoxin accumulation usually is not a major goal in sorghum breeding programs.

Keywords: aflatoxins; beer; biological control; ergot; fumonisins; grain mold; ochratoxin A; plant stress; trichothecenes; zearalenone

Introduction

Sorghum is the world's fifth largest cereal crop after maize, wheat, rice, and barley. Its origin is probably eastern African, with reports of domestication in Egypt as early as 8000 BC. It is still cultivated as a food crop throughout much of Africa. Beer in Africa often is malted with sorghum instead of barley, and the varieties within the crop are numerous. In some areas the stalks, which are used for roofing and fencing, may be of more value than the grain, with the lower leaves stripped by hand later in the season to feed livestock. Outside of Africa, sorghum is cultivated primarily in the Americas and South Asia. South Asians also use the crop for food, but in the Americas the most common use is for animal feed. Currently, the countries growing the most sorghum are the United States, India, Mexico, and Nigeria, with the United States, Argentina, and Australia the largest exporters. If the stalk is of economic value, then the plants commonly grow to 2–3 m in height, but if grain production is the primary goal, then converted dwarf lines, which dominate in the United States, that are ∼1 m tall are preferred. These plants usually yield more grain as well since more of their photosynthate can be used to fill the grain and since the stronger stalk can support a heavier grain head.

Sorghum is a crop prized for its drought hardiness, a property in which it is exceeded as a grain crop only by some of the millets. Thus, sorghum often is planted in marginal, hot, dry areas

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where other grain crops would produce at best a limited crop if any crop were produced at all. The area planted to sorghum has been declining for at least the last decade as farmers in developed countries plant short-season maize hybrids that mature before drought or temperature stress sets in. In less developed countries, maize is often preferred to sorghum by city dwellers and there has been considerable push by governments and aid agencies to meet this demand by expanding the area cultivated to maize to include areas that are marginal for maize production but are well adapted for sorghum production. Yet those who consume maize as their primary grain usually have much higher levels of esophageal cancer than do those who consume sorghum as their primary grain (Isaacson, 2005), probably due to increased levels of fumonisins in the diet (Rheeder *et al.*, 1992).

The expansion of maize cultivation to more marginal lands and climates has been accompanied by increased risk of contamination with mycotoxins such as aflatoxins and fumonisins. Conditions that stress plants make them more susceptible to fungal infection and render them more likely to be contaminated by one or more mycotoxins. For example, in farmers' fields in Nigeria, maize was more highly contaminated than either sorghum or pearl millet and there were more maize samples that exceeded international trade standards for safety than there were of either sorghum or pearl millet (Bandyopadhyay *et al.*, 2007). Like maize, following a sorghum crop with wheat can increase the risk of *Fusarium* head blight in the wheat and concomitant contamination with trichothecenes (Campagna *et al.*, 2005). With the various debilitating syndromes of both humans (Shephard, 2014; Turner, 2014) and domesticated animals (Jans *et al*., 2014) that are associated with mycotoxin contamination, planting maize instead of sorghum could result in both a net loss in available calories and increased problems such as immune system weakening, rendering those affected more susceptible to other infections, or developmental stunting that limits the potential for longer, more productive lives.

Sorghum is not free of mycotoxin contamination, although it usually is much less contaminated than maize, e.g., Bandyopadhyay *et al.* (2007) and da Silva *et al.* (2006). Under poor storage conditions, sorghum grain is quite amenable to colonization by mycotoxigenic fungi. Indeed sorghum grain is the preferred substrate for cultivation of *Aspergillus flavus* strains that are now being used commercially (AflaSafe) as biological control agents (Cotty, 2006). In the field, conditions that result in wet grain at harvest (Mall *et al.*, 1986; Reddy *et al.*, 1985) or that stress the plants, e.g., drought or poor nutrition, can result in increased mycotoxin contamination of the sorghum grain. The toxins that have been most commonly reported from sorghum are aflatoxins and fumonisins. There are reports of zearalenone and occasional reports of a variety of other toxins, but often these reports are not backed up with current chemical identification procedures and/or documentation of a toxin-producing fungus from the reported sample. In some cases, either the taxonomy or the chemical analysis is wrong. For example, claims that *Fusarium verticillioides* produces zearalenone or that *F. nygamai* produces deoxynivalenol are most generously explained as the result of an unknowing analysis of a mixed culture, e.g., Yassin *et al.* (2010), and render all of the other data in the manuscript suspect by association. Some other compounds are produced by fungi commonly found on sorghum but are not known to be associated with any prominent diseases or toxicoses of humans or domesticated animals, e.g., moniliformin. Mycotoxins in sorghum are regulated based on the final use of the crop. In developed countries this use usually is for animal feed, and the limits are usually the same as those for maize being used for this purpose.

Sorghum/Fungal Interactions

The most prominent plant disease in most locations where sorghum is grown is grain mold. This disease is complex in its etiology and the fungal components of the complex vary by location and

sometimes even by plant. Members of this complex often include mycotoxigenic fungi that are capable of producing toxins as a part of the disease process. Resistance to grain mold has been a long-term goal of many sorghum breeding programs, but selection usually is for resistance to the disease rather than resistance to infection by any single fungus or group of related fungi, and progress has been slow.

Of five *Fusarium* species associated with grain mold, *F. thapsinum* was the most virulent toward sorghum seedlings followed by *F. andiyazi*, then *F. verticillioides*, and finally *F. nygamai* and *F. pseudonygamai* (Leslie *et al.*, 2005). At one time all five of these species would have been identified as "*Fusarium moniliforme*," which could explain some of the differences in pathogenicity and toxin production associated with various strains collected under this name. The identification of host germplasm resistant to *F. thapsinum*, and its identification as one of the most important members of the species complex that causes this disease, has been an important step forward. The extent of the disease and the particular members of the complex can determine whether and to what extent mycotoxin contamination is present in infected sorghum grains, e.g., Tesfaendrias *et al.* (2011).

Fungal plant interactions between sorghum and its fungal pathogens are neither extensively studied nor well understood. Chitinase, a commonly produced antifungal protein, and a number of other antifungal proteins are produced by sorghum in response to infection by *Aspergillus parasiticus* (Ratnavathi and Sashidhar, 2000, 2004).

Reducing Mycotoxin Contamination

Practices to mitigate mycotoxin contamination in sorghum are neither well refined nor intensively studied. Fungicide seed treatments increase the vigor of infected seedlings and may reduce fungal colonization and subsequent mycotoxin production (Hasan, 2001). Sorghum lines with more compact heads often have more mycotoxin problems because as insects attack these heads they leave holes in the grain that fungi can use to infect and because the grain in these heads is moister than that found in the more open head types (Reddy and Nusrath, 1986). Crop rotation likely plays a role in reducing or increasing fungal inoculum levels, but again the available studies (Jaime-Garcia and Cotty, 2010) suggest solutions rather than test them experimentally. Scouting the crop for grain mold is insufficient as apparently healthy grain also can be contaminated with various mycotoxins (Bowman and Hagler, 1991). Harvesting the grain in a manner that does minimal damage to the grain certainly reduces both fungal colonization and mycotoxin contamination, e.g., Usha *et al.* (1994). Storing the grain under cool, dry, insect-free conditions is certainly important, but there is little published data, e.g., Elegbede *et al.* (1982), that focuses on grain quality and mycotoxin contamination during storage. In Ethiopia, increased levels of a number of mycotoxins in the grain are attributed to storage in underground pits in which there is little air circulation and in which the moisture of the grain usually increases with time (Ayalew *et al.*, 2006; Dejene *et al.*, 2004). Pit storage of sorghum also is practiced in Sudan (Abdalla *et al.*, 2002). Processing of the grain also is important. For example, extrusion cooking of milled sorghum in the presence of citric acid reduced detectable aflatoxins by 19–72% depending on the cooking temperature, the moisture of the sorghum flour, and the amount of citric acid added before cooking (Mendez-Albores *et al.*, 2009).

Fungal contamination of sorghum malt for beer often is a problem (Lefyedi *et al.*, 2005; Nkwe *et al.*, 2005), but can be reduced or eliminated by steeping the malt in 0.2% NaOH (Lefyedi and Taylor, 2006). Fumonisins at levels that are potentially high enough to be a health risk have been found in home-brewed beers in Africa that were malted with sorghum (Matumba *et al.*, 2011).

In Botswana, Nigeria, and South Africa, zearalenone was a problem in the local sorghum malt and sorghum beer, even when fumonisins and aflatoxins were not (Nkwe *et al.*, 2005; Odhav and Naicker, 2002; Okoye, 1986).

Developing economic and health rationales for acquiring evidence of toxin contamination and the advantages of reducing the contamination are probably needed to spur research that develops or adapts good agricultural practices for the mitigation of mycotoxin contamination of sorghum. A number of potential biological control agents have been examined, but in general these have not progressed much beyond initial identification stages. Strains of the bacteria *Rhodococcus erythropolis*, *Bacillus subtilis*, and *Pseudomonas fluorescens* and the fungus *Trichoderma viride* may reduce or completely inhibit growth of *A. flavus* and the production of aflatoxins under laboratory conditions, but it is not clear whether these agents would have much effect at the levels at which they could be delivered under field conditions (Reddy *et al.*, 2010). Eugenol, an aromatic component of clove oil, at 8 mg/g inhibits growth of *A. flavus* and the production of aflatoxin in stored sorghum grain (Komala *et al.*, 2012), but this compound also has toxic effects of its own that are likely to limit its use.

A number of novel plant products, e.g., zimmu (Karthikeyan *et al.*, 2007), 2-hydroxy-4-methoxybenzaldehyde isolated from *Decalepis hamiltonii* (Mohana and Raveesha, 2010), leachate of steeped sorghum seeds (Arafa *et al.*, 2003), some sorghum seed proteins (Ghosh and Ulaganathan, 1996), and thyme oil (Kumar *et al.*, 2009), also have been tested for antifungal activity and reductions of mycotoxin production with success at least at the laboratory level. Fermentation of moldy sorghum grain by *Lactobaccillus* spp. in conjunction with seeds of the weed *Senna tora* (syn. *Cassia tora*) reduced ergosterol (an indicator of fungal biomass) by 76%, fungal spore counts by up to 96%, and fumonisin B_1 and aflatoxin B_1 to nondetectable levels (Siruguri *et al.*, 2009).

Particular Mycotoxins in Sorghum

The major toxins associated with sorghum are relatively few in number and are discussed in somewhat more detail here, with reports, by country, of their occurrence given in Table 20.1. The mycotoxins may be reported as single contaminants or as multiple contaminants of individual samples, e.g., Shetty and Bhat (1997). Mycotoxigenic fungal species associated with sorghum are geographically widespread and often the same as those recovered from other cereal grains, but not necessarily well documented to occur on sorghum (Table 20.2).

Aflatoxins

Aflatoxin is the most intensively studied of all of the mycotoxins that are known to occur on sorghum. Colonization of sorghum by *A. flavus* may occur before the flag leaf emerges (Usha *et al.*, 1994), but such colonization does not necessarily mean that aflatoxin will be produced. Even when aflatoxin is produced, it often accumulates to amounts that are 10–100× lower than what accumulates in maize. Consumption of a diet composed primarily of sorghum rather than maize would reduce the aflatoxin exposure of West African subsistence farmers by four times, and if the grain consumed were pearl millet instead of maize, then the reduction would be eight times (Bandyopadhyay *et al.*, 2007). Thus, determining the level of aflatoxin contamination is important in addition to determining if aflatoxin is present. In many of the references cited (Table 20.1), the amount of aflatoxin present in most of the samples would not have been regarded as an acute health threat or subject to any

Table 20.1 Countries for which published reports of mycotoxins on sorghum are available

Mycotoxin	Country	Reference
Aflatoxin	Australia	Alisaukas (1974), Blaney and Williams (1991)
	Botswana	Nkwe *et al.* (2005), Siame *et al.* (1998)
	Brazil	da Silva *et al.* (2000)
	Colombia	Cespedes and Diaz (1997)
	Costa Rica	Garcia *et al.* (1988)
	Cuba	Escobar and Regueiro (2002)
	Egypt	El-Geddawy (2005), Ibrahim *et al.* (1998)
	Ethiopia	Ayalew *et al.* (2006)
	India	Reddy *et al.* (1983, 1985, 2011), Shetty and Bhat (1997), Shetty *et al.* (1994), Thirumala-Devi *et al.* (2002)
	Malawi	Matumba *et al.* (2011)
	Nigeria	Uraih and Ogbadu (1980), Bandyopadhyay *et al.* (2007)
	Pakistan	Lutfullah and Hussain (2012)
	South Africa	Odhav and Naicker (2002)
	Tunisia	Ghali *et al.* (2009a, 2010)
	Uganda	Kitya *et al.* (2010)
	United States	Hagler *et al.* (1987)
	Venezuela	Fernandez-Surumay *et al.* (2000)
Cyclopiazonic acid	India	Balachandran and Parthasarathy (1996)
Deoxynivalenol	Cameroon	Roger (2011)
	Ethiopia	Ayalew *et al.* (2006)
	South Africa	Lefyedi *et al.* (2005)
	United States	Bowman and Hagler (1991), Hagler *et al.* (1987)
Enniatins	Tunisia	Oueslati *et al.* (2011)
Ergot alkaloids	Australia	Blaney *et al.* (2010, 2011)
Fumonisins	Botswana	Nkwe *et al.* (2005), Siame *et al.* (1998)
	Brazil	Alves dos Reis (2010), da Silva *et al.* (2000)
	Burundi	Munimbazi and Bullerman (1996)
	Cameroon	Roger (2011)
	Ethiopia	Ayalew *et al.* (2006)
	India	Bhat *et al.* (1997), Das *et al.* (2010), Raghavender and Reddy (2009), Shetty and Bhat (1997)
	South Africa	Lefyedi *et al.* (2005)
	Tunisia	Ghali *et al.* (2009b)
	United States	Trucksess *et al.* (2000)
	Zimbabwe	Gamanya and Sibanda (2001)
Gliotoxin	Brazil	Keller *et al.* (2012)
Moniliformin	South Africa	Rabie *et al.* (1978)
Nivalenol	Ethiopia	Ayalew *et al.* (2006)
Ochratoxin A	Ethiopia	Ayalew *et al.* (2006)
	India	Shetty *et al.* (1994), Thirumala-Devi *et al.* (2002)
	Nigeria	Elegbede *et al.* (1982)
	Pakistan	Anjum *et al.* (2011)
	South Africa	Odhav and Naicker (2002)
	Tunisia	Ghali *et al.* (2009c)
	United States	Harvey *et al.* (1987)
T-2 toxin	India	Reddy *et al.* (1983), Shetty *et al.* (1994)
Zearalenone	Australia	Blaney and Williams (1991)
	Botswana	Nkwe *et al.* (2005)
	Ethiopia	Ayalew *et al.* (2006)
	India	Gupta and Singh (1994)
	Japan	Aoyama *et al.* (2009)
	Nigeria	Elegbede *et al.* (1982), Okoye (1986)
	South Africa	Lefyedi *et al.* (2005), Odhav and Naicker (2002)
	United States	Bowman and Hagler (1991), Hagler *et al.* (1987)

Table 20.2 Toxigenic fungal species with published associations with sorghum by country

Species	Country	Reference
Aspergillus flavus	Botswana	Nkwe *et al.* (2005)
	Brazil	da Silva *et al.* (2000, 2004)
	Burundi	Munimbazi and Bullerman (1996)
	Colombia	Diaz *et al.* (2009)
	Egypt	Arafa *et al.* (2003), Ibrahim *et al.* (1998)
	Hungary	Hasan (2001)
	India	Navi (2005), Reddy *et al.* (2011)
	South Africa	Odhav and Naicker (2002)
	Sudan	Elzupir *et al.* (2009)
	United States	Jaime-Garcia and Cotty (2010)
Claviceps africana	Australia	Blaney *et al.* (2006, 2011)
	India	Blaney *et al.* (2006)
	United States	Blaney *et al.* (2006)
Fusarium andiyazi	Australia	Petrovic *et al.* (2009)
	India	Sharma *et al.* (2011)
	South Africa	Marasas *et al.* (2001)
	United States	Marasas *et al.* (2001)
Fusarium equiseti	India	Sharma *et al.* (2011)
Fusarium graminearum	Australia	Blaney and Dodman (2002)
	Egypt	Ibrahim *et al.* (1998)
	South Africa	Tesfaendrias *et al.* (2011)
Fusarium proliferatum	Argentina	Carrillo *et al.* (2001)
	Brazil	da Silva *et al.* (2004)
	Burundi	Munimbazi and Bullerman (1996)
	India	Sharma *et al.* (2011)
	Korea	Lim *et al.* (2001)
	Serbia	Krnjaja *et al.* (2011)
	South Africa	Tesfaendrias *et al.* (2011)
Fusarium sacchari	India	Sharma *et al.* (2011)
Fusarium semitectum	Argentina	Saubois *et al.* (1999)
	Japan	Aoyama *et al.* (2009)
Fusarium subglutinans	Argentina	Saubois *et al.* (1999)
	Serbia	Krnjaja *et al.* (2011)
Fusarium thapsinum	Australia	Petrovic *et al.* (2009)
	Egypt	Klittich *et al.* (1997)
	India	Sharma *et al.* (2011)
	Italy	Moretti *et al.* (1995)
	Korea	Lim *et al.* (2001)
	Philippines	Klittich *et al.* (1997)
	South Africa	Klittich *et al.* (1997), Tesfaendrias *et al.* (2011)
	Thailand	Klittich *et al.* (1997)
	United States	Klittich *et al.* (1997), Tesso *et al.* (2010)
Fusarium verticillioides	Botswana	Nkwe *et al.* (2005)
	Brazil	da Silva *et al.* (2004)
	India	Navi (2005)
	Japan	Aoyama *et al.* (2009)
	Korea	Lim *et al.* (2001)
	Nigeria	Anthony *et al.* (2009)
	Serbia	Krnjaja *et al.* (2011)
	Zimbabwe	Gamanya and Sibanda (2001)

regulation for food safety, even if aflatoxin was present. Rapid detection techniques (Gilbert and Pascale, 2014) are becoming critical for effective detection and control of aflatoxin contamination in food. ELISA techniques are among those that hold the most hope for an accurate, fast, and inexpensive detection method (Zheng *et al.*, 2005). These tests need to be validated for sorghum before using with this crop, however, as test kits validated on maize may not be accurate when they are used with sorghum.

Strains of *A. flavus* associated with sorghum in India are capable of producing aflatoxins under laboratory conditions when inoculated on substrates such as cereals—barley, maize, rice, wheat, and sorghum; oil seeds—peanuts and sesame; and pulses—mung beans and horse gram (Reddy *et al.*, 2011). Similarly, the polyphenols in sorghum grain are capable of countering the mutagenicity of aflatoxin B_1 in the Ames *Salmonella* test for mutagenicity (Mosovska *et al.*, 2010). Such control suggests that including sorghum in the diet also might help reduce damage caused by aflatoxins introduced from other sources. Thus, there is no evidence that the *Aspergillus* strains from sorghum are genetically distinguishable from the strains that colonize other hosts, that these strains have any particular host specificity for sorghum, or that the spectrum of aflatoxins synthesized differs from what is found for other hosts.

Protein content in sorghum grain is positively associated with infection by *A. flavus* and negatively associated with starch content. As the starch content declines upon infection, these correlations suggest that *A. flavus* metabolizes the starch in the sorghum grain for growth and aflatoxin production and that the protein in the seed is used only slightly, if at all, in these processes (Bhadraiah and Ramarao, 1982; Komala *et al.*, 2012; Ratnavathi and Sashidhar, 2003). Sorghum lines with red pericarps are less susceptible to infection with *A. flavus* and to aflatoxin production than are those with a white pericarp (Ratnavathi and Sashidhar, 2000, 2003). Sorghum malt and opaque sorghum beer from southern Malawi were universally contaminated with aflatoxin (Matumba *et al.*, 2011), and grain used for malt production was contaminated with *A. flavus* in South Africa (Odhav and Naicker, 2002). The highest levels were in the malt, suggesting that the malting conditions were appropriate for growth of *Aspergillus* and the production of the toxin (Matumba *et al.*, 2011). Thobwa, a nonalcoholic sweet beverage made with maize and either sorghum or finger millet had even higher levels of aflatoxin than did the sorghum beer.

Fumonisins

Fumonisins are a family of chemically related molecules with varying levels of toxicity, but of which fumonisin B_1 is the most important. These toxins are known to be produced in sorghum grain, but usually at much lower levels than found in maize. Fumonisins are produced at significant levels (>1 μg/g) by relatively few species of *Fusarium*, all belonging to the *Gibberella fujikuroi* species complex (Desjardins, 2006; Leslie and Summerell, 2006), with *F. verticillioides* and *F. proliferatum* the two species with strains that generally produce high levels of fumonisins. Both *F. verticillioides* and *F. proliferatum* may occur on sorghum, but other members of the *G. fujikuroi* species complex are much more common and often produce high levels of moniliformin rather than fumonisins. Many of the species in the *G. fujikuroi* species complex are very difficult to distinguish from one another without molecular analyses (Leslie and Summerell, 2006), and diagnoses of any of the fungi in this group based solely on morphological characters should be regarded as suspect, e.g., Alves dos Reis *et al.* (2010), Das *et al.* (2010), and Karthikeyan *et al.* (2007). Strains identified as "*F. moniliforme*" are particularly suspect as that name is no longer valid and its description fits a number of different *Fusarium* species (Seifert *et al.*, 2003).

Sorghum (and millets) is often recommended as "safer" alternatives to maize and peanuts in diets of subsistence farmers in Africa who are commonly exposed to high levels of aflatoxins (Hell and Mutegi, 2011). However, fumonisin intoxication in humans who had consumed sorghum was reported in India in 1995 (Bhat *et al.*, 1997; Raghavender and Reddy, 2009), and the abilities of fumonisins to induce apoptosis in some human cell cultures may be responsible for the abdominal distress reported (Schmelz *et al.*, 1998). Nonetheless, sorghum is usually less heavily contaminated with fumonisins than is maize, e.g., da Silva *et al.* (2006) and Bandyopadhyay *et al.* (2007). Fumonisins also occur infrequently in sorghum syrup in the United States and at a level that is considerably below the threshold required for any regulatory action (Trucksess *et al.*, 2000).

Fumonisin B_1 and strains of *F. nygamai* that synthesize fumonisins have been proposed as control agents for plant parasitic *Striga* spp. that attack sorghum (Kroschel and Elzein, 2004). Given that *F. nygamai* is a known sorghum pathogen (Leslie *et al.*, 2005; Nelson *et al.*, 1992) and the toxicity of fumonisins, I do not think that this control measure should be encouraged. These results do, however, provide a possible reason for the persistence of the fumonisin production character in some of the *Fusarium* species that can both colonize sorghum as an endophyte and produce fumonisins. The phytotoxicity of fumonisins has led to them being proposed as a biological control for a number of weed species (Abbas and Boyette, 1992), but with the results never followed up.

The ability of fumonisins to bind to proteins and to form acylated derivatives when part of fried foods may make these compounds more difficult to detect in some situations (Scott, 2012). The adsorbent cholestyramine binds fumonisins and may be an effective adsorbent when added to the diet to reduce fumonisin uptake (Scott, 2012).

Trichothecenes

There are a number of toxicologically important trichothecenes, including deoxynivalenol, nivalenol, diacetoxyscirpenol, and T-2 toxin. These toxins are produced by *Fusarium* species more commonly associated with cooler weather than is found in most sorghum growing parts of the world. Reports are typically of chemical analyses of one or more trichothecenes present in a sorghum substrate—grain, couscous, beer, etc.—but the works routinely lack mycological backup and there are no *Fusarium* strains in major culture collections recovered from sorghum contaminated with trichothecenes that have been shown to produce these toxins in culture. It is quite possible that the *Fusarium* strains that produce trichothecenes under these hot, dry conditions belong to a different species than the better known species from temperate climates.

Zearalenone

Strictly speaking, zearalenone is not a mycotoxin because it is not known to be toxic in any reasonable dose. It is, however, estrogenic and can significantly alter reproductive metabolism of some mammals, even though the zearanol derivatives are popular cattle growth promotants (Hidy *et al.*, 1977). Zearalenone is most commonly synthesized by *Fusarium graminearum* and related fungi, e.g., *Fusarium culmorum*, but is not synthesized by *F. verticillioides* or other members of the *G. fujikuroi* species complex (Desjardins, 2006; Leslie and Summerell, 2006). If zearalenone is produced by *F. graminearum* or *F. culmorum* then it usually is accompanied by the synthesis of one or more trichothecenes, e.g., nivalenol or deoxynivalenol. However, there are reports in the literature of situations where zearalenone is the only toxin identified as being produced (Aoyama *et al.*, 2009;

Nkwe *et al.*, 2005). Unambiguous detection of zearalenone can be difficult and careful attention must be paid to the methodology used to make sure that other compounds are not misidentified as zearalenone (Seitz *et al.*, 1975; Smith *et al.*, 2004).

The patterns of toxin biosynthesis might be different for warm-weather and tropical *Fusarium* spp. than it is for species usually found in temperate or even colder climates and could be due to differences in the species present and/or the physiological capabilities of the strains present. Whether species that produce just zearalenone without producing trichothecenes has been described already remains an open question.

Ochratoxin A

Ochratoxin A is the most important member of the ochratoxin family of secondary metabolites and is produced by several species of *Aspergillus* and *Penicillium*. It is a common contaminant of wheat, barley, oats, and rye, and less common in maize and much less common in more tropical grains such as sorghum and millets. Ochratoxin A is a carcinogen and a neural and renal toxin, and is particularly problematic for poultry. Sorghum in mixed poultry feeds may be contaminated with ochratoxin A, e.g., Anjum *et al.* (2011), but it is not clear whether the low levels of ochratoxin A associated with sorghum grain are due to production on the sorghum grain or if the sorghum has acquired the toxin from other feed components that are much more heavily contaminated with ochratoxin A than it is.

Other Toxins

There are scattered reports of other toxins being found associated with sorghum, including ergot alkaloids and gliotoxin, and some reports of compounds, such as moniliformin and fusaproliferin, which are suspected, but not proven toxins occurring either on sorghum or being produced in culture by fungi recovered from sorghum.

Ergot alkaloids. Sorghum grain diseased with *Claviceps africana* may be contaminated with a variety of ergot alkaloids, the most prevalent of which is dihydroergosine. Alkaloids produced by *C. africana* cause hyperthermia in cattle (Blaney *et al.*, 2010). The sorghum ergot alkaloids in general, and dihydroergosine in particular, are toxic to cattle and swine (Blaney *et al.*, 2000a,b, 2011) and result in feed refusal and little or no milk production at sufficiently high levels and reduced weight gain at lower levels (Blaney *et al.*, 2000a,b). There is considerable intraspecific variation within *C. africana* in terms of the amount and type of alkaloids produced by any given strain, and alkaloid production is more consistent on inoculated plants than it is in the available laboratory media (Blaney *et al.*, 2006). The alkaloids can be recovered from contaminated grain, sclerotia, and the honeydew produced on the infected sorghum head (Blaney *et al.*, 2003). The spectrum of alkaloids produced by *C. africana* is different from the alkaloid spectrum produced by *C. purpurea*, which colonizes cereals such as rye under cool, wet conditions, with the *C. africana* alkaloids generally perceived as less toxic than those produced by *C. purpurea*. An ELISA assay (Molloy *et al.*, 2003) is available for the most common of the ergot alkaloids, dihyrdoergosine, with the other alkaloids usually detected by HPLC. Making silage from the infected plants reduces the amount of dihydroergosine in the final material (Blaney *et al.*, 2010).

Moniliformin. Moniliformin is a small molecule with a cyclobutane ring structure (Cole *et al.*, 1973; Springer *et al.*, 1974). This compound is synthesized at high levels by strains of *F. thapsinum*

from sorghum and is very toxic to ducklings (Leslie *et al.*, 2005; Lim *et al.*, 2001). The toxicity to poultry could potentially limit the use of sorghum as poultry feed, and further work to better define this risk is needed. *Fusarium pseudonygamai* also synthesizes large amounts of moniliformin, but is more commonly recovered from pearl millet than it is on sorghum. Onyalai, a human disease associated with the consumption of pearl millet, may be associated with moniliformin given the symptoms found in laboratory animals that consumed contaminated grain associated with this disease outbreak (Rabie *et al.*, 1975).

Non-ribosomal peptides (beauvericin and enniatins). Non-ribosomal peptides are common secondary metabolites of a number of *Fusarium* species. Many of these species have been reported from sorghum. In Tunisia (Oueslati *et al.*, 2011), enniatins were present in sorghum cereals and processed foods sold in the market, but no beauvericins were reported from these same samples.

Gliotoxin. Gliotoxin is synthesized by a number of *Aspergillus* species, primarily *Aspergillus fumigatus*, and has immunosuppressive and apoptotic effects in cell cultures. In Brazil (Keller *et al.*, 2012), sorghum was less contaminated than maize both before and after the production of silage for cattle feed.

Fusaproliferin. Fusaproliferin (Ritieni *et al.*, 1995) is produced by *F. proliferatum* (Moretti *et al.*, 1996), a common fungal contaminant of sorghum. In Tunisia, however, no fusaproliferin was detected in sorghum grain or processed foods purchased in the market (Oueslati *et al.*, 2011).

Conclusions

The fungal genera *Aspergillus* and *Fusarium* are responsible for most of the mycotoxins that significantly contaminate sorghum. Members of these genera are widespread, as are the reports of their toxins in sorghum. In some cases these toxins accumulate to levels that are toxic to humans or domesticated animals, but in most cases these toxins, if they are present at all, occur at levels below those that trigger regulatory concerns for trade and health. Beyond good agricultural practices and good storage practices, relatively little is done to manage or remediate mycotoxin contamination in sorghum. Developing sorghum lines that are resistant to grain mold probably is the most cost-effective approach to reducing mycotoxin contamination in sorghum at this time, especially if the goal is to reduce mycotoxin intake by the poorest people in the world who rely on sorghum as their primary calorie source.

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21 Toxigenic Fungi and Mycotoxins in the Rice Grain Chain and Strategies to Reduce Them

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Abstract

Rice is a staple food for almost half of the world's population and it is the most widely grown cereal in the world. Toxigenic fungi and their mycotoxins produced on rice are a significant problem worldwide, especially in underdeveloped and developing countries where rice is the staple food. *Aspergillus* and *Penicillium* are the most common toxigenic fungi found in rice, and aflatoxin B_1 and ochratoxin A are the most commonly recovered mycotoxins from this grain. The major concern is the level of mycotoxin contamination that may occur in traditionally stored rice in underdeveloped and developing countries. Moisture content, the level of field infection, and storage conditions in these countries all contribute to the high levels of toxigenic fungi and their mycotoxins. Efforts to inhibit the growth of these fungi and reduce mycotoxin contamination have had little success. Fungal contamination strongly influences the ultimate quality of rice and rice end-products and impacts food safety. Thus, more serious attention to this problem should be given by various government and nongovernment authorities. Setting maximum permissible levels of the most potent and commonly occurring mycotoxins in rice, e.g., aflatoxin B_1, is an important place to begin, especially in the countries where rice is the staple food.

Keywords: aflatoxins; *Aspergillus*; bran; detection methods; good agricultural practices; grain quality; ochratoxin A; *Penicillium*; risk assessment; trichothecenes

Introduction

Rice is the staple food for almost half of the world's population and is the world's most widely cultivated crop. About 700 million tons (mt) of rice are produced globally every year (FAO, 2011). It provides 20% of the world's dietary calories, compared to wheat (19%) and maize (5%) (Wenefrida *et al.*, 2009). Due to steady increases in population, even the top rice-producing countries are now beginning to import rice.

The main use for rice is for human consumption. It normally is milled (both polished and unpolished rice) and then cooked by boiling or steaming. The inedible rice hull (or husk) can be used as fuel, fertilizer, or insulation, while the bran is a source of cooking oil (Makun *et al.*, 2007). Brown rice contains bran, a moist and oily inner layer that may contain an extractable oil.

Contamination of rice by toxigenic fungi enhances the production of mycotoxins, which are secondary metabolites produced by some fungi (Turner *et al.*, 2008). Some of the mycotoxins

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are lethal while others have adverse health effects, e.g., weakening the immune system (Turner, 2014) or causing allergies, that are not necessarily specific to just the offending toxin. Mycotoxin contamination increases when conditions are conducive for fungal growth. Generally, stored rice is a potential target for fungal growth and mycotoxin production (Turner *et al.*, 2008).

Identification of the fungi present in a commodity and the determination of a mycotoxin profile associated with various fungal species are critical in reducing the number of mycotoxins to be analyzed (Frisvad *et al.*, 1989). Yeast extract sucrose (YES) agar is recommended for the production of mycotoxins *in vitro*, acidic systems such as toluene/ethyl acetate/90% formic acid as a general eluent, and an acidic anisaldehyde mixture as a spray reagent for mycotoxin detection in thin layer chromatography (TLC) systems. These methods are still effective for detecting most neutral and acidic mycotoxins.

In this chapter we review the presence, diversity, and detection of toxigenic fungi and the level of mycotoxins in rice. Our goal is for this information to be used to provide safe rice for human consumption and to reduce the threat of mycotoxins, especially in the underdeveloped and developing countries, where rice is the staple food.

Toxigenic Fungi in Rice

Aspergillus, *Penicillium*, *Fusarium*, and *Alternaria* were the most important toxigenic fungi isolated from rice (Pitt *et al.*, 1994; Reddy *et al.*, 2008; Shetty *et al.*, 1994), although the dominant fungus recovered varies by region (Table 21.1). Moreover, rice is harvested at very high moisture levels, between 33% and 50% (Zinedine *et al.*, 2006), which favor the growth of toxigenic fungi. Infected rice grains may be discolored, of lower quality, and contaminated with mycotoxins (Reddy *et al.*, 2008). *Penicillium* and *Alternaria* were the major genera of fungi isolated from harvested rice and *Aspergillus*, *Penicillium*, and *Eurotium* were the major fungi isolated from stored rice (Sakai *et al.*, 2005). *Fusarium* contamination of paddy rice also is common (Maheshwar and Janardhana, 2010; Pacin *et al.*, 2002; Tonon *et al.*, 1997).

Aspergillus spp. are the most common contaminants in stored rice and infestation by the rice weevil (*Sitophilus oryzae*) increases the incidence (Choudhury *et al.*, 1999; Kumar *et al.*, 2008; Prasad *et al.*, 1987). *Aspergillus* is one of the storage fungi that contribute to the deterioration of stored products, such as rice. Parboiling renders rice even more susceptible to *Aspergillus* infection (Bandara *et al.*, 1991). Infestation by rice with *Aspergillus* spp., primarily by *A. flavus* and *A. parasiticus*, can reduce quality and contaminate seeds with aflatoxins under poor storage conditions. In India, the fungi most commonly isolated from flood-affected paddy fields and farmer's storage structures were *A. flavus*, *A. nidulans*, *A. fumigatus*, and *A. candidus* with the incidence of *A. flavus* increasing during storage (Reddy, 1990). *Aspergillus sydowii* commonly is associated with rice in the field (73%), on threshing floors (65%), or from storage facilities (78%) (Kumar *et al.*, 2008).

Contamination of rice with *Penicillium* species was highest in Japan (Sakai *et al.*, 2005), Argentina, and Paraguay (Tonon *et al.*, 1997) as *Penicillium* commonly was found on foodstuffs in the field, storage, raw materials, and finished products. In Japan, *Penicillium* was the most common fungal genera found in rice with *P. citrinum* (66% of samples) and *P. islandicum* (50% of samples) the major internal contaminants of paddy rice (Sakai *et al.*, 2005). *Penicillium islandicum* was responsible for brownish discolored rice and *P. citrinum* was responsible for yellowish rice (Udagawa and Tatsuno, 2004).

Fusarium species were the third most frequent fungal contaminants of rice after *Aspergillus* and *Penicillium*, but the losses it causes are still significant. Scab, or *Fusarium* head blight, of rice occurs

Table 21.1 Fungi isolated from rice worldwide

Country	Type of rice	Most common fungi isolated	Reference(s)
China	Polished	*A. flavus*	Jayaraman and Kalyanasundaram (1990)
Egypt	Paddy	*A. flavus*, *A. fumigatus*, *A. sydowii*, *A. terreus*, *A. ochraceus*, *Alternaria tenuis*, *F. oxysporum*, *P. chrysogenum*	Hafez *et al.* (2004)
India	Milled	*A. flavus*, *A. fumigatus*, *A. nidulans*, *A. niger*, *F. oxysporum*, *P. citrinum*	Chary and Reddy (1987), Reddy *et al.* (2009)
Japan	Stored	*P. islandicum*	Sakai *et al.* (2005)
Korea	Polished	*A. candidus*, *P. citrinum*, *F. proliferatum*	Park *et al.* (2005)
Malaysia	Milled	*A. candidus*, *A. flavus*, *A. fumigatus*, *A. niger*, *A. versicolor*, *P. citrinum*	Kumar *et al.* (2008), Salleh and Farhana (2009), Udagawa (1976)
Nepal		*F. fujikuroi*, *F. graminearum*, *F. semitectum*, *F. oxysporum*	Desjardins *et al.* (2000)
Nigeria		*Alternaria*, *Aspergillus*, *Curvularia*, *Fusarium*, *Penicillium*	Makun *et al.* (2007)
Northern Argentina and southern Paraguay	Harvested	*A. flavus*, *A. niger*, *F. semitectum*	Tonon *et al.* (1997)
Sri Lanka	Parboiled	*Aspergillus* spp., *Penicillium* spp.	Bandara *et al.* (1991)
Uganda	Milled	*A. candidus*, *A. flavus*, *A. niger*, *E. rubrum*, *P. citrinum*, *Talaromyces* spp.	Taligoola *et al.* (2004)
Vietnam		*A. flavus*, *A. fumigatus*, *A. oryzae*, *A. candidus*, *A. niger*, *A. glaucus*, *Fusarium* spp., *P. citrinum*, *P. islandicum*	Trung *et al.* (2001)

in many rice-growing countries, especially in temperate regions in Asia. Scab symptoms on rice are similar to those caused by *Fusarium* species on other cereals, such as wheat and barley. A study of Nepalese rice found that all untreated samples of rice seeds with husks and 60% of the individual untreated seeds with husks were infested with *Fusarium* species. Seed infection levels generally were less than 10% following surface disinfestation (Desjardins *et al.*, 2000). Milled rice was less contaminated than paddy rice, but the number of species and fungal colony forming units (CFUs) usually was higher in untreated rice than in parboiled rice (Jayaraman and Kalyanasundaram, 1990). Fungal contamination was lowest in polished rice, intermediate in rice bran, and highest in rice hulls (Lima *et al.*, 2000).

Occurrence of Mycotoxins in Rice

Mycotoxins can enter food chains as contaminated plant products, or as carry-through in other products, e.g., milk or meat, from animals that have consumed contaminated feed. The most frequently encountered mycotoxin in rice is aflatoxin, but numerous other compounds such as fumonisin, ochratoxin A, and trichothecenes produced by numerous species of *Aspergillus*, *Fusarium*, and *Penicillium* also are recovered from rice (Table 21.2). Contamination of rice by mycotoxins may occur in the field prior to harvest or during storage. Delayed harvest, inadequate drying, and improper storage are all major contributors to mycotoxin contamination of rice. Interim outdoor storage after milling, especially in tropical areas, provides ideal conditions for fungal growth and mycotoxin

Table 21.2 Occurrence of mycotoxins in rice in different countries

Mycotoxins	Incidence rate	Concentration range (μg/kg)	Type of rice sample	Country	Reference
Aflatoxin B_1	33/252	5–50	Polished rice	China	Zhen-zhen (1989)
	337/525	0.5–3.5	Milled rice	India	Reddy *et al.* (2009)
	31/40	28–960	Rice bran	India	Jayaraman and Kalyanasundaram (2009)
	59/71	Mean 1.89		Iran	Mazaheri (2009)
	97/196	20–1640		Nigeria	Makun *et al.* (2007)
	35/51	Mean 3.3		Vietnam	Nguyen *et al.* (2007)
Aflatoxin G_1	156/329	n.d. to >1000	Parboiled rice	Sri Lanka	Bandara *et al.* (1991)
Total aflatoxins ($B_1 + B_2 + G_1 + G_2$)	24/81	0.45–11.4	Long grain rice	Austria	Reiter *et al.* (2010)
	36/37	0.99–3.9	Dehusked, brown	China	Liu *et al.* (2006)
	15/15	4.4–35	Bran	Iran	Zaboli *et al.* (2010)
	65/69	n.d. to 2.7	Polished	Philippines	Sales and Yoshizawa (2005)
	9/9	0.03–8.7	Brown		
	3/5	0.14–0.24	Basmati	Qatar	Abdulkadar *et al.* (2004)
	29/40	1–21	Bran	UK	Scudamore *et al.* (1999)
Citrinin		49–92		India	Tanaka *et al.* (2007)
		700–1130		Japan	
	2/13	Mean 0.38		Vietnam	Nguyen *et al.* (2007)
Fumonisin	1/4	0.028	Basmati	UK	Patel *et al.* (1996)
Fumonisin B_1	3/29	0.8–0.9		Argentina	Lerda *et al.* (2005)
	2/88	4.4–7.1	Polished	Korea	Park *et al.* (2005)
Ochratoxin A	13/31	n.d. to 12.5		Chile	Vega *et al.* (2009)
	2/60	0.1–5.3		Iran	Azizi and Azarmi (2009)
	5/60	n.d. to 6.0	Polished	Korea	Park *et al.* (2005)
	18/20	0.02–32		Morocco	Zinedine *et al.* (2006)
	26/100	0.08–47		Portugal	Juan *et al.* (2008)
	24/25	0.01–2.2	Parboiled	Nigeria	Williams *et al.* (2008)
	56/140	24–1160		Nigeria	Makun *et al.* (2007)
	6/42	0.09 –3.5		Portugal	Pena *et al.* (2005)
	2/5	1.65–1.95	Basmati	Qatar	Abdulkadar *et al.* (2004)
	5/63	n.d. to 27	Nonorganic	Spain	Gonzalez *et al.* (2006)
	6/20	n.d. to 7.1	Organic		
	0/17	n.d.	Brown	Taiwan	Lin *et al.* (2005)
	0/32	n.d.	Cooked		
	4/16	n.d. to 2.3		Tunisia	Ghali *et al.* (2008)
	27/96	n.d. to 150		Tunisia	Zaied *et al.* (2009)
		1.0–19		UK	Miraglia and Brera (2000)
	2/25	21.3–26.5		Vietnam	Trung *et al.* (2001)
	35/100	n.d. to 2.78		Vietnam	Nguyen *et al.* (2007)
Deoxynivalenol	4/124	0.12–2.9		Japan	Tanaka *et al.* (2007)
	1/5	140	Basmati	Qatar	Abdulkadar *et al.* (2004)
Nivalenol	15/124	0.2–2.2		Japan	Tanaka *et al.* (2007)
Zearalenone	3/60	n.d. to 106		Iran	Azizi and Azarmi (2009)
	93/196	24–1170		Nigeria	Makun *et al.* (2007)
	2/5	0.18–1.41	Basmati	Qatar	Abdulkadar *et al.* (2004)
	0/5	n.d.		Taiwan	Liao *et al.* (2009)

n.d., not detected.

biosynthesis. Improper storage during the rainy season is a particularly acute problem (Nguyen *et al.*, 2007).

Although storage is a major factor influencing the production of mycotoxins, a study of aflatoxin production in rice bran in Iran found no significant linear correlation between the concentration of aflatoxins B_2, G_1, and G_2 and the length of storage (Zaboli *et al.*, 2010). Instead, they found that aflatoxin concentrations in stored rice samples were lower than in samples that had not been stored (Zaboli *et al.*, 2010). Although, the reason for this difference is unknown, the inhibition of aflatoxin production may be due to the presence of synthetic pesticides (Paranagama *et al.*, 2003; Zaboli *et al.*, 2010) in storage or storage conditions that completely prevent any metabolism by the mycotoxin-producing fungi (Prasad *et al.*, 1987). Rice bran also may contain enzymes that inhibit the production of aflatoxins (Brown *et al.*, 1999).

Aflatoxins are a group of related mycotoxins produced by *Aspergillus* spp. with aflatoxin B_1 and G_1 the most frequently encountered in quantities sufficient to be toxic. These compounds are carcinogenic and are correlated with the incidence of liver cancer (Castegrano and Pfohl-Leszkowicz, 1999). Aflatoxins in rice usually are present at lower levels in field samples, than they are in samples that have been stored (Makun *et al.*, 2007). In countries such as Japan, where good storage facilities are available, postharvest contamination of rice with mycotoxins is rare (Sugita-Konishi *et al.*, 2006; Tanaka *et al.*, 2007). Stored paddy rice can be contaminated with aflatoxins (Kumar *et al.*, 2008; Pawan *et al.*, 1990; Prasad *et al.*, 1987) with rice at the various drying stages and at the stage preceding milling containing the highest levels of aflatoxins (Kumar *et al.*, 2008).

Rice of visibly poor quality or with off colors or bran is almost always more heavily contaminated with mycotoxins, with poorer grades sometimes contaminated in 100% of the cases and at relatively high levels, i.e., >1000 μg/kg (Bandara *et al.*, 1991). Thirty-five percent of raw rice bran and parboiled raw rice bran samples were contaminated with aflatoxin B_1, with toxin levels higher in the bran of the parboiled rice (Jayaraman and Kalyanasundaram, 1990; Kumar *et al.*, 2008). Over 60% of rice bran samples from India contained aflatoxin B_1 at lower levels (<50 μg/kg); one-third of these contaminated samples were in the 50–500 μg/kg range and another third had 500–2000 μg/kg (Elangovan *et al.*, 1999).

Contamination of rice bran could occur during the milling process if stale bran is present on the machinery or if there is a poor milling environment (Jayaraman and Kalyanasundaram, 2009). Rice bran is used mostly for animal feed and the storage conditions for this material often are poor with limited ventilation and hygiene and unsanitary conditions all contributing to favorable conditions for aflatoxin production. Another factor contributing to mycotoxin contamination is the time lag between bran production, processing, marketing, transport, and storage which also increases the opportunity for the rice bran to be colonized by toxigenic fungi and the production of mycotoxins to occur (Jayaraman and Kalyanasundaram, 2009; Reddy *et al.*, 2008). Contamination of rice bran in animal feed with aflatoxins, especially cattle feed, can potentially pose a human health hazard because of the conversion of the aflatoxins in the feed to aflatoxin M_1, which is secreted with the milk.

The quality of rice also is important in mycotoxin contamination. In Sri Lanka, parboiled rice contained aflatoxins more frequently and at higher levels than raw milled rice (Bandara *et al.*, 1991). Parboiled unpolished brown rice (with bran), in particular, is an ideal medium for fungal growth and mycotoxin biosynthesis when it is improperly stored (Bandara *et al.*, 1991). The presence of more fat, protein, and fiber in the unpolished brown rice (with bran) probably enhances fungal growth and increases subsequent toxin levels (Sales and Yoshizawa, 2005).

Citrinin is a toxic secondary metabolite produced by some strains of *Aspergillus*, *Penicillium*, and *Monascus* (Abd-Allah and Ezzat, 2005). Citrinin alters the uptake of amino acids and their incorporation into protein by plant cotyledons (Abd-Allah and Ezzat, 2005). It also induces

chromosome abnormalities, such as chromosome breakage, polyploidy, anaphase bridges, and lagging chromosomes in onion (Sinha *et al.*, 1992), and decreased seed germination (Sriram *et al.*, 2000). *Penicillium viridicatum* produced the highest amount of citrinin on glucose–ammonium nitrate salts broth and rice grains (Abd-Allah and Ezzat, 2005). A study on rice from South Vietnam found that *P. citrinum* produced citrinin at moderate levels (Trung *et al.*, 2001). The maximum amounts of citrinin detected in rice in the Vietnamese provinces of Binh Dinh and Phu Yen in the rainy season were 0.42 and 0.38 μg/kg, respectively, whereas no citrinin was found in rice samples taken during the dry season (Nguyen *et al.*, 2007).

Fumonisin contamination was detected in rough rice, polished rice, and rice cakes in Korea (Chung and Kim, 1995; Kim *et al.*, 1998); rice in China (Trucksess, 2000); and unpolished rice imported into Germany from various countries (Usleber *et al.*, 1997). Fumonisins cause leukoencephalomalacia (LEM) in horses, pulmonary edema in swine, neural tube defects in laboratory animals and humans, and are associated with esophageal cancer in humans (Desjardins, 2006).

Ochratoxin A produced by *Aspergillus ochraceus* and *Penicillium verrucosum* is a mycotoxin that is resistant to typical processing and may persist in foodstuffs even after mold destruction (Alldrick, 1996). Ochratoxin A contamination in rice was found in various countries such as Egypt (Abdelhamid, 1990), Vietnam (Trung *et al.*, 2001), Korea (Park *et al.*, 2005), Portugal (Pena *et al.*, 2005), Morocco (Zinedine *et al.*, 2006), Chile (Vega *et al.*, 2009), and Nigeria (Williams *et al.*, 2008). Ochratoxin A production might be related to the high amino acid content of wild rice which could explain the absence of ochratoxin A in white rice in contrast to the results from brown, basmati, aromatic, and wild rice samples (Pena *et al.*, 2005).

Contamination of rice by zearalenone and trichothecenes does not usually occur in the absence of scab. In Korea, low incidences and levels of zearalenone, deoxynivalenol, and nivalenol occur in polished rice purchased from retail markets (Park *et al.*, 2005). Recently, an intensive survey for zearalenone, deoxynivalenol, and nivalenol was conducted during rice processing in Korea. Blue-tinged rice and colored rice usually are removed by a biosensor during the processing. None of the polished rice intended for human consumption contained mycotoxins, but other rice samples including brown rice, blue-tinged rice, and colored rice were heavily co-contaminated with zearalenone, deoxynivalenol, and nivalenol (Lee *et al.*, 2011). Some of the contaminated samples contained >1 μg/g of deoxynivalenol or nivalenol. In Japan, a typhoon resulted in rice lodging in the field in 1998, and the unpolished rice was brown. Samples of this unpolished rice were analyzed for the presence of trichothecenes, and deoxynivalenol (0.12–2.9 μg/g), nivalenol (0.2–2.2 μg/g), and 4-acetyl-nivalenol (1.9 μg/g) were detected (Tanaka *et al.*, 2004a). In addition, an intensive survey for trichothecenes in rice was conducted in Japan (Tanaka *et al.*, 2004b). Of the rice samples, 4 of 124 were positive for deoxynivalenol (average 0.7 μg/kg) and 15 of 124 were positive for nivalenol (average 0.6 μg/kg) (Tanaka *et al.*, 2004b).

In southwest Germany, rice samples from retail markets were contaminated with deoxynivalenol and 3-acetyl-deoxynivalenol, but not nivalenol or 4-acetyl-nivalenol (Schollenberger *et al.*, 1999). In the southern United States, low levels of zearalenone and deoxynivalenol have been detected in rice (Abbas *et al.*, 2000). Neither deoxynivalenol nor nivalenol were detected in Nepalese (Desjardins *et al.*, 2000) or Saudi Arabian (Al-Julaifi and Al-Falih, 2001) rice samples.

Detection and Analysis of Mycotoxins in Rice

Due to the varied chemical structures of mycotoxins, it is not possible to use a single standard protocol to detect all of them. Practical requirements for high-sensitivity analyses and the need for a specialist

laboratory make routine analyses difficult. Simple detection methods that are fast, inexpensive, and portable and can be used by nonscientific personnel are needed and lacking. The use of various rapid screening methods, e.g., test kits and TLC, is increasingly being required by legislation for rapid analyses of mycotoxins in foods and feeds. Although the results are semiquantitative, the technique significantly reduces the number of samples that need to be analyzed in a more rigorous manner.

The analysis of a rice sample for mycotoxins follows a protocol typical for most cereal grains:

i. isolation of the mycotoxin from the sample, usually by a liquid–liquid extraction with suitable organic solvents;
ii. cleanup to remove potentially interfering components and to concentrate the mycotoxin in a solid phase extraction, a sorbent with chemically immobilized functional groups, or an immunoaffinity sorbent commonly is used; and
iii. analytical separation and quantification via a chromatographic or electrophoretic technique that may include a derivatization step to increase method sensitivity.

In rice, methods have been developed for aflatoxins (Nguyen *et al.*, 2007), beauvericin (Logrieco *et al.*, 1998), deoxynivalenol (Ehrlich and Lillehoj, 1984), fumonisins (Nelson *et al.*, 1991), ochratoxin A (Daradimos *et al.*, 2000), T-2 toxin (Pascale *et al.*, 2003), and zearalenone (Liao *et al.*, 2009). These protocols differ from one another in terms of extraction, cleanup columns, and chromatographic techniques. Rapid, quantitative methods are being developed for the simultaneous determination of mycotoxins in several types of matrices, with minimal sample preparation and cleanup (Frenich *et al.*, 2009).

Chromatographic methods, either gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS), are the best analytical approaches for reliable and accurate mycotoxin analyses, even at very low concentration levels (Frenich *et al.*, 2009). Detection by reverse-phase LC is satisfactory for all tested mycotoxins at levels close to or below the relevant maximum European Union-permitted or -recommended levels (Lattanzio *et al.*, 2007).

The analysis of mycotoxins in rice has been benefited from overall improvements in separation science. These improvements include the following:

- Reduced volume of extracting solvents.
- Simplified cleanup protocols through the development of multifunctional columns that contain both lipophilic (nonpolar) and charged (polar) active sites which retain compounds that may interfere with the analytical method while allowing the analyte(s) to pass through (Khayoon *et al.*, 2010).
- Development of ultra-high performance LC (UHPLC), which improves sensitivity, resolution, and speed (separation times of about 2 minutes) relative to conventional HPLC (Frenich *et al.*, 2009; Pastor-Montoro *et al.*, 2007).
- Troublesome derivatization procedures for some mycotoxins are expected to be replaced by the use of detectors that rely on evaporative light scattering or the capacitance-coupled conductivity.

Strategies to Reduce Mycotoxin Contamination in Rice

Toxigenic fungi, especially *Aspergillus*, are found in almost all rice samples worldwide, so proper management of rice during production and storage is critical. Adoption of good agricultural practices (GAP) including proper harvesting, storage, and transportation conditions with careful sorting and

processing helps reduce mycotoxin contamination levels (Williams *et al.*, 2008). Milled rice is usually low in mycotoxins because the toxin-containing bran fraction is removed during milling (Jayaraman and Kalyanasundaram, 1990). Moisture content is the main factor associated with mycotoxin contamination in rice. As fungi usually infect rice in the field, rapid drying of the crop after harvest and proper storage are critical to reducing contamination of the grain.

Biocontrol agents are another method to reduce mycotoxin contamination. Aflatoxin accumulation by *A. flavus* in rice scab was reduced by the presence of *A. kawachii* and *A. shirousamii* (Kumar *et al.*, 2008; Lee and Kim, 1989). The viability of conidia of *P. viridicatum*, the fungus that produced the most citrinin, was significantly reduced by *Trichoderma hamatum* (Abd-Allah and Ezzat, 2005). Competitive exclusion approaches, such as those of Atehnkeng *et al.* (2008) and Cotty *et al.* (2008), also should be tested to see if they can be used to reduce aflatoxin contamination in rice.

In 1995, the Codex Alimentarius Commission adopted safety and quality criteria for rice produced for human consumption (FAO, 2004b), including mycotoxin contamination levels. The Joint Expert Committee on Food Additives (JECFA—Scientific Advisory Body of the World Health Organization WHO) and the Food and Agriculture Organization (FAO) have evaluated hazards associated with aflatoxins, ochratoxins, patulin, fumonisins, zearalenone, and some of the trichothecenes, including deoxynivalenol (JECFA, 2000; WHO, 2002). Maximum permissible levels of mycotoxins are not standardized across countries (FAO, 2004a), with many countries completely lacking regulations on mycotoxin contamination. In general, maximum allowable limits are lower in the EU than anywhere else in the world. In some poorly developed countries, regulations are important only for export/import purposes and are effectively unenforced in domestic markets, which are essentially unregulated. Assessing the health risk posed by mycotoxins goes beyond the setting of a maximum allowable contamination level in one or more foodstuffs. A diet high in a foodstuff containing a low level of a mycotoxin may pose a higher risk than a diet that contains low amounts of a highly contaminated foodstuff. Determining potential mycotoxin exposure levels is the most important part of any risk assessment process.

Conclusions

The presence of fungi such as *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, and certain species of Mucoraceae in rice can lead to the presence of mycotoxins produced by these fungi that may cause serious toxicity and illness in humans and domesticated animals. Aflatoxin is the major mycotoxin contaminant of rice worldwide, although other compounds, e.g., ochratoxin A and trichothecenes, can be locally important in some years. As fungal contamination has significant impact on the quality of rice, rice end-products, and food safety, more attention is needed by governmental and nongovernmental agencies to further improve the quality and safety of consumed rice. As a beginning, the establishment of a maximum permissible level of the most potent and frequently found mycotoxin in rice, aflatoxin B_1, should be established in all countries where rice is a major staple food.

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22 Global Risk Maps for Mycotoxins in Wheat and Maize

Paola Battilani and Antonio F. Logrieco

Abstract

This chapter maps the worldwide risks for mycotoxin contamination with a focus on deoxynivalenol in wheat and on fumonisins and aflatoxins in maize. A modeling approach was taken with meteorological data as the input. FAO databases were used both to define crop distribution and as a meteorological data source. Simple existing models developed to predict mycotoxin contamination at harvest were adapted and used for the global forecasting. Risk maps were drawn by overlapping a layer of countries with significant wheat/maize-growing areas, a North and South latitude filter (60° and 55°, respectively, for wheat and maize), and the risk as assessed by predictive criteria. The idea of mapping mycotoxin risk in wheat and maize worldwide is ambitious because of the large amount of data required. Predictive maps are drawn as mean maps and do not consider annual and local variations, but instead stress major problems in particular areas. Annual local surveys, in specific years, may suggest a different picture of the mycotoxin risk. These discrepancies are expected because of the simple modeling approach adopted and because only meteorological data have been taken into account. Nevertheless, these simple models could provide the basis for global comparisons. The long-term goal is to study the occurrence of mycotoxins and related fungi, to monitor crop phenology and cropping systems, and to store meteorological data in a broadly accessible manner.

Keywords: aflatoxins; deoxynivalenol; disease prediction models; ear rot; ecology; epidemiology; *Fusarium* head blight; fumonisins; insects; surveys; weather

Introduction

The principal fungi associated with mycotoxin contamination are species belonging to one of the *Aspergillus*, *Fusarium*, and *Penicillium* genera that occur on cereals. *Aspergillus flavus* and *Aspergillus parasiticus* are the two species responsible for producing the widely studied aflatoxins that can be detected in many crops, with maize and peanuts cited as at particularly high risk of contamination. After aflatoxins, the most commonly reported mycotoxins in raw agricultural commodities are those produced by various *Fusarium* species. Deoxynivalenol usually is produced by *Fusarium graminearum* or *Fusarium culmorum* and is frequently found in cereal grain, especially in wheat and maize and sometimes together with other trichothecenes or zearalenone. Finally there

Mycotoxin Reduction in Grain Chains, First Edition. Edited by John F. Leslie and Antonio F. Logrieco.

are the fumonisins, which usually are produced by *Fusarium verticillioides* or *Fusarium proliferatum* and are most frequently detected in maize and its derivatives.

All of these mycotoxin-producing fungi infect host crops and produce mycotoxins in the field, so the occurrence of different toxins in the grain is related to agronomic practices, the geographic location where the crop is grown, and meteorological conditions. Postharvest contamination also is possible, but usually results only when grain is stored under unsuitable storage conditions. The major postharvest toxin is ochratoxin A, which is produced by *Aspergillus ochraceus* and *Penicillium verrucosum*.

All of these mycotoxins are regulated in maize and wheat in many countries. European Union (EU) regulations for food contaminants were first published in 2006 (European Commission, 2006), and then revised the following year for *Fusarium* toxins (European Commission, 2007). The limits are 4000 μg of fumonisin B_1 and fumonisin B_2, 1750 μg of deoxynivalenol, and 5 μg of aflatoxin B_1 per kg of unprocessed maize for human consumption; 1250 and 1750 μg of deoxynivalenol per kg of unprocessed soft and durum wheat, respectively; and 5 μg of ochratoxin A per kg of unprocessed cereal.

Global surveys have been conducted to define both fungal distribution and mycotoxin contamination, but with different approaches. Synthesizing the available data is difficult because of their heterogeneity and the spot distribution of samples analyzed. To build a picture of the global risk posed by the main mycotoxins, an approach other than simply merging all of the available data was necessary.

The objective of this chapter is to describe some epidemiological and ecological aspects of toxigenic fungi involved in wheat and maize diseases and the models developed to predict the level of risk for mycotoxin contamination of these grains at harvest (de Wolf and Paul, 2014). We did not consider the important role that cropping system can play in determining the level of mycotoxin contamination. Crop distributions were mapped, and historical meteorological data were selected from worldwide databases based on the time when the crops of interest would be susceptible to these fungi. Available predictive models were run with meteorological data as the input and the model output was mapped to obtain a picture of the risk level posed by key mycotoxins worldwide.

Fusarium Head Blight of Wheat

Causal Agents and Symptoms

Fusarium head blight of wheat and other small cereals is a severe disease worldwide that can significantly reduce crop yield (Windels, 2000). An etiological characteristic of *Fusarium* head blight is the co-occurrence, or the quick succession, of several *Fusarium* species usually referred to as a "complex" (Logrieco *et al*., 2007), and as many as nine different *Fusarium* species may be recovered from a single fragment of infected tissue or as many as 17 different *Fusarium* species from freshly harvested grain samples collected in a limited geographic area. However, only a relatively few of these species are regarded as pathogenic and, generally, only one or a few species dominate at any particular location. However, if the dominant pathogenic *Fusarium* species are absent, then strains of the less pathogenic or more opportunistic *Fusarium* species that also can produce considerable amounts of mycotoxins may be found instead. Thus, the toxigenic profile of a contaminated crop is a product not only of the dominant pathogenic *Fusarium* species, but also of the opportunistic species that compose the rest of the "complex." The pathogen most commonly associated with head blight of wheat and other small grains worldwide is *F. graminearum* (teleomorph—*Gibberella*

Table 22.1 Major *Fusarium* species involved in ear diseases of cereals and their cardinal values of temperature (T) and water activity (a_w) for growth and mycotoxin production (FAO/IAEA, 2001; Leslie and Summerell, 2006; Magan and Lacey, 1984)

		Growth		Toxins		
Fusarium species	Mycotoxin(s)	T	a_w min	T_{opt}	a_w opt	A_w min
F. culmorum	Type B trico	20–25	0.90	25–28	0.97	
	Zearalenone			17–28	0.97	
F. graminearum	Type B trico	24–28	0.90	25–28	0.97	0.92
	Zearalenone			17–28	0.97	
F. poae	Type A trico	20–25		20–25	0.99	
F. proliferatum	Fumonisins	30	0.87	15–30	0.98	0.90
F. subglutinans	Fumonisins	15–25		15–30	0.98	
	Moniliformin			25–30		
F. verticillioides	Fumonisins	30	0.87	15–30	0.98	0.90

Trichothecenes: Type A trico—diacetoxyscirpenol, HT-2 toxin, monoacetoxyscirpenol, and neosolaniol, T-2 toxin; Type B trico—deoxynivalenol (vomitoxin), diacetyl deoxynivalenol, fusarenone-X, monoacetyl deoxynivalenols, and nivalenol.

zeae), while *F. culmorum*, *F. avenaceum* (teleomorph—*Gibberella avenacea*), and *F. poae* are more often associated with *Fusarium* head blight in cooler regions, e.g., northern Europe (Table 22.1).

Toxin Formation

From the mycotoxicological point of view, *Fusarium* head blight is the greatest concern in wheat, because it can lead to mycotoxin accumulation, primarily in scabby grain, included in human foods and animal feeds. This fungus also can contaminate forage and straw, which poses an additional risk for livestock. The most frequently encountered *Fusarium* mycotoxin associated with *Fusarium* head blight is deoxynivalenol, also termed vomitoxin, and its mono- (3-acetyl deoxynivalenol and 15-acetyl deoxynivalenol) and diacetylated (3,15-diacetyl deoxynivalenol) derivatives, produced by strains of *F. graminearum* and *F. culmorum*. Other type B trichothecenes reported to occur in *Fusarium* head blight-afflicted grains include nivalenol and its monoacetylated derivative fusarenone-X and the diacetylated derivative (4,15-diacetyl nivalenol), synthesized by strains of *F. cerealis* (syn. *F. crookwellense*), *F. graminearum*, *F. culmorum* and, particularly in northern areas, also by *F. poae*. Zearalenone often co-occurs with deoxynivalenol and usually is produced by strains of *F. graminearum* and *F. culmorum* (Logrieco *et al*., 2007).

In some *Fusarium* head blight epidemics, strains of *F. sporotrichioides* and *F. poae* may be found and produce type A trichothecenes, e.g., T-2 toxin, HT-2 toxin, and diacetoxyscirpenol (Torp and Langseth, 1999). In northern European countries and Canada, as a consequence of the widespread distribution of *F. avenaceum*, both the increasing and consistent occurrence of moniliformin and enniatins are being reported in *Fusarium* head blight-afflicted grains (Logrieco *et al*., 2002).

Epidemiological Notes

Wheat is susceptible to *Fusarium* head blight at flowering, and meteorological conditions near anthesis are crucial for infection. *Gibberella zeae* ascospores are splash-borne and windborne, and rain during anthesis is needed to initiate a *Fusarium* head blight epidemic. A warm, moist

environment characterized by frequent precipitation and/or heavy dew favors fungal growth, infection, and disease development in head tissues. No other pests or diseases are known to alter *Fusarium* head blight severity. Visible symptoms can appear on individual kernels, single ear spikelets, or entire heads, all leading to scab of the kernels. Salmon-orange mold may grow on heads exposed to continuous rain after flowering. Important epidemiological issues have arisen recently and include an apparent shift in the community of *Fusarium* species on infected heads in Europe towards *F. graminearum* and away from *F. culmorum* (Osborne and Stein, 2007). The reason for this shift is not known, but may include an increase in no-till cultivated maize in areas where maize has not traditionally been a major crop.

Mycotoxins in Maize

Maize is one of the crops most susceptible to mycotoxins wherever it is grown. Maize may be colonized by strains from numerous *Fusarium* spp., including *F. graminearum, F. verticillioides* (teleomorph—*Gibberella moniliformis*), *F. proliferatum* (teleomorph—*Gibberella intermedia*), and *F. subglutinans* (teleomorph—*Gibberella subglutinans*), all of which can cause maize ear and/or stalk rot. Maize also can be colonized by mycotoxin-synthesizing strains from *Aspergillus* section *Flavi*. The dominant species in any given year depends upon the meteorological conditions in the regions of cultivation. In fact, the optimal ecological conditions for growth and mycotoxin production differ for these important genera. *Fusarium graminearum*, *F. verticillioides*, and *A. flavus* usually become more common as the climate goes from cool and wet to hot and dry and from higher latitudes to lower ones.

Fusarium Ear Rot of Maize

Causal Agents and Symptoms

Fusarium verticillioides is the fungus most frequently isolated from maize. It is associated with disease at all stages of plant development and may even be endophytic. Colonization of the ear is crucial for mycotoxin production. Two maize ear rots have been described: *pink ear rot* or *Fusarium* ear rot, usually caused by members of the *Liseola* section, such as *F. verticillioides*, *F. proliferatum*, and *F. subglutinans,* and *red ear rot* or *Gibberella* ear rot, usually caused by species in the *Discolor* section, with *F. graminearum* the most common species. Pink ear rot is common in moderately dry, warm climates, while red ear rot is common in years and locations characterized by frequent rainfall and lower temperatures during the summer and early autumn (Logrieco *et al*., 2002).

Pink Fusariosis or Fusarium Ear Rot

Causal agents and symptoms. The species commonly isolated from maize "pink ear rot" are *F. verticillioides*, *F. proliferatum*, and *F. subglutinans*. In maize "pink ear rot," the kernels may be infected through the silks as the silks emerge to be pollinated, but the pathogen also may colonize the ear from the tip via external infections. In association with a more generalized colonization that originates from the ear's tip, a "random kernel rot" type of symptom also can occur. In this case, apparently randomly scattered individuals or groups of kernels, usually tan to brown, develop white-pink mycelia under wet conditions (Warfield and Gilchrist, 1999).

Toxin formation. High levels of fumonisin B_1 frequently are reported in freshly harvested maize ears infected with *F. verticillioides* or *F. proliferatum. Fusarium proliferatum* can grow and synthesize fumonisins under drier grain conditions than are suitable for *F. verticillioides* (Marín *et al*., 1995). These differences in response to temperature and A_w suggest that the frequency of these species may be governed by weather conditions and that these variables could impact fungal succession on maize ears and the onset of maize ear rot. In maize pink ear rot, which often is associated with high levels of fumonisin B_1 contamination, the co-occurrence of moniliformin, beauvericin, and/or fusaproliferin produced by *F. proliferatum* and/or *F. subglutinans*, but not *F. verticillioides*, also has been reported (Logrieco and Moretti, 2008).

Epidemiological notes. The *F. verticillioides*–maize pathosystem is complex, and the relative importance of different inoculum sources is not settled (Munkvold and Desjardins, 1997). There are three main pathways for *in planta* kernel infection: (i) air- or splash-borne infection by conidia through silks or wounds; (ii) systemic growth of the pathogen via the stalk; and (iii) infection through a wound made by a spore-carrying insect(s). As few macroconidia usually are produced by these three species, microconidia are commonly the most important infectious spore produced.

In the first pathway, the inoculum consists of conidia produced on crop residues and on tassels (Rossi *et al*., 2009). These conidia are air or splash dispersed and infect ears through silks or wounds (Ooka and Kommendahl, 1977). Such infections almost always cause a primary disease infection and, in this context, may be the most important.

The second pathway involves the systemic growth of the pathogen within the plant and is thought to be of the least importance (Marín *et al*., 1996; Munkvold and Carlton, 1997).

In the third pathway, conidia reach ears or are dispersed on ears, via spore-carrying insects, mainly European corn borer larvae, *Ostrinia nubilalis* (Le Bars *et al*., 1994), which can burrow in systemically infected stalks and penetrate through bracts.

Symptoms of *Fusarium* ear rot are visible at the tip of the ear and consist of a white-pink mold colony on the silks that may grow with the silks from the tip of the ear to its base. As the ear ripens, the fungal mycelia darken and become less prominent. Following European corn borer attacks, kernels damaged by the insects often are covered by fungal growth. Production of fumonisins in grain before harvest has been documented under many environmental conditions. Nevertheless, optimal conditions for the development of maize ear rot by *F. verticillioides* are warm to hot temperatures and dry conditions at silk emergence and during grain-filling (Munkvold, 2003).

Red Ear Rot or Gibberella Ear Rot

Causal agents and symptoms. The most common species causing maize "red ear rot" are *F. graminearum*, *F. culmorum*, *F. cerealis*, and *F. avenaceum*. In maize "red ear rot" the pathogen, most commonly *F. graminearum*, usually begins to grow from the tip of the ear with a reddish mold colony covering most of the ear. The blue-black perithecia of the teleomorph, *G. zeae*, are commonly observed on infected husks and ear shanks. The severity of the disease, and the occurrence and prevalence of the causal species, may vary by region and year. This variation depends primarily on climatic parameters, e.g., temperature, relative humidity, rain, and location, and also on the farming system, e.g., tillage, crop rotation, fertilization, planting area, irrigation, and disease and pest control.

Toxin formation. In relation to disease severity, it is possible to find, unexpectedly, very high levels of mycotoxins. Maize red ear rot with a high concentration of deoxynivalenol occurs often in years with a cool rainy autumn. Long season hybrids, e.g., FAO class 500–700, are particularly

vulnerable. Although zearalenone may accumulate in *Fusarium*-infected cereals before harvest, this toxin also may be synthesized in storage if proper drying and storage practices are not followed (Channaiah and Maier, 2014).

Epidemiological notes. *Gibberella* ear rot and deoxynivalenol contamination occur worldwide, but are more severe in cool humid areas. *Fusarium graminearum* overwinters in maize debris and other crop residues. The disease is favored by cool, wet weather at silk emergence. Ascospore dispersal is promoted by rainfall, high humidity (>60%, with an optimum at 80%), and temperature >25°C. Heavy rainfall late in the season increases disease severity and mycotoxin production.

The fungus infects silks, grows into the ear, and progresses down the ear during grain fill. Infections also may occur at the base of the ear when heavy rainfall occurs late in the season. The most visible symptoms on ears are the reddish mold at the tip of the ear.

Aspergillus Ear Rot

Causal Agents and Symptoms

Aspergillus flavus and *A. parasiticus* are the causal agents of *Aspergillus* ear rot in maize. These fungi are morphologically similar, with *A. flavus* the most common on maize. Damage to the ear caused by this fungus is very limited in most years, with only a few kernels visibly infected. The disease is more serious in geographic areas with high temperatures and drought conditions. Infected kernels often are covered by a yellow-green mold. Spores contribute to the visible symptoms and they may turn dark green, brown, or black, but retain more yellow color than do colonies of other fungi like *Penicillium* or *Trichoderma* spp. Injured kernels are best suited for fungal sporulation. The fungus also may be present in kernels with no visible sporulation, although these kernels often appear to be dull or discolored.

Toxin Formation

Aspergillus-infected material is always of concern due to its potential contamination with aflatoxins. *Aspergillus flavus* produces only aflatoxins B_1 and B_2, while *A. parasiticus* produces all four principal aflatoxins—B_1, B_2, G_1, and G_2 (Diener *et al*., 1987). However, Gabal *et al*. (1994) described a large number of *A. flavus* strains that produce aflatoxin G_1 and a smaller group that produces aflatoxin G_2. The International Agency for Research on Cancer classified aflatoxin B_1 as a class 1 carcinogen because of its demonstrated carcinogenicity to humans. All aflatoxins are regulated in most countries throughout the world, including Europe, in various products but almost always in maize and milk (European Commission, 2006, 2010).

Epidemiological Notes

Aspergillus ear rot is a serious disease when high temperatures and drought occur and maize is growing in the field, e.g., the southern United States and many parts of Africa, where *A. flavus* is a common pathogen and aflatoxin contamination is a chronic problem. Spores of *Aspergillus* spp. are airborne and insects can contribute to their dispersal. Maize kernels may be colonized with *A. flavus* shortly after silk emergence. The fungus can colonize silk tissues and grow down the ear. Colonization of the silks and kernel surfaces occurs soon after silk emergence and may continue and

increase throughout the season. Internal kernel infections are usually low, but may occur directly through the silks or through cracks and intercellular gaps. Under conditions of high temperature and low a_w, *A. flavus* becomes very competitive and may be the most common fungal species in the soil (Payne, 1998).

Ecology and Modeling

Ecology of Fusarium *spp.*

The factors contributing to the development of *Fusarium* head blight and ear rot and subsequent mycotoxin contamination include temperature (T), relative humidity (RH), amount and timing of rainfall (R), wind (W) patterns, and niche competition. The influence of meteorological factors on *Fusarium* diseases is complicated by the fact that strains of *Fusarium* spp. may cause disease individually or as part of a species complex. In general, the optimal conditions for the development of *Fusarium* head blight of small grains include moderately warm temperatures and rain at anthesis. The same general conditions are necessary for the development of *F. graminearum* ear rot of maize, including high rainfall during maturation (Munkvold, 2003). Warm temperatures and moderate rainfall are more conducive for infections by *F. verticillioides* and other species in the *Liseola* group. The influence of climatic factors on *Fusarium* spp. pathogenic to cereals has been reviewed by Doohan *et al*. (2003), who focused on the climatic distribution of species. Further contributions (Table 22.2) were made by Ramirez *et al*. (2006), Hope *et al*. (2005), Llorens *et al.* (2004), and Marín *et al*. (2004).

Ecology of Aspergillus *spp.*

Aspergillus flavus can grow across a wide range of temperatures. Optimal growth of *A. flavus* on most substrates occurs between 19°C and 35°C, with minimum and maximum temperatures of 12°C and 43°C, respectively. Conidia of *A. flavus* germinate very rapidly at $a_w > 0.90$ with an almost linear increase with time at 25°C. However, at lower levels of a_w, germination can be very slow (Marín *et al*., 1998). The range for a_w conditions at optimal temperatures for germination is generally broader than the range for mycelial growth (Magan and Lacey, 1984; Marín *et al*., 1998). The optimum temperature for aflatoxin B_1 synthesis was first reported as 24–32°C depending on the substrate (Northolt *et al*., 1977) with other investigations later broadening this range to 20–35°C. Generally, 28°C is accepted as the optimum temperature for aflatoxin production (Sanchis and Magan, 2004). Water activity (a_w) also has a significant impact on fungal growth and mycotoxin production. *Aspergillus* strains can grow and produce mycotoxins at a_ws as low as 0.73 and 0.85, respectively (Sanchis and Magan, 2004). The minimum a_w for *A. flavus* to grow on maize is 0.73 with a temperature of 26–32°C. Aflatoxin production limits are similar within this temperature range at a_ws from 0.85 to 0.89.

Predictive Models in Wheat

Fusarium head blight is a serious disease worldwide and models are available to predict (i) the need for and timing of fungicide sprays to control the disease, (ii) the dynamics of the disease and

Table 22.2 *Fusarium* species and mycotoxins occurring in cereals

Fusarium species	Climate			Mycotoxins
	Cool–temperate, rain[a]	Warm–temperate, rain[b]	Warm, dry[c]	
F. acuminatum	–	±	±	MON, ENs, BEA
F. arthrosporioides	+	+	–	ENs, BEA
F. avenaceum	++++	+	++	MON, ENs
F. cerealis	+	+	±	NIV, FUS, ZEN, ZOH
F. culmorum	+	+++	++	DON, ZEN, ZOH, NIV
F. equiseti	+	++	+	DAS, ZEN, ZOH
F. graminearum	++	++++	+	DON, NIV, ZEN, AcDON, FUS
F. langsethiae	++	+	–	T2, HT2, NEO, MAS, DAS, BEA, ENs
F. oxysporum	–	±	–	MON
F. poae	+++	++	++	NIV, DAS, FUS, ENS, BEA
F. proliferatum	–	–	±	FB_1, FB_2, FUP, MON, BEA
F. solani	–	±	–	–
F. sporotrichioides	++	+	±	T2, HT2, NEO
F. subglutinans	–	±	–	MON, BEA, FUP
F. tricinctum	+	+	+	MON, ENs, BEA
F. verticillioides	–	–	±	FB_1, FB_2, FB_3

Abbreviations: AcDON, monoacetyl deoxynivalenols (3-AcDON, 15-AcDON); BEA, beauvericin; DiAcDON, diacetyl deoxynivalenol (3,15-AcDON); DAS, diacetoxyscirpenol; DON, deoxynivalenol (vomitoxin); ENs, enniatins; FB_1, fumonisin B_1; FB_2, fumonisin B_2; FB_3, fumonisin B_3; FUP, fusaproliferin; FUS, fusarenone-X (= 4-acetyl-NIV); HT2, HT-2 toxin; MAS, monoacetoxyscirpenol; MON, moniliformin; NEO, neosolaniol; NIV, nivalenol; T2, T-2 toxin; ZEN, zearalenone; ZOH, zearalenols (α and β isomers).

[a]Scandinavian countries, Russia, Canada.

[b]Central European countries, northern United States, Argentina.

[c]Southern European countries, Mediterranean countries, central United States.

deoxynivalenol accumulation during ripening, and (iii) deoxynivalenol contamination at harvest (Table 22.3).

- Moschini and Fortugno (1996) used linear regression to correlate meteorological variables with the incidence of *Fusarium* head blight at harvest. The time period considered was from 8 days before until 26–32 days after heading (defined as 539 degree days, with a base of 0°C). The weather variables considered were rain, RH $> 83\%$, and $T_{\min}$ and $T_{\max}$.
- Hooker *et al.* (Hooker and Schaafsma, 2005; Hooker *et al.*, 2002; Schaafsma and Hooker, 2007) used multiple meteorological parameters to predict deoxynivalenol contamination at harvest. They considered rain and $T_{\min}$ 4–7 days prior to heading, and at least one parameter from amongst rain, RH, T_{avg} and $T_{\max}$ from days 3 to 18 after heading. They also considered rain near harvest. Agronomic practices also were included with cultivar weighted the most heavily.
- de Wolf *et al.* (2003) used a stepwise logistic regression to make predictions. For 7 days prior to anthesis they followed rain and temperature between 15°C and 30°C. For the 10 days following anthesis they measured the number of days with temperatures between 15°C and 30°C and RH $> 90\%$.
- Weather radar data on rain were used by Detrixhe *et al.* (2003) to simulate leaf wetness duration as the parameter most strongly related to disease outbreaks.
- FHB Wheat (Rossi *et al.*, 2003) is a model based on system analysis and more detailed input data are requested. Hourly data on air temperature, RH, and rain are inputs and the daily risk

Table 22.3 Models developed worldwide to predict mycotoxin contamination in wheat and maize

Model	Country	Authors	Prediction
Wheat			
Predictive index	Argentina	Moschini and Fortugno (1996)	FHB incidence at harvest
DONcast[a]	Canada	Hooker *et al*. (2002)	DON at harvest
Logistic DON	USA	de Wolf *et al*. (2003)	FHB epidemics
Weather radar data	Belgium	Detrixhe *et al*. (2003)	FHB infection
FHB wheat	Italy	Rossi *et al*. (2003)	Dynamic of DON risk
Neural network	Czech Republic	Klem *et al*. (2007)	DON at harvest
FUsaProg	Switzerland	Musa *et al*. (2007)	DON at harvest
Maize			
Fusarium ear rot	Italy	Battilani *et al.* (2003)	Daily FUM risk
DONcast	Canada	Schaafsma and Hooker (2007)	FUM at harvest
Logistic FUM	Italy	Battilani *et al.* (2008a)	FUM at harvest
Logistic AFB_1	Italy	Battilani *et al.* (2008b)	AFB_1 at harvest
FUMAgrain	Italy	Maiorano *et al*. (2009)	FUM at harvest

[a] http://www.weatherinnovations.com/DONcast.cfm. FHB, *Fusarium* head blight; DON, deoxynivalenol; FUM, fumonisins; and AFB_1, aflatoxin B_1.

for *Fusarium* head blight and deoxynivalenol contamination risk are predicted from anthesis to harvesting. This model is integrated into a decision support system in which the role of the host species (soft and durum wheat) and variety, previous crop, and soil tillage also are included (Rossi *et al.*, 2007),

- A neural network is a different approach for modeling that was used by Klem *et al.* (2007) to predict deoxynivalenol levels. April T_{avg} and sum of rain, as well as T_{avg} and the sum of rain 5 days prior to anthesis were considered, together with the previous crop, with the last two parameters identified as the most important.
- A decision support system was prepared in Switzerland by Musa *et al.* (2007). This system considers several cropping factors including previous crop, soil and straw management, and wheat variety susceptibility to *Fusarium* head blight together with prevailing weather conditions to make predictions.

The published models are all based on very different approaches, but they all confirm the central role played by weather data. Narrow time periods around crop anthesis were commonly considered and, in general, the prediction was reasonably good when using only relatively simple models.

Modeling in Maize

Forecasting deoxynivalenol and fumonisin contamination in maize is more difficult than in wheat, because the ear is exposed to infection for a longer period and wounding, primarily by insect pests, plays an important role and can significantly change the level of contamination. Only a few models have been developed for maize (Table 22.3).

- A prototype mechanistic model was published in 2003 (Battilani *et al*., 2003), but no published validation of this model is available. The model uses hourly meteorological data (air temperature, RH, and rain) as inputs and predicts *Fusarium* ear rot and fumonisin contamination risks on a

daily basis, from silking to harvesting. The resulting relational diagram was later updated by Maiorano *et al*. (2009), who added a sub-model to account for European corn borer effects. European corn borer damage was estimated based on a summation of degree days (base 10°C) from January 1.

- A preliminary model was developed for maize by using procedures similar to the DONcast predictive model (Schaafsma and Hooker, 2007). Several climatic variables were measured in the period 10 days before and 14 days after silking: $T_{min} < 15$°C, $T_{max} > 34$°C, and rain > 2 mm were considered as negative, negative, and positive, respectively, when observed between 4 and 10 days before or between 2 and 8 days after silking. $T > 34$°C was considered as favorable for fumonisin biosynthesis when measured between 4 days before and 2 days after silk emergence, while rain was considered negative between 8 and 14 days after silking. The severity of borer attacks to the ear was included as a significant predictor of fumonisin contamination in the model (Hooker and Schaafsma, 2005; Schaafsma and Hooker, 2007).
- The cropping system is important in predicting fumonisin contamination, as confirmed by several authors (Battilani *et al*., 2008a; Schaafsma and Hooker, 2007). Battilani *et al*. (2008a) developed a model based on logistic regression to quantify the effects of sand content in soil, previous crop, maize hybrid, sowing and harvest times, nitrogen manure applied, and grain humidity at harvest. All the parameters considered influence fumonisin contamination, but the model developed could explain ~60% of the observed variation with the atitude of the growing area, season length of the hybrids, and the available growing weeks, i.e., the number of weeks between sowing and harvest.
- A similar approach was used by Battilani *et al*. (2008b) to estimate the probability of aflatoxin B_1 contamination in maize at harvest. An aridity index was computed, on a rolling 10-day base, to summarize meteorological conditions. The aridity indexes of the last 10 days of June, and the first and the last 10 days of August were chosen by the stepwise approach as relevant. The model gave 64% correct predictions and acceptable warning on aflatoxin B_1 contamination in maize. Thirteen percent of the predictions were underestimates, but only half of these were related to fields with aflatoxin B_1 levels >5 μg/kg.

No models predicting deoxynivalenol levels in maize are available.

Global Crop Distribution and Meteorological Conditions

Global Distribution of Maize and Wheat

Data on the global distribution of selected crops were extracted from FAOSTAT, a database managed by FAO (http://faostat.fao.org/site/291/default.aspx). FAOSTAT provides access to over 3 million time series and cross-sectional data points relating to food and agriculture. It contains a full matrix of integrated and compatible statistics that cover 200 countries, 15 years, and more than 200 primary products and input items related to production, trade, resources, consumption, and prices.

Countries that contained ≥0.2% of the world's land used to grow wheat or maize were selected for modeling. Wheat-growing areas were limited to between 60° North and South latitude, while maize was limited to latitudes between 55° North and South. No restrictions were placed on altitude because the latitude limit for cultivation results from an interaction between latitude and altitude and a general rule, applicable worldwide, is not available.

Table 22.4 World meteorological data available in FAOCLIM

Variable	Number of stations	EMD[a]
Mean temperature	20,828	48
Mean minimum temperature	11,550	65
Mean maximum temperature	11,544	65
Rainfall	27,375	42

[a]EMD is the effective maximum distance (in km) from the estimated point to the nearest station; it is half the distance between two stations if the stations are homogeneously distributed.

Climatic Database

The relevant role of meteorological/ecological parameters was confirmed for all of the diseases described. Consequently, the risk of mycotoxin contamination in wheat and maize can be assessed, on a global level, by starting with a description of meteorological conditions in the crop-growing areas.

Reliable data collected from meteorological stations well-distributed worldwide, with a sufficiently long history, were used to describe the areas and as data for modeling to predict the risk of mycotoxin occurrence. We used the FAOCLIM database (ftp://ext-ftp.fao.org/SD/SDR/Agromet/New_LocClim/) as the source for our meteorological data. This database contains monthly data for up to 14 observed and computed agro-climatic parameters, of which average temperature and rainfall are the most commonly available (Table 22.4). The time series differ significantly in length and accuracy relative to continent. The average length of each data series is ∼50 years, with some series exceeding 200 years (the longest rainfall series covers 299 years). At the other extreme, 185 rainfall series include only a single year. Eighty percent of the series exceed 20 years and ∼45% are longer than 50 years. Ninety percent of the rainfall time series and 86% of temperature series have no gaps, i.e., on average a missing data point occurs no more than once every 10 years for each rainfall time series.

Temperature and rain data were extracted from FAOCLIM for all of the stations in the geographic area of cultivation for the selected crops and for the time periods considered appropriate for mycotoxin production. This data set was used to predict global risks for mycotoxin contamination in wheat and maize.

Global Risk Maps for Mycotoxins in Wheat and Maize

Risk of Deoxynivalenol Contamination of Wheat

Previous models of *Fusarium* head blight in wheat (de Wolf and Paul, 2014; de Wolf *et al*., 2003; Hooker *et al*., 2002; Klem *et al*., 2007; Moschini and Fortugno, 1996) all agree that the meteorological conditions around heading or anthesis are important. In particular, rainfall during the week before and temperature during the week before and after crop heading/anthesis are crucial. All of the studies used daily, and sometimes hourly, data. This level of precision is not available globally and we used monthly average meteorological data as our input.

Globally, April and May, respectively, were assumed to be the heading months up to 45° North latitude and between 45° and 60° North latitude, with October and November the corresponding

months in the Southern hemisphere. Conditions that determine the risk of deoxynivalenol contamination in wheat at harvest were taken as rainfall >15 mm and temperatures of 15–30°C during April up to 45° North latitude, and in May with higher latitudes, with October and November substituted for Southern latitudes.

Risk of Fumonisin Contamination in Maize

Predictions of fumonisin contamination in maize with only meteorological parameters were evaluated in only one paper (Schaafsma and Hooker, 2007), and the importance of pest attacks was emphasized. Similar to the conditions used to predict *Fusarium* head blight in wheat, the authors defined the 10 days before and the 14 days after silk emergence as crucial and considered rain and temperature to be the critical meteorological parameters. Based on this study, conditions that determine the presence of fumonisin B_1 were identified as rainfall >15 mm and temperatures between 15°C and 34°C during July/January and rainfall >15 mm in August/February, with July/January considered the months for silk emergence.

Risk of Aflatoxin B_1 Contamination in Maize

The approach used by Battilani *et al*. (2008b) was followed to predict aflatoxin contamination, as this is the only published predictive model for aflatoxins in maize. Temperature and rain data were organized as climatograms, and a graphic display of temperature and rain commonly used by meteorologists and climatologists to represent the general climate of a region (Weltzien, 1983) prepared monthly. Two parameters were computed: AURC, the area under the rain curve, and AUTC, the area under the temperature curve. The overlapping of the two areas was calculated as

$$\mathrm{AI} = \mathrm{AURC} - \mathrm{AUTC}.$$

AI, the aridity index, is a measure of aridity with values <0 indicating aridity at the site being evaluated. The months relevant for the prediction were June–September and December–March, depending on the hemisphere. Mean monthly temperatures >20°C in at least two of these months were a mandatory condition. With the logistic regression model (Battilani *et al*., 2008b), the probability of maize kernel contamination >2 μg/kg, which would lead to contamination greater than the legal limit of 5 μg/kg for maize destined for human consumption (European Commission, 2006), was computed by using aridity index values as the input data.

Logistic regression is a multivariate technique for estimating the probability that an event occurs. This regression is useable whenever the dependent variable is binary and enables the estimation of the probability of an event that occurs (dependent variable) based on independent variables. The probability (P) values are scaled from 0 to 1, with $P < 0.3$ as a low risk, P between 0.3 and 0.7 as a middle risk, and $P > 0.7$ as a high risk.

Geostatistical Data Analysis

The data selected for modeling were based on criteria described for risk assessment and a geostatistical approach was taken to prepare the data set for mapping. The geostatistical approach is

appropriate when the variables are continuous and have a strict geographic dependence. In particular, we used the kriging method to obtain the best linear unbiased estimates of spatially dependent data (Isaaks and Srivastava, 1989). Kriging is a means of locally averaging the weights of data from sampled locations that surround an unsampled location based on statistical similarity to the unsampled location. This approach can differentiate variables on a geographic basis and, as user-friendly output, be used to draw maps in which the gradient of the variables is highlighted. The parameters, temperature and rain in the selected time period and areas, were entered into the geostatistical analysis module of ArcView 8.2.

Cross-validation was used to check the accuracy of estimates obtained by kriging. Cross-validation consists of using the spatial model to predict a value at each sampled point and then comparing the predictions with the observed data. The difference between the predicted and the observed values is the error of the estimate. The results of the spatial analysis are represented as raster maps with each country's administrative boundaries as a cartographic base. A raster data set is composed of cells all of the same size. Each cell, or pixel, is a square that represents a specific portion of an area. Cells are arranged in rows and columns, to produce a Cartesian matrix. All of the locations in the studied area are covered by the matrix and a specific value is assigned to each cell by kriging.

Maps were drawn and their reliability confirmed by cross-validation; the errors were acceptable for all parameters. Temperature was estimated more accurately than rain, as expected, due to the higher variation in rainfall between neighboring locations. The kriging models were efficient and effective and could explain a portion of the variance.

Risk Maps for Mycotoxins in Wheat and Maize

Risk maps are the result of overlapping different layers, as previously described. The first layer consists of countries that contain $\geq$0.2% of the world's land planted to wheat or maize. In the second layer, the North and South latitude filter was imposed, 60° and 55°, respectively, for wheat and maize. Finally, the risk assessed by predictive criteria was drawn on the resulting area.

Risk Map for Deoxynivalenol in Wheat

The global risk map (Figure 22.1a) for deoxynivalenol contamination at harvest in wheat divides into low/absent risk and high risk. Seven countries—Canada (4.4%), Kazakhstan (5.7%), Australia (6%), the United States (10%), China (10.5%), Russia (11.5%), and India (12.4%)—collectively represent ~60% of the world's wheat-growing area. All of these countries include high-risk areas for deoxynivalenol contamination, according to the model, except for Canada. However, due to the large size of these countries, the risk could be quite different at different locations within the country. Other high-risk areas occur in South Africa, Ethiopia, Mexico, Brazil, and Argentina.

Risk Maps for Fumonisin B_1 and Aflatoxin B_1 in Maize

India (5.1%), China (18.4%), and the United States (19.6%) also are important countries for maize production and together with Mexico (4.5%) and Brazil (8.9%) collectively represent ~60% of the global area planted to maize. As for the map of deoxynivalenol contamination in wheat, the risk map for fumonisin B_1 contamination at harvest in maize worldwide (Figure 22.1b) is divided

into low/absent or high, but the global risk map for aflatoxin B_1 contamination (Figure 22.1c) has low/absent, middle, and high-risk regions.

In India, the risk for fumonisin B_1 contamination is high while the risk for aflatoxin B_1 contamination is low/absent. A similar scenario is predicted in China, except for the northwestern part of the country, where the risk for aflatoxin B_1 contamination is high. The risk for fumonisin

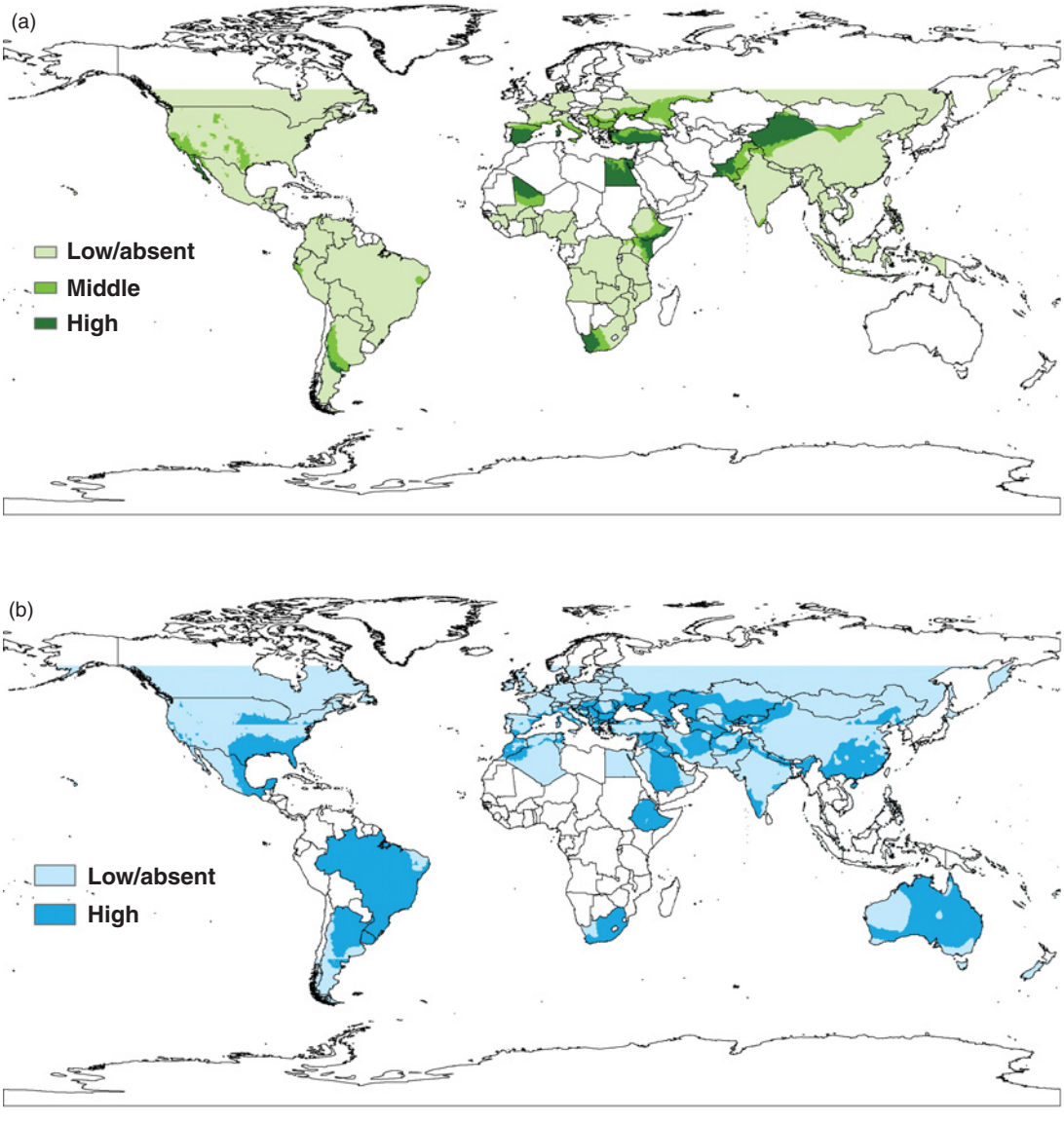

Figure 22.1 (a) Global risk of deoxynivalenol contamination in wheat. The prediction is based on monthly data for mean temperature and rain occurring near heading. (b) Global risk of fumonisin contamination in maize. The prediction is based on monthly data for mean temperature and rain occurring near silk emergence. (c) Global risk of aflatoxin B_1 contamination in maize. The prediction is based on the aridity index during heading and ear ripening. (For a color version, see the color plate section.)

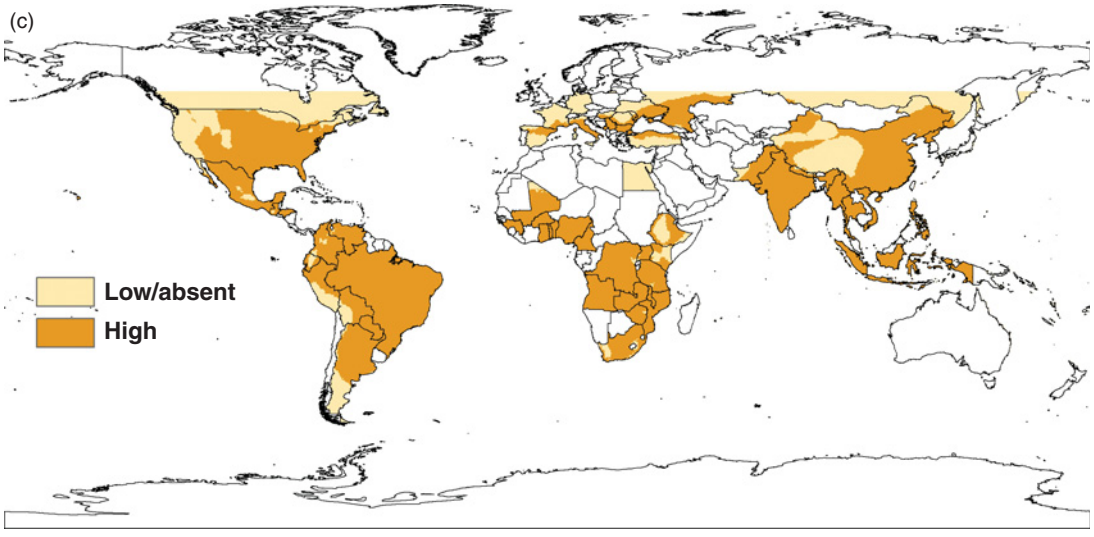

Figure 22.1 (*Continued*)

B_1 contamination also is high in the United States, Mexico, and Brazil. In general, the risk for fumonisin B_1 contamination is high in all of the world's maize-growing areas except the highest latitudes. The middle risk areas for aflatoxin B_1 are limited to smaller areas. The Mediterranean basin is a suitable area for *A. flavus*, and Spain, Turkey, and Egypt are all predicted low-risk areas for aflatoxin B_1 contamination. Other areas associated with high risk for aflatoxin B_1 contamination are found in Africa, particularly in northern Mali close to the Sahara desert, Ethiopia and Kenya along the Somalia border, and in southwestern South Africa. In the Americas, only California and part of Argentina are low-risk areas for aflatoxin B_1.

Closing Comments

Fungi are heavily influenced by microclimate and the shorter the time interval used for meteorological data collection, the more precise the impact on fungal growth can be described. The idea of mapping the global mycotoxin risks in wheat and maize is ambitious because of the large amount of data required and because of the broad vision of potential problems that results. Monthly data were used instead of daily/hourly meteorological data, historical mean data were used instead of data collected in the current year, predicted instead of observed crop growth stages were considered, and whole countries instead of only the relevant growing areas inside a country were used to run the models. These modifications were adaptations to the limited data detail available and/or to the lack of data. Consequently, the predictive maps (Figure 22.1) are mean maps and do not consider annual and local variations. Instead, they stress the major problems in certain geographic areas from a global perspective. No validation of these risk maps is planned, primarily because available data are very detailed for some areas and almost completely absent for others.

The relationship of yearly data to the mean meteorological conditions of a geographic area for estimating mycotoxin contamination risks has been discussed in both Battilani *et al.* (2008b) and Schaafsma and Hooker (2007). From this perspective the mean maps in this chapter have limited

value for any particular geographic point and should instead be used to identify areas with common problems. This approach also can help predict the effect of atypical meteorological conditions in an area or draw conclusions regarding the effect of climate change. Local surveys in specific years may suggest a different picture of the mycotoxin risk. This variation is expected because of the very simple approach followed in constructing the current models and because only meteorological data have been taken into account. Nevertheless, the approach followed could form the basis for a global approach with common milestones.

This chapter was designed as the basis for a global project to study the occurrence of mycotoxins and related fungi, to monitor crop phenology and cropping systems, and to store meteorological data for a shared analytical approach. This data set could be used to develop and validate new models with particular areas of interest. Such a data set must contain:

- the year in which the survey was conducted;
- a geo-reference for the grain sample(s);
- clear description of the methodology applied, both for the sampling and the analysis; the limit of quantification of the analytical method(s) used, and the level of precision of fungal identification

so that researchers with different objectives can all work with a common data set. Both very precise data collected in small areas and less detailed information coming from broader areas are important for such analyses, but their precision level must be clearly defined. The best approach in the future is to standardize protocols for surveys with different levels of precision that can be implemented as feasible. MYCORED is a good point from which to begin developing such protocols with a worldwide consensus that is also supported by the International Society for Mycotoxicology.

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Index

Mycotoxin Reduction in Grain Chains, First Edition. Edited by John F. Leslie and Antonio F. Logrieco.

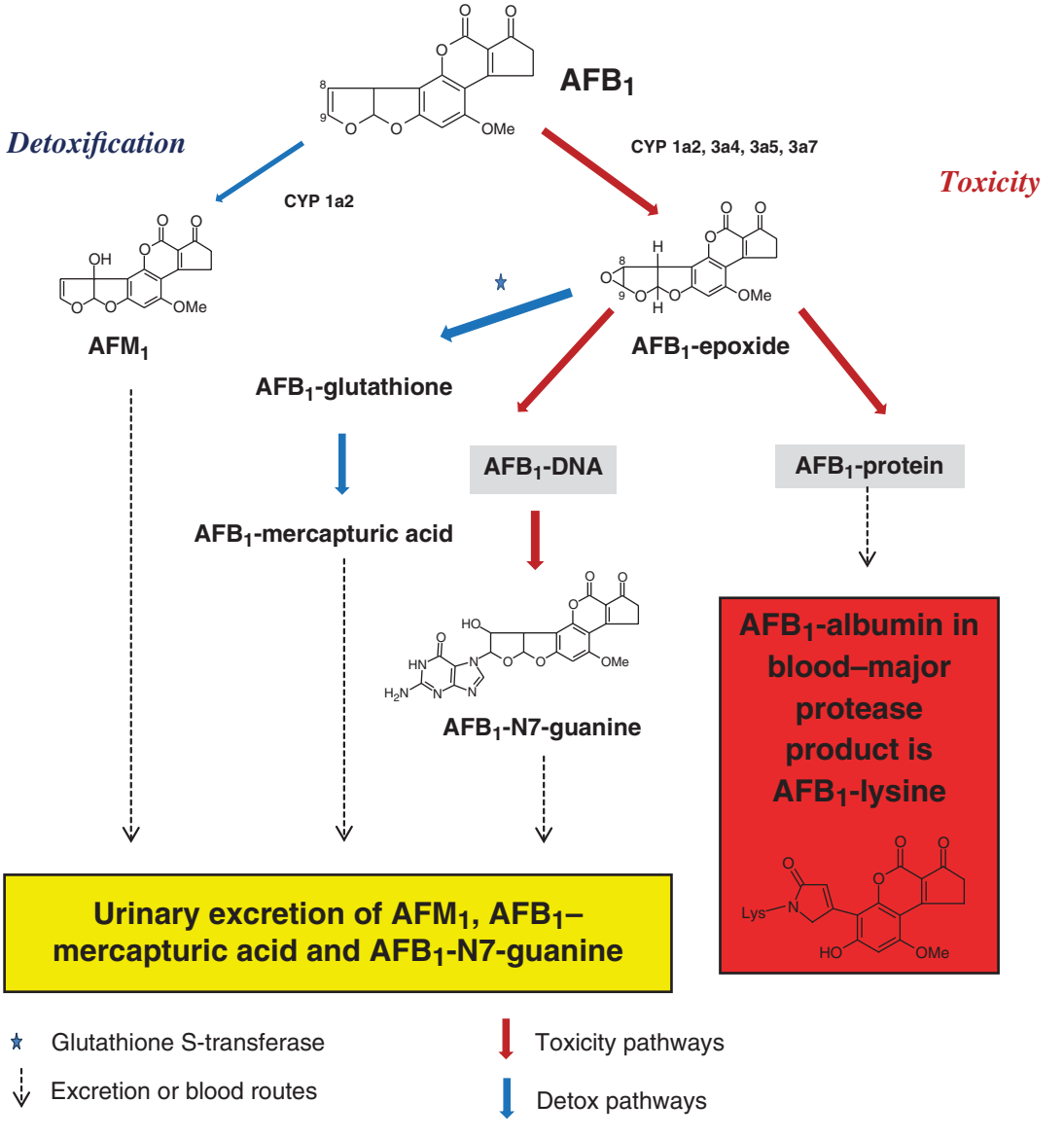

Plate 10.1 Simplified overview of important steps and key metabolites in the established metabolic pathways for aflatoxin B_1 (AFB_1) and biomarkers used to assess the efficacy of aflatoxin intervention studies [modified from Turner *et al.*, 2012]. This set of metabolites and enzymes excludes many other pathways and is simplified here to focus on those important in understanding chemoprevention. AFM_1, aflatoxin M_1.

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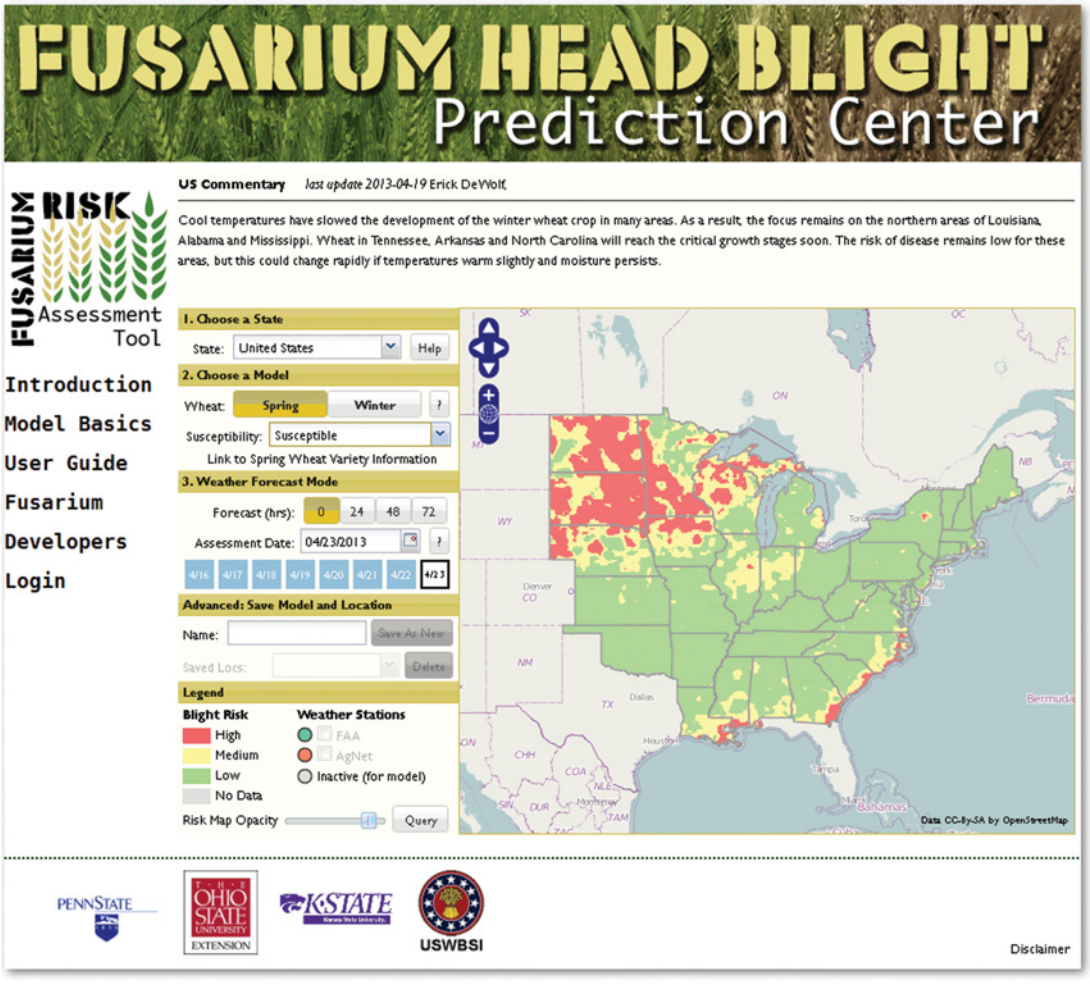

Plate 17.2 Prediction models for *Fusarium* head blight of wheat caused by *Fusarium graminearum* are available for public use via the Internet (www.wheatscab.psu.edu). The user interface displays daily estimates of disease risk for 30 states east of the Rocky Mountains in the United States. The prediction models deployed through the site were developed through the collaboration of researchers at Kansas State University, the Pennsylvania State University, and the Ohio State University. This interface for the model was designed by the Center of Environmental Informatics at the Pennsylvania State University.

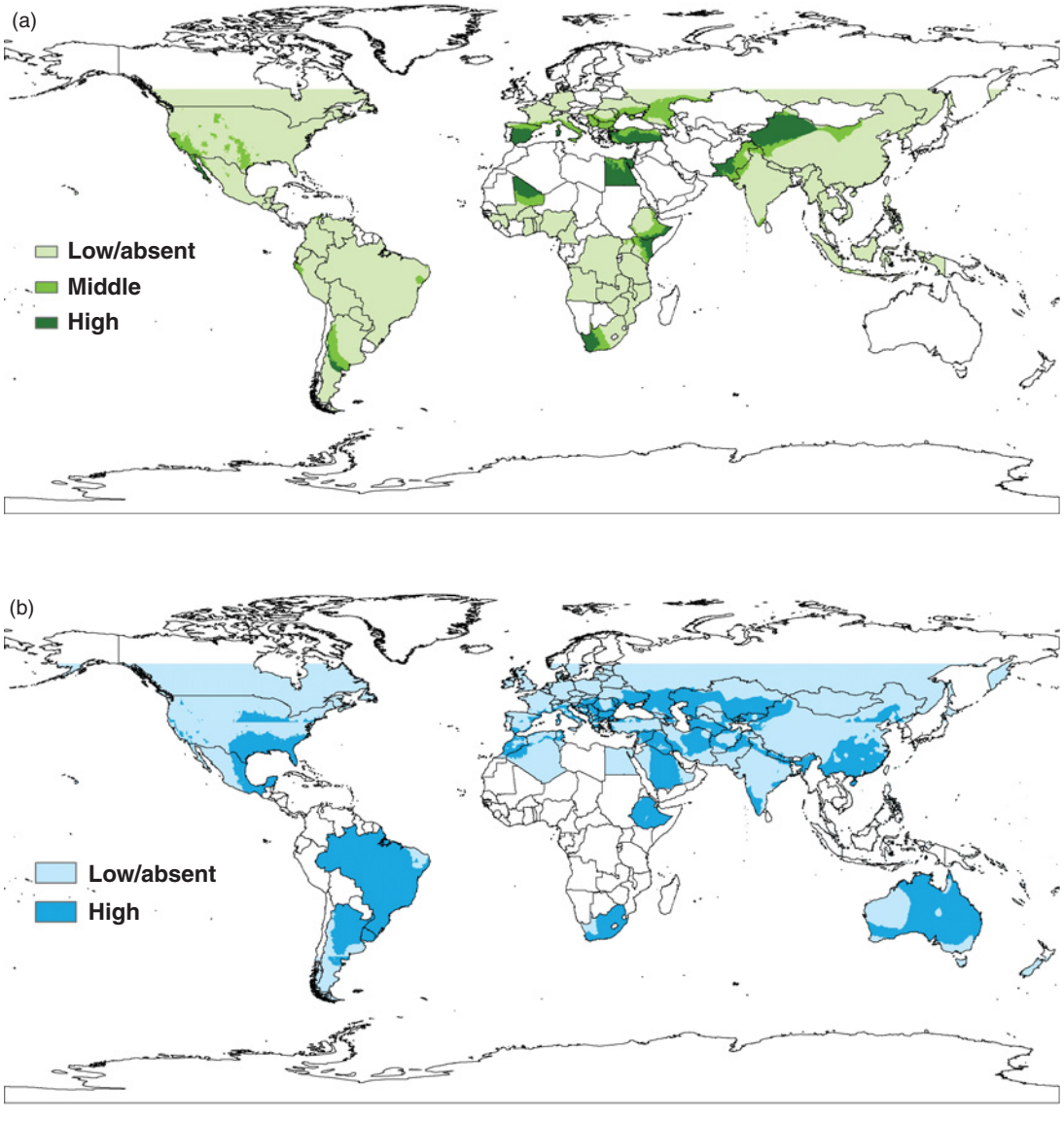

Plate 22.1 (a) Global risk of deoxynivalenol contamination in wheat. The prediction is based on monthly data for mean temperature and rain occurring near heading. (b) Global risk of fumonisin contamination in maize. The prediction is based on monthly data for mean temperature and rain occurring near silk emergence. (c) Global risk of aflatoxin B_1 contamination in maize. The prediction is based on the aridity index during heading and ear ripening.

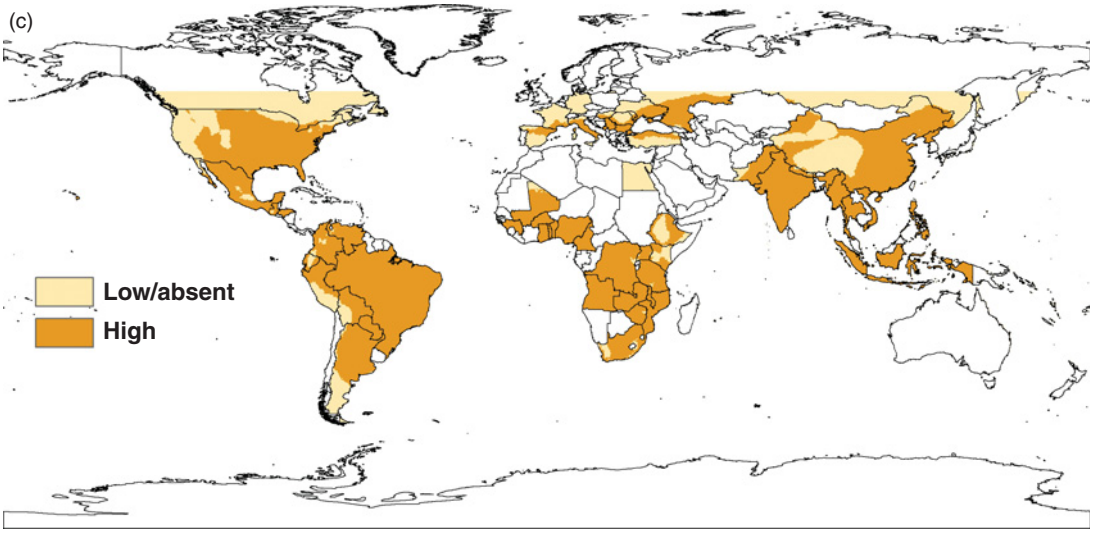

Plate 22.1 (*Continued*)