Università degli Studi di Torino

Tesi di Dottorato di Ricerca in Scienze Biologiche
e Biotecnologie Applicate

PhD Thesis in Biological Sciences
and Applied Biotechnologies

Elucidation of the production of secondary metabolites, including mycotoxins, in fungal pathogens

Silvia Valente

Tutor: **Davide Carmelo Spadaro**

XXXII Cycle: 2016 – 2019

Table of contents

Abbreviations

A

a_w = water activity
AFs = aflatoxins
AFB1 = aflatoxin B1
AFB2 = aflatoxin B2
AFG1 = aflatoxin G1
AFG2 = aflatoxin G2
ATMT = *Agrobacterium tumefaciens* mediated transformation

B

BGC = biosynthetic gene cluster

C

CFU = colony forming units
CRISPR = clustered regularly interspaced short palindrome repeats
crRNA = CRISPR-RNA
CYA = Czapek yeast extract broth

G

gRNA = guide RNA

H

HR = homologous recombination

M

MEA = Malt Extract Agar
MMEJ = micro-homology-mediated end joining
MPA = Meat Peptone Agar

N

NHEJ = nonhomologous end-joining
NRPS = non-ribosomal peptide synthetase

P

PAM = Protospacer Associated Motif
PDA = Potato Dextrose Agar
PKS = polyketide synthase

R

RNP = ribonucleoprotein

S

SM = secondary metabolite

T

TALEN = transcription activator-like effector nuclease
TC = terpene cyclase
T-DNA = transfer DNA
TF = transcription factor
tracrRNA = Trans-activating crRNA
TS = terpene synthase

Y

YES = Yeast Extract Sucrose

Z

ZFN = zinc-finger nuclease

Chapter 1

Introduction: biosynthesis of fungal secondary metabolites

Fungal secondary metabolites (SMs): definition and roles

Under favourable conditions, many fungi produce an impressive array of natural products. These compounds are also defined as secondary metabolites (SMs) because conversely to primary metabolites such as vitamins, carbohydrates, proteins and lipids, they are not necessary for the growth, development or reproduction of the producing organism (Frisvad et al., 2004; Keller, 2015; Medina et al., 2015).

Secondary and primary metabolites have been also defined as exometabolites and endometabolites respectively. Indeed, SMs exported outside the cell or deposited in the cell wall (Frisvad, 2015), while endometabolites are not secreted and are used for macromolecular or SMs biosynthesis.

Despite their name, "secondary" metabolites play a central role in many biological processes, which were extensively reviewed in last years (Fox and Howlett, 2008; Keller, 2019; Macheleidt et al., 2016).

Among the secondary metabolites produced by fungi, antibiotic and antifungal compounds are essential for producing organism for competition for space and the occupation of ecological niche. These organisms may live as saprophytes in soil or in other matrices and are surrounded by other competing organisms such as bacteria, fungi and insects (Fox and Howlett, 2008). Therefore, SMs could be considered as chemical weapons to defence themselves and allow the survival in the specific environment.

More generally, SMs are essential for the interspecies communication and defence that represent only a way to (competitively) communicate with other microorganisms. As a matter of fact, some natural products may have a role in the quorum sensing processes, such as butyrolactone I in *Aspergillus terreus*

(Macheleidt et al., 2016). Moreover, many natural products are produced *in vitro* consequently to the co-cultivation of microorganisms, such as *A. nidulans* and *Streptomyces* spp. (Fischer et al., 2016; Guzmán-Chávez et al., 2018). The interaction between these organisms led to the discovery of many SMs and represents an extensively studied example of microbial communication.

SMs, especially pigments, may also protect fungi from UV light. The most known pigments found in fungal spores or hyphae are melanins, which give colours to the conidia and protect fungi from UV damages (Calvo et al., 2002; Keller, 2019).

Additionally, SMs are important for fungal development in different ways. First, SMs are usually produced by filamentous fungi during cell differentiation and development, especially in conjunction with sporulation. Secondly, deletion or overexpression of SM biosynthetic genes usually lead to change in fungal development and sexual and asexual structure formation (Calvo et al., 2002; Keller, 2019).

Other SMs play a role in virulence both for human and plant pathogens. Examples for that are the melanins which were found to safeguard pathogenic fungi from host defence molecules, and are consequently recognized as virulence factors in the human pathogen *A. fumigatus* and in the plant pathogen *Magnaporthe grisea* (Keller, 2019; Macheleidt et al., 2016). Moreover, virulence of several other plant pathogens is enhanced or mediated by SMs produced (Macheleidt et al., 2016). The study of their biosynthesis in plant pathogens represents a good target to reduce their production and improve crop protection.

In summary, the heterogeneity of natural products (known and uncharacterized) reflects the multitude of functions for organisms. It is very likely that, together with the SMs potential, their roles in fungal life cycle and its fitness in the environment are underestimated and will be highlighted in further studies.

Importance of fungal SMs: from antibiotics to mycotoxins

The great interest on fungal SMs arise both on the huge plethora and in the chemical diversity of natural products produced.

Since the discovery of penicillin, thousands of compounds produced by microorganism including fungi have been described. Considering only fungal species belonging to *Aspergillus* and *Penicillium* genera (approximately 700 fungal species according to Samson et al. 2014 and Visagie et al., 2014) over 3000 characterized compounds have been recognized (Frisvad, 2015).

Many of these SMs have antibacterial, antifungal or anti-tumour activity and are therefore are extremely important for medicine and pharmaceutical applications (Katz and Baltz, 2016). For example, *P. chrysogenum* is the main producer of the most famous and economically important antibiotic penicillin, *P. griseofulvum* can produce the antifungal compound griseofulvin (Banani et al., 2016), *P. solitum* and *A. terreus* are able to produce statins, cardiovascular drugs (Frisvad, 2015; Frisvad et al., 2004; Katz and Baltz, 2016). Furthermore, many other novel secondary metabolites are bot produced by this group of fungi when grown under laboratory conditions (Fischer et al., 2016; Schwenk et al., 2016; Wiemann and Keller, 2014).

Many other compounds produced by fungi are instead toxicity for human and animals, these compounds falls under the category of mycotoxins definition. This particular class of natural products is produced by certain classes of microfungi and characterized by high toxicity to humans or vertebrate animals in very low concentration (Taevernier et al., 2016). Over 300 mycotoxins have been described, most of them are produced by five genera: *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria* and *Claviceps* (Nicholson, 2004). Relatively few mycotoxins have agricultural relevance such as aflatoxins, ochratoxins, *Fusarium* toxins, tremorgenic toxins, ergot alkaloid and patulin. There are numerous review articles on mycotoxins and their fungal producers (Abrunhosa et al., 2016; Ismaiel and Papenbrock, 2015; Richard, 2007; Sanzani et al., 2016). Regulations for the presence of these

compounds on several food commodities have been introduced by national and international organizations in order to ensure safety of foods for human and animal consumption (Battilani et al., 2008; van Egmond and Jonker, 2008).

Mycotoxins can be found in a wide variety of harvested commodities, such as grains, fruits and nuts, on feed and on derived or processed food products such as meat, cheese, milk, juices. Therefore, mycotoxins are currently considered the most important chronic dietary risk factor, more than food additives or pesticide residues (Ismaiel and Papenbrock, 2015; Richard, 2007; Shi et al., 2017; Yu et al., 2015).

In some cases, detrimental and beneficial effects of SMs can be produced by the same fungal species, making it necessary for an in-depth analysis of the biosynthetic pathway in the producer strains (Banani et al., 2016; Hoffmeister and Keller, 2007; Mattern et al., 2015). The study of secondary metabolism represent a source of novel metabolites and a possibility to enhance the production of beneficial compounds (Brakhage, 2013; Katz and Baltz, 2016). Moreover, by studying and characterising the fungal secondary metabolism it will be possible to interrupt or avoid the production of toxic metabolites, and to rapidly detect mycotoxigenic strains and mycotoxins and elucidate the role of SM on pathogenicity of fungal pathogens.

Fungal pathogens in post-harvest and SMs produced

The main post-harvest diseases associated with fresh and dried fruits are caused by the growth of spoilage fungi, especially *Penicillium* and *Aspergillus* species. Due to their tolerance to low temperatures and low free water content (water activity or a_w) these fungi are often found in wide range of agricultural commodities and they can grow both in field and during storage causing significant food losses (Sanzani et al., 2016). The development of mold does not represent only a problem in term of food losses, but also because these ascomycetes can produce and accumulate harmful mycotoxins.

The favourable conditions for fungal growth and mycotoxin production are variable depending on fungal strain studied and chemical composition of products (Jackson and Al-Taher, 2008; Medina et al., 2015; Sanchis and Magan, 2004). Usually environmental conditions allowing mycotoxin production are narrow compared to condition which allow the growth, as shown for *A. flavus* (Ojiambo et al., 2018). Moreover, some mycotoxins are usually produced in the field (e.g. ochratoxin on grapes) or in postharvest (e.g. patulin on apples), while other mycotoxins can be produced both in field and in post-harvest (e.g. aflatoxins on nuts) (Battilani et al., 2008).

The reduction of mycotoxigenic species in the field and in post-harvest as well as the avoidance of mycotoxin biosynthesis and accumulation represent a challenge a far as food safety is concerned.

Classes of secondary metabolites

From a chemical point of view, despite their enormous diversity, all SMs arise from a limited number of precursors of the primary metabolism, that is acetyl-coA and amino acids (Fig. 1) (Brakhage, 2013; Keller, 2019; Nielsen and Nielsen, 2017). These precursors are usually polymerized by core enzymes responsible for the synthesis of initial carbon structure, and later modified by several tailoring enzymes. Due to that, SMs are usually classified based on the core enzymes which are the milestone of their biosynthesis. Polyketides are produced by polyketide synthases (PKSs) from acyl-CoAs, non-ribosomal peptides are generated from amino acids by non-ribosomal peptide synthetases (NRPSs) and terpene synthases and terpene cyclases (TSs and TCs, respectively) synthetize terpenes from activated isoprene units. Many SMs are also hybrids between these classes and are therefore synthesized from two synthases or hybrid synthetase (Keller, 2019; Nielsen and Nielsen, 2017).

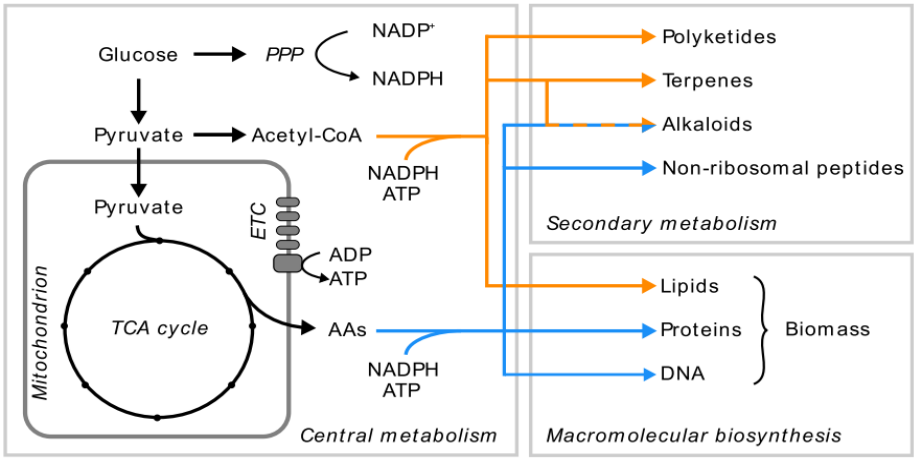


Figure 1 – Schematic overview of interconnection between primary and secondary metabolism. PPP: Pentose Phosphate Pathway. ETC: Electron Transport Chain. TCA: Tricarboxylic Acid. AAs: Amino Acids. (Nielsen and Nielsen, 2017).

Biosynthesis of secondary metabolites and related gene clusters

The availability of data from fungal genome sequencing projects together with the development of bioinformatic tools has greatly facilitated the characterization of SMs biosynthesis pathways in fungi. Through this genetic information, it was possible to characterize the genes responsible for the biosynthesis, export, and transcriptional regulation of natural products that are often arranged close to each other in the genome, in so called biosynthetic gene clusters (BGCs) (Keller, 2015, 2019; Mattern et al., 2017).

The typical structure of BGC include at least a synthase that build the SM carbon structure (e.g. PKS, NRPS, TCs and TSs) and tailoring enzymes (e.g. methyltransferases, p450 monooxygenases, hydroxylases and epimerases) which are able to modify the newly formed backbone structure (Fig. 2). Some BGCs can contain transcription factors that may act as positive or negative regulators of the cluster. Other genes could be found associated in the gene cluster such as genes

encoding proteins which can protect the producer from the toxic effect of the SM produced and other genes with unknown or unclear function (Keller, 2019).

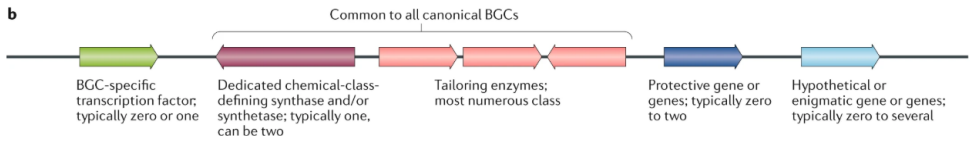


Figure 2 – Minimal composition of biosynthetic gene clusters (BGCs): a chemically defining synthase and/or synthetase and tailoring enzymes. Some BGCs may contain cluster-specific transcription factors, protective genes and genes encoding hypothetical proteins (Adapted from Keller, 2019).

Study and characterization of biosynthetic gene clusters (BGCs)

Traditionally, the study and discovery of SM biosynthetic genes required time-consuming techniques, such as the development of mutants blocked in the production of SMs, the construction of genomic DNA libraries to complement mutants and consequently screening and chromosome walking techniques (Cacho et al., 2015). For example, the elucidation of BGC of lovastatin required to obtain and screen over 6000 mutants (Hendrickson et al., 1999), while 200 mutants were chemically analysed to identify the PKS responsible for the biosynthesis of sterigmatocystin in *A. nidulans* (Yu and Leonard, 1995). Alternatively, differential hybridization analysis techniques allowed to isolate genes involved in SM biosynthesis. However, also with this approach the generation of genomic DNA libraries was necessary and required the generation of thousands of *Escherichia coli* transformants (e.g. approximately 20000 and 5000 to find aflatoxin and verrucosidin related genes, respectively) (Aranda et al., 2002; Feng et al., 1992).

Currently, there is no use of these laborious and time-consuming strategies. Conversely, the omics and bioinformatic approaches to mine and characterize BGCs represent the milestone strategies to study the biosynthesis of natural products.

Among these omics techniques, we can further distinguish between tools that allow finding genes in the gene cluster and techniques to associate the BGCs with specific metabolite production.

Genome mining

As previously indicated, several sequencing projects have been conducted, such as the 1000 Fungal Genome project supported by the Joint Genome Institute (http://1000.fungalgenomes.org). Therefore, the genome of many fungi is currently available; more than one thousand fungal genome sequences can be found in genomic portals such as MycoCosm (Grigoriev et al., 2014; Yu et al., 2015).

Additionally, the minimal structure of BGCs has been elucidated, allowing the development of bioinformatic algorithms to recognize them in the genome. SMURF and antiSMASH were the first tools to predict BGCs in fungal genomes developed in 2010 and 2011 respectively. These bioinformatic software recognize core biosynthetic genes, such as polyketide synthases (PKSs) or non-ribosomal peptidase synthetases (NRPSs), and the neighbouring genes which could be involved in the regulation, export and modification of the natural products. Many other programs and databases have been developed and an exhaustive list can be found in the review of Weber and Kim (2016).

Three different approaches can be used, depending on the starting information available, to link SMs with BGCs (Fig. 3) (Cacho et al., 2015; Kjærbølling et al., 2019; Nielsen and Nielsen, 2017). Briefly, a target approach which adopt homologous research can be used when BGC for the same or a similar compound is known in other fungal species. Conversely, when only the chemical structure of SM is known, it will be possible, by retro-biosynthesis, to predict the enzymes required for the production of certain metabolite, and therefore scan the genome for a BGC putatively involved in the pathway. Finally, by using comparative genomics it is

possible to compare BGCs of producer fungi and narrow down the number of putative clusters involved in the biosynthesis of the studied compound.

In addition to this techniques, untargeted approaches can be used to find all the putative BGCs in a sequenced genome, allowing to highlight the full genetic potential of specific fungal species.

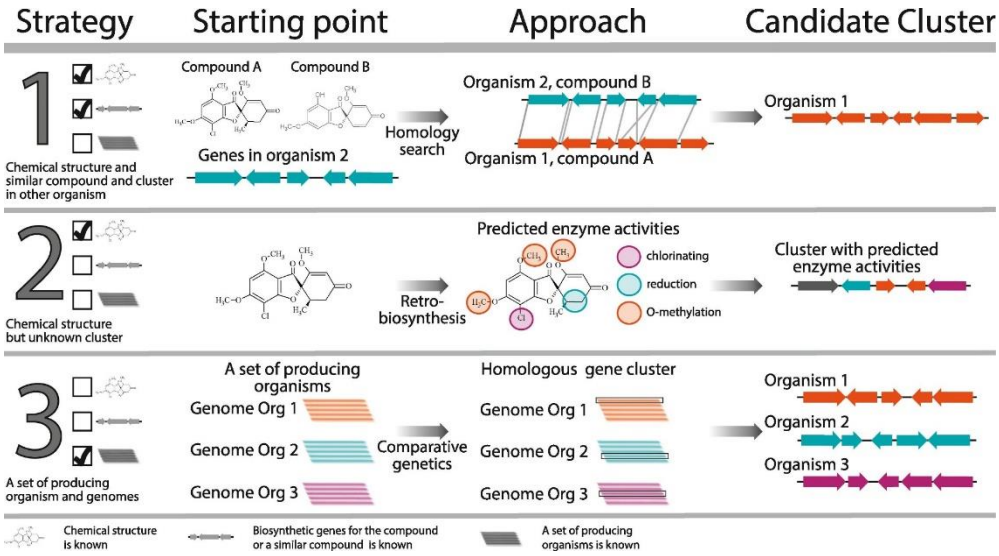


Figure 3 – Strategies to link SM to BGCs using whole genome sequences. 1 = Homology search, 2 = Retro-biosynthesis, 3 = Comparative genomics (Kjærbølling et al., 2019).

Connect gene clusters and SM

Thanks to gene mining approaches, thousands of gene clusters have been described in fungal genomes, a number which is higher compared to the chemically identified compounds (Yu et al., 2015). Most of these uncharacterized BGCs are not actively expressed under laboratory conditions, therefore are usually referred as “silent” BGCs. Special effort was made during last years to characterize the silent BGCs, that represent a source of novel compound with a potential for industrial value (Fischer et al., 2016). In certain cases, BGC prediction leads to misidentification of

BGC due to the difficulty to distinguish between functional gene cluster or unfunctional one that is mutated , that do not produce any compound.

Genome-based techniques allow both to confirm the role of putative genes in BGCs identified and to fill the gap between BGCs found and SMs produced. These techniques developed to characterize gene clusters can be divided in two main categories: tools to investigate silent or active BGCs (Fig. 4).

Most of these techniques have been developed to induce expression of silent BGC, such as cultivation on different media or screening of different organisms, epigenetic or chromatin remodelling, promoter exchange, overexpression of specific and global regulators, ribosome of RNP and PKS engineering. Several reviews provide a detailed description of these techniques (Fischer et al., 2016; Guzmán-Chávez et al., 2018; Kjærbølling et al., 2019; Mattern et al., 2015; Wiemann and Keller, 2014; Yaegashi et al., 2014).

To identify and characterize active BGC, the main strategy adopted is the deletion of synthase genes. By knocking out the putative core genes, it is expected to observe the lack of chromatographic peaks corresponding to the metabolite studied and potentially to identify new peaks corresponding to the accumulation of intermediates (Kjærbølling et al., 2019).

This approach can be adopted only if the organism is cultivable and if effective system to genetically manipulate the species is available. Otherwise, the heterologous expression in other host is a viable alternative system (Keller, 2019).

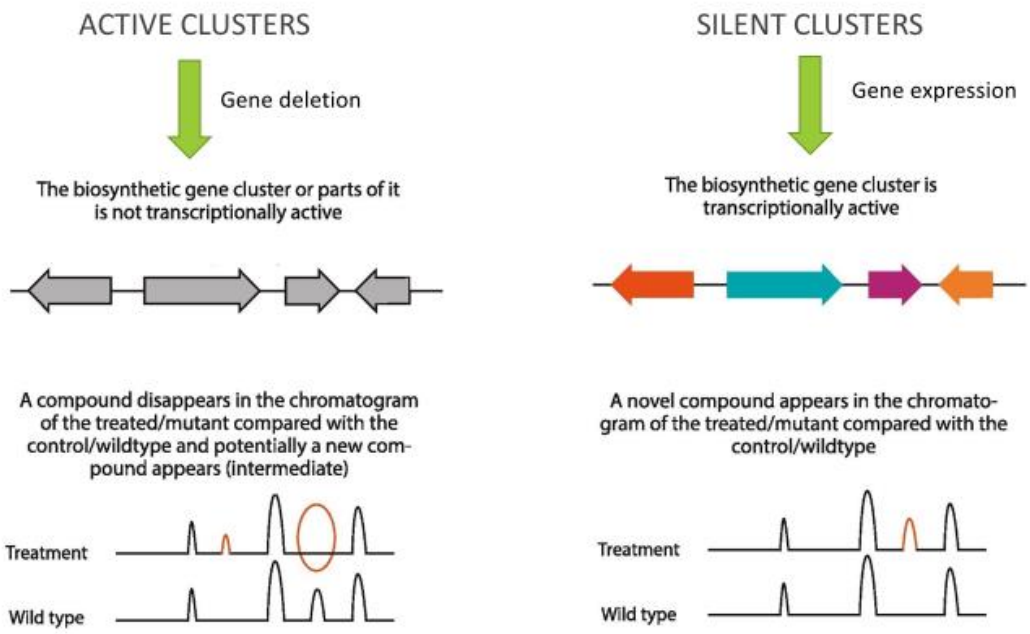


Figure 4 – Investigation of BGC in the native host. For actively expressed gene cluster the gene deletion lead to lack of SM production, in which a compound disappears in the chromatogram of the treated/mutant compared with the control/wild type and potentially a new compound appears (intermediate). Strategies for activating silent gene cluster lead to the production of new compounds, which correspond to a new peak in the chromatogram (Adapted from Kjærbølling et al., 2019).

Strategies for gene knockout in fungi

There are several ways to inactivate a gene of interest. Usually, knockout approaches are based on the recombination of a donor sequence of DNA with the gene of interest, leading to the loss of function of this gene. The DNA inserted generally confers antibiotic resistance to the mutant strains, making the selection of transformed strains possible. Alternatively, through homologous recombination with upstream and downstream region of a target gene it is possible to delete the gene without inserting any foreign DNA. In this case, appropriate methods to screen mutants are necessary, and in most cases they are usually based on auxotrophy (such

as uridin auxotrophy caused by the removal of the *pyrG* gene) or on different morphology such as white colour of conidia (Wang et al., 2017). Finally, with availability of specific techniques of gene editing it is possible to induce a single mutation in the gene of interest leading to loss of its function. In this case the selection can be performed by using antibiotic resistance genes or genes involved in primary or secondary metabolism.

Conversely to bacteria, fungi are more complex to transform, both because of the multicellularity and because of the presence of the cell wall. Since the '70s, methods have been developed to transform fungi and several reviews give an extensive overview on the available methodologies (He et al., 2017; Li et al., 2017; Wang et al., 2017). Among them, the main adopted are protoplast mediated transformation, *Agrobacterium tumefaciens* mediated transformation (ATMT) and CRISPR-Cas9.

Protoplasts are obtained through the lytic removal of the cell wall and exposing them to polyethylene glycol to allow the foreign DNA to penetrate the protoplast membrane into the cell and fusing with the fugal DNA (He et al., 2017; Li et al., 2017). The main components of the cell wall are shared among fungi, but its composition is variable depending on fungal species. Furthermore, the composition could vary depending on fungal structure. Fungal conidia are more resistant to lysis compared to hyphae, due to the presence of hydrophobic rodlets (Erwig and Gow, 2016). Therefore, the main hurdles to obtain protoplasts are the selection of lytic enzymes which effectively degrade cell wall and the optimization of growth condition to obtain material accessible to the enzymes used. Moreover, the optimization of the regeneration of protoplasts once the cell wall has been removed is another point which should be taken into consideration (Li et al., 2017). The main advantage of the protoplasts system is that it is relatively easy to perform transformations once established for a certain fungal species.

A. tumefaciens is a gram-negative bacterium largely found in soil and certain strains can induce tumours on tree plants. In plant systems, *A. tumefaciens* acetosyringone

induce the expression of *vir* genes to produce a portion of DNA (usually referred as transfer DNA or T-DNA) that is then cut and transferred into the host. The T-DNA is integrated in the genome leading to the biosynthesis of opines required for bacterial growth and production of hormones which eventually cause the tumours (Wang et al., 2017). By engineering *A. tumefaciens* strains, it is possible to take advantage of this efficient system of transformation, and to integrate foreign DNA into plant and fungal genomes. The system requires to obtain binary vectors, the induction of *vir* genes through acetosyringone, optimization of a co-colture of *A. tumefaciens* with the fungus and finally the removal of bacterial cells through addition of antibiotics. Overall, The ATMT requires much more efforts compared to protoplasts but its main advantage is having a higher target transformation efficiency (Li et al., 2017). In the classical method (protoplasts) used to genetically manipulate fungi, the foreign DNA is inserted randomly in the genome, due to the error-prone nonhomologous end-joining (NHEJ) repair system, with only 10% of cases homologous recombination (HR) occur. Efficiency of homologous recombination can be increased though deleting genes involved in the NHEJ (Schuster and Kahmann, 2019). However, additional transformation rounds and selection procedures are required. With ATMT the frequency of homologous recombination is increased and therefore it is one of the most used method for genetic manipulation in fungi.

Several types of nucleases have been engineered to achieve precise double strand break of the DNA, such as TALEN, ZFN and CAS9. Among them, Cas9 could be considered the most promising. In prokaryotes, Cas9 is part of the adaptative immune system against viruses. When the enzyme is associated with two short sequences of RNA (defined crRNA and tracrRNA) in the so-called ribonucleoprotein (RNP) it is able to recognize the alien DNA and cause a double strand break (Tong et al., 2019). For genetic manipulation, crRNA and tracrRNA can be combined in one sequence called guide RNA (gRNA). To induce the deletion

of target genes it requires only a gRNA which recognize the desired site of deletion and the endonuclease. Multiple DSB repair pathways are available, ranging from NHEJ, micro-homology-mediated end joining (MMEJ) and HR (Hoshijima et al., 2016; Seol et al., 2018). Therefore, these pattern of repair can lead to diverse genome editing events such as indels, in-frame deletion/insertions, random deletion, nucleotide substitutions and so on, as described in Fig. 5 (Tong et al., 2019).

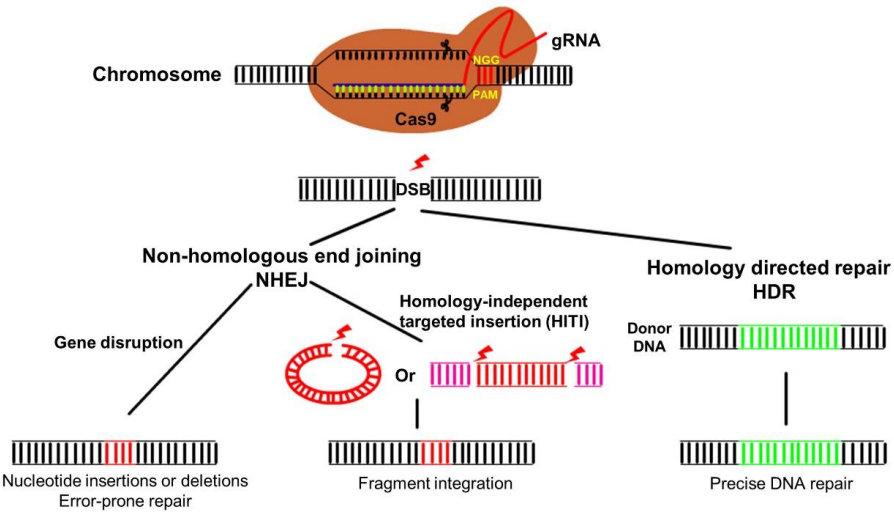


Figure 5 – CRISPR-Cas9 system and kind of editing induced due to the different repair system of the DNA (Wang and Coleman, 2019).

The introduction of foreign DNA (plasmid expressing gRNA and/or CAS9, gRNA) or of the RNP in its complex (Cas9 associated with gRNA) is facilitated by classical approaches such as protoplast formation or through *A. tumefaciens*. This represent the main disadvantage of the technique, despite that, the great specificity led CRISPR-Cas9 to be the most promising tools to genetically manipulate fungi. This is confirmed by several authors who summarized the main strategies used to perform CRISPR-Cas9 in fungi and explain in details its main advantages (Ceasar et al., 2016; Mei et al., 2019; Schuster and Kahmann, 2019; Shi et al., 2017; Song et al., 2019; Tong et al., 2019; Wang and Coleman, 2019).

Characterization of SMs produced by fungal pathogens: three case studies

In this thesis, the secondary metabolism of three postharvest pathogens was investigated. The main targets were mycotoxigenic *Penicillium* and *Aspergillus* species isolated from different food matrices (fresh fruits and nuts); their ability to produce mycotoxins was assessed *in vitro* and *in vivo*. The biosynthesis of three SMs was investigated using different strategies (Fig. 6), ranging from the study of environmental conditions which promote mycotoxin production (Chapter 4) to bioinformatic and genetic tools to find and characterize related gene clusters (Chapters 2, 3).

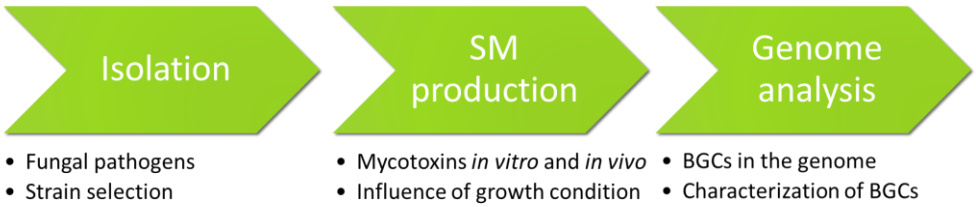


Figure 6 – Workflow to study and characterize SMs produced by fungal pathogens.

To facilitate the understanding of the strategies used to investigate fungal SMs, a brief overview of the three main case studies included in our work is presented.

Penicillium griseofulvum *and griseofulvin on apples*

P. griseufulvum cause apple blue mold in postharvest. The annotation of its genome in 2016 allowed the identification of several putative SMs BGCs including that putatively involved in the biosynthesis of griseofulvin (Banani et al., 2016). The griseofulvin gene cluster was previously identified in *P. aethiopicum* but the putative transcription factors found in the BGC were not characterized (Chooi et al., 2010). The first goal of the present thesis was to investigate the regulation of

griseofulvin biosynthesis in *P. griseofulvum* using deletion mutants obtained through ATMT. Additionally, promoter analysis allowed to identify many putative global regulators involved in the biosynthesis and the role of the availability of carbon and nitrogen was investigated *in vitro*. Furthermore, the role of griseofulvin for virulence was assessed by inoculating apples with the wild type and deletion mutants for the PKS (unable to produce griseofulvin) and for the putative regulatory genes.

Penicillium polonicum *and verrucosidin on chestnuts*

P. polonicum is a ubiquitous fungus, able to contaminate different food matrices especially meat, grains and nuts. The strain X6 was previously isolated from chestnut and it was able to produce *in vivo* and *in vitro* a neurotoxin called verrucosidin (Prencipe et al., 2018b). The biosynthesis of verrucosidin was poorly investigated, therefore in the third chapter a putative verrucosidin gene cluster was searched in the genome of *P. polonicum*. Many putative BGCs were found and their activity was invistigated by RT-qPCR. Then, two gene clusters were further characterized through target deletions of PKS. To do that, a method to obtain *P. polonicum* protoplasts was developed and optimized and the deletion was mediated by CRISPR-Cas9 *in vitro*.

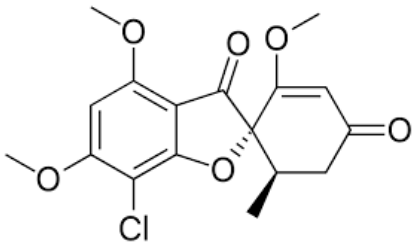
Aspergillus flavus *and aflatoxins on hazelnuts*

Aflatoxins represent the most dangerous mycotoxins for human health, and they are produced mainly by *A. flavus* and *A. parasiticus*. The presence of *A. flavus* and *A. parasiticus* on hazelnuts is well documented as well as the contamination of hazelnuts and derived products with aflatoxins (Aycicek et al., 2005; Baltaci et al., 2012; Golge et al., 2016; Gürses, 2006; Kabak, 2016; Ozay et al., 2008; Prelle et al., 2012; Şimşek et al., 2002). Growth conditions, including temperature of processing

and storage, available water on nuts (water activity or a_w) and chemical composition of products, largely influence aflatoxin biosynthesis (Kumar et al., 2017). The main strategy used to avoid fungal contamination and aflatoxin production is drying, which allow reducing moisture content of nuts and consequently a_w. In the fourth chapter the influence of two important drying parameter (time and temperature of drying) on aflatoxin production was evaluated. Furthermore, the influence of drying treatments on the reduction and viability of fungal spores was investigated.

Chapter 2

Elaborated regulation of griseofulvin biosynthesis in *Penicillium griseofulvum* and its role on conidiation and virulence



Silvia Valente [a], Agnese Cometto [a], Edoardo Piombo [a], Giovanna Roberta Meloni [a], Ana-Rosa Ballester [b], Luis González-Candelas [b] and Davide Spadaro [a]

[a] Dept. Agricultural, Forest and Food Sciences (DISAFA), Università degli Studi di Torino, Largo Paolo Braccini 2, 10095 Grugliasco, Italy.

[b] IATA-CSIC – Instituto de Agroquímica y Tecnología de Alimentos. Calle Catedrático Agustín Escardino 7, Paterna, 46980-Valencia, Spain.

Submitted to International Journal of Food of Microbiology

Abstract

Penicillium griseofulvum, the causal agent of apple blue mold, can produce *in vitro* and on apple a broad spectrum of secondary metabolites (SM), including patulin, roquefortine C and griseofulvin. Among them, griseofulvin is known for its antifungal and antiproliferative activity, and has received interest in many sectors, from medicine to agriculture. The biosynthesis of SM is finely regulated by filamentous fungi and can involve global regulators and pathway specific regulators, which are usually encoded by genes present in the same gene cluster as the backbone gene and tailoring enzymes. In the griseofulvin gene cluster, two putative transcription factors were previously identified, encoded by genes *gsfR1* and *gsfR2*, and their role has been investigated in the present work. Analysis of *P. griseofulvum* knockout mutants lacking either gene suggest that *gsfR2* forms part of a different pathway and *gsfR1* exhibits many spectra of action, acting as regulator of griseofulvin and patulin biosynthesis and influencing conidia production and virulence on apple. The analysis of *gsfR1* promoter revealed that the regulation of griseofulvin biosynthesis is also controlled by global regulators in response to many environmental stimuli, such as carbon and nitrogen. The influence of carbon and nitrogen source on griseofulvin production was further investigated and verified, revealing a complex network of responses and confirming the central role of *gsfR1* in many processes in *P. griseofulvum*.

Key words

Transcription factor, knockout, gene cluster, regulation, secondary metabolites, apple blue mold

1. Introduction

Blue mold of pome fruit is one of the most important postharvest diseases of apple fruit. The major causal agent is *Penicillium expansum*, but many other *Penicillium* spp. can be found together causing blue mold, such as *P. griseofulvum, P. aurantiogriseum, P. crustosum, P. verrucosum, P. carneum, P. commune, P. brevicompactum, P. solitum* and *P. puberulum* (Moslem et al., 2010, 2013; Spadaro et al., 2011; Wu et al., 2019).

These species, as other *Penicillium* spp., can produce a wide range of secondary metabolites (SMs), which are so called because they are not essential for primary metabolism and growth, but they play an essential role in the interactions, such as competition or communication with other microorganisms, and in many other cellular processes (Brakhage, 2013b). Many of these SMs have industrial value, such as antibiotics and antitumor compounds, while other metabolites can be toxic for humans and animals and are defined as mycotoxins.

In apples, the major concern is related to the presence of patulin, which is a mycotoxin with acute and chronic toxicity, characterized by stability in acid environment and during thermal processes, and therefore can be found in apples, apple juices and other derived products (Reddy et al., 2010).

Among causal agents of blue mold, *P. griseofulvum* is able to produce elevated amount of patulin, even higher compared to *P. expansum*, up to 4,500 µg/kg *in vivo*. The European residue limit for patulin is between 10 and 50 µg/kg depending on food commodities (Banani et al., 2016; Moslem et al., 2013; Spadaro et al., 2008).

P. griseofulvum can also produce other well-known mycotoxins such as roquefortine C and cyclopiazonic acid, and a well characteristic secondary metabolite called griseofulvin (Banani et al., 2016). Griseofulvin was initially recognized as an antibiotic, this antifungal compound is classified as a potential carcinogenic for humans by the International Agency on Research on Cancer (World Health Organization, 2001). Nevertheless, griseofulvin is commonly found in many

medical and veterinary products to treat cutaneous dermatophyte infections and it was recognized in 2017 as an essential medicine by the World Health Organization for its antifungal properties (World Health Organization, 2012). Moreover, its role against cancer cells has been investigated by many researchers (Mauro et al., 2013; Rathinasamy et al., 2010).

Because of its antiproliferative activity, griseofulvin and derived compounds have also been investigated for their potential use against fungal pathogens, such as *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Magnaporthe grisea*, *Corticium sasaki*, *Puccinia recondita*, *Blumeria graminis* f. sp. *hordei*, *Alternaria solani*, *Fusarium solani* and *Colletotrichum gloeosporioides*, and showed inhibitory activity both *in vitro* and *in vivo* (Bai et al., 2019; Ribeiro et al., 2018; Stierle and Stierle, 2015; Tang et al., 2015).

Due to the importance of griseofulvin in a wide range of applications, ranging from medicine to agriculture, griseofulvin biosynthetic genes were previously identified in *P. aethiopicum* (Chooi et al., 2010). The putative griseofulvin gene cluster consists of 13 genes, including a nonreducing polyketide synthase (PKS), tailoring enzymes and two putative transcription factors. The gene cluster was later determined in *P. griseofulvum* in which 10 genes of the cluster were found in the same order, but genes encoding for a reductase (*gsfK*), an hydrolase (*gsfH*) and a transcription factor (*gsfR2*) were not located in the same genomic region (Banani et al., 2016).

The putative genes encoding the backbone enzyme (*gsfA*), *O*-methyltransferases (*gsfB, gsfC, gsfD*) halogenase (*gsfI*), dehydrogenase /reductase (*gsfE*) and cytochrome P450 (*gsfF*) were confirmed by producing deletion mutants (Cacho et al., 2013; Chooi et al., 2010), but the role of the putative transporter as well as the regulatory genes remains to be investigated.

Regulatory genes are an interesting target for mutagenesis because silencing or promoting their expression can provide significant enhancement of production of compounds with industrial value.

In this work, the regulation of griseofulvin biosynthesis was investigated by characterizing knockout mutants for the putative transcription factors encoded by *gsfR1* and *gsfR2* and comparing them with wild type and deletion mutants for the backbone gene of the cluster, *gsfA*. However, the regulation of SM biosynthesis is complex and involves not only pathway-specific regulators, which are commonly encoded by genes located inside the gene cluster, but also global regulators expressed in response to various environmental stimuli (Brakhage, 2013). A second approach aimed to study the involvement of global regulators of the cluster was therefore investigated, particularly by evaluating the effect of carbon and nitrogen sources on *P. griseofulvum* growth and on griseofulvin biosynthesis. The findings reported here led to a better understanding of griseofulvin biosynthesis and its role in the growth and virulence of *P. griseofulvum*.

2. Materials and Methods

2.1. Fungal strain

Penicillium griseofulvum Dierck strain PG3 was previously isolated from rotten apples in Piedmont, Italy (Spadaro et al., 2011) and grown on Potato Dextrose Agar (PDA, Merck KGaA, Darmstadt, Germany) with 50 µg/mL streptomycin (Merck) in the dark at 25 °C for 7-10 days. *P. griseofulvum* transformed strains were grown on PDA containing 500 µg/mL of hygromycin B (ThermoFischer Scientific, Waltham, MA USA) under the same conditions. Conidial suspensions were obtained by adding 5 mL of sterile water with 0.01 % (w/v) Tween-20 and gently scraping the surface of fungal cultures grown on Petri plates. The final conidia concentration was measured using a hemocytometer and adjusted to the required

concentration, depending on the type of experiment. Conidial suspension of wild type and transformed strains were maintained in glycerol (40%) at -80 °C.

2.2 Bioinformatic analysis

The promoter regions of the genes *gsfR1* and *gsfA* were extracted from the genome of *P. griseofulvum* PG3 (Genbank: GCA_001561935.1), and scanned for transcription factor (TF) binding using the CIS-BP database with default parameters (http://cisbp.ccbr.utoronto.ca/TFTools.php). *P. chrysogenum* was used as model species when performing the analysis. Identified *P. chrysogenum* TFs were blasted against PG3 proteome to check for their presence in *P. griseofulvum*, and they were then identified by blasting against NCBI non-redundant protein database.

2.3 Obtaining of knockout mutants

Knockout mutants were obtained as previously described (Ballester et al., 2015), by amplifying 5' (promoter) and 3' (terminator) flanking regions of *gsfA*, g*sfR1* and *gsfR2* genes from the genomic DNA of *P. griseofulvum* PG3 with specific primers pairs (O1/O2 and A3/A4, Tab. S1) containing a tail for USER cloning (Frandsen et al., 2008). Amplified fragments were mixed with pRFHU2 vector pre-digested with *PacI* and *NtBbVCI* (New England Biolabs, Ipswich, MA, USA) and treated with USER enzyme (New England Biolabs). Five µL of the USER mixture was directly used to transform chemically competent cells of *Escherichia coli* strain DH5α, with heat shock protocol, then bacterial cells were recovered and plated on Luria Bertani Agar (LB, Miller, Merck) supplemented with 25 µg/mL of kanamycin (ThermoFischer Scientific). Resistant transformants were screened by colony PCR and plasmid DNA for positive colonies containing both promoter and terminator using NucleoSpin® Plasmid EasyPure kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. The correct integration of promoter and terminator of genes was confirmed by sequencing the amplified fragments with

primers pairs RF1/RF6 and RF2/RF5 (Tab. S1). Then, 10 ng of plasmid were introduced by electroporation into electro-competent *A. tumefaciens* AGL1 cells and *A. tumefaciens* mediated transformation of *P. griseofulvum* was conducted as described by Buron-Moles et al. (2012). Transformants were maintained on PDA with 500 µg/mL of hygromycin B, according to the results of a sensitivity assay performed on PDA for *P. griseofulvum*.

2.4 DNA and RNA extraction

DNA of *P. griseofulvum* PG3 and knockout mutants was obtained using E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-tek, Norcross, GA, USA) or using the slightly modified protocol for isolation of high molecular weight DNA from fungal mycelium (Möller et al., 1992). Briefly, a conidial suspension (10^5 conidia/mL) was inoculated in 50 mL of Glucose Yeast Peptone medium (GPY; 4% w/v glucose, 0.5% w/v yeast extract and 0.5% w/v peptone) and incubated on a rotary shaker (200 rpm) at 24 °C for 2 days. Fungal mass was collected, dried and frozen in liquid nitrogen. DNA extraction from 0.5 g of frozen mycelium was performed in 50 mL tubes containing 5 mL TES (100 mM Tris HCl, pH 8.0, 10 mM EDTA, 2% SDS), 25 µL proteinase K, and 50 µL ß-mercaptoethanol. DNA concentration and purity were checked by spectrophotometer (Nanodrop 2000, Thermo Scientific, Wilmington, USA).

RNA extraction was performed according to Ballester et al. (2015) with some adjustments. First, 5 µL of a conidial suspension (10^6 conidia/mL) were inoculated on PDA with a cellophane membrane and incubated at 24 °C in the dark. One hundred mg of mycelium were collected in 2 mL tube at 5, 7 and 10 dpi and frozen in liquid nitrogen. Two tungsten beads (diameter: 2.7 mm) were added to the mycelium that was crushed using TissueLyser (Qiagen, Hilden, Germany) for 1 min at 20.00 Hz speed. Then, 750 µL of extraction buffer (100 mM Tris-HCl pH 8.0; 100 mM lithium chloride, 10 mM EDTA pH 8.0; 1% SDS; 1% PVP-40; 1% β-

mercaptoethanol) and 375 µL of phenol:chloroform:isoamyl alcohol (25:24:1, v:v:v) were added and the mixture was vortexed and incubated at 65 °C for 10 min. After cooling, 375 µL of phenol:chloroform:isoamyl alcohol (25:24:1) were added and the extract was vortexed. The homogenate was centrifuged at 13,800 x *g* for 10 min and the aqueous phase was re-extracted with 750 µL of phenol:chloroform:isoamyl alcohol. Nucleic acids were precipitated by adding 1/10 volume of 3 M sodium acetate pH 5.2 and 2 volumes of cold 100% ethanol and then incubated at -20 °C for 60 min. The precipitated RNA was pelleted by centrifugation for 15 min at 13,800 x *g*. After washing with 500 µL of 70% ethanol, the pellet was dissolved in 600 µL of TES buffer (10 mM Tris–HCl pH 8; 5 mM EDTA pH 8; 0.1% SDS) and precipitated overnight at −20 °C with 200 µL of 12 M lithium chloride. After centrifugation at 13,800 x *g* for 60 min, the pellet was washed with 500 µL of 70% ethanol, suspended with 250 µL of 3 M sodium acetate, pH 6.0, to remove residual polysaccharides and washed with 70% ethanol. Finally, RNA was dissolved in 50 µL of DEPC-water and its concentration was measured spectrophotometrically and verified by gel electrophoresis.

DNase treatment was performed using TURBO DNA-free™ Kit (Thermo Fischer Scientific). The samples were diluted 1:2 in a final volume of 50 µL and rigorous DNase treatment followed. First-strand cDNA was conducted with High Capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific) using 1 µg of total RNA.

2.5 PCR and qPCR

Upstream and downstream fragments of *gsfR1*, *gsfR2* and *gsfA* genes were amplified by PCR with Top-Taq DNA polymerase (Bioron Diagnostics GmbH, Römerberg, Germany) and a T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). The cycling conditions of the PCR were 5 min at 94 °C, followed by 35 cycles of 15 s at 94 °C, 20 s at 58 °C, 90 s at 72 °C and a final extension of 5 min at 72 °C. The PCR

mixture contained 1X PCR Buffer, 0.2 mM dNTPs, 0.4 µM of each primer, 0.5 U of polymerase and 10 ng of genomic DNA of *P. griseofulvum.* Taq DNA Polymerase (Qiagen) was used to perform the other PCRs. To confirm the correct integration of T-DNA in the genome, several PCRs were conducted to amplify the hygromycin cassette and *gsfA*, *gsfR1* and *gsfR2* genes. The cycling conditions of the PCRs were 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 45 s at 58 °C, 90 s at 72 °C and 5 min at 72 °C.

To verify the efficiency of the DNase treatment, a PCR was performed using ITS1 and ITS4 universal primers. The cycling conditions of the PCR were 3 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C and 5 min at 72 °C.

RT-qPCRs were performed with StepOne™ and StepOnePlus™ Real-Time PCR System with Power SYBR™ Green PCR Master Mix (ThermoFischer Scientific); cycling conditions were 5 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C. In order to determine the number of insertions in the genome of the transformants, the $2^{\Delta\Delta cq}$ method (Pfaffl, 2001) was used using genomic DNA of samples and comparing the amplification of β-tubulin gene or histone H3, with the amplification of the promoter of the corresponding gene target of deletion.

To determine the expression of *gsfA* and *gsfR1* genes in Δ*gsfR1* mutants, cDNA was added in the reaction instead of gDNA.

All primer sequences used in the PCR and qPCR reactions are listed in Tab. S1.

2.6 Characterization of mutants in vitro

To check growth rate conidiation, and griseofulvin production, deletion and ectopic mutants were compared with wild type *P. griseofulvum* PG3 by inoculating 5 µl of spore suspension (1 x 10^6 conidia/mL) on PDA and incubating the plates at 25 °C in the dark. Some mutants were also inoculated on Malt Extract Agar (MEA, 2% malt

extract, 2% glucose, 0.1 % peptone, 2% agar) and Meat Peptone Agar (MPA, 2.5% meat extract, 0.25% peptone, 2% agar) under the same conditions. Colony diameter (cm), number of asexual spores (conidia/plate) and griseofulvin production (µg/plate) were measured up to 10 days post inoculation (dpi). At least 5 plates were inoculated for each strain and every assay was performed three times.

2.7 Characterization of mutants in vivo

Apples 'Golden Delicious' were purchased from a local supermarket and stored at 4 °C until use. Fruits were surface disinfected with sodium hypochlorite (1% as chlorine), washed with deionized water and allowed to air dry before inoculation. Then 21 fruits for each treatment were inoculated by wounding in three points at the equatorial region of the fruits with a sterile needle (3 mm depth, 3-4 mm wide) (Spadaro et al., 2013). Ten µl of conidial suspension (1 x 10^8 conidia/mL) of each strain were pipetted into each wound, while controls were inoculated with deionized water. Wounded apples were placed in plastic trays, covered with a transparent polyethylene film and stored at room temperature for 3-14 days.

2.8 Griseofulvin and patulin extraction

Griseofulvin and patulin produced *in vitro* were extracted simultaneously from the mycelium and the medium by washing the plates with 3 mL of methanol. The plates were gently scraped and then placed in rotary shaken at 450 rpm for 15 min. The liquid was then collected and centrifuged (13000 x *g*) at room temperature for 5 min. After that, the supernatant was filtered through a 0.45 µm syringe filter and analysed by HPLC.

Griseofulvin was extracted from rotten apples as previously described by Banani et al. (2016) with some modifications. Briefly, the decayed portion around the inoculation sites from 7 apples was collected and combined. Ten g of each sample were weighed and then placed in a centrifuge tube with 10 drops of pectinase

enzyme solution (Sigma Chemical Co., St Louis, USA; 5 U/g juice) and 10 mL of water were added. The mixture was left at 38 °C for 2 hours and then centrifuged at 3,600 *x g* for 5 min. Five mL of clear liquid were placed into a clean tube and griseofulvin was extracted with 5 mL of ethyl acetate (three times). The organic phase was evaporated to dryness using rotary evaporator and the residual dissolved in 500 µL of MeOH:H_2O (1:1, v/v) and transferred into a HPLC vial for HPLC-MS/MS analysis.

2.9 Chemical analyses

To assess the production of griseofulvin and patulin on PDA, the analyses were carried out using the method previously described by Banani et al (2016) with few modifications. A Waters HPLC system equipped with a 600 Consolider pump, a 717 plus Autosampler and a 2996 photodiode array detector was used. The analytical column used was a Kinetex 5 µm Biphenyl 100Å column (150 x 4.6 mm; Phenomenex, Torrance, CA, USA) coupled to a SecurityGuard ULTRA Cartridges UHPLC Biphenyl guard column (4.6 mm; Phenomenex). The chromatography conditions were: flow rate of 1 mL/min on a linear gradient of 5 to 95 % solvent B in 30 min followed by isocratic 95 % solvent B for another 10 min (solvent A: acid water pH 4.0 with acetic acid; solvent B: acetonitrile); the injection volume was 20 µL. The spectra were acquired between 200 nm and 600 nm wavelength, and patulin and griseofulvin quantifications was performed at 294 nm and 304 nm, respectively. Patulin and griseofulvin were identified in the samples by comparing the retention time and UV-vis spectra with those of chemical standards.

Comparison of griseofulvin production on different growth media and *in vivo* was analysed by liquid chromatography coupled with mass spectrometry. The HPLC-MS/MS system consisted of a binary pump and a vacuum degasser (1260 Agilent Technologies, Santa Clara, CA, USA) connected to a Varian auto-sampler Model 410 Prostar (Hansen Way, CA, USA), equipped with a 20 µL loop and coupled with

a Varian 310-MS TQ Mass Spectrometer. The sample was injected (10 µL) onto Luna C18(2) (150mm x 2mm i.d. 3µm, Phenomenex) and eluted under a flow of 200 µL/min. The mobile phase was an isocratic mixture of ACN:HCOOH 0.05% (60:40, v/v) for 5 min.

The mass spectrometer was equipped with an electrospray ionization (ESI) source operating in positive ion mode. Griseofulvin detection and quantitation were carried out using multiple reaction monitoring (MRM) mode by selecting three transitions: m/z 353 > 285 CE 18 eV (monitoring), m/z 353 > 215 CE 16 eV (quantification) and m/z 353 > 165 CE 16 eV (monitoring). The collision gas (Ar) pressure was set at 2 mbar for all experiments.

Quantification was performed by external calibration using a calibration curve for each mycotoxin. The calibration curves were prepared by dilution of standard solutions of griseofulvin and patulin.

2.10 Statistical analysis

All statistical analyses were performed with one-way ANOVA followed by Duncan test using IBM SPSS statistics software version 24 (SPSS Inc., Chicago, IL, USA), $p < 0.05$ was considered significant. The REST 2009 Software (Qiagen) was used for the statistical analysis of RT-qPCR and qPCR results.

3. Results

3.1 Knockout mutants for gsfR1 *and* gsfR2 *and their role in griseofulvin biosynthesis*

The role of putative transcription factors for griseofulvin biosynthesis encoded by *gsfR1* and *gsfR2* was assessed by obtaining deletion mutants and they were compared with the wild-type strain and a deletion mutant for *gsfA*, the gene encoding the PKS.

The deletion of genes was mediated by *A. tumefaciens* transformation, which delivered a T-DNA containing a hygromycin resistance cassette flanked by the promoter and terminator of target genes. Due to the presence of these flanking regions, homologous recombination led to replacement of genes with the antibiotic resistance cassette (Figs. 1-2, S1).

The knockout mutants were then assessed by amplifying the hygromycin resistance cassette and target gene by PCR (Figs. 1 B, 2 B, S1 B). As expected, the deleted genes were not amplified in knockout mutants, while hygromycin resistance cassette was only absent in the wild type PG3. Ectopic strains had a functional copy of the target gene and the antibiotic resistance cassette, due to non-target integration into the genome of the T-DNA.

To confirm the right insertion of the cassette, PCRs with primer pairs designed on the hygromycin resistance cassette and on 3'UTR or 5'UTR of deleted gene were performed (Figs. 1 C, 2 C, S1 C). These primer pairs can only amplify if the T-DNA has been introduced by homologous recombination at the target sites in the 3'UTR and 5'UTR flanking regions. As expected, the PCR fragments were amplified in the knockout mutants, and amplification failed in the wild type PG3 and in the ectopic mutants.

The number of integrations in the genome was determined by qPCR by measuring the difference between the quantification cycle (Cq) of target and reference genes in the mutant and in the control wild-type strain (Pfaffl, 2001) (Figs. 1 D, 2 D, S1 D).

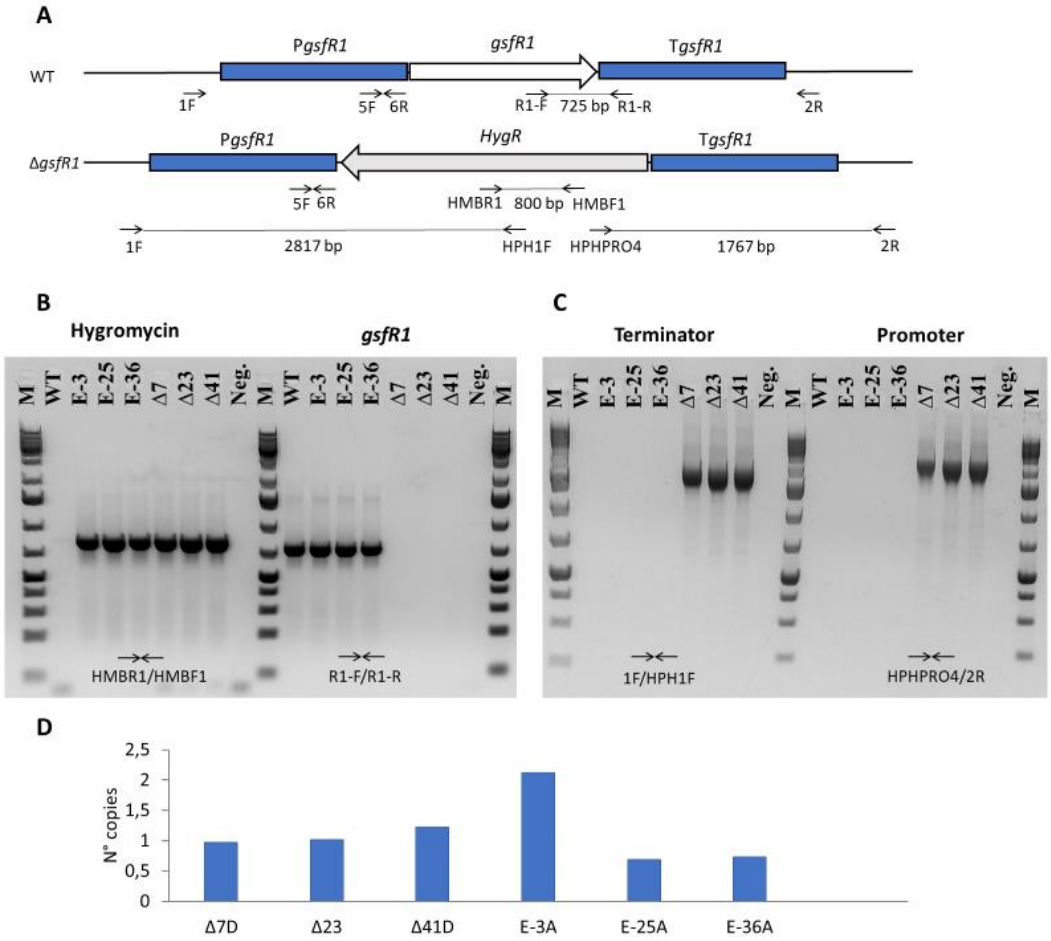


Figure 1 – PCR analysis of *gsfR1* mutants. Schematic presentation of the *gsfR1* locus in the wild type and deletion mutants, primers used are presented as arrows (**A**). Amplification of hygromycin resistance cassette (*HygR*) and *gsfR1* gene (**B**); confirmation of orientation of inserted T-DNA (**C**); copies number of T-DNA inserted in *P. griseofulvum* (**D**) determined by qPCR on gDNA using primers 5F/6R and histone H3 gene as reference gene. M= GeneRuler 1Kb Plus DNA ladder, WT = wild type *P. griseofulvum*, Δ = deletion mutants for *gsfR1* and E = ectopic strains, Neg. = negative control (PCR mix without DNA).

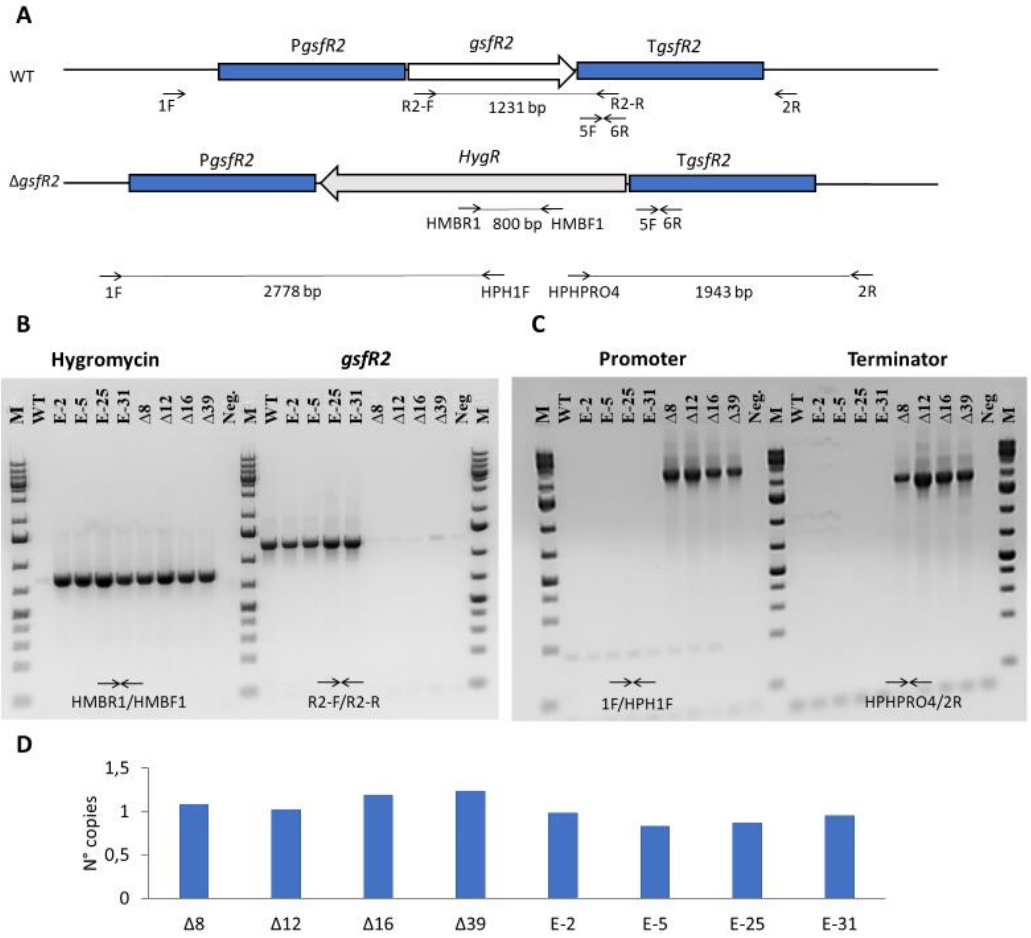


Figure 2 – PCR analysis of *gsfR2* mutants. Schematic presentation of the *gsfR2* locus in the wild type and deletion mutants, primers used are presented as arrows (**A**). Amplification of hygromycin resistance cassette (*HygR*) and *gsfR2* gene (**B**); confirmation of orientation of inserted T-DNA (**C**); copies number of T-DNA inserted in *P. griseofulvum* (**D**), obtained through qPCR on gDNA using primers 5F/6R and histone H3 gene as reference gene. M= GeneRuler 1Kb Plus DNA ladder, WT = wild type *P. griseofulvum*, Δ = deletion mutants for *gsfR2* and E = ectopic strains, Neg. = negative control (PCR mix without DNA).

Three mutants with one single event of integration and two ectopic strains were selected for each knockout experiment and they were characterized *in vitro* both phenotypically and chemically.

Concerning the effect of gene deletion on phenotype, it was shown that the deletion of the polyketide synthase did not affect the phenotype (Fig. S2). The knockout mutants for *gsfR1* showed the same growth rate as the wild type strain (Fig. 3A), but exhibited colonies with a markedly less green colour, caused by a marked reduction in conidiation *in vitro* (Fig. 3 B, C). On the other hand, knockout *gsfR2* mutants were similar to the wild type in both growth and sporulation (Fig. 5).

The ability of knockout mutants to produce griseofulvin was assessed on fungal cultures growing on PDA. Mutants lacking the *gsfA* gene were no longer able to produce griseofulvin *in vitro* (Fig. S2 B), while knockout mutants for the regulator gene *gsfR1* were found to produce higher quantities of griseofulvin compared to wild-type strain, with an average of about 30%, 50% and 110% higher production compared to wild-type strain at 5, 7 and 10 dpi (Fig. 4 A, Fig. 9).

The production of patulin was also investigated and deletion of *gsfR1* showed to influence also the regulation of this mycotoxin, especially at 5 dpi, reaching over 3000 µg/plate in all mutants compared to 2300 µg/plate produced by *P. griseofulvum* PG3, which was significantly different according to Duncan's statistical analysis ($p < 0.05$) (Fig. 4 B).

The knockout of *gsfR2* did not affect griseofulvin or patulin production, nor conidiation or growth rate, and were comparable with the wild type and knockout and ectopic mutants (Figs. 5, 6).

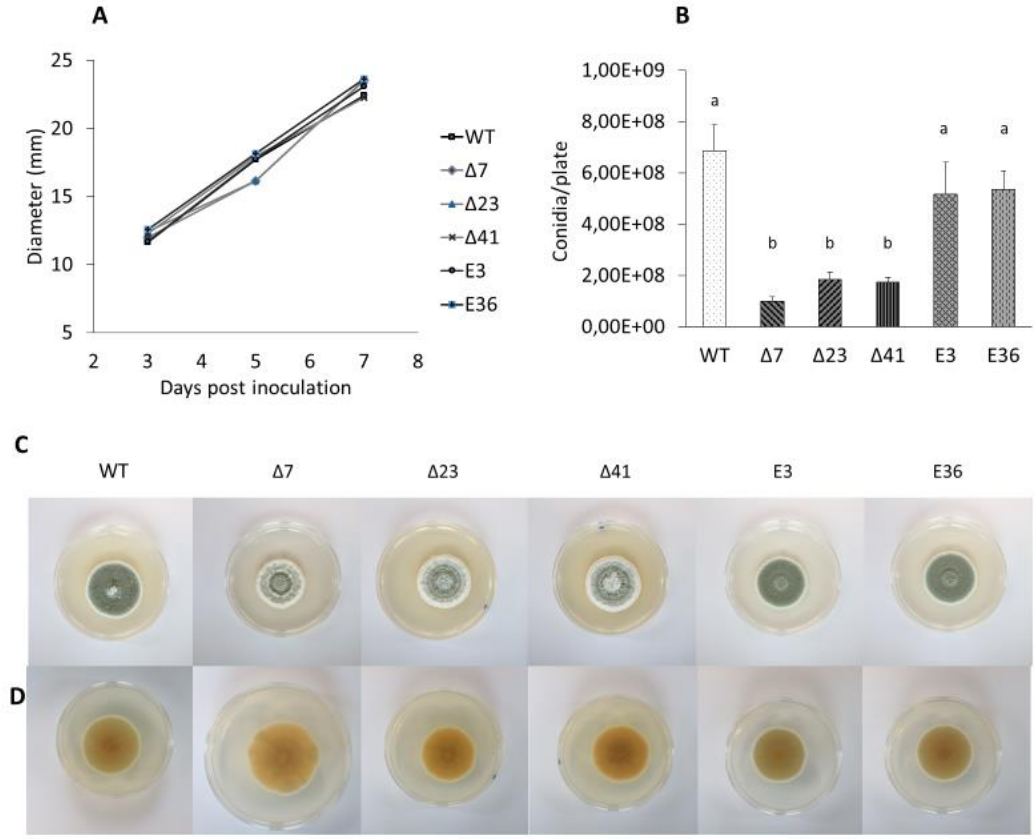


Figure 3 – Effect of *gsfR1* deletion on *P. griseofulvum* growth *in vitro.* Colony diameter (**A**) and conidia production (**B**) from 3 to 7 dpi. Plate view (front **C** and reverse **D**) at 7 dpi on PDA. WT = wild type *P. griseofulvum*, Δ = deletion mutants for *gsfR1* and E = ectopic strains. Values followed by the same letter are not statistically different by Duncan's multiple range test ($p < 0.05$).

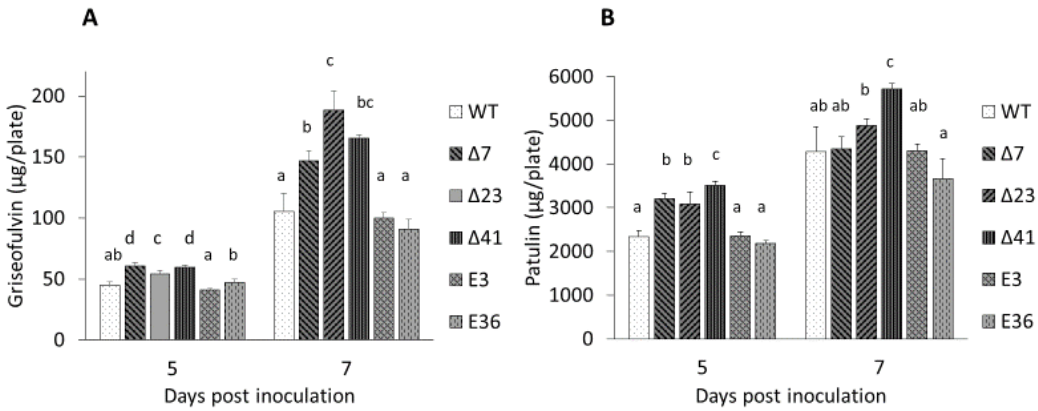


Figure 4 - Effect of *gsfR1* deletion on griseofulvin and patulin production. Griseofulvin (**A**) and patulin (**B**) production were measured at 5 and 7 dpi. WT = wild type *P. griseofulvum*, Δ = deletion mutants and E = ectopic strains. Values followed by the same letter are not statistically different by Duncan's multiple range test ($p < 0.05$).

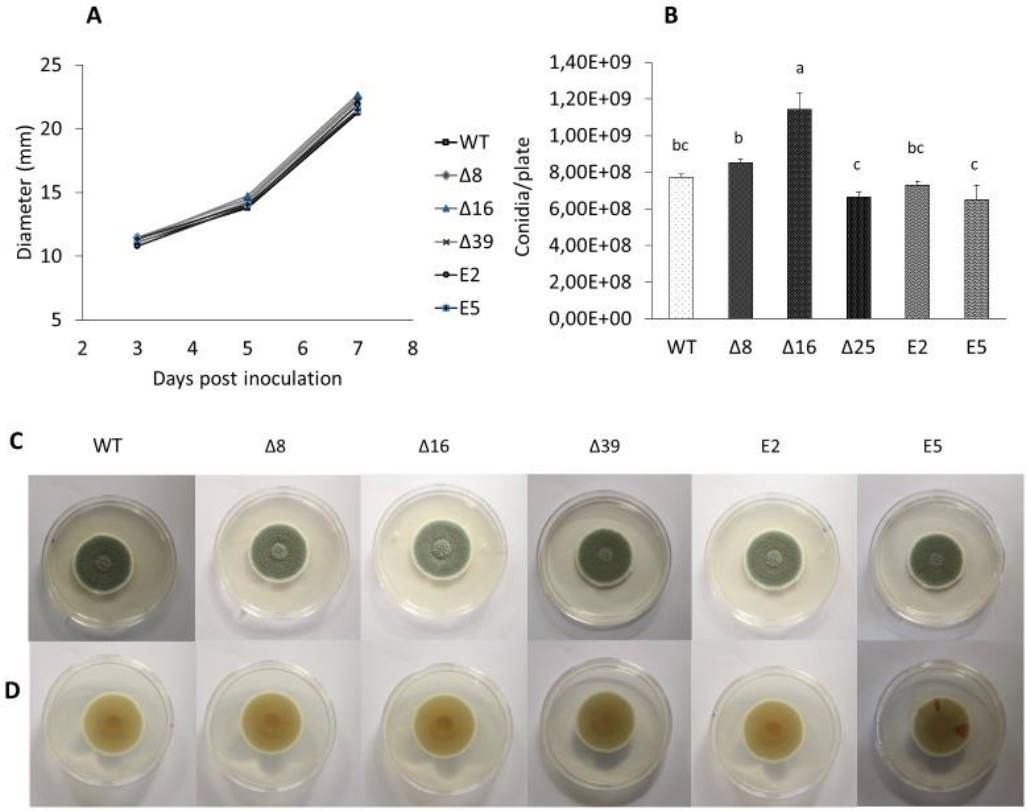


Figure 5 – Effect of *gsfR2* deletion on *P. griseofulvum* growth *in vitro.* Colony diameter (**A**) and conidia production (**B**) from 3 to 7 dpi. Plate view (front **C** and reverse **D**) at 7 dpi on PDA. WT = wild type *P. griseofulvum*, Δ = deletion mutants for *gsfR2* and E = ectopic strains. Values followed by the same letter are not statistically different by Duncan's multiple range test ($p < 0.05$).

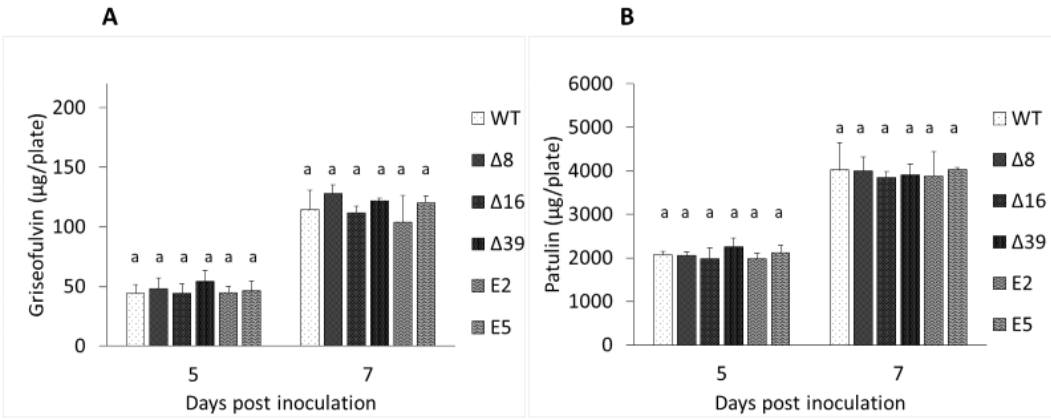


Figure 6 - Effect of *gsfR2* deletion on griseofulvin and patulin production. Griseofulvin (**A**) and patulin (**B**) production were measured at 5 and 7 dpi. WT = wild type *P. griseofulvum*, Δ = deletion mutants and E = ectopic strains. Values followed by the same letter are not statistically different by Duncan's multiple range test ($p < 0.05$).

3.2 GsfR1 negatively regulates griseofulvin biosynthesis on PDA

The observation that the deletion of *gsfR1* led to a higher griseofulvin production *in vitro* led us to hypothesize that this gene could encode a negative regulator of griseofulvin biosynthesis. To examine this hypothesis, the relative expression of the polyketide synthase gene (PKS) of three mutants and two ectopic strains was investigated by RT-qPCR. The Δ*gsfR1* deletion mutants showed an increased expression of *gsfA* compared to the wild type, both at 5 and 7 dpi, while ectopic mutants exhibited a similar gene expression to the wild type (Fig. 7).

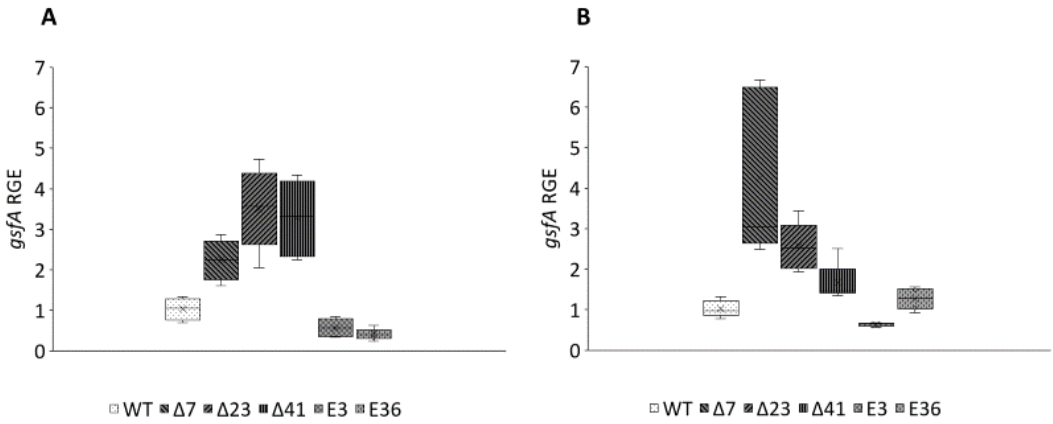


Figure 7 – Relative gene expression (RGE) of *gsfA* gene, at 5 (**A**) and 7 (**B**) dpi on PDA. WT = wild type *P. griseofulvum*, Δ = deletion mutants for *gsfR1* and E = ectopic strains. The expression is relative to the expression of the β-tubulin gene.

3.3 Virulence and griseofulvin production on apples

To evaluate the effect of griseofulvin on pathogenicity of *P. griseofulvum*, the virulence of three knockout mutants was compared with that of the wild-type strain and one ectopic mutant on apples (Fig. 8). *ΔgsfA* mutants, which were not able to produce griseofulvin, showed a slightly reduced virulence (Fig. 8 A). In this assay, the inoculation with E-A ectopic mutant, which contains 4 insertions of the T-DNA, led to a strong reduction of virulence on apples. However, *gsfR2* mutants showed a similar virulence compared to the wild-type strain over a period of 14 days after inoculation (Fig. 8 B). On the contrary, Δ*gsfR1* strains were slightly more virulent (Fig. 8 C) compared to the *P. griseofulvum* parental strain. Nevertheless, the ectopic mutant showed an enhanced virulence as well, although it was less virulent than the mutant in the first stages of the infections. For this reason, we cannot exclude that the enhanced virulence of the mutants was at least partly due to the transformation itself.

To check if there is a relationship between increased virulence and production of griseofulvin *in situ*, the mycotoxin was extracted from apples inoculated with *gsfR1* mutants and quantified. Results showed a significant increased production of griseofulvin from knockout mutants at 10 dpi (Fig. 8 D), while at 14 dpi *gsfR1* mutants produced less griseofulvin compared to wild-type strain.

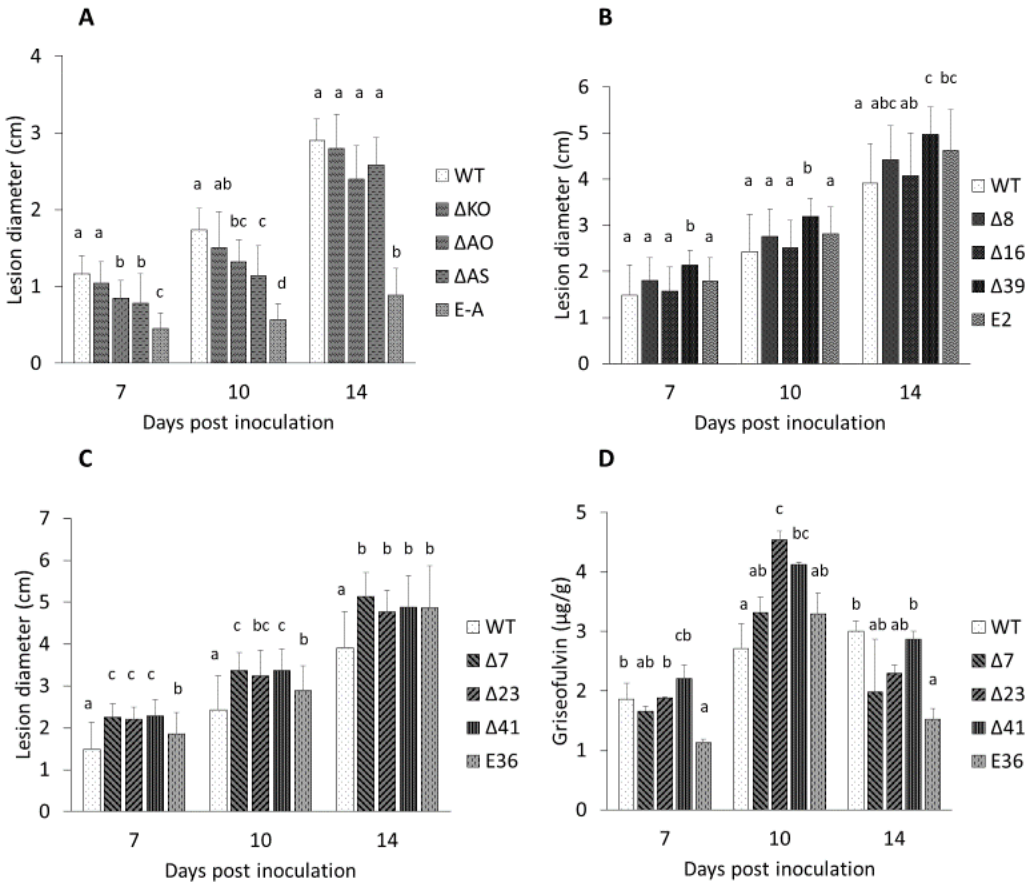


Figure 8 - Effect of gene deletions on virulence and griseofulvin production *in vivo*. Lesion diameter caused by the wild-type strain was compared with rot diameter of knockout ad ectopic strains for *gsfA* (**A**) *gsfR2* (**B**) and *gsfR1* (**C**). Griseofulvin production (µg/g of apple) was compared between wild type and Δ*gsfR1* strains (**D**). Measurements were taken at 7, 10 and 14 dpi. WT = wild type *P. griseofulvum*, Δ = deletion mutants and E = ectopic strains. Values followed by the same letter are not statistically different by Duncan's multiple range test ($p < 0.05$).

3.4 Global regulators in griseofulvin biosynthesis

By using information on the CIS-BP database, the promoters of *gsfR1* and *gsfA* were scanned in order to find putative binding sites for global regulators. Eighty-one and seventy-five transcription factors were predicted to bind the promoters of *gsfR1* and *gsfA*, respectively. Thirteen transcription factors seem to bind only the promoter of *gsfR1*, while 7 are typical of *gsfA* and 68 were predicted to regulate both genes, ensuing a complex scenario (Tab. S2).

3.5 Carbon and nitrogen have a strong effect on in vitro *conidiation and griseofulvin production*

Among the different stimuli, the availability of nutritional sources, especially nitrogen and carbon, is a key factor for the biosynthesis of many SMs and for the growth and differentiation of filamentous fungi. Because of this, it is not surprising that we found many putative TFs involved in carbon or nitrogen consumption, which could bind the promoter of *gsfR1* and *gsfA* (Tab. S2).

We focused our attention on the effect of carbon and nitrogen on griseofulvin production. To do that, two *gsfR1* deletion mutants and one ectopic strain were grown for ten days on three different media (PDA, MEA and MPA); one knockout mutant for the polyketide synthase gene was also included as control (denoted as KO). These three media are characterized by different C/N ratios: PDA is composed of potato extract and dextrose and has the highest carbon content, while on MEA and MPA, peptone was added, increasing the nitrogen content, which is ten times higher on MPA (thanks to the addition of meat extract) compared to MEA (Núñez et al., 2000). As expected, carbon and nitrogen showed a huge impact both on griseofulvin and conidia production (Fig. 9).

Conidiation of the wild-type strain was reduced in both media containing peptone, with a stronger reduction on MEA (Fig. 9 A), and a significant reduction of

griseofulvin production was observed in MPA (23%) compared to PDA or MEA (Fig. 9 B).

Conidiation of the *gsfA* knockout mutant, lacking the gene encoding the polyketide synthase, was similar to that observed for the wild-type strain in the three media tested. However, the knockout mutant was not able to produce griseofulvin in any condition.

When focusing on *gsfR1* mutants, a more complex scenario was observed. Deletion of *gsfR1* gene led to a significant reduction of conidia on MEA and PDA compared to wild type, ectopic strain and *gsfA* mutants, suggesting that this gene could play a role in the direct or indirect regulation of conidia production. Considering griseofulvin production, a significant increase of griseofulvin production was observed on PDA (Fig. 4 and 9 B). Instead, on the other two media, richer in nitrogen, there was a significantly reduced production compared to wild type.

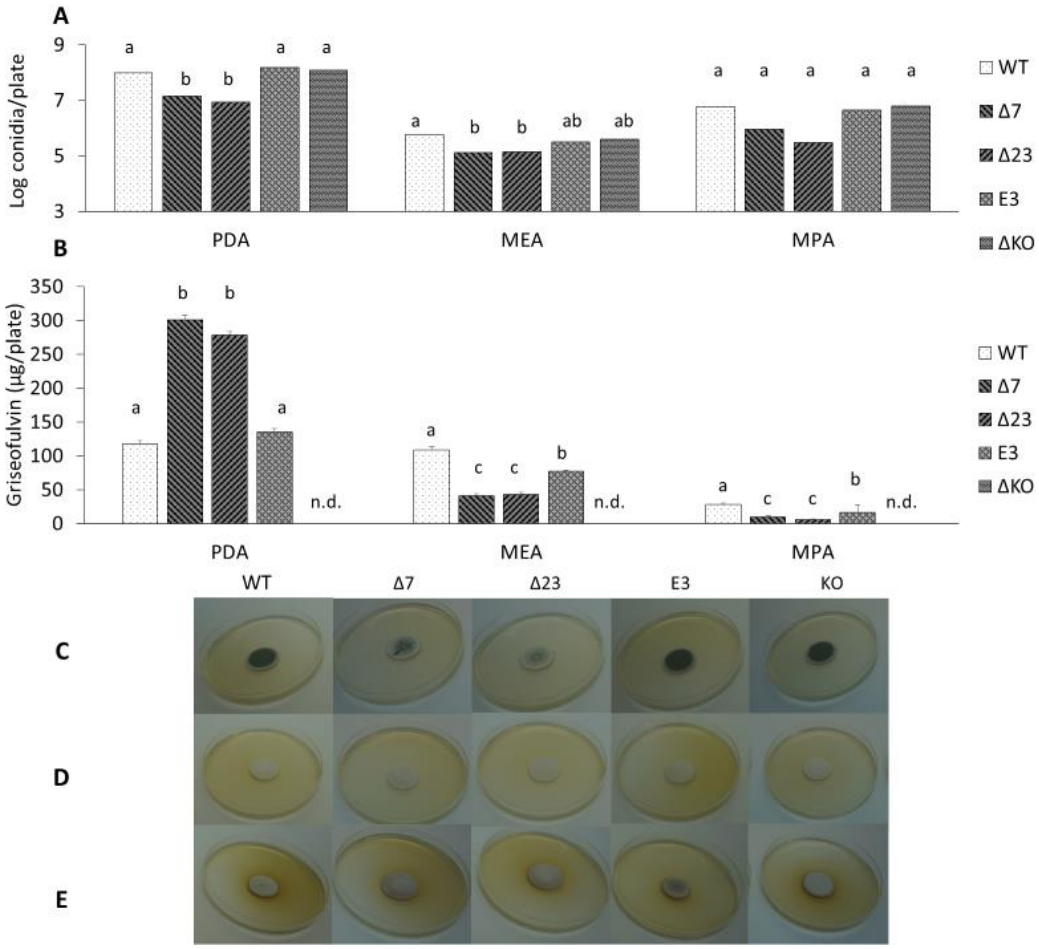


Figure 9 – Effect of carbon and nitrogen on conidiation and on griseofulvin production *in vitro*. Logarithm of conidia per plate (**A**) and griseofulvin produced (**B**) were measured at 10 dpi in three solid media (PDA, MEA and MPA). Colony view on PDA (**C**), MEA (**D**) and MPA (**E**). WT = wild type *P. griseofulvum*, Δ7, Δ23 = deletion mutants for *gsfR1*, E3 = ectopic strain and ΔKO = deletion mutant for *gsfA*. Values followed by the same letter are not statistically different by Duncan's multiple range test ($p < 0.05$); n.d. = non detectable.

4. Discussion

4.1 Role of transcription factors GsfR1 and GsfR2 in griseofulvin biosynthesis

The griseofulvin gene cluster previously identified in *P. aethiopicum* includes two genes, *gsfR1* and *gsfR2*, encoding for putative transcription factors. The *gsfR2* gene in *P. griseofulvum* was located in a separated genomic region of the biosynthetic gene cluster, differently from *P. aethiopicum*. Previous reports demonstrated the mode in which some TFs regulate the expression of a specific gene cluster even when they are located outside the cluster itself. This was the case of both *fum21* and *zfr1*, that are located inside and outside the gene cluster respectively, and regulate fumonisin biosynthesis in *Fusarium verticilloides* (Brown et al., 2007; Flaherty and Woloshuk, 2004). In this work, our aim was to elucidate the role of putative transcription factors in griseofulvin biosynthesis by generating deletion mutants. Deletion mutants for the *gsfA* core biosynthetic gene encoding a polyketide synthase lacking the ability to produce griseofulvin were obtained and used as control (Chooi et al., 2010).

Our results show that the *gsfR1* gene encodes a putative transcription factor not only acts on griseofulvin biosynthesis but also plays an essential role as an important regulator of *P. griseofulvum* development and secondary metabolism. GsfR1 seems to regulate griseofulvin biosynthesis as a negative regulator of the cluster when tested *in vitro* on PDA medium and on apples. Indeed, the deletion of this regulatory gene led to an increase in griseofulvin production under these conditions with a higher expression of the *gsfA* gene *in vitro.* Conversely, on media supplemented with peptone, *gsfR1* gene positively regulates the griseofulvin biosynthesis, as all mutant strains were able to produce significantly less amount of the compound compared to wild type. Similar results were obtained with the *mtfA* gene encoding the TF of aflatoxin in *Aspergillus flavus* and *Aspergillus parasiticus*, revealing that

the regulatory activity of this class of enzymes is largely dependent on environmental conditions (Zhuang et al., 2016).

A differential regulation of patulin production was also revealed in knockout mutants of *gsfR1* , which displayed increased patulin production at 5 dpi *in vitro.* This result suggests that *gsfR1* could be involved in regulation of other SMs produced by *P. griseofulvum*, demonstrating a multiplicity of downstream pathways that are regulated by the same transcription factor.

A crosstalk between different SM gene clusters has been largely recognized. For instance, in *Aspergillus nidulans* the putative transcription factor encoded by *scpR* controls the expression of *inpA* and *inpB*, two genes involved in the biosynthesis of a NRP-like compound, and the same TF is also involved in regulation of asperfuranone biosynthesis, a polyketide compound (Bergmann et al., 2010).

The interconnection of signals and responses is also manifested by the complex pattern of TFs that can putatively bind the promoters of *gsfR1* and *gsfA*, triggering the activation or repression of griseofulvin biosynthesis and regulating at the same time other SM gene clusters and many aspects of the biology of *P. griseofulvum*, such as conidiation and virulence. Surprisingly, in the recently released genome of griseofulvin-producer *Xylaria flabelliformis* (Mead et al., 2019) the gene *gsfR1* was missing, pointing to a possible regulation of griseofulvin biosynthesis independently of *gsfR1* action.

Considering the second putative transcription factor of the cluster, the deletion of *gsfR2* seems to have no effect on growth and SMs production of *P. griseofulvum*, and it is likely that the gene is involved in a different biosynthetic pathway. Indeed, the gene *gsfK* encoding for putative reductase is located next to *gsfR2*, and its deletion in *P. aethiopicum* did not affect the production of griseofulvin (Cacho et al., 2013). These findings led to the assumption that both *gsfK* and *gsfR2* are probably involved in the biosynthesis of another SM. Moreover, in *X. flabelliformis*

(Mead et al., 2019), *gsfJ* and *gsfG* genes were missing, suggesting that these two genes are not necessary for griseofulvin production.
Therefore, considering also that the knockout of *gsfR2* does not impair griseofulvin production, we suggest that the core genes for the biosynthesis of this metabolite are only 7 instead of 13.

4.2 Role of global regulators in griseofulvin biosynthesis

Secondary metabolism gene clusters are usually regulated by several global regulatory proteins in response to many environmental stimuli, such as light, carbon and nitrogen levels, pH and redox status, iron concentration and signalling from other organisms (Brakhage, 2013; Hoffmeister and Keller, 2007). Many of these global regulators are well characterized and their putative binding sites in the promoter regions of genes could be detected.
One of the transcription factors predicted to regulate *gsfR1* and not *gsfA* is KXG54396.1, a *P. griseofulvum* gene similar to *mtfA*, whose deletion or overexpression in *A. flavus* causes a reduction if aflatoxins production (Zhuang et al., 2016). Environmental conditions influence the regulatory activity of the gene in *A. flavus*, where the deletion increases conidiation *in vitro* and reduces it *in vivo* (Zhuang et al., 2016).
Other transcription factors putatively binding *gsfR1* and not *gsfA* include NsdD, which is necessary for sexual development and represses asexual one (Han et al., 2001), a quinic acid utilization activator, an homolog of the fluconazole resistance protein and *stuA*, a gene able to regulate cell pattern formation (Miller et al., 1992), carbon metabolism, effector expression and the synthesis of penicillin and several mycotoxins (IpCho et al., 2010; Sigl et al., 2011; Yang et al., 2018). Another transcription factor, KXG45700.1, was similar to LreA, a protein involved in conidiation and response to light (Igbalajobi et al., 2019), while KXG46600.1 was similar to Res2, necessary for meiosis (Zhu et al., 1997). KXG47124.1 has 60%

identity with the transcription factor Msn2 of *A. parasiticus*, involved in conidiation, hyphal growth and on occasionally stress response in several fungi (Liu et al., 2013; Song et al., 2018; Tian et al., 2017). KXG48399.1 is homologous to another transcription factor *flbC* related to asexual development, whose knock-out causes delayed conidiation in *A. nidulans* (Kwon et al., 2010). The *gsfR1* promoter was predicted to be bound also by homologs of PAP1, SebA and NapA, transcription factors involved in oxidative stress response (Asano et al., 2007; Dinamarco et al., 2012; Ikner and Shiozaki, 2005). The presence of the binding sites for this group of transcription factors suggest a complex regulation of *gsfR1*, that is markely influenced by sexual and asexual development and environmental conditions.

The promoter of *gsfA* had 7 unique binding sites for transcription factor, including SreP, a repressor of siderophore biosynthesis in presence of high iron concentrations (Haas et al., 1997). Other transcription factors putatively regulating *gsfA* included two regulators of filamentous growth, a transcriptional activator of gluconeogenesis, a pathway necessary in *A. nidulans* for growing with certain carbon sources metabolized via Acetyl-CoA (Hynes et al., 2007), and a homolog of *gal4*, necessary in *Saccharomyces cerevisiae* for growth using galactose or lactose as only carbon sources (Riley et al., 1987). These data suggest the possible increase in *gsfA* expression, and therefore griseofulvin production, in the presence of difficult carbon sources, but further experiments would be required to check this possibility.

Several transcription factors binding the promoters of both *gsfR1* and *gsfA* have not been characterized yet. However, among them, there were homologs of the well-known global regulators AreA and CreA, which are respectively a positive and a negative transcription factor acting in response to nitrogen or carbon sources (Katz et al., 2008; Wilson and Arst, 1998). Moreover, the binding sites of NirA and the carbon catabolite repressor MIG1 were also found in the promoters of both genes, increasing the possibilities that production of griseofulvin being heavily influenced by carbon and nitrogen availability (Burger et al., 1991; Randhawa et al., 2018).

Binding sites for genes regulating the utilization of different carbon and nitrogen sources are also present. These include: *facB*, regulating acetate utilization (Todd et al., 1998), *argR*, regulating arginine metabolism (Dubois et al., 1987), *nirA*, regulating nitrate utilization (Burger et al., 1991), *amdR*, regulating amides, omega amino acids and lactams catabolism (Andrianopoulos and Hynes, 1990), and the quinic acid utilization activator (Wheeler et al., 1996). Other two transcription factors, the cutinase transcription factor 1 beta and SKN7, increase the expression of cutinases and the resistance to ROS respectively, suggesting a role of the griseofulvin cluster in pathogenesis (Cao et al., 2009), as shown in the pathogenicity trials.

In addition to those indicated above, other identified transcription factors were related to sexual and asexual development, heat shock, stress resistance, cellobiose response, sulphur catabolism and assimilation, iron homeostasis and adhesion, and hyphal growth (Tab. S2).

Both promoters of *gsfR1* and *gsfA* also contain binding sites similar to those recognized by Apa-2 (Chang et al., 1993), strengthening the hypothesis that the griseofulvin regulatory system is somewhat similar to the aflatoxins one, regulated by MtfA.

4.3 Global regulation of griseofulvin biosynthesis is triggered by carbon and nitrogen

Nitrogen is a central element for the life of living organisms, and many specific regulatory genes are expressed when nitrogen sources such as ammonium, glutamate or glutamine are scarce. These genes encode transcription factors that repress the utilization of nitrogen sources that are easy to assimilate, in the so-called mechanism “Nitrogen Metabolism Repression” (NMR). All these genes are responsible for the activation of pathways required for the uptake of alternative nitrogen sources (Marzluf, 1997; Tudzynski, 2014). AreA is one of the main

enzymes involved in NMR, and putatively regulate griseofulvin gene cluster, acting both on *gsfR1* and *gsfA*. Similarly, the global repressor in response to carbon, CreA, was found to be putatively involved in the regulation of griseofulvin biosynthesis, together with transcription factors that modulate the utilization of different carbon sources.

The effect of carbon and nitrogen sources on growth, conidiation and griseofulvin biosynthesis was investigated *in vitro* on three different media, with different C/N ratios. Núñez and collaborators (2000) used MEA and MPA to evaluate the effect of carbon and nitrogen on production of verrucosidin, showing that MEA induced a higher production of this compound and that the type of growth medium had significant influence on mycotoxin production. The wild type strain showed a similar pattern of griseofulvin production on PDA and MEA, while in MPA a significant reduction was observed. This result is reasonable because carbon is essential for building the polyketide structure.

On the other hand, the deletion of *gsfR1* seems to have a differential effect on MEA and MPA. In contrast to what observed on PDA plates, the production of griseofulvin drops drastically in deletants compared to wild type. The high nitrogen concentration in these media likely triggered the NMR, resulting in the activation of SM gene clusters. This could explain the higher production of griseofulvin in the wild type compared to *gsfR1* deletion mutants. This result also indicates that GsfR1 can regulate griseofulvin biosynthesis both in a positive and negative manner, depending on external stimuli.

4.4 Regulation of griseofulvin biosynthesis and conidiation

Fungal development and secondary metabolism are deeply connected and many examples have demonstrated that some compounds (especially mycotoxins) can be secreted at the time of conidiation, while other SM such as linoleic-acid in *A. nidulans* or zearalenone in *Fusarium graminearum* can induce sporulation, and

some of them are required for formation of sexual ad asexual spores and for their survival (e.g. pigments) (Calvo et al., 2002). Knockout mutants for *gsfR1* produce less conidia compared to the parental strain, but this behaviour is not surprising since the deletion of regulatory genes is often associated with reduced conidiation, such as for deletion of *aflR* TF for aflatoxin biosynthesis (Wilkinson et al., 2004). The reduced conidiation at first was thought to be related to the antifungal properties of griseofulvin, which is highly produced on PDA by knockout mutants. From this perspective, the role of *gsfR1* is to avoid an excessive production of griseofulvin, which is known to have an antifungal activity and could have toxic effects on the producer. Nevertheless, a strongly reduced production of conidia was observed in MEA and MPA, where the mutants exhibited a similar and lower griseofulvin production, respectively, compared to production on PDA. Consequently, the reduced conidiation of *gsfR1* mutants cannot be linked with the antifungal activity of griseofulvin, but instead it seems to be the result of both the influence of the culture media and the effect of deletion of *gsfR1* itself.

Concerning the culture media, MEA and MPA were supplemented with peptone. The malt extract added in MEA provides carbon and nitrogen content, while in MPA the meat extract contributes to richness in nitrogen. In previous studies on aflatoxin biosynthesis it was shown that simple sugars support fungal growth, sporulation and aflatoxin production, unlike complex sugars and peptone, which strongly repress aflatoxin production (Calvo et al., 2002; Yao et al., 2018).

The reduced number of conidia produced by all tested strains in MEA and MPA appears therefore to be related to the nutrient composition of these media. Instead, the reduced conidiation of knockout mutants in all tested media could be explained as a consequence of a direct or indirect positive regulation of conidiation driven by g*sfR1*. In this regard, many TFs have a role in co-regulating spore production and mycotoxin formation. MtfA, RtfA, NsdC, RafA and StuA all regulate aflatoxin production, conidiation and sclerotia development in *A. flavus* (Yao et al., 2018).

Furthermore, the regulator of patulin biosynthesis was recently proposed to be connected with the development of *P. expansum*, even though the authors suggests that this is only a remote possibility that have to be proved (Snini et al., 2016). Additionally, *gsfR1* is putatively bound by transcription factors involved in asexual and sexual development, that strongly supports this model. Further studies are needed to investigate the role of *gsfR1* in regulation of sporulation or conidiation, in addition to already known global transcription factors.

4.5 Role of griseofulvin on pathogenicity of P. griseofulvum

Considering that SM are not essential for primary metabolism of fungi, along the fact that they confer several advantages to the producer organism, it is reasonable to assume that they could have a role in pathogenicity of fungal pathogens. The relationship between SMs production and pathogenicity has been extensively investigated previously, and several examples linking SMs and mycotoxins with pathogenesis are well documented (Macheleidt et al., 2016; Scharf et al., 2014). Considering causal agents of blue molds in apples, previous studies have investigated the role of patulin in infection and development of of *P. expansum*, but contrasting results emerged. For instance, Ballester et al. (2015) and Li et al. (2015) pointed out that patulin produced by fungal pathogens is not essential for apple infection, and the deletion of *patK*, *patL*, and *patN* genes involved for patulin biosynthesis, did not affect the infection and virulence. Conversely, Barad et al. (2013) and Snini et al. (2016) observed a direct correlation with virulence, because mutants with reduced production of patulin were less virulent on apples. These contrasting results obtained in the same pathosystem can be explained by the use of different strains from different countries and by the great quantity variable factors involved in *in vivo* trials such as temperature, pH, cultivar sensitivity and storage conditions (Barad et al., 2016b). Among these factors, apple variety plays an important role as demonstrated by Snini et al. (2016). Taking into account the

previous works reported, patulin could be considered a virulence factor (connected to severity of disease), instead of a pathogenicity one (linked to the ability to cause the disease), contributing to the establishment of the disease when the pathogen has already colonized the fruit (Barad et al., 2016b). Indeed, the addition of patulin directly into the wound completely restored the wild-type phenotype in less virulent strains (Snini et al., 2016).

According to results of the current work, griseofulvin may be involved in virulence and not pathogenicity. Indeed, the deletion mutants for the *gsfA* gene are still able to induce the disease, even if they are not able to produce griseofulvin, but the infected apples exhibit reduced lesions. This supports the idea that griseofulvin alone should not be considered a pathogenicity factor but can act to enhance virulence.

ΔgsfR1 mutants were slightly more virulent compared to the wild type, while Δ*gsfR2* strains were comparable to the wild type both in griseofulvin production and in pathogenicity. Considering that Δ*gsfR2* mutants displayed no differences in virulence compared to the wild type, the differences on development of rot diameter of knockout mutants for *gsfA* and *gsfR1* genes could be related to griseofulvin production. The differences seen in the development of rot diameter on apples by Δ*gsfA* and Δ*gsfR1* mutants could suggest a role of griseofulvin production in virulence of *P. griseofulvum*. Nevertheless, the ectopic strain showed increased virulence compared to the wild type and therefore further studies are necessary to understand the molecular mechanism/s underlying this increased production.

Concerning the griseofulvin produced *in vivo* by Δ*gsfR1* mutants a higher production was observed at 10 dpi (similarly to what observed on PDA plates) and a lower production at 14 dpi (similarly to what observed on MEA and MPA plates). In establishing the interaction with the host, *P. griseofulvum* would activate a series of well characterized responses, such as biosynthesis of gluconic acid and ammonium depletion, in order to improve the acidification of apples and the

biosynthesis of polygalacturonases responsible for cell wall degradation of fruits (Barad et al., 2016a; Prusky et al., 2004). Reduced nitrogen availability in the site of infection, together with high availability of simple sugars, can trigger the activation of SM gene clusters, including griseofulvin, which is also enhanced in presence of ROS and cutinases according to the findings of our work. This hypothesis is supported by the fact that *gsfR1* mutants produced less griseofulvin compared to wild type after 14 days, so it is reasonable to assume that positive regulation of griseofulvin biosynthesis is activated at this point. Moreover, if GsfR1 acts as a negative regulator, it is reasonable to observe an earlier production of griseofulvin in the Δg*sfR1* knockout mutants, as it was found in infected apples by day 10, when the production of griseofulvin was higher in the knockout mutants. To our knowledge, the current work presents the first attempt to investigate the role of griseofulvin in virulence of *P. griseofulvum* and further investigation is necessary to further prove its involvement.

5. Conclusions

This work led to a better understanding of the complex regulation of griseofulvin biosynthesis. The role of putative transcription factors was also investigated. Based on our results, *gsfR2* is not involved in the biosynthesis pathway and it is probably part of another gene cluster. The putative reductase *gsfK*, while *gsfG* and *gsfJ* are putatively not essential for griseofulvin biosynthesis due to their absence in the genome of the producer species *X. flabelliformis*. Therefore, the griseofulvin gene cluster should be considered as composed of 7 genes, instead of 13, as previously described.

The gene *gsfR1*, on the other hand, is involved in griseofulvin biosynthesis, acting as a negative regulator of the cluster on PDA and on apples. However, in different culture conditions, characterized by nitrogen and complex sugars richness, *gsfR1* could act as a positive regulator of griseofulvin biosynthesis. These findings suggest

that *gsfR1* can trigger different responses depending on external stimuli, especially nitrogen and carbon availability. The deletion of *gsfR1* has a significant impact on many aspects of *P. griseofulvum*, ranging from secondary metabolism to virulence and conidiation. It is remarkable to notice that *gsfR1* seems to be involved in the regulation of patulin biosynthesis, which *P. griseofulvum* can produce in high amounts on apples. This finding could lead to new strategies to limit the production of patulin on apples.

Concerning pathogenicity of *P. griseofulvum*, the deletion mutants for *gsfA* were less virulent than the wild-type strain, while the deletion mutants for *gsfR1* were slightly more virulent *in vivo* suggesting a direct role of griseofulvin on virulence of *P. griseofulvum*. Until now, this is the first study on the role of griseofulvin in pathogenicity, and further investigations are needed to confirm this data and to highlight the mechanisms of action involved.

gsfr1 is also involved in the asexual reproduction of *P. griseofulvum*, as reduced conidiation was observed compared to the wild type in all *in vitro* conditions tested. In conclusion, g*sfR1* represents a good example of how the regulation of SM biosynthesis and fungal development can be complex and extremely interconnected.

Acknowledgments

We thank Prof. Maria Lodovica Gullino for reviewing and editing of the manuscript and Dr. Houda Banani for setting up the knockout on *P. griseofulvum*. This work was supported by Fondazione Cassa di Risparmio di Cuneo (progetto SMART APPLE – Innovative and SMART technologies for sustainable apple production) and by the Spanish Ministry of Science, Innovation and Universities (AGL2017-88120-R, AEI/FEDER, UE).

Author contributions

Silvia Valente: Conceptualization, Investigation, Formal analysis, Writing - original draft; Agnese Cometto: Investigation, Formal analysis, Writing - original draft; Edoardo Piombo: Conceptualization, Formal analysis, Software, Writing - original draft; Giovanna Roberta Meloni: Investigation, Formal analysis, Writing - original draft; Ana-Rosa Ballester: Conceptualization, Investigation, Formal analysis, Writing - review & editing; Luis González-Candelas: Conceptualization, Resources, Writing - review & editing; Davide Spadaro: Conceptualization, Resources, Writing - review & editing.

Supplementary material

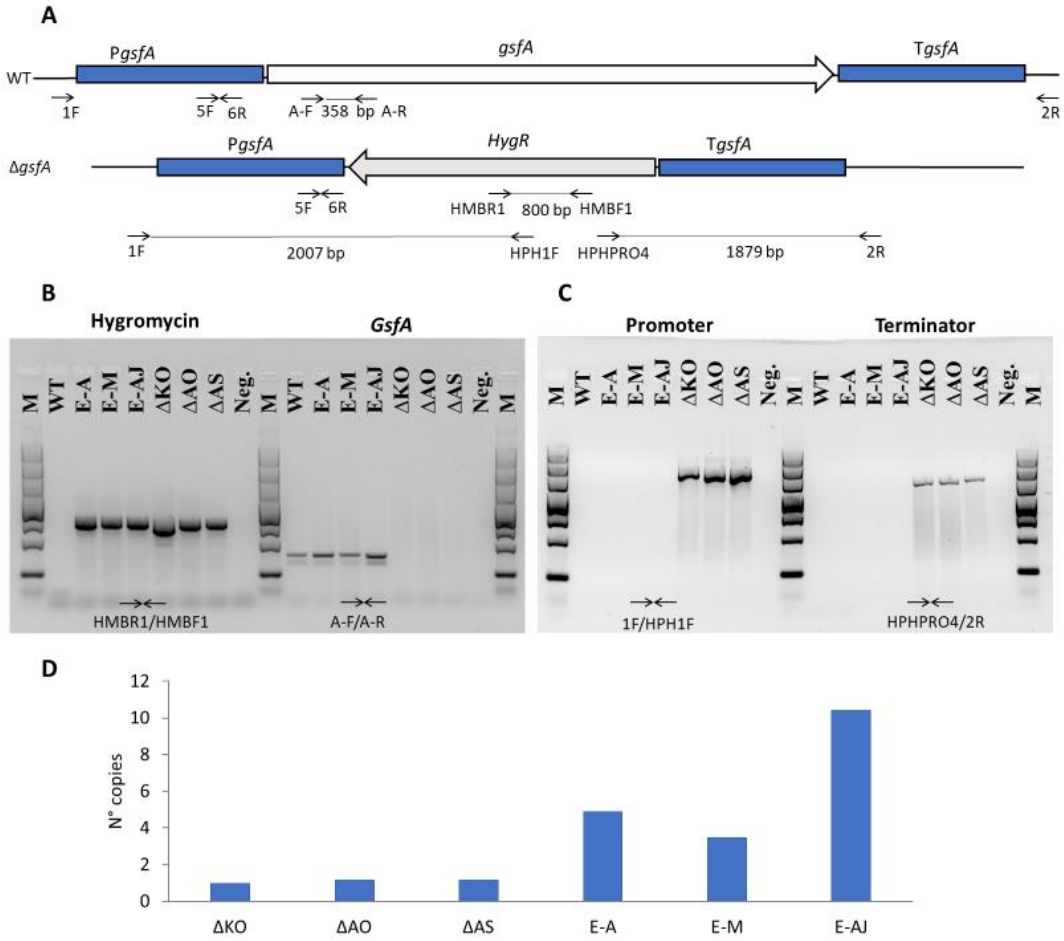


Figure S1 – PCR analysis of *gsfA* mutants. Schematic presentation of the *gsfA* locus in the wild type and deletion mutants, primers used are presented as arrows (A). Amplification of hygromycin resistance cassette (*HygR*) and *gsfA* gene (B); confirmation of orientation of inserted T-DNA (C); copies number of T-DNA inserted in *P. griseofulvum* (D), determined by qPCR on gDNA using primers 5F/6R and β-tubulin gene as reference gene. M= GelPilot Wide Range Ladder, WT = wild type *P. griseofulvum*, Δ = deletion mutants for *gsfA* and E = ectopic strains, Neg. = negative control (PCR mix without DNA).

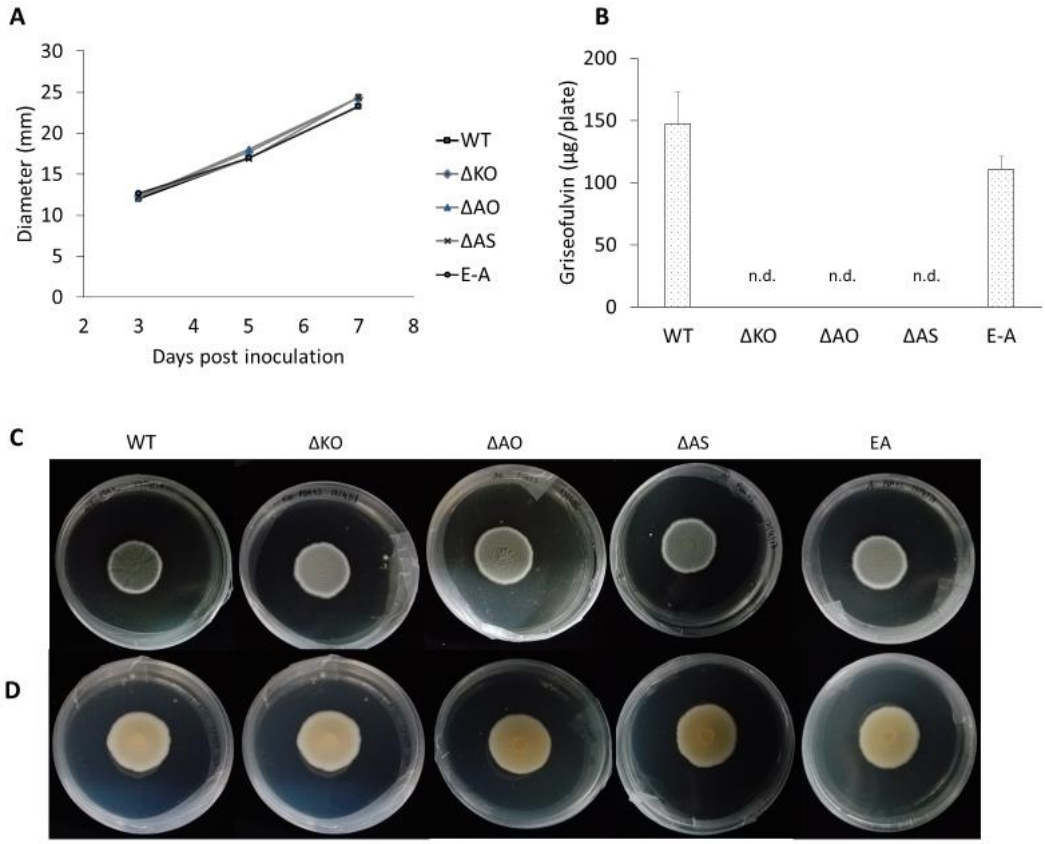


Figure S2 – Effect of *gsfA* deletion on *P. griseofulvum.* Colony diameter (**A**) and griseofulvin production (**B**) from 3 to 7 dpi. Plate view (front **C** and reverse **D**) at 7 dpi on PDA. WT = wild type *P. griseofulvum*, Δ = deletion mutants for *gsfA* and E = ectopic strains, n.d. = non detectable.

Table S1 – List of primers used in this work.

Name	Primer sequence (5'→3')	Description
Construction and analysis of knockout mutants		
O1- *gsfR1*	GGTCTTAAUGTCGGCATCGGACGAATTATAG	Insertion of *gsfR1* promoter in pRFHU2. Tail for USER reaction in red.
O2- *gsfR1*	GGCATTAAUCATCTTTCTGAGGAGGGAGAGA	
A3- *gsfR1*	GGACTTAAUGCGCGAAACGGTATTTCTAT	Insertion of *gsfR1* terminator in pRFHU2. Tail for USER reaction in red.
A4- *gsfR1*	GGGTTTAAUGTGTGAGCGTGAATGGTAATG	
O1- *gsfR2*	GGTCTTAAUTCGGTGTCCACGTTTCTATTC	Insertion of *gsfR2* promoter in pRFHU2. Tail for USER reaction in red.
O2- *gsfR2*	GGCATTAAUTTGCTTGCCACGGTTATCT	
A3- *gsfR2*	GGACTTAAUGACGAGCCAGTTCTCCTAATG	Insertion of *gsfR2* terminator in pRFHU2. Tail for USER reaction in red.
A4- *gsfR2*	GGGTTTAAUCTCCTTGCCCGATGTCTTATC	
O1- *gsfA*	GGTCTTAAUGCACACCTGAGCAAGATCAA	Insertion of *gsfA* promoter in pRFHU2. Tail for USER reaction in red.
O2- *gsfA*	GGCATTAAUTATTGCAGCTGCCGAGAGTA	
A3- *gsfA*	GGACTTAAUGGTCAATTCGGCGCACTTAA	Insertion of *gsfA* terminator in pRFHU2. Tail for USER reaction in red.
A4-*gsfA*	GGGTTTAAUTCGAGAGGGCAAGAATGTGT	
RF2	TCTCCTTGCATGCACCATTCCTTG	Confirmation of terminator fusion into pRFHU2 plasmid
RF5	GTTTGCAGGGCCATAGAC	
RF1	AAATTTTGTGCTCACCGCCTGGAC	Confirmation of promoter fusion into pRFHU2 plasmid
RF6	ACGCCAGGGTTTTCCCAGTC	
1F-*gsfR1*	CCCAACATGAGTGAAAGCATAAC	Confirmation of orientation of inserted T-DNA. Upstream region of *gsfR1*
1F-*gsfR2*	TCGCACCAGAGCAAACATAC	Confirmation of orientation of inserted T-DNA. Upstream region of *gsfR2*
1F-*gsfA*	TGATAGAGCATTCGCGGTCC	Confirmation of orientation of inserted T-DNA. Upstream region of *gsfA*

HPH1F	ACGAGGTCGCCAACATCTTCTTCT	Confirmation of orientation of inserted T-DNA. Hygromycin cassette.
2R-*gsfR1*	AGTGCAGTCGGTCAACAATAC	Confirmation of orientation of inserted T-DNA. Downstream region of *gsfR1*
2R-*gsfR2*	GCTAGACGATGCGACACTACTA	Confirmation of orientation of inserted T-DNA. Downstream region of *gsfR2*
2R-*gsfA*	TTGGGAAACTCGTCGACCTG	Confirmation of orientation of inserted T-DNA. Downstream region of *gsfA*
HPHPRO4	GCACCAAGCAGCAGATGATA	Confirmation of orientation of inserted T-DNA. Hygromycin cassette.
HMBF1	CTGTCGAGAAGTTTCTGATCG	Amplification of hygromycin resistance cassette
HMBR1	CTGATAGAGTTGGTCAAGACC	
R1-F	CTTGCGGGTCTCGAAGTAAA	Amplification of *gsfR1* gene
R1-R	CCGTCATCCGAATGTCTATCTG	
R2-F	TGCGAATACAACGGCCGATA	Amplification of *gsfR2* gene
R2-R	CATGAGAACCTCACGGAGAAA	
A-F	GCTTTGGTTTGGTTGTCGAT	Amplification of *gsfA* gene
A-R	CATCCCCTGGATCTTTCTCA	
Determination of number of integration and qPCR		
5F-*gsfR1*	GGATCTATCCATGTCGGAACAC	Determine number of integrations of T-DNA in *gsfR1* mutants and calculate relative expression of *gsfR1*
6R-*gsfR1*	CCTCACAGCCTGTTTGGTTA	
5F-*gsfR2*	GAAGCCGGATGATTTAGAGGAG	Determine number of integrations of T-DNA in *gsfR2* mutants
6R-*gsfR2*	CATGAGAACCTCACGGAGAAA	

5F-*gsfA*	TGCTAGAGTGCGTGACAGT	Determine number of integrations of T-DNA in *gsfA* mutants
6R-*gsfA*	AGGACGCTCTCAAAAGGTGG	
PeHis3_F2	TCTCCGCTTCCAGTCCTCTG	Amplification of histone H3 to determine number of integrations of T-DNA in *gsfR1* and *gsfR2* mutants
PeHis3_R2	TTGGTGTCCTCGAAGAGAGAGAC	
Pgr_tub_1F	CGAGTTGACCCAGCAGATGT	Amplification of β-tubuline to determine number of integrations of T-DNA in *gsfA* mutants and to calculate relative expression of *gsfR1* and *gsfA*.
Pgr_tub_2R	GTCTGGACGTTGTTGGGGAT	
gsfA-F	AAAAGACGGACAGTGACAGC	Calculate relative expression of *gsfA*.
gsfA-R	TATCCGCGATTTCACACTCG	

Table S2 – Transcription factors putatively binding the promoter of *gsfR1* and *gsfA*. It is reported the name of homolog and the regulated processes in other organisms with references when available.

Protein	Homolog	Regulated processes	Reference
Transcription factors binding only the promoter of *gsfR1*			
KXG45700.1	LreA	Conidiation, response to light	(Igbalajobi et al., 2019)
KXG46329.1	Fluconazole resistance protein	resistance to fluconazole	
KXG46595.1	NsdD	activation of sexual development	(Han et al., 2001)
KXG46600.1	res2	Meiosis	(Zhu et al., 1997)
KXG47124.1	Msn2	Conidiation, hyphal growth and stress response	(Liu et al., 2013; Song et al., 2018; Tian et al., 2017)
KXG47905.1	quinic acid utilization activator	Quinic acid utilization	(Wheeler et al., 1996)
KXG48399.1	FlbC	conidiation	(Kwon et al., 2010)
KXG48752.1	Fluconazole resistance protein 3	Fluconazole resistance	
KXG48995.1	hypothetical protein		
KXG49526.1	Transcription factor PAP1		
KXG53661.1	Transcription factor		
KXG54396.1	MtfA (ID 50%)	regulation of aflatoxin production	(Zhuang et al., 2016)
KXG54691.1	StuA	Regulation of cell pattern formation	(Miller et al., 1992)
Transcription factors binding only the promoter of *gsfA*			
KXG46092.1	gal4	Using galactose or lactose as only carbon sources	(Riley et al., 1987)
KXG47012.1	Hypothetical protein		

KXG47549.1	SREP	repressor of siderophore biosynthesis in presence of high iron concentrations	(Haas et al., 1997)
KXG47976.1	Zinc finger, C2H2-type/integrase, DNA-binding		
KXG51025.1	transcriptional activator of gluconeogenesis	transcriptional activator of gluconeogenesis	(Hynes et al., 2007)
KXG51359.1	Filamentous growth regulator	Filamentous growth	
KXG52025.1	Achaete-scute transcription factor related		
Transcription factors putatively binding the promoters of *gsfR1* and *gsfA*			
KXG45164.1	Basic-leucine zipper (bZIP) transcription factor		
KXG45306.1	Zinc finger, C2H2		
KXG45454.1	Transcription factor		
KXG45545.1	Hypothetical protein		
KXG46064.1	RlmA	response to cell wall stress, asexual development	(Damveld et al., 2005; Kovács et al., 2013)
KXG46347.1	RosA	represses sexual development	(Vienken et al., 2005)
KXG46776.1	Homeodomain-like		
KXG46828.1	Quinic acid utilization activator	Quinic acid utilization	(Wheeler et al., 1996)
KXG46909.1	Non-histone chromosomal protein 6		
KXG46994.1	Aflatoxin biosynthesis regulatory protein		(Chang et al., 1993)
KXG47056.1	High mobility group, HMG-I/HMG-Y		
KXG47172.1	Winged helix-turn-helix transcription repressor		
KXG47315.1	Protein of unknown function DUF3468		
KXG47412.1	NirA	Nitrate induction	(Burger et al., 1991)
KXG47486.1	Tbf1	Coordinated ribosomal protein (RP) gene expression	(Hogues et al., 2008)

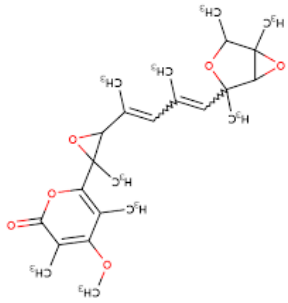
KXG47556.1	Protein of unknown function DUF3468		
KXG47599.1	CRZ1	Calcineurin pathway	(Cramer et al., 2008)
KXG47656.1	C6 transcription factor		
KXG47688.1	ARO80	catabolism of aromatic amino acids	(Lee et al., 2013)
KXG47847.1	AmdA	acetate induction	(Lints et al., 1995)
KXG47936.1	Hypothetical protein		
KXG47959.1	Hypothetical protein (RfeB)		
KXG48139.1	PrnA		
KXG48212.1	Homeodomain-like		
KXG48341.1	Activator of stress genes		
KXG48355.1	GATA transcription factor nreB		
KXG48533.1	Putative transcription factor kapC		
KXG48617.1	Protein of unknown function DUF3468		
KXG48682.1	Biofilm and cell wall regulator 1	Biofilm and cell wall	(Nobile and Mitchell, 2005)
KXG48765.1	Homeodomain		
KXG48781.1	Activator of stress genes 1	stress response	
KXG48784.1	Hypothetical protein		
KXG48884.1	AtfA	Spore germination	(Wolfers et al., 2015)
KXG48955.1	Hypothetical protein		
KXG48983.1	TATA-box binding protein		
KXG49138.1	Hypothetical protein		
KXG49157.1	facB	Regulation of genes involved in acetate utilization	(Todd et al., 1998)
KXG49345.1	acu-15	Positive regulator of acetate induction	(Bibbins et al., 2002)
KXG50499.1	carbon catabolite repressor		

KXG50601.1	Hypothetical protein (Sfp1)		
KXG50650.1	ARO80	catabolism of aromatic amino acids	(Lee et al., 2013)
KXG50787.1	transcription factor Prf		
KXG50810.1	HMG box protein		
KXG50911.1	Hypothetical protein		
KXG51030.1	cef1	pre-mRNA splicing and cell cycle control	(Kellner et al., 2014)
KXG51076.1	C6 transcription factor		
KXG51406.1	cellobiose response regulator 2	cellobiose response	
KXG51472.1	Heat shock factor (HSF)-type	Heat response	
KXG51622.1	Fungal transcriptional regulatory protein		
KXG51830.1	Hypothetical protein		
KXG52055.1	CCAAT-binding transcription factor, subunit B		
KXG52059.1	bZIP transcription factor FlbB		
KXG52166.1	C6 transcription factor		
KXG52228.1	Zinc finger, C2H2-like		
KXG52264.1	Basic-leucine zipper (bZIP) transcription factor		
KXG52366.1	Forkhead transcription factor (Sep1)		
KXG52406.1	Hypothetical protein		
KXG52464.1	DNA damage and replication checkpoint protein Rfx1		
KXG52761.1	Respiration factor		
KXG52955.1	Hypothetical protein		
KXG53008.1	stress response transcription factor rrA/Skn7	response to oxidative stress	(Cao et al., 2009)
KXG53655.1	Acetamidase regulatory protein	catabolism of certain amides, omega amino acids and lactams	(Andrianopoulos and Hynes, 1990)

KXG53834.1	Homeodomain-like		
KXG53935.1	Cutinase transcription factor 1 beta		
KXG54406.1	MCM1	Induces mating, growth rate, pathogenesis and appressorium formation	(Zhou et al., 2011)
KXG54419.1	AreA	nitrogen response	(Katz et al., 2008)
KXG54528.1	Zinc finger, C2H2-like		
KXG54658.1	Transcription factor		

Chapter 3

Elucidation of verrucosidin biosynthesis in *Penicillium polonicum* through CRISPR-Cas9



Silvia Valente [a,b], Edoardo Piombo [a,b], Volker Schroeckh [c], Giovanna Roberta Meloni [a,b], Thorsten Heinekamp [c], Axel A. Brakhage [c,d], Davide Spadaro [a,b];

[a] AGROINNOVA – Centre of Competence for the Innovation in the Agroenvironmental Sector, Largo Paolo Braccini 2, 10095 Grugliasco, Italy

[b] Dept. Agricultural, Forest and Food Sciences (DISAFA), Università degli Studi di Torino, Largo Paolo Braccini 2, 10095 Grugliasco, Italy

[c] Dept. Molecular and Applied Microbiology, Leibniz Institute for Natural Product Research and Infection Biology – Hans Knöll Institute (HKI), Beutenbergstr. 11a, 07745 Jena, Germany

[d] Dept. Microbiology and Molecular Biology, Institute for Microbiology, Friedrich Schiller University Jena, Germany

Manuscript in preparation

Abstract

Penicillium polonicum produces a neurotoxin called verrucosidin. This methylated α-pyrone polyketide inhibits the oxidative phosphorylation in mitochondria and thereby causing neurological diseases. Despite the importance of verrucosidin as a toxin, its biosynthetic genes have not been characterized yet. Here, by using bioinformatic methods we screened the *P. polonicum* genome for presence of biosynthetic gene clusters. Sixteen putative clusters for the biosynthesis of α-pyrone polyketides were identified based on similarity analysis with polyketide synthase for the biosynthesis of aurovertin (AurA) and citreoviridin (CtvA). Two clusters were further selected based on the putative function of genes in the clusters. These two clusters were transcribed in *Penicillium* spp. able to produce verrucosidin *in vitro,* as seen through RT-qPCR.

To prove the importance of the analysed genes for the verrucosidin gene cluster, we applied the clustered regularly interspaced short palindrome repeats (CRISPR) technology to *P. polonicum*. CRISPR-Cas9 reconstituted *in vitro* was used to induce target gene deletions in *P. polonicum*. This deletion approach allowed to identify and characterize the verrucosidin gene cluster. Deletion mutants for the PKS encoded by *verA* were no longer able to produce verrucosidin *in vitro*, while they displayed morphological characteristic comparable with the wild-type strain. Other identified BGCs are putatively involved in the biosynthesis of related α-pyrone polyketides and are therefore a valuable source of novel compounds.

Keywords

Secondary metabolites, *Penicillium*, mycotoxins, CRISPR/Cas, alpha-pyrone polyketides

1. Introduction

Penicillium polonicum is a ubiquitous fungus, found as contaminant of food matrices, such as meat (Sunesen and Stahnke, 2003; Wigmann et al., 2018), nuts (Prencipe et al., 2018b) and fresh fruits such as berries and strawberries (Jensen et al., 2013; Santini et al., 2014), or as saprophyte in diverse environments (Sonjak et al., 2006). *P. polonicum* is able to produce a variety of secondary metabolites (SMs). When they exhibit toxic effects on animals and humans they are called mycotoxins. The most characteristic compounds produced by *P. polonicum* are penicillic acid, verrucosidin, aspterric acid, verrucofortine, cyclopenins and nephrotoxic glycopeptides (Frisvad et al., 2004). Among them is verrucosidin, a neurotoxic compound initially isolated from *Penicillium verrucosum var. cyclopium*, and later from other *Penicillium* species (Burka et al., 1983; Frisvad et al., 2004).

From a chemical point of view, verrucosidin is a highly reduced polyketide, composed of a methylated α-pyrone, a polyene linker and an epoxidated tetrahydrofuran ring. Many others polyketides sharing the α-pyrone structure, such as citreoviridin and aurovertins have been extensively studied because of their ability to inhibit mitochondrial oxidative phosphorylation by binding the F1-ATPase β-subunit, resulting in cytotoxicity and potentially antitumor activity (Li et al., 2018).

Verrucosidin acts on the central nervous system, causing neurological diseases. These effects were previously found in cattle and experimentally confirmed in mice (Fink-Gremmels et al., 1991; Hodge et al., 1988). Verrucosidin is also considered the most cytotoxic and genotoxic compound compared to other tremorgenic mycotoxins (Núñez et al., 2000; Sabater-Vilar et al., 2003). Based on its cytotoxicity, verrucosidin has been studied for its effect against cancer cells (Park et al., 2007; Thomas et al., 2013), making this compound interesting for medical purposes. Despite the importance of this metabolite to human health and its potential benefit in medicine, only few studies have been conducted to elucidate its

biosynthesis. Nuñez and co-workers (2000) determined favourable growth conditions of *P. polonicum* for verrucosidin production, while Aranda et al. (2002) screened verrucosidin-producer strains to develop a molecular probe to identify mycotoxigenic fungi present in foodstuff, that was further used by Rodriguez et al. (2012) to design a TaqMan probe. Because the biosynthesis of structurally related α-pyrones polyenes such as citreoviridin, aurovertins and aspernidgulenes has been elucidated (Li et al., 2018; Lin et al., 2016, 2019), this information could form the basis to identify genes responsible for verrucosidin biosynthesis.

The availability of bioinformatic tools and sequenced fungal genomes (Nielsen and Nielsen, 2017; Weber and Kim, 2016) opened the possibility to identify biosynthetic genes based on similarity. Moreover, central biosynthetic genes like polyketide synthases and non-ribosomal peptide synthetases are often associated with other genes encoding tailoring enzymes which are required for the modification of the carbon structure, or genes encoding transporter, transcription factors and resistance proteins. Such gene clusters are named BGCs (Brakhage, 2013; Keller, 2019). Many of the BGCs found in the genomes are "silent" because they are not actively transcribed under laboratory conditions and are considered as a source of novel compounds (Bergmann et al., 2007). The deletion of member genes of BGCs is usually the main strategy to assign a gene cluster to a compound.

Among the techniques that have been used for deletion of genes, clustered regularly interspaced short palindrome repeats (CRISPR) technology is currently considered the most promising tool, especially for editing genomes of filamentous fungi (Deng et al., 2017; Krappmann, 2017; Song et al., 2019; Tong et al., 2019; Wang and Coleman, 2019; Weber et al., 2017). This system is derived from the prokaryotic immune system and it is based on the endonuclease Cas9. Cas9, when associated with small sequences of RNA called crRNA (CRISPR-RNA) and tracrRNA (Trans-activating crRNA), recognizes sequences called protospacers in the genome, adjacent to a conserved motif (Protospacer Associated Motif or PAM). Cas9 then

cuts the double strand of DNA (Tong et al., 2019). The most pronounced advantages of the CRISPR-Cas9 technology are the possibility of marker-free genome editing and its high efficiency in generating mutations at specific sites in the genome. For these reasons, CRISPR-Cas9 based genome editing became the method of choice in genome studies including filamentous fungi (Fang and Tyler, 2016; Krappmann, 2017; Nagy et al., 2017; Nielsen et al., 2017; Nødvig et al., 2015; Pohl et al., 2016; Weber et al., 2017).

Here, BGCs involved in the biosynthesis of α-pyrone polyketides in *P. polonicum* were identified and the expression of some of the identified PKS genes in *Penicillium* spp. in culture conditions promoting verrucosidin production was verified. Furthermore, a CRISPR-Cas9 method for *P. polonicum* was established and used to characterize the putative verrucosidin BGCs.

2. Materials and Methods

2.1 Fungal strains

P. polonicum strain X6 and *P. crustosum* strain CAL64 were isolated from chestnut production chain (Prencipe et al., 2018b). *P. aurantiogriseum* CBS 112021 was obtained from Westerdijk Fungal Biodiversity Institute. The strains were grown on Potato Dextrose Agar plates (PDA, Merck KGaA, Darmstadt, Germany) with 50 μg/mL streptomycin (Merck) in the dark at 25 °C for 7-10 days. *P. polonicum* mutants obtained were grown on PDA supplemented with 100 μg/mL of hygromycin B (ThermoFischer Scientific, Waltham, MA USA) in the same conditions. All the strains were maintained in glycerol stock at -80 °C.

2.2 Growth of Penicillium *spp.* in vitro

Conidial suspensions were obtained by adding 5 mL of sterile water with 0.01 % (w/v) Tween-20 and gently scraping the surface of fungal cultures grown on Petri

plates. The final conidia concentration was measured using haemocytometer and adjusted by dilution to different concentrations depending on each assay.

To evaluate verrucosidin production *in vitro*, 1 mL of conidial suspension (10^8 conidia/mL) was inoculated in 30 mL of Malt Extract broth (MEA broth, 2% malt extract, 2% glucose, 0,1% peptone) and in 30 mL Czapek yeast extract broth (CYA broth, 0.5% yeast extract, 3.5% Czapek broth). Flasks were kept at 26 °C for 10 days, 55% RH with 12 hours of light and 12 of dark. After 10 days of incubation, the fungal mycelium was separated from the liquid media through double layer of sterile gauze and half of the mycelium was immediately frozen in liquid nitrogen, stored at -80 °C and used for RNA extraction. The remaining fungal tissue was used for verrucosidin extraction.

2.3 Bioinformatic analyses

The primers VerF1 and VerR1, together with the TaqMan probe related to verrucosidin production (Rodríguez et al., 2012), were identified in the genome of *P. polonicum* strain IBT 4502 (GCA_002072265.1) by NCBI BLAST (Madden, 2013). To identify BGCs in the genomes of *P. polonicum* strain IBT4502 and *P. polonicum* strain hy4 (GCA_003344595.1), antiSMASH (Weber et al., 2015) was used and only clusters containing a putative polyketide synthase similar to both CtvA protein (Q0C9L7.1) and AurA (A0A0M4L8I7.1) were further considered (query coverage ≥ 50 % and evalue > e^{-5}). All genes found to be highly similar to the putative polyketide synthase genes were considered as part of the gene cluster while proteins with unknown function encoded by genes found far away from the core gene were omitted. The genes showing 70 % query coverage and identity with differentially expressed genes (DEG) of the study reported by Kim and collaborators (2016) were also reported. The proteins in these clusters were additionally blasted against *P. expansum* (ALJY00000000.1), and *P. crustosum* (GCA_902712905), to verify their presence in the genome of these *Penicillium* spp.. BLAST using Non

Redundant database and Interproscan (Quevillon et al., 2005) were used to find functional domains and predict a putative function.

2.4 DNA extraction and PCRs

Fungal DNA was extracted from mycelium using E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-tek, Norcross, GA, USA) with brief modifications. The mycelium was lysed by adding the required amount of lysis buffer, two tungsten beads and by using TissueLyser II (Qiagen, Hilden, Germany) at 20.00 Hz speed for 20 min. DNA concentration and purity were checked by spectrophotometer (Nanodrop 2000, Thermo Scientific, Wilmington, USA).

To amplify gDNA of *Penicillium* spp., Taq DNA Polymerase (Qiagen, Hilden, Germany) was used with PCR mixture containing 1X PCR buffer, 0.2 mM dNTPs, 0.4 µM of each primer, 0.5 U of polymerase and 10 ng of gDNA. PCR parameters to amplify fragments between 100-400 bp were: initial denaturation step at 95 °C for 3 min, 35 cycles of denaturation (30 s at 95 °C), primer annealing (30 s at 58 °C) and elongation (30 s at 72 °C), final elongation (5 min at 72 °C). PCR parameters to amplify fragments between 2-3 kbp were: initial denaturation step at 95 °C for 5 min, 35 cycles of denaturation (30 s at 95 °C), primer annealing (45 s at 65 °C) and elongation (3 min at 72 °C), final elongation (5 min at 72 °C).

To amplify DNA repair template from plasmid pUChph1 (Liebmann et al., 2004), the Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific, Germany) was used according to the manufacturer's instructions. PCR parameters were: initial denaturation step at 98 °C for 10 s, 30 cycles of denaturation (10 s at 98 °C), primer annealing (30 s at 60 °C) and elongation (20 s at 72 °C), final elongation (1 min at 72 °C). The amplified PCR product was loaded onto an agarose gel, cut out and extracted using Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA), according to the manufacturer’s instruction. All primer sequences used in the PCR are listed in Tab. S1.

2.5 RNA extraction and RT-qPCRs

RNA was extracted from 100 mg of fungal mycelium using Spectrum Plant Total RNA (Sigma-Aldrich, St. Louis, MO, USA), essentially following the manufacturer's instructions. Mycelium was placed in 2-mL tube with two tungsten beads and tubes were immersed in liquid nitrogen for 1 min. Then samples were immediately lysed using TissueLyser II at 20.00 Hz for 1 min.

DNase treatment was performed using TURBO DNA-free™ Kit (Thermo Fischer Scientific). The samples were diluted 1:2 in a final volume of 50 µL and rigorous DNase treatment followed. To verify the efficiency of the DNase treatment, a PCR was performed using ITS1 and ITS4 universal primers to verify the absence of fungal DNA. The cycling conditions of the PCR were 3 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C and 5 min at 72 °C.

First-strand cDNA was synthesised with High Capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific) using 1 µg of total RNA.

RT-qPCR was performed with StepOne™ and StepOnePlus™ Real-Time PCR System with Power SYBR™ Green PCR Master Mix (ThermoFischer Scientific); cycling conditions were 5 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C. In order to determine relative gene expression (RGE) the $2^{\Delta\Delta cq}$ method (Pfaffl, 2001) was used using cDNA of samples and comparing the amplification of β-tubulin gene, with the amplification of the target genes. *P. aurantiogriseum* was used as wild-type strain. All primer sequences used in RT-qPCR reactions are listed in Tab. S1.

2.6 Verrucosidin extraction

Verrucosidin produced *in vitro* was extracted from approximately 0.5 g of fungal mycelium. The mycelium was placed in 2 ml tubes and 1.5 mL of MeOH:chloroform (1:2, v/v) was added to the samples that was then subjected to ultrasounds for 30 min. After centrifugation at 5,500 rpm for 5 min, the liquid phase

was transferred into a new tube. The mycelium pellet was subjected again to two further extractions: the first with ethyl acetate and the second with isopropanol. The extracts were combined and concentrated at 45 °C (Eppendorf concentrator 5301). The dry extracts were then resuspended in 500 µL of H_2O: acetonitrile (1:1, v/v) and transferred into a HPLC vial for HPLC-MS/MS analysis.

2.7 Chemical analyses

The HPLC-MS/MS system consisted of a binary pump and a vacuum degasser (1260 Agilent Technologies, Santa Clara, CA, USA) connected to a Varian auto-sampler Model 410 Prostar (Hansen Way, CA, USA), equipped with a 20 µL loop and coupled with a Varian 310-MS TQ Mass Spectrometer. Ten microliters of each extract were analysed using a Pursuit XRs ULTRA 2.8 µm C18 (100x2 mm, Varian) column and a binary mixture as a mobile phase: solvent A and B were composed of 40% of 0.05% formic acid and 60% of acetonitrile, respectively. The isocratic mode was used at flow rate of 0.2 mL/min for 5 min. Since the verrucosidin standard is not commercially available, only a qualitative analysis could be performed. Verrucosidin qualitative analysis was performed using a mass spectrometer equipped with an electrospray ionization (ESI) source operating in positive ion mode and while Product Ion Scan (PS) mode was used for triploquadrupole: m/z 417→100-427. The collision gas (Ar) pressure was set at 2 mbar.

To characterize the metabolic profile of wild-type and knockout strains, samples were analysed using the same HPLC-MS/MS system. The LC was equipped with a C18 analytical column (Luna 3 µm, 150 x 2 mm, 100Å, Phenomenex, Torrance, CA, USA) and the chromatographic separation was achieved by gradient conditions for 45 min at a flow rate of 300 µl/min. Solvent A was water and solvent B was acetonitrile both containing 0.1% formic acid. The gradient was programmed as follows: 0–3 min isocratic 5% B, followed by a linear gradient to 100% B, ending at 40 min and from 40 to 45 min isocratic 100% B. Full-scan mass spectra were

acquired in the positive-ion mode over the m/z range from 100 to 700 using the TQ mass analyser.

2.8. Protoplasts preparation and CRISPR-Cas9 procedure

Protocols used to obtain protoplasts from *Aspergillus fumigatus* (Weidner et al., 1998), *Ophiostoma piceae* (Wang et al., 1999), *P. nalgiovense* (Fierro et al., 2004), *P. paxilli* (McMillan et al., 2003; Young et al., 1998), *P. chrysogenum* (Pohl et al., 2016), *P. crustosum* and *P. janthinellium* (Nicholson et al., 2015) were used as the basis to design a protocol for protoplast preparation of *P. polonicum*. Fungal mycelium was obtained by inoculating 2 mL of conidial suspension (1.25 x 10^8 conidia/mL) in 50 mL of YGG (8 g/L KCl, 16 g/L glucose, 6.6 g/L yeast nitrogen base, 1.5 g/L citric acid, 6 g/L KH_2PO_4, 2 g/L yeast extract). Flasks were shaken in a rotary shaker (180 rpm) at 26 °C for 20 h. Fungal mycelium was filtered through Miracloth (Merck) and washed with $MgSO_4$ 0.6 M. A pre-treatment was conducted mixing 5 g of mycelium in 50 mL tubes with 5 mM Na_2EDTA and 25 mM 2-mercaptoethanol; tubes were kept in horizontal position in a rotary shaker (80 rpm) at 30 °C for 20 min. Fungal mycelium was washed with 0.6 M $MgSO_4$ and then digested with 2 g of Vinotaste®Pro (Lamothe-Abiet, Canéjan, FR) and 0.1 g of Lysing Enzymes from *Trichoderma harzianum* (Sigma-Aldrich) suspended in 15 mL of Osmo Solution (1.2 M $MgSO_4$, 10 mM sodium phosphate buffer). The lytic solution was kept at 30 °C, 80 rpm. Every 30 minutes the mycellium-lysis solution was mixed using a serological pipette and visually checked under microscope; about 3-4 hours were necessary to digest the cell wall and obtain protoplasts. The suspension was filtered through Miracloth in order to separate and remove undigested mycelia. Protoplasts were then recovered using Trapping Buffer (0.6 M sorbitol, 0.1 M Tris-HCl pH 7) and by centrifuging for 25 min. The intermediate layer was separate and washed twice with STC Buffer (2.4 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5). All centrifugation steps were performed at 3,000

x *g* at 4 °C. Finally, protoplasts were resuspended in 1 mL of STC Buffer and kept on ice until transformation with PEG.

CRISPR RNAs were designed using Alt-R Custom Cas9 crRNA Design Tool (https://eu.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM) and are listed in Tab. S1. The off-target analysis was performed using Blast. Alt-R® CRISPR-Cas9 crRNA, Alt-R® CRISPR-Cas9 tracrRNAand Alt-R® S.p. Cas9 nuclease V3 was purchased from IDT (Integrated DNA Technologies, Inc., Coralville, IA, USA) and was combined to obtain ribonucleoprotein (RNP) complex according to the manufacturer's instructions. Briefly, crRNA (2 nM) and tracrRNA (2 nM) were heated at 95 °C for 5 min and cooled at room temperature to obtain gRNA. Three µL of each gRNA were mixed with 4 µL of Cas9 at room temperature for 20 min. The entire mixture of RNP together with 10 µL of repair template (3 µg) and 40 µL of PEG20 solution (20% PEG6000) were gently mixed with 80 µL of protoplasts (10^7 protoplasts/mL). As a control, instead of DNA ten µL of water were used. The mixtures were kept on ice for 30 min, then 900 µL of PEG60 solution (60 % of PEG6000) were added. After 30 min of incubation on ice, protoplasts were gently spread in Petri dishes with regeneration media (RM, Malt extract 2%, peptone 1%, glucose 2%, sucrose 0.8 M) supplemented with hygromycin or without the antibiotic for positive control.

2.9 Characterization of mutants in vitro

Mutants were selected on PDA supplemented with hygromycin B. Conidia were obtained in order to assure uniformity in the genetic material. The deletion event was confirmed by PCR and by sequencing PCR products.

Deletion mutants were compared with wild-type *P. polonicum* X6 by inoculating 5 µl of spore suspension (1 x 10^6 conidia/mL) on PDA and incubating the plates at 25 °C in the dark. Colony diameter (cm) and number of asexual spores (conidia/plate) were measured up to 7 days post inoculation (dpi). Additionally, deletion mutants

were inoculated in liquid media (CYA and MEA) as previously described in order to examine verrucosidin production.

2.10 Characterization of mutants in vivo

Apples cv. Ambrosia and cv. Opal were surface disinfected and wounded as described in Spadaro *et al.* (2013). Ten µl of conidial suspension (1 x 10^6 conidia/mL) of each strain were pipetted into each wound, while controls were inoculated with deionized Ringer solution. Inoculated apples were placed in plastic trays, covered with a transparent polyethylene film and stored for 14 days at room temperature.

3. Results

3. 1 Putative verrucosidin gene cluster

Taqman probe of Rodriguez and colleagues (2012) was localized in a retroviral peptidase of the A2A class in *P. polonicum* IBT 4502. The genes within 15,000 bp of the probe were identified as a MFS transporter, an Emopamil-binding, a protein of unknown function DUF3292 and 6 additional hypothetical proteins (Tab. S2).
All these proteins were also present also in *P. expansum*, *P. polonicum* strain hy4 and *P. crustosum*, except of the MFS transporter and two hypothetical proteins, which were only found in *P. polonicum* IBT 4502.
Because of the lack of PKS encoding genes in the genomic region recognized by the molecular probe, verrucosidin BGC was searched using as leads CtvA and AurA. Sixteen putative BGCs (Fig. 1) were found in the genomes of *P. polonicum* based on similarity analysis with polyketide synthase for the biosynthesis of aurovertin (AurA) and citreoviridin (CtvA). Ten of these clusters were found in both genomes of *P. polonicum* available, while clusters 4 (verrucosidin BGC), 12 and 13 were only

present in *P. polonicum* IBT 4502 and clusters 14, 15 and 16 in *P. polonicum* strain hy4 genome, that is deposited without annotation (Tab. S2).

The biosynthetic genes were also searched for in the available genomes of *P. expansum* strain NRRL 62431 isolated from hazel and *P. crustosum* strain CAL64 isolated from chestnuts. It was reported that some strains of *P. expansum* can produce verrucosidin (Kim et al., 2016). *P. crustosum* CAL64 was used as negative control, as this strain is unable to produce verrucosidin (Prencipe et al., 2018b).

The backbone genes encoding the putative PKS *cl5A*, *cl7A*, *cl8A*, *cl9A*, *cl11A* were exclusively identified in *P. polonicum* isolates, while *cl6A* and *cl10A* were missing in the genome of *P. expansum*.

The highly reducing (HR) PKS, found in the third, sixth and sixteenth clusters, contained an additional enoyl reductase (ER) domain, while *cl1A2*, *cl9A2*, *cl10A2*, *cl14A2* and *cl15A2* genes encoding small proteins with an enoyl reductase domain were found in the clusters 1, 9, 10, 14 and 15.

The genes identified were also compared with the DEGs previously identified (Kim et al., 2016) where *P. expansum* was cultured under two conditions; in one of these conditions verrucosidin was produced. The putative transcription factor and the transporter encoded by *verF* (or *cl4F*) and *cl10J3* respectively were under-expressed in *P. expansum* grown in verrucosidin-producing conditions. Conversely, the genes *cl2U1* (hypothetical protein), *cl3B* (methyltransferase), *cl6F* (transcription factor) and *cl15J1* (transporter) found in the second, third, sixth and fifteenth cluster, respectively, were overexpressed.

The third and the fourth clusters were further considered due to the presence of genes encoding putative tailoring enzymes involved in the verrucosidin biosynthesis (Fig. 1). The third cluster, present in all *Penicillium* spp., contained genes encoding putative methyltransferase (*cl3B*), serine hydrolase (*cl3D*), FAD-dependent oxidoreductase (*cl3C*) and ABC transporter (*cl3J*). The fourth cluster, which was found only in the genome of *P. polonicum* IBT 4502, had genes encoding putative

methyltransferase (termed *cl4B* or *verB*), monooxygenases FAD dependent (*cl4C1* and *cl4C2* or *verC1* and *verC2*), acyltransferase (*cl4G* or *verG*) and cytochrome P450 (*cl4H* or *verH*).

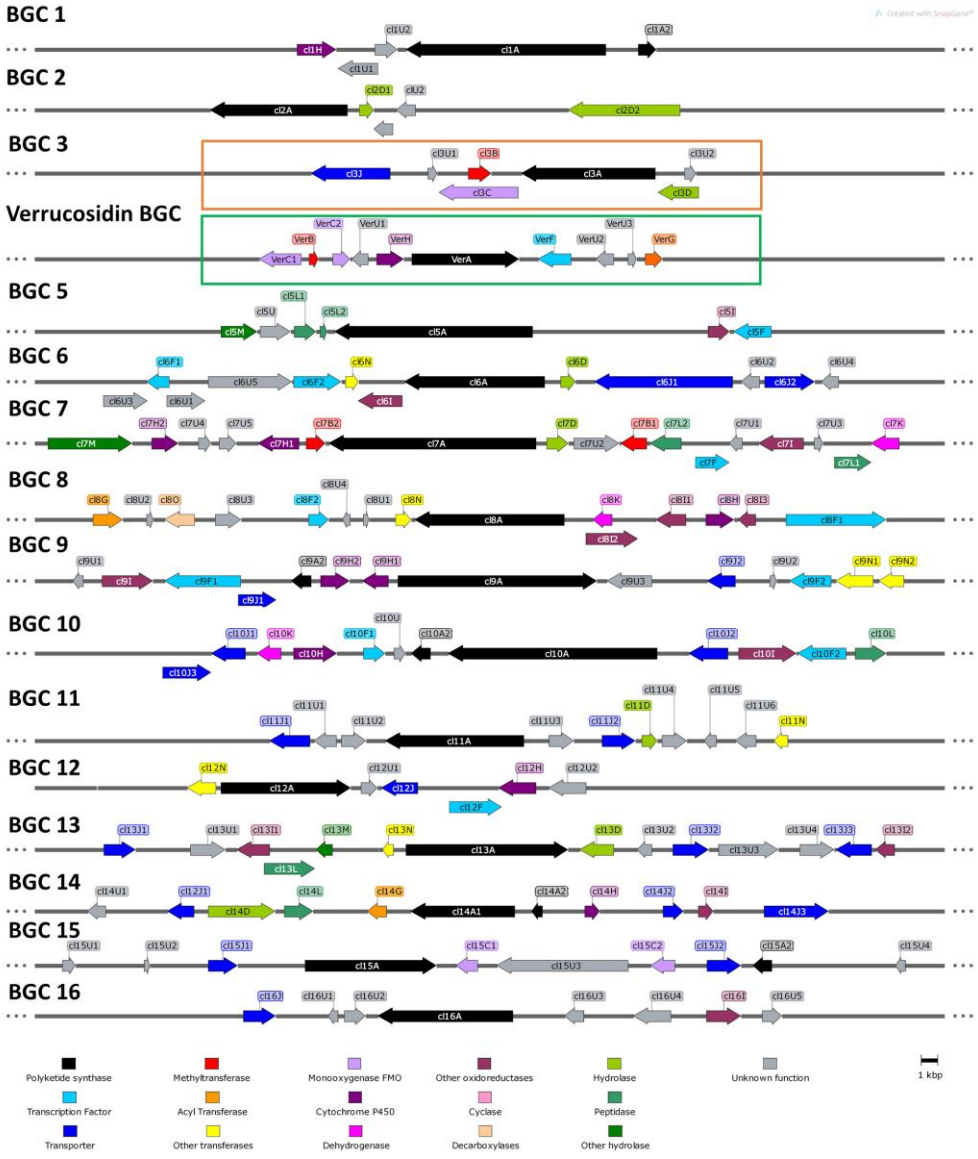


Figure 1 – Representation of 16 Biosynthetic Gene Clusters (BGCs) found in *P. polonicum* genomes. Genes are marked by arrows and putative function is given

by colours. The selected clusters for confirmation through CRISPR-Cas9 are labelled in a square.

3.2 Biosynthesis of verrucosidin and gene expression

P. polonicum X6, *P. aurantiogriseum* CBS 112021 and *P. crustosum* CAL64 were grown in induction media in order to monitor verrucosidin production. As shown in Figure S1, *P. polonicum* and *P. aurantiogriseum* were able to produce verrucosidin, while *P. crustosum* failed to produce this compound.

The fungal mycelium was additionally used for RNA extraction in order to evaluate the expression of some of the genes identified in the selected BGCs. Based on the presence of the BGCs available genomes of *P. polonicum*, we also found the putative genes in the genome of the producer strains *P. polonicum* X6 and *P. aurantiogriseum* CBS 112021 by PCR (Fig. S2 A). The genes encoding HR-PKS (*cl3A*) and methyltransferase (*cl3B*) of the third cluster were found in all the *Penicillium* spp. analysed. Finally, the *verA* gene encoding the putative HR-PKS of the fourth cluster was amplified from the genome of the verrucosidin producing strains *P. aurantiogriseum* CBS 112021 and *P. polonicum* X6. However, in the genome of *P. crustosum* CAL64 the gene was absent.

The genes *cl3A, cl3B* and *verA* were used as target to evaluate their expression. The results showed that *cl3A* and *cl3B* were actively transcribed both in verrucosidin-producers (Figs. 2, S2 B, C) and in *P. crustosum* (Fig. 2, S2 D), while *verA* was expressed both in *P. polonicum* and in *P. aurantiogriseum* (Fig. 2, S2 B, C).

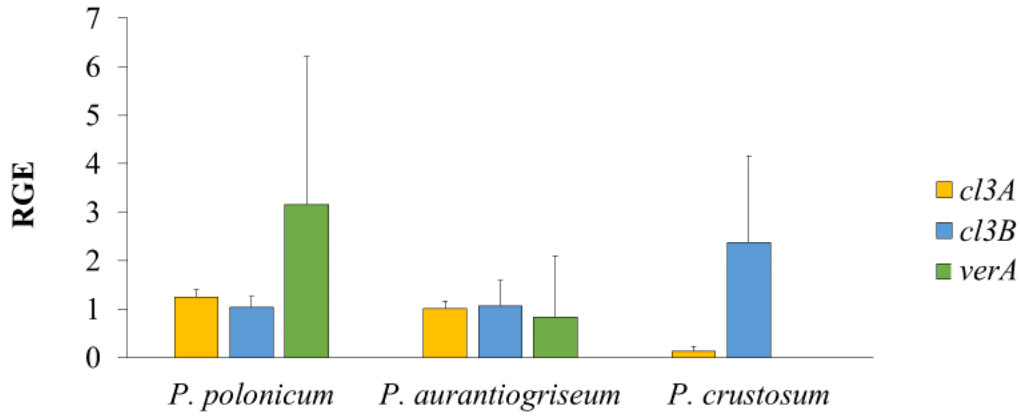


Figure 2 – Relative gene expression (RGE) of *cl3A*, *cl3B* and *verA* (*cl4A*) genes. RGE in *P. polonicum* X6, *P. aurantiogriseum* CBS 112021 and *P. crustosum* CAL64 was evaluated 10 days post inoculation on CYA broth. The expression is given relative to the expression of the β-tubulin gene. *P. aurantiogriseum* was used as wild-type strain.

3. 3 Deletion of PKSs through CRISPR-Cas9

To investigate the role of PKSs encoded by *cl3A* and *verA* in verrucosidin biosynthesis, the genes were deleted using CRISPR-Cas9. For this purpose, a method to obtain *P. polonicum* protoplasts was developed. The method allowed to obtain 1 x 10^7 protoplasts/mL starting from 5 g of mycelium.

In order to delete target genes with the endonuclease Cas9, the protospacers were designed based on the sequenced promoter and terminator of target genes in *P. polonicum* X6. To obtain the two deletion mutants, two sets of guide RNA (gRNA) were used allowing to cut both the promoter and the terminator and to excise each target gene. The endonuclease Cas9 was mixed *in vitro* with gRNA and a repair DNA template encoding the hygromycin resistance cassette. The repair DNA template was amplified using primers with an additional tail of 50 bp that at both flanking regions provided micro-homology for the DNA next to the PAM site. This

way, the correct integration of the repair DNA through micro-homology-mediated end joining (MMEJ) was achieved.

The obtained knockout mutants were assessed through PCR, by amplifying the hygromycin resistance cassette and target genes (Figs. 3 B, 3 C, 4 B, 4 C). The deleted genes were not amplified in knockout mutants, while the hygromycin resistance cassette was absent in the wild type X6. Amplification of hygromycin resistance cassette failed also in some of the mutants obtained and these strains were not further characterized. Some strains with ectopic integration were also identified, with a functional copy of the target gene and, in addition, the antibiotic resistance cassette (Figs. 3 C, 4 C).

Sixteen mutants for each deleted gene were randomly chosen and the correct integration of the repair template was confirmed using PCR with primer pairs designed for the *hph* gene and promoter and terminator of the deleted PKSs (Figs. 3 D, 3 E, 4 D, 4 E). These primer pairs can only amplify the corresponding DNA fragment if the foreign DNA had been introduced at the target sites. PCR fragments were amplified in some of the correct knockout mutants and, as expected, amplification failed for the wild type. In most cases, Δ*verA* mutants were the results of specific integration in the genome (Figs. 4 D, 4 E), while only 7 Δ*cl3A* mutants displayed a correct amplification of both regions upstream and downstream the insertion of the hygromycin resistance cassette (Figs. 3 D, 3 E).

Some of the positive transformants were further confirmed by sequencing of the amplified PCR products (Fig. S3).

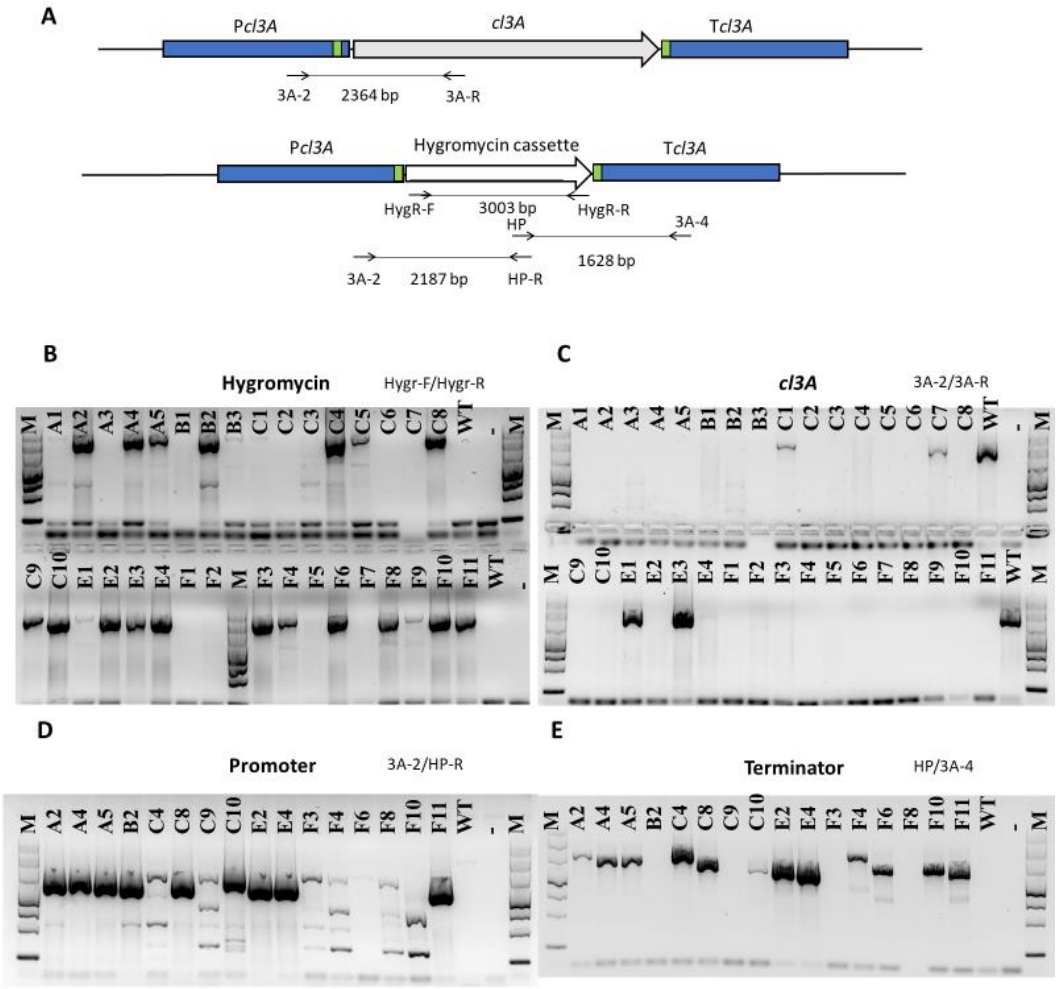


Figure 3 – PCR analysis of *cl3A* mutants. Schematic presentation of the *cl3A* locus in the wild type and deletion mutants, 50 bp of homology sequence are presented in green, primers used are labelled by arrows (**A**). Amplification of hygromycin resistance cassette (**B**) and *cl3A* gene (**C**); confirmation of orientation of inserted repair DNA (**D, E**); M = GelPilot Wide Range Ladder, WT = wild type *P. polonicum* X6, A-F = deletion mutants for *cl3A*, - = negative control (PCR mix without DNA).

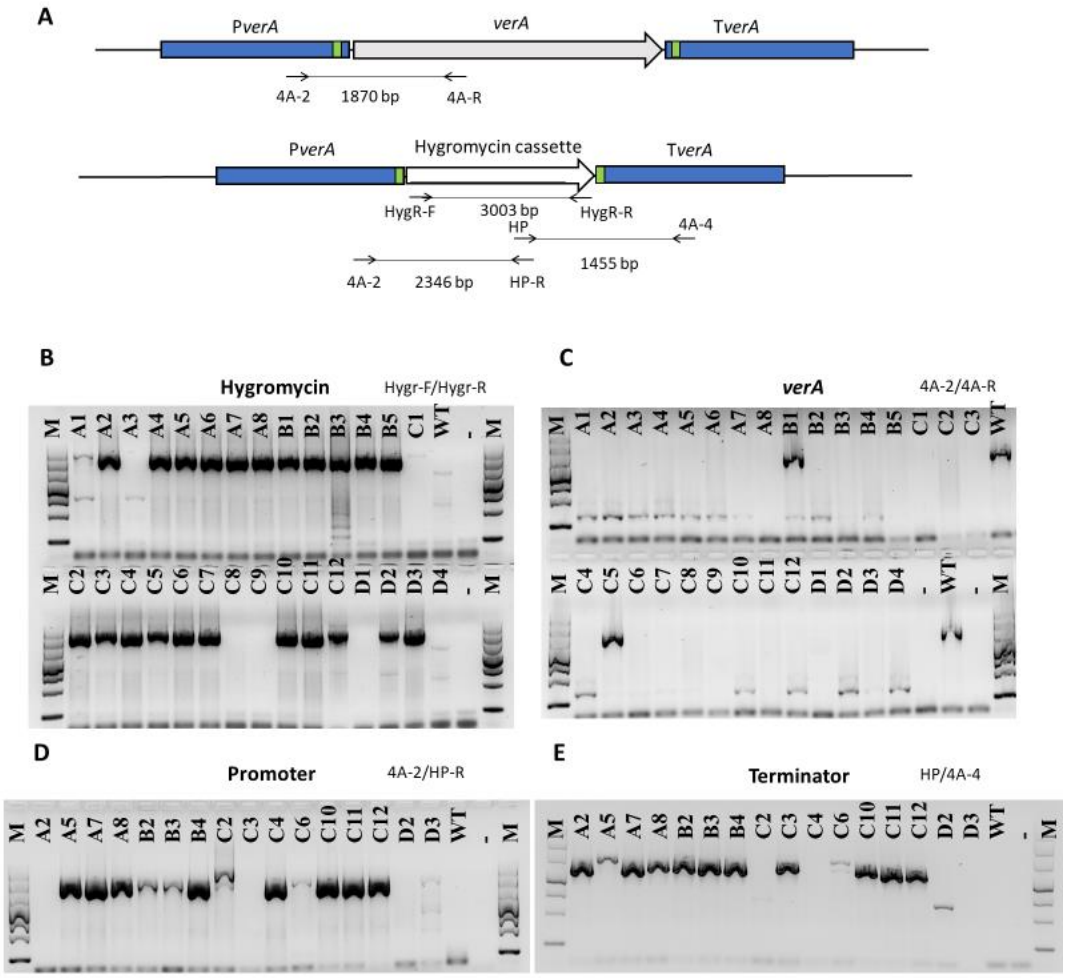


Figure 4 – PCR analysis of *verA* mutants. Schematic presentation of the *verA* (*cl4A*) locus in the wild type and deletion mutants, 50bp of micro-homology sequence are presented in green, primers used are marked by arrows (**A**). Amplification of hygromycin resistance cassette (**B**) and *verA* gene (**C**); confirmation of orientation of inserted repair DNA (**D, E**); M= GelPilot Wide Range Ladder, WT = wild type *P. polonicum* X6, A-D = deletion mutants for *verA*, - = negative control (PCR mix without DNA).

3. 4 Characterization of mutants

Three mutants per each deletion event displaying a correct integration of the *hph* gene were further phenotypically characterized *in vitro* and on apples. Compared to

wild-type strain, the mutant strains displayed the same ability to grow, except for strain Δ*cl3A*-C8 which was grew slightly slower compared to the other strains (Fig. 5 A). No significant differences in conidiation were detected (Fig. 5 B).

The ability of knockout mutants to produce verrucosidin was assessed on CYA broth and MEA broth. Mutants lacking the *verA* were no longer able to produce verrucosidin, while knockout mutants for *cl3A* were still able to produce this compound (Fig. 5). Furthermore, knockout mutants displayed different chromatographic profiles between 17 and 23 minutes in the chromatogram (Fig. 6).

The effect of gene deletion on virulence of *P. polonicum* was assessed on two cultivars of apples. On apples cv. Ambrosia *verA* deletion mutants displayed a slightly reduced virulence 7 days after the inoculation (Tab. S3) while this behaviour was not observed after 14 days of storage nor on apples cv. Opal (Figure 7).

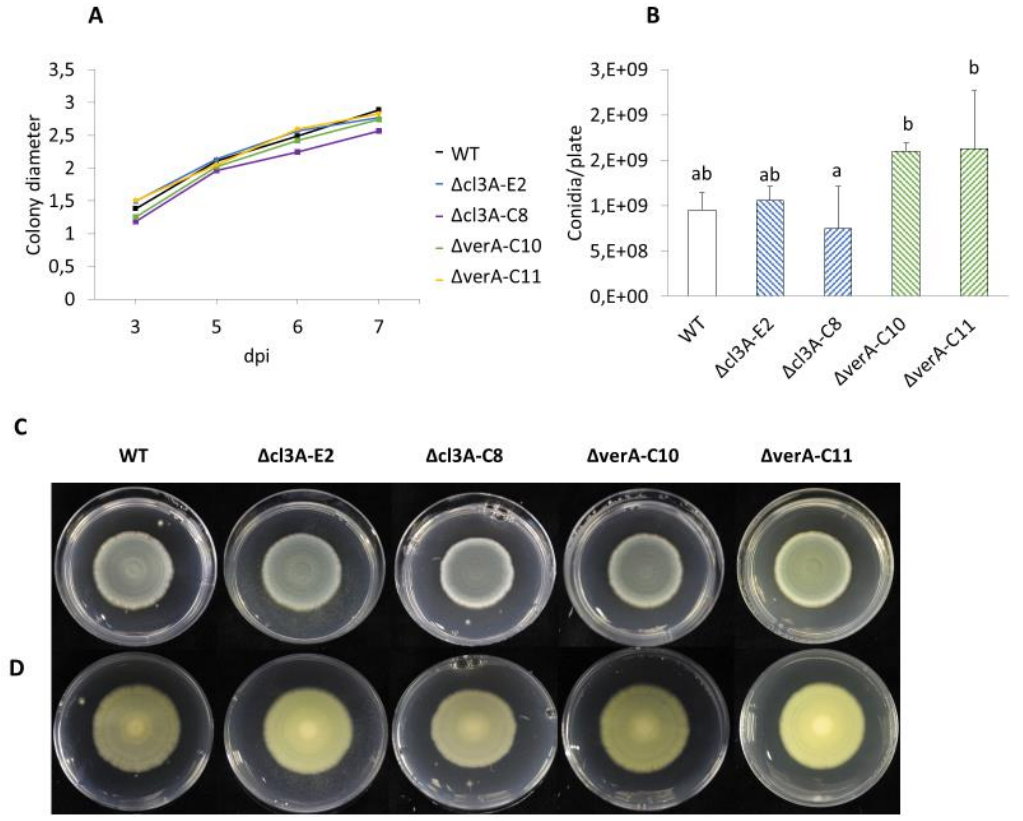


Figure 5 – Effect of *cl3A* and *verA* deletion on *P. polonicum* growth *in vitro.* Colony diameter from 3 to 7 days post inoculation (**A**) conidia production (**B**) and plate view

(front **C** and reverse **D**) 7 days after inoculation (dpi) on PDA. WT = wild type *P. polonicum* X6, Δcl3A = deletion mutants for *cl3A* and ΔverA = deletion mutants for *verA* (*cl4A*). Values followed by the same letter are not statistically different by Tukey's-b multiple comparison test ($p < 0.05$).

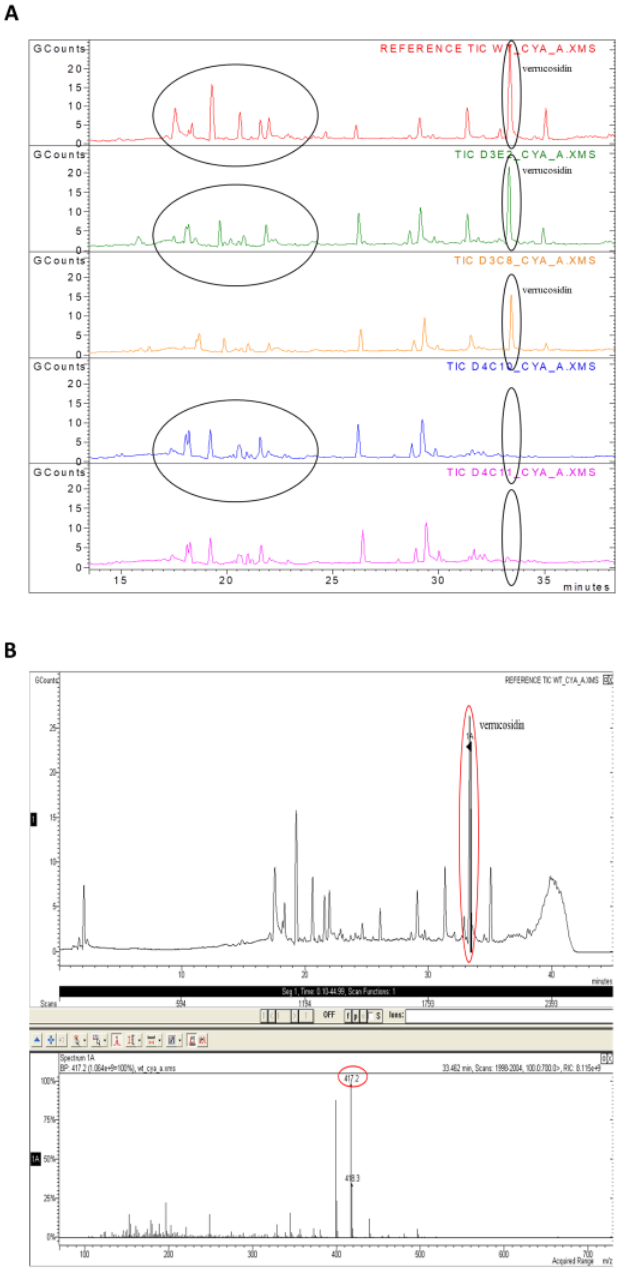


Figure 6 – Effect of *cl3A* and *verA* deletion on *P. polonicum* verrucosidin production *in vitro*. (**A**) Liquid chromatography-mass spectrometry (LC-MS) spectrum of wild type (in red), Δcl3A_E2 (in green), Δcl3A_C8 (in yellow), ΔverA_C10 (in blue) and ΔverA_C11 (in purple) extracts. On the right, differences in the chromatographic peaks of wild type and mutants are encircled. On the left, the chromatographic peak circled in black confirms the presence or absence of verrucosidin. (**B**) LC-MS spectrum of wild-type extract and mass spectrum related to the chromatographic peak circled in red, which confirms the identity of verrucosidin (MW = 417, retention time 33.34 min).

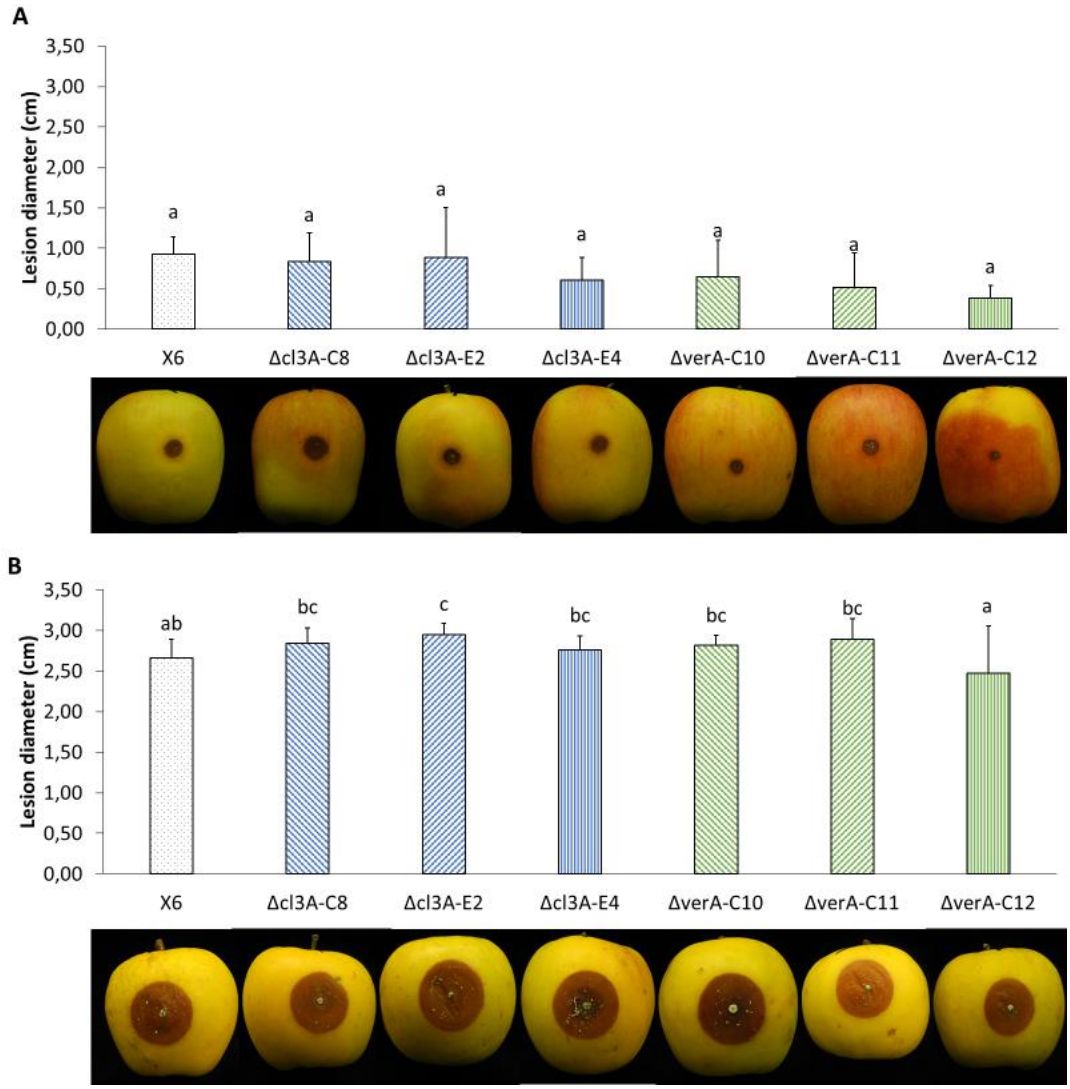


Figure 7 – Effect of *cl3A* and *verA* deletion on virulence of *P. polonicum in vivo.* Lesion dimeter (cm) and pictures of apples cv. Ambrosia (A) and Opal (B) were recorded 14 days after inoculation. WT = wild type *P. polonicum* X6, Δcl3A = deletion mutants for *cl3A* and ΔverA = deletion mutants for *verA* (*cl4A*). Values followed by the same letter are not statistically different by Tukey's-b multiple comparison test ($p < 0.05$).

4. Discussion

Bioinformatic and genetic tools were used to find the putative verrucosidin BGC in the genome of *P. polonicum*, a species known to be a verrucosidin producer (Frisvad et al., 2004). This was allowed by the availability of BGCs and fungal genomes deposited, combined with the development of many genetic tools to mine BGCs and characterize previously unknown or known compounds.

4.1 Genes around verrucosidin probe

The biosynthesis of verrucosidin has been poorly studied. Therefore, in this study we started with the use of molecular probes previously reported to screen the genomes of producer and non-producer *Penicillium* spp. (Aranda et al., 2002; Rodríguez et al., 2012). The probe itself was localized in a retroviral peptidase of the class A2. This class of proteins includes the proteases of leukemia viruses, immunodeficiency viruses, infectious anaemia viruses and mammary tumour viruses, as well as those encoded by several retrotransposons from fruit flies and yeast, and endogenous viral sequences in humans and other primates (Dunn et al., 2002). The presence of this gene in this site is probably due to an event of introgression after contact with a retrovirus, but it is not trivial to predict its function in relation to verrucosidin production. A gene close to the probe contains an emopamil-binding domain (*prO6*). A similar protein is a virulence factor in *Botrytis cinerea* (Gioti et al., 2008). Similar proteins are sterol isomerases in mammals (Bae et al., 2001; Silve et al., 1996) and plants (Grebenok et al., 1998). Interestingly, ergosterol was one of the metabolites produced by *P. aurantiogriseum* together with verrucosidin and verrucofortine reported in the study of Hodge and colleagues (1988).

However, the gene that is most likely involved in the production of verrucosidin is an MFS transporter gene whose product could play a role in the protection of the fungus against verrucosidin (Keller, 2019). In the citreoviridin BGC, for instance,

ctvE encodes an ATP synthase β-chain (Lin et al., 2016). The gene *prJ* coding for OQD71106.1, the MFS transporter, is not located within a gene cluster since the area is lacking a core biosynthetic gene such as a PKS or NRPS, but it could act as a protective gene against verrucosidin. As a matter of fact, it is possible that genes involved in the synthesis or regulation of the same metabolite are located in distant parts of a genome (Bergmann et al., 2010; Liu et al., 2015).

4.2 Many α-pyrone polyketide BGCs were found in Penicillium polonicum

The HR-PKS involved in the biosynthesis of citreoviridin and aurovertin share the same domains: KS-AT-DH-Met-KR-ACP (KS=ketosynthase, AT=acyltransferase, DH=dehydratase, Met=methyltransferase, KR=ketoreductase, ACP=acyl carrier protein), which are also found in the HR-PKS responsible for the biosynthesis of other similar compounds called aspernidgulenes (Lin et al., 2019) and fumigermin (Stroe et al., 2020). Therefore, we assumed that it is likely that the backbone gene of verrucosidin biosynthesis shares these structural domains.

Some of the HR-PKS found in these selected BGCs had an additional domain in their structure, such as *cl3A*, *cl6A* and *cl16A* showing an additional ER domain, which probably reduces a double bond in a single bond.

Considering that the biosynthesis of related α-pyrones polyketides only requires few enzymes, it is expected that verrucosidin biosynthesis is similarly concise. In particular, for the biosynthesis of aurovertin and citreoviridin, a methyltransferase is required for the methylation of the α-pyrone hydroxy group, a monooxygenase FAD dependent and a hydrolase are then involved in the oxidation and reduction of the epoxides to build the ring attached to the polyene linker; the others genes found in the BGCs are not essential for the production of these compounds (Li et al., 2018; Lin et al., 2016).

In this work, genes encoding methyltransferases were found only in the third, fourth and sixth clusters, these enzymes are probably involved in the methylation of the α-

pyrone hydroxy group. As suggested by Lin et al. (2019), the presence of a methyltransferase is a possible way to screen BGCs and distinguish between genes responsible for the biosynthesis of methylated and unmethylated polyenes. Additionally, we found that the *cl3B* gene was overexpressed in *P. expansum* making this cluster particularly interesting.

Furthermore, verrucosidin and citreoviridin differ only for the epoxidation of the tetrahydrofuran ring and for the presence of an extra oxygen in the polyene linker. It is expected that the verrucosidin BGC could include a gene encoding a cytochrome P450 or another monooxygenase responsible for the epoxidation of the ring. Genes encoding oxidoreductases were found in all clusters except in the second, confirming that these screened BCGs could be involved in verrucosidin biosynthesis.

Only clusters 3 and 4 contained genes encoding both HR-PKS and methyltransferase, monooxygenase FAD dependent and hydrolase, and were therefore considered the best candidates to investigate verrucosidin biosynthesis. Moreover, the gene cluster 4 was previously identified in *P. polonicum* by homologous search of aurovertin-type compounds BGCs (Li et al., 2018).

The differential expression of some of the investigated genes in *P. expansum* (Kim et al., 2016) suggests that they could play a role in the production of verrucosidin.

4.3 The gene expression study confirms that BGCs selected are not silent

P. aurantiogriseum and *P. polonicum* were able to produce verrucosidin *in vitro* as expected, while *P. crustosum* did not produce this compound. However, to date the production of this compound by *P. crustosum* has not been described (Frisvad et al., 2004).

The presence and the expression of representative genes belonging to the third and fourth BGC was evaluated in *P. polonicum*, *P. aurantiogriseum* and *P. crustosum*.

The clusters were active because of gene expression in both producer species (*P. polonicum* and *P. aurantiogriseum*).

Finally, the lack of *verA* only in *P. crustosum* suggests that this gene could encode the HR-PKS of verrucosidin biosynthesis. Furthermore, the gene expression analysis indicated that *verA* is actively transcribed in the verrucosidin-producers *P. polonicum* and *P. aurantiogriseum*.

4.4 CRISPR Cas9 gene deletion is efficient method to characterize BGCs

To delete *cl3A* and *verA* encoding putative HR-PKS responsible for the biosynthesis of verrucosidin, an *in vitro* method for CRISPR-Cas9 gene deletion was established in *P. polonicum*. Previously, in *P. chrysogenum* CRISPR-Cas9 was applied. Protoplasts of a strain harbouring the Cas9 gene on an AMA1 plasmid were transformed with a synthesized gRNA (Pohl et al., 2016).

Here, the method of Abdallah and collaborators (2017) was followed that transformed protoplasts of *A. fumigatus* with RNP. This strategy does not require the generation of plasmids or the expression of the endonuclease Cas9. It is rather based on transforming protoplasts with the endonuclease protein Cas9 together with crRNA and tracrRNA. This strategy requires both the accurate design of the crRNA and an efficient protocol to transform fungi.

Mutants for two putative PKS genes were obtained, confirming that the method is effective and could be used for gene editing in *P. polonicum*. The presence of some mutants displaying a wrong insertion of the foreign DNA, suggests that screening and confirmation of mutants are necessary. In particular, by sequencing the promoter and terminator regions in Δ*cl3A*-A2 strain and the terminator of Δ*verA*-B4 strain, an unexpected insertion was observed, suggesting that the endonuclease had cut the double strand DNA in the expected site, but MMEJ did not occur. To our knowledge, there were not previous reports about MMEJ occurring only at one excision site. Therefore, the integration of repair DNA could have occurred by

nonhomologous end-joining (Krappmann, 2017). The efficiency of homologous recombination can be improved by adjusting the length of the homology sequence or by changing the amount of donor DNA, as previously shown in *A. fumigatus* (Al Abdallah et al., 2017).

4.5 Characterization of verrucosidin BGC

The characterization of mutants obtained allowed to identify and characterize the verrucosidin gene cluster. In fact, deletion mutants for the HR-PKS encoded by *verA* were no longer able to produce verrucosidin *in vitro*. This gene cluster was previously hypothesized as verrucosidin cluster in *P. polonicum* by Li and colleagues (2018) using a bioinformatic approach but the function of the cluster was not experimentally confirmed. Surprisingly, the verrucosidin gene cluster was absent in the genome of *P. polonicum* strain hy4, demonstrating high variability in clusters between strains of the same species (Ballester et al., 2015). The biosynthetic genes were also absent in *P. expansum* strain NRRL 62431. This fungal genome was initially deposited as *P. aurantiogriseum* and later assigned to another species (Ballester et al., 2015). Due to the lack of verrucosidin gene cluster, we can conclude that both *P. expansum* strain NRRL 62431 and *P. polonicum* strain hy4 are not able to produce this SM.

In contrast, deletion mutants for *cl3A* gene were able to produce verrucosidin *in vitro* similarly to *P. polonicum* X6. The third gene cluster is likely involved in the biosynthesis of another α-pyrone type polyketide. Further investigations are needed to determine the SM related to this cluster, which was actively transcribed in culture conditions.

4.6 Gene deletion did not affect P. polonicum growth *in vitro* and *in vivo*

Secondary metabolites are known to play a central role in many biological processes such as growth development (Calvo et al., 2002) and pathogenesis (Macheleidt et

al., 2016; Scharf et al., 2014), which were extensively reviewed in last years (Fox and Howlett, 2008; Keller, 2019). In this work, the deletion of both *cl3A* and *verA* genes did not affect *P. polonicum*, both in terms of growth rate and conidiation on PDA. Similar results were obtained deleting the PKS encoded by *pksCT* responsible for the biosynthesis of citrinin (Ballester et al., 2015). Conversely, the deletion or the overexpression of the *pks4* gene affected fruit bodies formation in *Sordaria macrospora* (Keller, 2019). To evaluate the effect of genes deletion on virulence, *P. polonicum* was inoculated on two cultivars of apples. *P. polonicum* strains can be found as contaminants of apples in post-harvest (Andersen and Thrane, 2006) and the pathogenicity of *P. polonicum* X6 on apples was previously verified (data not shown). In this work wild-type strain and deletion mutants displayed a similar development of rot diameter. Slight differences on virulence of Δ*verA* mutants were observed on apple cv. Ambrosia while this behavior was not observed on apples cv. Opal. These results are not surprising, as the apple cultivar is known to be a key factor in the virulence, such as in the case of patulin which may be considered a cultivar-dependent aggressiveness factor (Snini et al., 2016). However, the reduced virulence of Δ*verA* mutants was not observed later in the infection, therefore further studies are needed to confirm these preliminary observations.

Conclusion

The verrucosidin biosynthesis was identified by bioinformatic and genetic approaches. Sixteen HR-PKS gene-containing BGCs were discovered in the available genomes of *P. polonicum*. These BGCs are probably involved in the biosynthesis of α-pyrone type polyketides which could have many biological activities, such as cytotoxic, antitumor, antimicrobial and anti-germination activities and are therefore a source of a novel compound (Li et al., 2018; Schäberle, 2016; Stroe et al., 2020). Based on the putative function of the genes present in the clusters, two of these BGCs were further characterized through deletion approach, in order

to investigate their involvement in verrucosidin biosynthesis. To delete the selected PKS genes, CRISPR-Cas9 was adopted to *P. polonicum*. This promising tool of gene editing allowed to obtain mutants by replacing the target genes with repair template sequences with 50 bp of homology to chromosomal sequences. This procedure does neither require plasmid construction nor the endogenous expression of Cas9 and allowed to obtain many mutants of *P. polonicum*. By applying this technology, the verrucosidin BGC was confirmed by creating deletion mutants. The availability of the CRISPR/Cas technology will allow to characterise the biosynthetic potential of this interesting fungus.

Acknowledgments

This work was supported by the German Academic Exchange Service (DAAD) with a scholarship in the program Research Grants - Short-Term Grants, 2019. We thank Christina Täumer from the Leibniz Institute for Natural Product Research and Infection Biology for technical assistance, and the students at the University of Torino who helped to characterize the mutants.

Supplementary material

A

Strain	MEA	CYA
P. polonicum X6	+	+
P. aurantiogriseum CBS 112021	+	+
P. crustosum CAL64	-	-

B

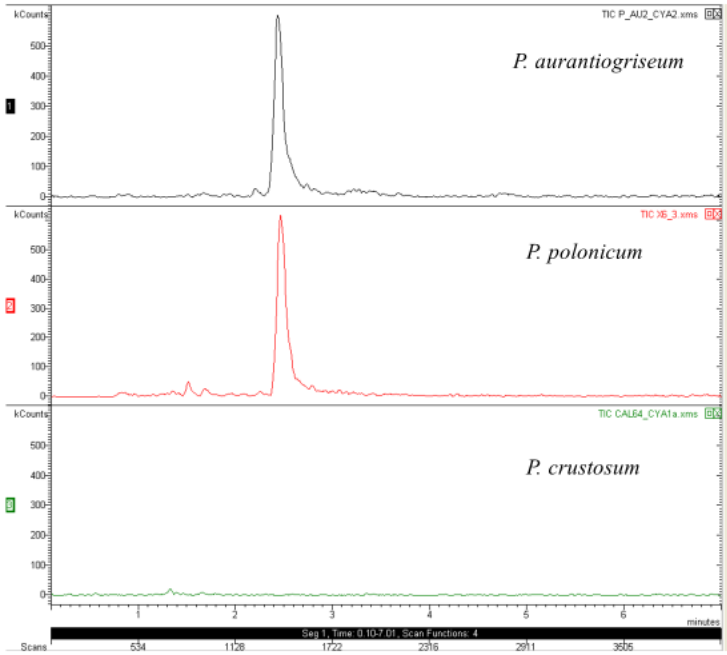


Figure S1 – Verrucosidin production *in vitro*. *P. polonicum*, *P. aurantiogriseum* and *P. crustosum* were inoculated in CYA and MEA broth. Presence (+) or absence (-) of verrucosidin production 10 days after inoculation (A) and chromatograms (B).

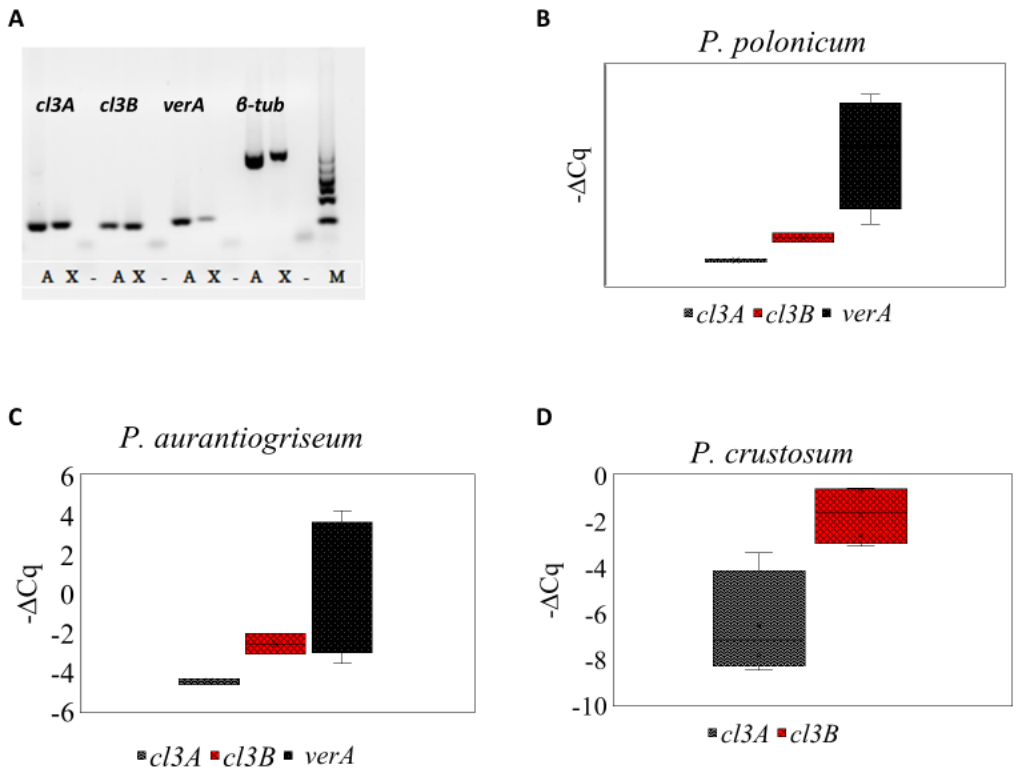


Figure S2 – Presence and expression of selected genes in *P. polonicum*, *P. aurantiogriseum* and *P. crustosum*. Amplification of selected genes from gDNA (**A**); X = *P. polonicum* X6, A = *P. aurantiogriseum* CBS121001, M = GelPilot 100 bp Ladder, - = negative control (PCR mix without DNA). Expression of *cl3A*, *cl3B* and *verA* (*cl4A*) genes in *P. polonicum* (**B**), *P. aurantiogriseum* (**C**) and *P. crustosum* (**D**). Gene expression is represented as the negative difference between Quantification cycle (Cq) of gene and Cq of reference gene (-ΔCq). β-tubulin was used as reference gene.

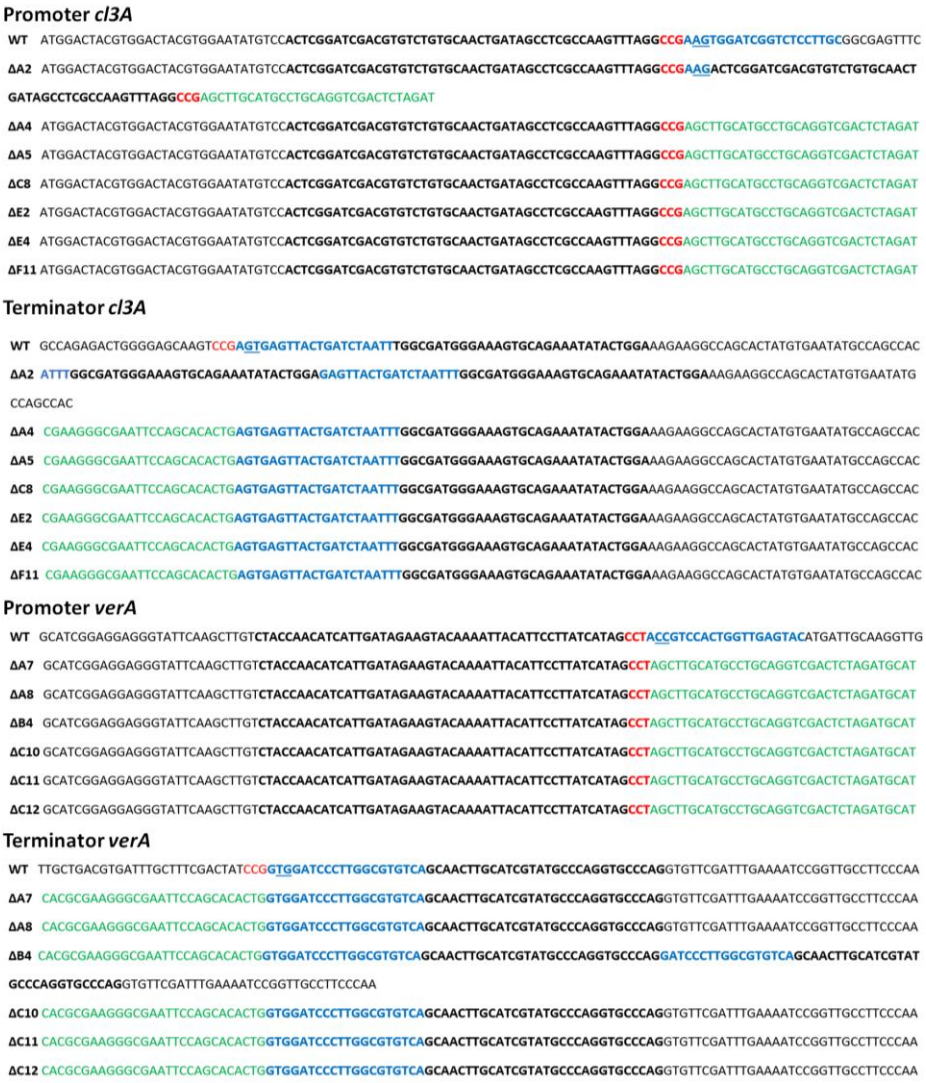


Figure S3 – Sequencing of deletion locus. Blu = protospacer, red = PAM, green = hygromycin cassette, in bold = 50 bp of homology, underline = expected site of cut, WT = *P. polonicum* X6, Δ = deletion mutants for *cl3A* or *verA* (*cl4A*) gene.

Table S1 – Primers and sequences used in this study

RT-qPCR and PCR with gDNA to verify presence of genes		
Name	**Sequence**	**Amplification**
3A-F	GGGTCCTTCTCAGCAGATTATAC	*cl3A* (111 bp)
3A-R	CAGCCTGTTTGCTGTCATTG	
4A-F	CCTCTATGTTGGGTCGATCAAG	*verA/ cl4A* (119 bp)
4A-R	ATAGCAGGTTTGGGAAGATGG	
3B-F	GCCGTTCAGGGTAATCTCTT	*cl3B* (114 bp)
3B-R	GACATGGTGCAATGCCATAC	
BTUB-F	CGAGTTGACCCAGCAGATGT	β-tubulin
BTUB-R	GTCTGGACGTTGTTGGGGAT	
Primers to amplify donor DNA (hygromycin resistance cassette)		
Name	**Sequence**	**Amplification**
H50P3-F	**ACTCGGATCGACGTGTCTGTGCAACTGATAGCCTCGCCAAGTTTAGG CCG**AGCTTGCATGCCTGCAGGTC	50 bp micro-homology with *cl3A* promoter and terminator in bold
H50P3-R	**TCCAGTATATTTCTGCACTTTCCCATCGCCAAATTAGATCAGTAACTC ACT**cagtgtgctggaattcgcccttc	

H50P4-F	**CTACCAACATCATTGATAGAAGTACAAAATTACATTCCTTATCATAGC CT**AGCTTGCATGCCTGCAGGTC	50 bp micro-homology with *verA* promoter and terminator in bold
H50P4-R	**CTGGGCACCTGGGCATACGATGCAAGTTGCTGACACGCCAAGGGATC CACC**AGTGTGCTGGAATTCGCCCTTC	
Primers to verify knockout mutants		
Name	**Sequence**	**Amplification**
Hygr-R	CAGTGTGCTGGAATTCGCCCTTC	Hygromycin resistance cassette (3003 bp)
Hygr-F	AGCTTGCATGCCTGCAGGTC	
3A-2	TGCTGACAAGACGCCAATAG	Promoter of *cl3A* and *verA*(*cl4A*)
4A-2	TGTGCAGTTGGCTTTCATTTC	
HP	CCGCAAGGAATCGGTCAATACA	
3A-4	CATACTTCTCGCCGCTACAG	Terminator of *cl3A* and *verA*(*cl4A*)
4A-4	GATGACCTCTTGGCGCTTAT	
HP-R	TGTATTGACCGATTCCTTGCGG	
Protospacer and Protospacer Adjacent Motif (PAM)		
Name	**Sequence – PAM**	**On target score**
CD.Cas9.GFZS9721.AB	GCAAGGAGACCGATCCACTT-CGG	80
CD.Cas9.PKBV4077.AB	AATTAGATCAGTAACTCACT-CGG	89
CD.Cas9.TPPB7515.AE	GTACTCAACCAGTGGACGGT-AGG	80
CD.Cas9.FWKX5170.AA	TGACACGCCAAGGGATCCAC-CGG	91

Table S2 – BGCs found in the genome of *P. polonicum*. In the next tables are reported genes found to be close to the molecular probe to detect verrucosidin-producer *Penicillium* spp. (Rodriguez et al., 2012) and 16 putative verrucosidin gene clusters found in *P. polonicum* strains IBT 4502 and hy4. Gene name are chosen according to previously identified genes in aurovertin biosynthesis: pr = probe, cl = cluster, ver = verrucosidin BGC, A = polyketide synthase, B = methyltransferase, C = Monooxygenase FMO, D = Hydrolase, E = Cyclase, F = Transcription Factor, G = Acyl Transferase, H = Cytochrome P450, I = Other oxidoreductases, J = Transporter**,** K = Dehydrogenase, L = Peptidase, M = Other hydrolase, N = Other transferases, O = decarboxylases, U = Unknown function. PKS in bold has similarity with *ctvA* and *aurA*. The name of protein in *P. polonicum* and presence (+) or absence (-) of the protein in the genome of other *Penicillium* spp. are reported. In putative function is reported the closest match considering BLAST and INTERPROSCAN. In notes are reported additional domains or gene overexpressed or underexpressed *in P. expansum* while producing verrucosidin (Kim et al., 2016).

Genes close to verrucosidin probe						
Name	***P. polonicum* IBT 4502**	***P. polonicum* hy4**	***P. expansum* NRRL 62431**	***P. crustosum* CAL64**	**Putative function**	**Notes**
prU1	OQD71346.1	+	-	-	Hypothetical protein	It has a PKS domain, part of an adenyl binding domain, but no PKS close blast matches and no predicted protein family membership
prU2	OQD71664.1	+	-	-	Hypothetical protein	
prJ	OQD71106.1	+	-	-	MFS transporter	
prL	OQD71618.1	+	+	+	Peptidase A2A, retrovirus, catalytic	Probe attaching here
prU3	OQD71889.1	+	+	+	Hypothetical protein	
prU4	OQD71177.1	+	+	+	Protein of unknown function DUF3292	
prU5	OQD70896.1	+	+	+	Hypothetical protein	
prU6	OQD71058.1	+	+	+	Emopamil-binding	
prU7	OQD71634.1	+	+	+	Hypothetical protein	
prU8	OQD71406.1	+	+	+	Hypothetical protein	

Cluster 1						
Name	***P. polonicum* IBT 4502**	***P. polonicum* hy4**	***P. expansum* NRRL 62431**	***P. crustosum* CAL64**	**Putative function**	**Notes**
cl1H	OQD68095.1	+	+	+	Cytochrome P450 oxydoreductase	
cl1U1	OQD68222.1	+	+	+	Hypothetical protein	
cl1U2	OQD67563.1	+	+	+	Calycin	
cl1A	**OQD67773.1**	**+**	**+**	**+**	**Polyketide synthase**	
cl1A2	OQD68121.1	+	+	+	Polyketide synthase, enoylreductase, alchool deydrogenase	

Cluster 2						
Name	***P. polonicum* IBT 4502**	***P. polonicum* hy4**	***P. expansum NRRL 62431***	***P. crustosum* CAL64**	**Putative function**	**Notes**
cl2A	**OQD63672.1**	**+**	**+**	**+**	**Polyketide synthase**	
cl2D1	OQD63761.1	+	+	+	Hypotetical protein with serin hydrolase domain	
cl2U1	OQD63370.1	+	+	+	Hypothetical protein	Overexpressed in verrucosidin producing conditions
cl2U2	OQD63792.1	+	+	+	Endosomal protein with GOLD domain (protein-protein interaction) and transmembrane domains	
cl2U3	-	-	-	+	Hypothetical protein	
cl2D2	OQD63654.1	+	+	+	Hypothetical protein with glycoside hydrolase domain	
cl2J	-	-	-	+	ABC transporter	

Cluster 3						
Name	***P. polonicum* IBT 4502**	***P. polonicum* hy4**	***P. expansum NRRL 62431***	***P. crustosum* CAL64**	**Putative function**	**Notes**
*cl3*J	OQD60362.1	+	-	+	ABC transporter	
*cl3U*1	OQD60333.1	+	-	+	Protein of unknown function DUF3716, Phospolipase A2, active site	
*cl3*C	OQD60328.1	+	+	+	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	
*cl3*B	OQD60332.1	+	+	+	S-adenosyl-L-methionine-dependent methyltransferase	Overexpressed in verrucosidin producing conditions
***cl3*A**	**OQD60358.1**	**+**	**+**	**+**	**Polyketide synthase**	Additional Enoyl reductase domain
*cl3*D	OQD60365.1	+	+	+	Serine hydrolase FSH	
*cl3U*2	OQD60340.1	+	-	-	Hypothetical protein	

Verrucosidin BGC (Cluster 4)						
Name	***P. polonicum* IBT 4502**	***P. polonicum* hy4**	***P. expansum NRRL 62431***	***P. crustosum* CAL64**	**Putative function**	**Notes**
cl4C1/ verC1	OQD69144.1	-	-	-	FAD/NAD(P)-binding domain-containing protein	Similar to CTVC
cl4B/ verB	OQD68888.1	-	-	-	Methyltransferase	Similar to CTVB
cl4C2/ verC2	OQD69870.1	-	-	-	Monooxygenase FAD-binding protein	Similar to CTVC
cl4U1/ verU1	OQD69720.1	-	-	-	Hypothetical protein with transmembrane domains	
cl4H/ verH	OQD69071.1	-	-	-	Cytochrome P450	
cl4A/ verA	**OQD69647.1**	**-**	**-**	**-**	**Polyketide synthase**	
cl4F/ verF	OQD68940.1	+	+	-	Transcription factor	Underexpressed in verrucosidin producing conditions
cl4U2/ verU2	OQD69889.1	+	+	-	Hypothetical protein	
cl4U3/ verU3	OQD69759.1	+	+	-	Hypothetical protein	
cl4G/ verG	OQD69357.1	+	+	-	Acyl-CoA N-acyltransferase	

Cluster 5						
Name	***P. polonicum* IBT 4502**	***P. polonicum* hy4**	***P. expansum NRRL 62431***	***P. crustosum* CAL64**	**Putative function**	**Notes**
***cl5*A**	**OQD63426.1**	**+**	**-**	**-**	**Polyketyde synthase**	
*cl5L*1	OQD63428.1	+	-	+	Peptidase C45, acyl-coenzyme A:6-aminopenicillanic acid acyl-transferase	
cl5I	OQD63430.1	+	+	+	Cytochrome b561	
*cl5L*2	OQD63436.1	+	-	-	Peptidase C45, acyl-coenzyme A:6-aminopenicillanic acid acyl-transferase	
cl5U	OQD63551.1	+	+	-	Hypothetical protein	
*cl5*F	OQD63610.1	+	-	-	Transcription factor	
cl5M	OQD63635.1	+	+	-	Alpha-L-fucosidase	

Cluster 6						
Name	***P. polonicum* IBT 4502**	***P. polonicum* hy4**	***P. expansum NRRL 62431***	***P. crustosum* CAL64**	**Putative function**	**Notes**
cl6J1	OQD61059.1	+	+	+	Cation transporting ATPase	
cl6U1	OQD61070.1	+	+	+	Translation elongation factor	
cl6A	**OQD61128.1**	**+**	**-**	**+**	**Polyketide synthase**	Additional Enoyl reductase domain
cl6U2	OQD61131.1	+	+	+	Eukaryotic translation initiation factor 2	
cl6D	OQD61132.1	+	+	+	Serine hydrolase FSH	
cl6J2	OQD61154.1	+	+	+	Sulfate transporter	
cl6U3	OQD61177.1	+	+	-	Hypotetical protein	
cl6U4	OQD61186.1	+	+	+	CENP-A-nucleosome distal centromere subunit CENP-L	
cl6I	OQD61187.1	+	+	+	Aldo/keto reductase	
cl6N	OQD61204.1	+	+	+	Hypotetical protein with Protein kinase-like domain	
cl6F1	OQD61216.1	+	+	+	DNA-binding HORMA	
cl6F2	OQD61240.1	+	+	+	Fungal transcriptional regulatory protein	Overexpressed in verrucosidin producing conditions
cl6U5	OQD61244.1	+	+	+	DNA-directed RNA polymerase	

Cluster 7						
Name	***P. polonicum* IBT 4502**	***P. polonicum* hy4**	***P. expansum NRRL 62431***	***P. crustosum* CAL64**	**Putative function**	**Notes**
cl7U1	OQD64350.1	+	-	-	Hypothetical protein with EthD domain	
cl7B1	OQD64367.1	+	-	-	O-methyltransferase	
cl7D	OQD64429.1	+	-	-	Alpha/beta hydrolase	
cl7L1	OQD64470.1	+	+	+	Peptidase M24	
cl7L2	OQD64500.1	+	-	-	Peptidase M24	
cl7U2	OQD64508.1	+	-	-	Acetate-CoA ligase	
cl7I	OQD64517.1	+	-	-	Monooxygenase	
cl7F	OQD64519.1	+	-	-	C6 finger transcription factor	
cl7H1	OQD64603.1	+	-	-	Cytochrome P450	
cl7K	OQD64659.1	+	-	-	SirQ protein	
cl7U3	OQD64682.1	+	-	-	Hypothetical protein	
cl7B2	OQD64738.1	+	-	-	Methyltransferase SirN-like protein	
cl7U4	OQD64754.1	-	-	-	Hypothetical protein	
cl7M	OQD64757.1	+	+	+	Protein phosphatase, Leucine rich repeats	
cl7A	**OQD64783.1**	**+**	**-**	**-**	**Polyketide synthase**	
cl7U5	OQD64845.1	+	-	-	Translation elongation factor eEF 1	
cl7H2	OQD64894.1	+	+	+	Cytochrome P450	

Cluster 8						
Name	**Protein in *P. polonicum* strain IBT 4502**	***P. polonicum strain hy4***	***P. expansum* strain *NRRL 62431***	***P. crustosum* strain CAL64**	**Putative function**	**Notes**
cl8A	**OQD61269.1**	**+**	**-**	**-**	**Polyketide synthase**	
cl8F1	OQD61286.1	+	+	+	Transcription factor jumonji	
cl8N	OQD61288.1	+	-	-	Aminoglycoside phosphotransferase	
cl8I1	OQD61304.1	+	-	-	FAD-dependent isoamyl alcohol oxidase	
cl8G	OQD61318.1	+	+	+	Acyl-CoA N-acyltransferase	
cl8U1	OQD61322.1	+	+	+	Hypothetical protein	
cl8I2	OQD61330.1	+	-	-	Oxidoreductase, NAD(P)-binding protein	
cl8K	OQD61332.1	+	-	-	Zinc-binding dehydrogenase	
cl8H	OQD61338.1	+	-	-	Cytochrome P450	
cl8I3	OQD61339.1	+	-	-	Oxidoreductase, NAD(P)-binding protein	
cl8U2	OQD61365.1	+	-	-	Hypothetical protein	
cl8O	OQD61370.1	+	+	+	Oxalyl-CoA decarboxylase	
cl8U3	OQD61375.1	+	+	+	Hypothetical protein	
cl8F2	OQD61382.1	+	-	-	Fungal transcriptional regulatory protein	
cl8U4	OQD61435.1	-	-	-	Hypothetical protein	

Cluster 9						
Name	***P. polonicum* IBT 4502**	***P. polonicum* hy4**	***P. expansum NRRL 62431***	***P. crustosum* CAL64**	**Putative function**	**Notes**
*cl9*H1	OQD62930.1	+	-	-	Cytochrome P450	
*cl9U*1	OQD62937.1	+	-	-	Hypothetical protein	
*cl9*J1	OQD62943.1	+	-	-	MFS general substrate transporter	
cl9N1	OQD62951.1	+	-	+	Hypothetical protein with protein kinase-like domain	
*cl9*J2	OQD62954.1	-	+	+	Amino acid transporter, transmembrane	
cl9N2	OQD62956.1	+	-	-	Calcium/calmodulin-dependent protein kinase II isoform	
cl9I	OQD62974.1	+	-	-	NAD(P)-binding protein	
*cl9*A2	OQD63049.1	+	-	-	GroES-like protein, enoylreductase	
*cl9*H2	OQD63117.1	+	-	-	Cytochrome P450	
cl9U2	OQD63150.1	+	-	-	Hypothetical protein	
*cl9*F1	OQD63214.1	+	+	-	Hypothetical potrein with Zn2C6 DNA binding domain	
*cl9*F2	OQD63223.1	+	-	+	Fungal transcriptional regulatory protein	
cl9U3	OQD63280.1	+	-	-	Hypothetical protein	
***cl9*A**	**OQD63284.1**	**+**	**-**	**-**	**Polyketide synthase**	

Cluster 10						
Name	***P. polonicum* IBT 4502**	***P. polonicum* hy4**	***P. expansum NRRL 62431***	***P. crustosum* CAL64**	**Putative function**	**Notes**
cl10I	OQD60326.1	+	+	+	Taurine catabolism dioxygenase TauD/TfdA	
*cl10*A2	OQD60329.1	+	+	+	Polyketide synthase, enoylreductase, alchool deydrogenase	
cl10U	OQD60337.1	+	+	+	Hypotetical protein	
cl10K	OQD60359.1	+	+	+	acyl-CoA dehydrogenase	
*cl10*J1	OQD60366.1	+	+	+	Amino acid/polyamine transporter I	
*cl10*J2	OQD60369.1	+	+	+	Sucrose/H+ symporter	
*cl10*F1	OQD60370.1	+	+	+	Fungal transcriptional regulatory protein	
*cl10*H	OQD60373.1	+	+	+	Cytochrome P450	
*cl10*F2	OQD60384.1	+	+	+	Transcription factor	
*cl10*J3	OQD60393.1	+	+	+	Sucrose/H+ symporter	Underexpressed in verrucosidin producing conditions
***cl10*A**	**OQD60396.1**	**+**	**-**	**+**	**Polyketide synthase**	
cl10L	OQD60403.1	+	+	+	Peptidase M24	

Cluster 11						
Name	***P. polonicum* IBT 4502**	***P. polonicum* hy4**	***P. expansum* *NRRL 62431***	***P. crustosum* CAL64**	**Putative function**	**Notes**
cl11J1	OQD65718.1	+	+	+	Transport protein	
cl11U1	OQD65662.1	-	+	+	Hypothetical protein	
cl11U2	OQD65737.1	+	+	+	Cyclin-like	
cl11A	**OQD66219.1**	**+**	**-**	**-**	**Polyketyde synthase**	
cl11U3	OQD65636.1	+	-	-	Polysaccharide synthase	
cl11J2	OQD66164.1	-	-	-	MFS transporter	
cl11D	OQD65818.1	-	+	+	alpha/beta-hydrolase	
cl11U4	OQD65688.1	+	+	+	Autophagy-related protein 3	
cl11U5	OQD65906.1	+	+	+	Hypothetical protein	
cl11U6	OQD66060.1	-	+	+	Hypothetical protein	
cl11N	OQD65617.1	-	+	+	Hypothetical protein/Glycosyl transferase	

Cluster 12						
Name	***P. polonicum* IBT 4502**	***P. polonicum* hy4**	***P. expansum* *NRRL 62431***	***P. crustosum* CAL64**	**Putative function**	**Notes**
cl12N	OQD59992.1	-	-	-	Putative aminotransferase	
cl12A	**OQD59991.1**	-	-	-	**Polyketyde synthase**	
cl12U1	OQD59995.1	-	-	-	putative toxin biosynthesis protein	
cl12J	OQD59999.1	-	+ (vaguely similar)	-	MFS transporter	
cl12F	OQD59997.1	-	-	-	Fungal specific transcription factor	
cl12H	OQD59998.1	-	-	-	Cytochrome P450	
cl12U2	OQD59994.1	-	-	-	NAD dependent epimerase/dehydratase	

Cluster 13						
Name	***P. polonicum* IBT 4502**	***P. polonicum* strain *hy4***	***P. expansum* *NRRL 62431***	***P. crustosum* CAL64**	**Putative function**	**Notes**
cl13J1	OQD61546.1	-	-	-	Putative transporter	
cl13U1	OQD61463.1	-	-	-	Hypothetical protein	
cl13I1	OQD61510.1	-	+	+	Fatty acid hydroxylase	
cl13L	OQD61571.1	-	-	+	Amidase family protein	
cl13M	OQD61479.1	-	+	+	Acyl-CoA thioesterase	
cl13N	OQD61620.1	-	-	-	3-amino-3-carboxypropyl transferase	
cl13A	**OQD61552.1**	**-**	**-**	**-**	**Polyketyde synthase**	
cl13D	OQD61622.1	-	+	-	Hydrolase	
cl13U2	OQD61573.1	-	-	-	Hypothetical protein DUF3632	
cl13J2	OQD61591.1	-	+	+	MFS transporter	
cl13U3	OQD61653.1	-	+	-	Hypothetical protein with NACHT domain	
cl13U4	OQD61560.1	-	-	+	Hypothetical protein DUF3176	
cl13J3	OQD61616.1	-	-	-	MFS transporter	
cl13I2	OQD61608.1	-	-	-	Hypothetical protein/NADP-binding	

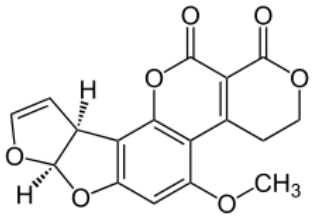
Cluster 14						
Name	***P. polonicum* IBT 4502**	***P. polonicum* hy4**	***P. expansum NRRL 62431***	***P. crustosum* CAL64**	**Putative function**	**Notes**
cl14U1	+	QPIC01000001_1_region004_polonicum_gene196	+	+	Allantoicase	
cl12J1	+	QPIC01000001_1_region004_polonicum_gene197	+	+	Major facilitator superfamily, general substrate transporter	
cl14D	-	QPIC01000001_1_region004_polonicum_gene198	+	-	Glycoside hydrolase	
cl14L	-	QPIC01000001_1_region004_polonicum_gene199	+	-	Peptidase C45, acyl-coenzyme A:6-aminopenicillanic acid acyl-transferase	
cl14G	+	QPIC01000001_1_region004_polonicum_gene200	'	-	CoA-dependent acyltransferases	
cl14A1	-	QPIC01000001_1_region004_polonicum_gene201	-	-	**Polyketyde synthase**	
cl14A2	+	QPIC01000001_1_region004_polonicum_gene202	+	-	Polyketide synthase, enoylreductase	
cl14H	+	QPIC01000001_1_region004_polonicum_gene203	+	-	cytochrome P450	
cl14J2	-	QPIC01000001_1_region004_polonicum_gene204	+	+	Major facilitator superfamily, general substrate transporter	
cl14I	+	QPIC01000001_1_region004_polonicum_gene205	+	+	Cytochrome b561/ferric reductase transmembrane	
cl14J3	-	QPIC01000001_1_region004_polonicum_gene206	-	-	ABC transporter	

Cluster 15						
Name	***P. polonicum* IBT 4502**	***P. polonicum* hy4**	***P. expansum NRRL 62431***	***P. crustosum* CAL64**	**Putative function**	**Notes**
cl15U1	-	QPIC01000002_1_region002_polonicum_gene37	-	-	Hypothetical protein	
cl15U2	+	QPIC01000002_1_region002_polonicum_gene38	+	+	Hypothetical protein DUF1993	
cl15J1	+	QPIC01000002_1_region002_polonicum_gene39	+	+	Amino acid/polyamine transporter I	Overexpressed in verrucosidin producing conditions
cl15A	**-**	**QPIC01000002_1_region002_polonicum_gene40**	**-**	**-**	**Polyketyde synthase**	
cl15C1	-	QPIC01000002_1_region002_polonicum_gene41	-	-	Hypothetical protein/FAD-linked oxidoreductase	
cl15U3	-	QPIC01000002_1_region002_polonicum_gene42	-	-	Hypothetical protein	
cl15C2	-	QPIC01000002_1_region002_polonicum_gene43	-	-	Hypothetical protein/FAD-dependent monooxygenase	
cl15J2	-	QPIC01000002_1_region002_polonicum_gene44	-	+	Sucrose/H+ symporter	
cl15A2	-	QPIC01000002_1_region002_polonicum_gene45	-	+	Polyketide synthase, enoylreductase, alchool deydrogenase	
cl15U4	-	QPIC01000002_1_region002_polonicum_gene46	-	-	Hypothetical protein	

Cluster 16						
Name	***P. polonicum* IBT 4502**	***P. polonicum* hy4**	***P. expansum NRRL 62431***	***P. crustosum* CAL64**	**Putative function**	**Notes**
cl16J	+	QPIC01000004_1_region001_polonicum_gene191	+	+	Putative transporter	
cl16U1	+	QPIC01000004_1_region001_polonicum_gene192	+	+	Hypothetical protein/ribosomal	
cl16U2	+	QPIC01000004_1_region001_polonicum_gene193	+	+	Hypothetical protein	
cl16A	**-**	**QPIC01000004_1_region001_polonicum_gene194**	**+**	**-**	**Polyketide synthase**	Additional ER domain
cl16U3	+	QPIC01000004_1_region001_polonicum_gene195	+	+	Hypotetical protein/Heat shock protein	
cl16U4	+	QPIC01000004_1_region001_polonicum_gene196	+	+	Hypothetical protein/Lipid binding	
cl16I	+	QPIC01000004_1_region001_polonicum_gene197	+	+	Malic oxidoreductase (NADP-dependent)	
cl16K	+	QPIC01000004_1_region001_polonicum_gene198	+	+	Aldolase-type TIM barrel	

Chapter 4

Effect of drying temperature on *Aspergillus flavus* growth and aflatoxin production on artificially inoculated hazelnuts



Silvia Valente [a,b], Giovanna Roberta Meloni [a,b], Simona Prencipe [b], Nicola Spigolon [c], Marco Somenzi [c], Mauro Fontana [c], Maria Lodovica Gullino [a,b], and Davide Spadaro [a,b]

[a] AGROINNOVA – Centre of Competence for the Innovation in the Agroenvironmental Sector, Largo Paolo Braccini 2, 10095 Grugliasco, Italy

[b] Dept. Agricultural, Forest and Food Sciences (DISAFA), Università degli Studi di Torino, Largo Paolo Braccini 2, 10095 Grugliasco, Italy

[c] SOREMARTEC ITALIA S.r.l., Piazzale Ferrero 1, 12051 Alba (CN), Italy

Published at Journal Food Protection (2020), https://doi.org/10.4315/JFP-20-061

Abstract

Aspergillus flavus may colonise hazelnuts and produce aflatoxins in field and during storage. The main purpose of this study was to investigate the influence of drying temperature and exposure times on the viability and ability of *A. flavus* to produce aflatoxins during the drying process and storage. Hazelnuts were inoculated with *A. flavus* and dried at different temperatures to reach 6% moisture content and a_w 0.71, a commercial requirement to avoid fungal development and aflatoxin contamination. Hazelnuts were dried at 30, 35, 40, 45, and 50 °C and subsequently stored at 25 °C for 14 days. After drying at 30, 35 and 40 °C, an increased number of *A. flavus* was evident, with the highest concentration at 35 °C ($6.1 \pm 2.4 \times 10^6$ *A. flavus* CFU/g). At these temperatures, aflatoxins were detected only at 30 °C and 35 °C. Aflatoxins, however, were higher after drying at 30 °C with a concentration of 1.93 ± 0.77 μg/g for aflatoxin B1 (AFB1) and 0.11 ± 0.04 μg/g for aflatoxin B2 (AFB2). After 14 days of storage, the highest *A. flavus* concentration and the highest level of mycotoxins were detected in samples treated at 35 °C ($8.2 \pm 2.1 \times 10^7$ *A. flavus* CFU/g, 9.30 ± 1.58 μg/g and 0.89 ± 0.08 μg/g for AFB1 and AFB2, respectively). In hazelnuts dried at 45 °C or 50 °C no aflatoxins were found both after drying and storage, and a reduction of *A. flavus* viable conidia was observed, suggesting that a shorter and warmer drying is essential to guarantee the nut safety. The lowest temperature that guarantees the lack of aflatoxins should be selected to maintain the organoleptic quality of hazelnuts. Therefore, 45°C should be the recommended drying temperature to limit *A. flavus* growth and aflatoxin contamination on hazelnuts.

Keywords

aflatoxins; hazelnuts; *Aspergillus flavus*; temperature; drying

1. Introduction

Hazelnut (*Corylus avellana* L.) is mainly cultivated in warm temperate areas, such as Turkey which is the first world producer, covering approximately 65% of the world production (675,000 tons in 2017), followed by Italy (approximately 13%), Azerbaijan, the United States, and China (Food and Agriculture Organization of the United Nations (FAO), 2020; Köksal, 2000). The abundance of nutrients in hazelnuts, such as lipids and carbohydrates, makes them susceptible to decay and mould development (Gallo et al., 2016; Özilgen and Özdemir, 2001). Several factors can affect fungal contamination on hazelnut, such as the variety, the composition and the presence of damages on shells caused by insects and environmental or processing conditions. Besides, other factors can favour fungal development, including temperature and humidity in the field, methods and time of harvest, processing of hazelnuts (e.g. dehulling and drying methods), time and storage conditions (Fontana et al., 2014; Özdemir and Devres, 1999).

Most fungi isolated from nuts, including hazelnuts, belong to *Aspergillus* and *Penicillium* genera (Gürses, 2006; Khosravi et al., 2007; Kumar et al., 2008; Ozay et al., 2008; Prencipe et al., 2018b, 2018a; Rodrigues et al., 2012; Siahmoshteh et al., 2017; Şimşek et al., 2002). These ascomycetes are often present in the field, in the soil and in food matrices as saprophytes. Due to their tolerance to low humidity and temperature, they may remain latent during pre-harvest and develop later during storage.

Aspergillus and *Penicillium* spp. are characterized by their ability to produce several secondary metabolites, including mycotoxins. In particular, *Aspergillus* section *Flavi* may produce aflatoxins, which are highly toxic and carcinogenic compounds. The most important mycotoxins are aflatoxins B1 (AFB1) and B2 (AFB2), mainly produced by *A. flavus* and *A. parasiticus*, and aflatoxin G1 (AFG1) and G2 (AFG2), synthesized by *A. parasiticus* (Perrone et al., 2014; Prelle et al., 2012; Prencipe et al., 2018a). The European Commission (European Commission, 2010a) imposed

maximum levels of aflatoxins in foodstuffs, including nuts. For hazelnuts intended for direct consumption, limits are fixed at 5 µg/kg for AFB1 and 10 µg/kg for total aflatoxins. Aflatoxins are very stable compounds which are not degraded during roasting or other thermal treatments (Shapira and Paster, 2004; Siciliano et al., 2016, 2017). For this reason, it is very important to prevent conditions favourable to aflatoxin production both in field and during the postharvest phase (Ozay et al., 2008; Özdemir and Devres, 1999).

The most common technique to reduce fungal growth and prevent mycotoxins in nuts is drying, preferably within 72 hours after harvesting, to a moisture content (5 −8% depending on nuts) that corresponds to a low water activity, a_w (CAC-Codex Alimentarius Commission, 2005; Kader, 2013). The European Commission (European Commission, 2002) established the maximum moisture content for nuts in 12% for shelled hazelnuts and 7% for dehulled nuts. Commercially, hazelnuts are dried until 10% and 6% moisture content for shelled and dehulled hazelnuts, respectively (Wang et al., 2018a). In Turkey, nuts are conventionally dried outdoor for a period ranging from 4 days to 2–3 weeks depending on the weather conditions, before being stored at room temperature for 12 months (Baltaci et al., 2012; Turan, 2018). This prolonged and uncontrolled drying process usually promotes fungal contaminations that can result in fungal growth and aflatoxin production during storage. The use of drying machines is preferable because of the speed and homogeneity in reaching a low moisture content (Ozay et al., 2008; Turan and İslam, 2018). Air temperature, relative humidity and time can be controlled with drying machines. Drying temperatures commonly used by growers range from 30 to 40 °C (López et al., 1998; Ozay et al., 2008; Wang et al., 2018a). Nevertheless, air drying machines could result in physical damage, such as shell-cracks that could reduce the quality and safety of the kernels (Wang et al., 2018b). Moreover, several studies highlighted the influence of drying processes on hazelnuts quality, including lipid oxidation, fatty acid profile, enzymatic activity and kernel browning (López et al.,

1997a, 1997c, 1997b; Mousadoost et al., 2018; Özdemir and Devres, 1999; Turan, 2018; Turan and İslam, 2018; Wang et al., 2018a). Previously, Ozay *et al.* (Ozay et al., 2008) evaluated the effect of drying parameters on aflatoxin contamination on hazelnuts. The influence of drying techniques both on fungal growth and on aflatoxin production has not been investigated on hazelnuts so far.

The aim of this study was to investigate the effect of two significant drying parameters, temperature and drying time, on *A. flavus* viability and aflatoxin production on shelled hazelnuts. The time to reach the commercial threshold of 6% of moisture content at 30 °C, 35 °C, 40 °C, 45 °C and 50 °C was determined. Fungal growth and aflatoxin concentration were determined on hazelnuts inoculated with a conidial suspension of *A. flavus*, able to produce aflatoxins *in vitro* and *in vivo*. After drying, hazelnuts were stored at 25 °C for 14 days to simulate commercially adopted practices.

2. Materials and Methods

2.1. Hazelnuts and sample preparation

Fresh shelled hazelnuts 'Tonda Gentile Romana' (30 kg), provided by Soremartec Italia s.r.l., were harvested in Viterbo (Latium, Central Italy) in 2018. Hazelnuts were stored with shells in sealed polypropylene plastic bags at 4 °C for later use. Hazelnuts used for drying assays were dehulled one day before the experiment and kept in plastic bags at 4 °C overnight.

Raw hazelnuts were analysed for absence of AFs (AFG1, AFG2, AFB1 and AFB2) using the method detailed below. Sampling of hazelnut was done according with the Commission Regulation (EU) No. 178/2010 (European Commission, 2010b): 15 incremental samples were collected randomly from the lot and then mixed thoroughly to achieve a well homogenized aggregate sample of about 3 kg.

2.2. Determination of moisture content, water activity and drying conditions

The drying experiments were performed in a drying and heating chamber (Binder M115-230V) while, after drying, an environmental testing chamber (Panasonic MLR-352H-PE) was used for storing hazelnuts at 25 °C for 14 days. Moisture content of nuts was determined by weighing samples before and after drying at 103 °C for 14 hours (Prencipe et al., 2018c). Water activity (a_w) was measured by using an electronic hygrometer AquaLab Series 3TE (Decagon Devices Inc., Pullman, WA, USA), which adopted the chilled-mirror technique at 25 ◦C.

In order to mimic the moisture conditions favouring the fungal development, hazelnuts were soaked with sterile water for 2-14 hours. Hazelnuts were, later, inoculated with *A. flavus*, and fungal growth was visually observed after 7 days at 25 °C. Fourteen hours of soaking were adopted in the subsequent experiments.

To determine the time needed to reach approximately 6% of moisture content in healthy hydrated hazelnuts, they were dried in drying chamber using 5 temperatures (30 °C, 35 °C, 40 °C, 45 °C and 50 °C). Nuts were placed in aluminium trays on a paper towel and were covered with non-woven fabric sheet to promote drying. After 8 −72 hours their moisture content was determined. Furthermore, the water activity of hazelnuts dried at 6% of moisture content was determined.

For each treatment, the analyses were performed in three biological replicates and two technical replicates of 15 hazelnuts. A negative control was added. The analyses were performed twice.

2.3. Fungal strain and hazelnut inoculation

An aflatoxigenic strain of *Aspergillus flavus*, AFSP4, was used to inoculate hazelnuts in all experiments (Prencipe et al., 2018a). The strain was grown on Potato Dextrose Agar (PDA, Merck, Darmstadt, Germany) at 30 °C for seven days. In order to collect the spores, 5 mL of sterile deionized water containing 0.1% Tween (Merck, Darmstadt, Germany) were added to each plate and the colony surface was gently scraped. Conidia were pelleted using a centrifuge at 4,452 x *g* for 10 min and

resuspended in deionized water with 0.1% Tween and their number was counted with Bürker chamber and adjusted to a final concentration of $1x10^7$ spores/mL.
Before spore inoculation, hazelnuts were surface disinfected by immersion in a 1% sodium hypochlorite solution for 10 min and rinsed in sterile water for 10 min. Then, hazelnuts were soaked for 14 hours and dried for 10 min on paper towel under a laminar-flow hood. Hazelnuts were inoculated by stirring (ARE Hot Plate Stirrer, VELP Scientifica, Velate, Italy) in 200 mL of *A. flavus* spore suspension for 20 min, and then dried on paper towel in laminar-flow hood for 10 min. Inoculated and untreated hazelnuts were used as control.

2.4. Effect of drying on Aspergillus flavus *growth*

To evaluate the effect of drying temperatures on *A. flavus* growth, hazelnuts were dried at 30 °C, 35 °C, 40 °C, 45 °C and 50 °C. The relative humidity during drying was 60±5 %. For each drying condition, three biological replicates were used together with one negative control (hazelnuts not inoculated and dried at the same conditions). Biological replicates and the negative control were placed in separate aluminium trays.
A. flavus CFU/g hazelnuts and aflatoxins content were determined immediately after drying and after 14 days of storage at 25 °C and 70±5 % relative humidity. To count the number of viable *A. flavus* CFU/g, each technical replicate of 15 nuts was washed in a beaker placed on a stirrer with 100 mL of sterile water containing 0.1% Tween for 60 min. Serial dilutions of the washing water were plated on Yeast Extract Sucrose Agar (YES Agar) and on PDA. Agar plates were kept at 30 °C for 3 days and fungal colonies were visually counted, and the numbers of CFU/g nuts were calculated, by considering the weight of each sample.

2.5. Effect of drying on aflatoxin production

The chemical analyses were performed immediately after drying and after 14 days of storage at 25 °C in three biological replicates and two technical replicates. Each technical replicate consisted of 15 hazelnuts. Each replicate was placed into a 50 mL polypropylene tube and 30 mL of acetone/water (60/40 v/v) were added. The mixture was vortexed at high speed for 1 min and aflatoxins were extracted by sonication for 30 min. An aliquot (10 mL) of centrifuged extract (4,452 x *g* for 15 min) was filtered through a Whatman No. 4 filter and then the analytes were extracted twice with 10 mL ethyl acetate. The organic layer was evaporated to dryness and recovered with 1 mL of methanol/water (30/70 v/v).

Aflatoxin analysis was carried out using an Agilent 1100 series Quaternary pump LC system (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent 1100 series fluorescence detector (Agilent Technologies) set for excitation wavelength of 360 nm, and emission wavelength of 425 nm (quantification) and 455 nm (monitoring). The AFB1 signal enhancement was performed through an electrochemical bromine derivatization cell (Kobra Cell, R-Biopharm, Darmstadt, Germany). Analytes were separated using a reversed phase Eclipse Plus C18 (3.5 µm, 4.6 x 100 mm, Agilent Technologies) column at 25 °C. The isocratic mobile phase comprised water:methanol:acetonitrile (40:32:28, v/v/v) containing potassium bromide (119 mg/L) and 4 M nitric acid (350 µL/L) at flow rate of 1 mL/min for 15 min. The injection volume into the HPLC system was 100 µL. Retention times of 5.6, 7.0, 8.5, and 10.9 min were registered for AFG2, AFG1, AFB2, and AFB1, respectively.

AFs were quantified by external calibration curve. The standard solutions were prepared by diluting mixture of AFB1, AFB2, AFG1 and AFG2 using concentrations ranging from 2 to 500 ng/mL. Standards of the aflatoxins were purchased from Sigma-Aldrich (St. Louis, MO, USA) in crystallized form.

The limit of detection (LOD) of the method was 0.42 ng/g, 0.49 ng/g, 0.33 ng/g and 0.25 ng/g for AFG2, AFG1, AFB2, and AFB1, respectively. The recovery of AFs

was determined at three concentrations in the hazelnut matrix. The blank samples were spiked with the standards of AFB1, AFB2, AFG1 and AFG2 at low (5 µg/kg), middle (20 µg/kg) and high concentration (50 µg/kg) in three replicates. The average recoveries for AFB1, AFB2, AFG1 and AFG2 were 90.3 ± 3.6%, 89.6 ± 2.2%, 94.6 ± 1.1% and 98.1 ± 5.4%, respectively. The calculated limits of quantification (LOQ) were 2.10, 1.71, 0.99, and 1.00 µg/kg for AFB1, AFB2, AFG1, and AFG2, respectively.

2.6. Statistical Analysis

Statistical analyses were performed using IBM SPSS statistics software Inc. version 25 (Chicago, IL, USA), for variance analysis (one-way analysis of variance) using the Duncan test with $P \leq 0.05$. Data on *A. flavus* CFU/g hazelnuts were logarithmically transformed before statistical analysis.

3. Results

3.1. Effect of drying time on moisture content

The average moisture content of fresh shelled hazelnuts used in this study was 13.2 ± 0.7%, while dehulled hazelnuts had an average moisture content of 8.5 ± 1.2%, corresponding to a water activity of 0.82. Freshly harvested hazelnuts were already at a low moisture content before drying, due to the dry cropping season. As it was not possible to obtain consistent results about fungal development in fresh hazelnuts inoculated with *A. flavus* and stored at 25 °C for 14 days (data not shown), hazelnuts were soaked in water for 2 to 14 hours to increase the moisture content and consequently the water activity prior to drying (Table S1). After 14 days of storage at 25 °C, fungal growth was observed on hazelnuts soaked for at least 8 hours (Fig. S1). For the drying assay, hazelnuts were soaked for 14 hours to reach 35.9 ± 1.5%

moisture content and a_w of 0.94 ± 0.02 to create conditions suitable for *A. flavus* growth.

To calculate the time required to reach about 6–7% moisture content using different drying temperatures, the moisture was determined 8 to 72 hours after the treatments (Table 1). We observed that at 30 °C, 35 °C, 40 °C, 45 °C and 50 °C, there was a need of 72 h, 33 h, 28 h, 23 h and 20 h respectively, to dry hazelnuts. The water activity of samples dried at these conditions was calculated (Table 1), after the drying treatment hazelnuts had a final a_w of 0.71± 0.05.

Table 1. Moisture content (%) of hydrated hazelnuts dried at different temperatures for 8−72 hours. The time points used to reach around 6% of moisture content are in bold and for them the water activity was measured (in brackets). Values are expressed as mean values ± SD (3 replicates of 15 nuts per experiment, 2 repetitions of the experiment).

Drying Time (h)	**30 °C**	**35 °C**	**40 °C**	**45 °C**	**50 °C**
8	31.8 ± 2.4	18.7 ± 0.3	17.6 ± 0.2	15.1 ± 0.4	14.8 ± 0.1
20	-	-	-	-	**6.2 ± 0.1 (0.71 ± 0.06)**
23	27.8 ± 1.0	9.2 ± 0.7	7.8 ± 0.7	**5.6 ± 0.5 (0.67 ± 0.04)**	4.3 ± 0.3
28	26.0 ± 0.6	7.9 ± 0.4	**6.6 ± 0.4 (0.74 ± 0.03)**	4.6 ± 0.3	-
33	23.5 ± 0.3	**6.5 ± 0.2 (0.73 ± 0.03)**	5.2 ± 0.1	3.6 ± 0.1	-
50	15.4 ± 2.9	4.2 ± 0.1	3.4 ± 0.1	2.5 ± 0.1	-
56	12.6 ± 0.1	3.8 ± 0.1	3.1 ± 0.1	2.3 ± 0.0	-
72	**6.4 ± 0.3 (0.72 ± 0.03)**	-	-	-	-

3.2. Effect of drying temperatures on A. flavus growth

A. flavus was determined as CFU/g in the samples dried at different temperatures. A reduction of *A. flavus* growth was observed with the increase of temperature (Fig. 1). Fungal growth was higher in hazelnuts dried between 30 °C and 40 °C. In hazelnuts dried at 35 °C, *A. flavus* reached 6.1 x 10^6 CFU/g, while at 45 °C and 50 °C, *A. flavus* growth was significantly lower compared to the samples dried at 30 °C, 35 °C and 40 °C and to the inoculated untreated hazelnuts (3.9 x 10^4 CFU/g) (Fig. 1A). No CFU were found on uninoculated nuts used as negative controls (data not shown).

Similarly, after 14 days of storage at 25 °C, hazelnuts dried at 45 °C and 50 °C showed a lower number of *A. flavus* (CFU/g) compared to control and samples dried at lower temperatures (Fig. 1B). Hazelnuts dried between 30 °C and 40 °C exhibited a significant increase of fungal growth after the storage period, with the highest *A. flavus* concentration at 35°C (8.2 x 10^7 CFU/g) (Fig. 1B).

FIGURE 1

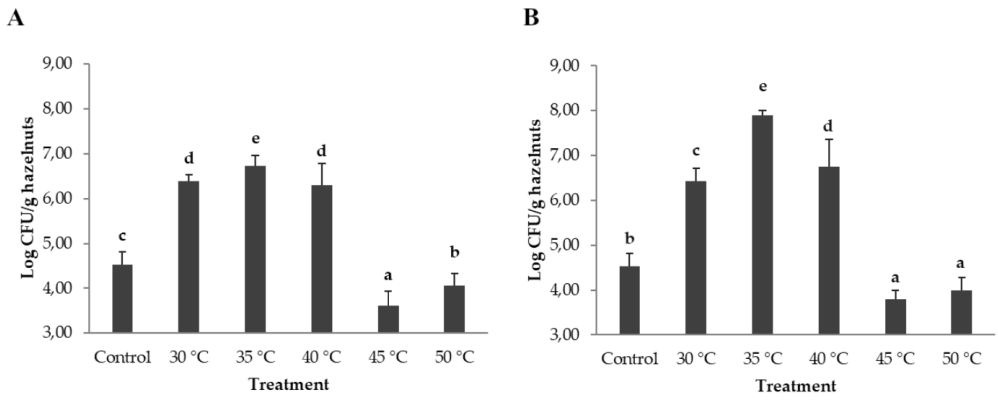


Figure 1. Log_{10} of CFU of *A. flavus* per gram of hazelnuts counted both after drying at different temperatures to reach approximately 6% of moisture content (A) and after 14 days of storage at room temperature (B). Control refers to inoculated samples undried and not stored and used directly to count inoculated *A. flavus* spores. Values followed by the same letter at the same time are not statistically

different by Duncan's multiple range test ($P \leq 0.05$). The statistical analysis was performed considering separately values obtained after drying and after storage.

3.3. Effect of drying temperatures on aflatoxin production

No natural aflatoxin contamination was detected on raw hazelnuts used in this study. Aflatoxin production was evaluated in hazelnuts after drying at different temperatures and after 14 days of storage at 25 °C. The strain of *A.flavus* used in this study produces AFB1 and AFB2 (Prencipe et al., 2018a).

As shown in Fig. 2A, production of both toxins (AFB1, AFB2) started already during drying at 30 °C with a concentration of 1.93 ± 0.77 μg/g for AFB1 and 0.11 ± 0.04 μg/g for AFB2. A lower toxin concentration was measured at 35 °C. At 40 °C, 45 °C or 50 °C no aflatoxins were detected immediately after drying.

Fourteen days after the drying treatment, aflatoxin production was observed in the samples dried at 30 °C, 35 °C and 40 °C (Fig. 2B). The highest level of mycotoxins detected was at 35 °C (9.30 ± 1.58 μg/g and 0.89 ± 0.08 μg/g for AFB1 and AFB2, respectively). Similar aflatoxin production was found for the samples dried at 30 °C for both time points tested.

For samples dried at 40 °C, aflatoxin production was found only after 14 days of storage (Fig. 2B), with a concentration of 1.57 ± 0.62 μg/g for AFB1 and 0.15 ± 0.06 μg/g for AFB2. No aflatoxins were found in samples dried at 45 °C and 50 °C, both after drying and after storage.

FIGURE 2

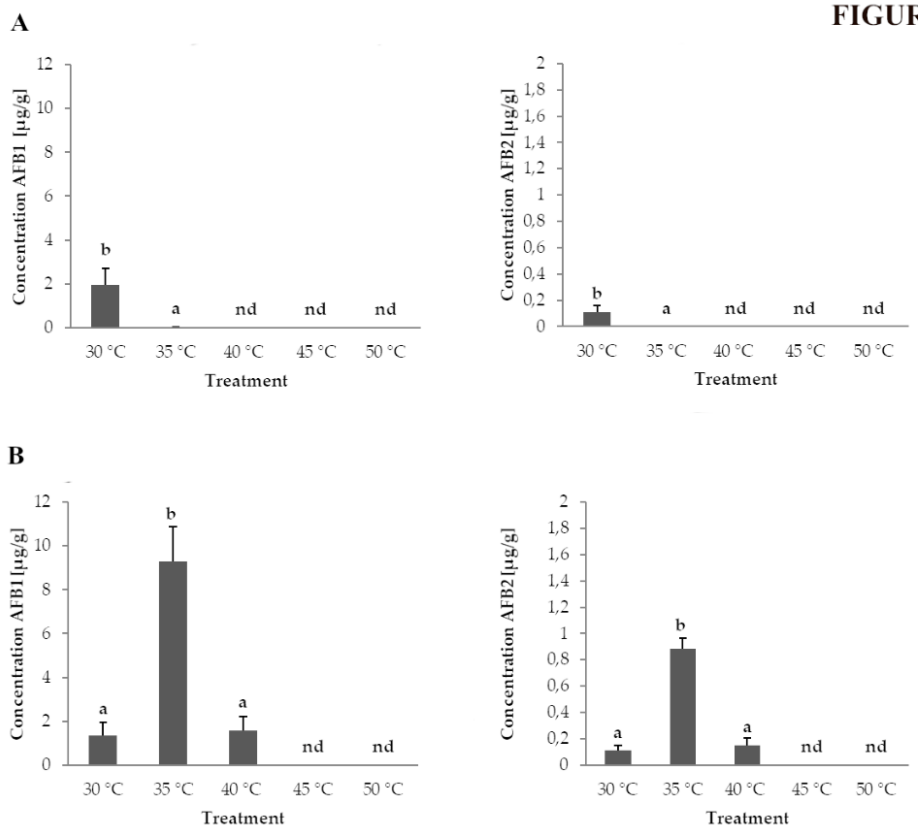


Figure 2. Concentration (µg/g) of AFB1 and AFB2 present in inoculated hazelnuts dried at different temperatures to reach 6% of moisture content. (A) shows data from hazelnuts collected immediately after drying; (B) shows data collected from hazelnuts stored for 14 days at 25 °C. N.d. = non detected. Values followed by the same letter at the same time are not statistically different by Duncan's multiple range test ($P \leq 0.05$). The statistical analysis was performed considering separately values obtained after drying and after storage.

4. Discussion

Hazelnuts can be contaminated with mycotoxigenic fungi at various steps from harvesting to the final product, especially after dehulling, and drying is commonly used to reduce the moisture content of the nut, to avoid the fungal growth. Due to the dry climate conditions in the field before harvest, shelled hazelnuts used in this work had a moisture content of 13.2%, which is similar to the values measured in Oregon hazelnuts (Wang et al., 2018a), but lower compared to those measured (about 25%) in hazelnuts used in other studies (López et al., 1997a; Ozay et al., 2008; Turan, 2018, 2019). This low amount of water corresponds to a water activity of 0.82, that is not favourable for fungal development and mycotoxin production, because close to the minimum a_w that ranges from 0.80 to 0.83 for fungal growth and is 0.85 for aflatoxin production (Abdel-Hadi et al., 2012; Giorni et al., 2011; Medina et al., 2017a). To investigate the effect of drying temperatures and drying time on fungal growth and aflatoxin production, the development of *A. flavus* was promoted by increasing moisture content and consequently water activity of hazelnuts. Hydrated hazelnuts with final a_w of 0.94 are suitable for *A. flavus* growth (Giorni et al., 2011), and this was confirmed in our work by *A. flavus* growth on hazelnuts.

Hazelnuts were dried at increasing temperatures to determine the time required to reach 6% of moisture content, which is commercially adopted to prevent fungal growth and mycotoxin occurrence (Wang et al., 2018a). As expected, higher temperatures required a shorter time of drying, however, at 30 °C, three days of drying were necessary (Mousadoost et al., 2018). The drying times tested were similar to those commercially used for hazelnuts (Ozay et al., 2008; Turan, 2018).

Significant differences were observed in *A. flavus* growth among different drying treatments: a higher growth (measured as CFU/g) of *A. flavus* was found in the samples dried between 30 °C and 40 °C. Hazelnuts treated at 35 °C showed the highest fungal growth and aflatoxin production. These results were in agreement

with those reported in literature, showing that the optimal temperature for *A. flavus* growth ranges from 30 °C to 35 °C (Shapira and Paster, 2004), but could vary depending on the nature and composition of the substrate and the fungal strain studied (Gallo et al., 2016; Giorni et al., 2011; Lahouar et al., 2016). Indeed, in peanuts and sorghum the optimal temperature for fungal growth was 37 °C (Lahouar et al., 2016; Liu et al., 2017). In soybean and in dried Brazil nuts, however, the highest fungal growth was observed at 30 °C (Pratiwi et al., 2015). On rice, the optimal temperature was predicted to be between 30 and 32 °C (Mousa et al., 2011) and on chestnuts (Prencipe et al., 2018c) the highest fungal colonization was observed after 7 days of drying at 30 − 35 °C, while at 45 and 50 °C no mould growth was detected. Similar results were observed in the present study on hazelnuts, however drying times were modified according to the temperature of drying used in order to reach the same moisture content. Our data discourage the use of 30 or 35 °C to dry hazelnuts, because these temperatures are favourable for *A. flavus* growth and because longer times are required to reach a moisture content unfavourable for fungal growth. At 45 and 50 °C, a significant reduction of *A. flavus* growth on hazelnuts was achieved. These high temperatures are therefore suitable to reduce the viability of fungal spores on hazelnuts, avoiding mould growth during storage.

Mycotoxin production is often promoted under suboptimal growth conditions as an adaptive response (Prencipe et al., 2018c; Schmidt-Heydt et al., 2008, 2009). For example, in chestnuts, drying at 40 °C resulted in a significant reduction of fungal growth but also in a higher production of aflatoxins (Prencipe et al., 2018c). Moreover, aflatoxin production is generally blocked at temperatures above 42 °C, as confirmed by negative regulation of genes involved in their biosynthesis (Liu et al., 2017). As expected, *A. flavus* growth was dramatically reduced at 45 °C or 50 °C and the remaining spores were unable to produce aflatoxins. These results were consistent with those obtained by other researchers which showed that fungal development occurred in a wider range of a_w and temperatures, compared to

aflatoxin production (Abdel-Hadi et al., 2012; Giorni et al., 2011; Medina et al., 2017a, 2017b; Sanchis and Magan, 2004). Our results confirm that higher drying temperature are suitable to reduce aflatoxin contamination. The use of high temperature for drying is also connected with a lower time of the process, which is a benefit from the industrial point of view.

Interestingly, *A. flavus* was able to produce aflatoxins after drying at 30 °C, and, to a lower extent, at 35 °C. This could be explained by the longer time for drying until 6% moisture (Aldars-García et al., 2015). At 30 °C, 72 hours of drying were used, while, at 35 °C 33 h were sufficient.

After 14 days of storage at 25 °C, an increased aflatoxin production was observed for hazelnuts previously dried at 35 °C and 40 °C. In these conditions, an increase of *A. flavus* growth was evident. These results highlight the need to control also storage conditions, such as humidity and temperature, to avoid fungal contamination and proliferation. Indeed, during storage, a modification of these two parameters can cause a rapid increase in water activity and a proliferation of filamentous fungi (Fontana et al., 2014; Khosravi et al., 2007; Marín et al., 2012; Özilgen and Özdemir, 2001), which could result in aflatoxin production.

In conclusion, drying temperatures from 30 °C to 40 °C associated to longer times of drying can promote fungal development and aflatoxin production, while 45 °C and 50 °C seem to be optimal temperatures for drying hazelnuts, because of the reduction of *A. flavus* and the lack of aflatoxins both after drying and storage. Wang at al. (Wang et al., 2018a) and Turan and Islam (Turan and İslam, 2018) suggest to dry hazelnuts with higher temperature (43-49 °C and 45 °C, respectively). For other nuts, the best drying temperatures were found to be above 40 °C: 42 °C for peanuts (Liu et al., 2017) and 45 °C for chestnuts (Prencipe et al., 2018c). In this work, hazelnuts were artificially inoculated, while fungal growth and aflatoxin production were promoted by adjusting the moisture content, as a result, high level of aflatoxins were produced especially between 30 °C and 40 °C. Despite the change of the

natural conditions, drying at 45 °C and 50 °C led to a significant reduction of *A. flavus* and to inhibition of aflatoxins production. Further studies are required to confirm that the adoption of these temperatures on non-inoculated nuts, results in the improvement of safety of nuts (both in terms of reduction of aflatoxin contamination and *A. flavus* presence) and in the maintenance of the nutritional qualities of nuts.

In previous reports, the use of temperatures above 40°C permitted to maintain the chemical characteristics of hazelnuts (López et al., 1997a; Mousadoost et al., 2018; Wang et al., 2018a). For instance, the use of drying machines at 45 °C to dry Turkish hazelnuts cv. Levant provided products with a better oxidative stability over 12 month of storage at room temperature (Turan, 2018). Instead, it is not advisable to dry hazelnuts at temperatures higher than 50 °C because the frequency of rancidity reactions increases with a damage in hazelnut quality (Wang et al., 2018a). By considering that an increase of the temperature of drying could affect the organoleptic quality of hazelnut, the lowest temperature that guarantees the absence of aflatoxins should be selected. Therefore, 45°C should be the recommended drying temperature to limit *A. flavus* growth and aflatoxin contamination on hazelnuts.

This is the first study concerning the effect of drying temperatures both on *A.flavus* growth and AFs production on hazelnuts. The information gained could be used to implement drying strategies to avoid sun drying and to limit the development of aflatoxigenic fungi and of aflatoxins production. Moreover, the control of the environmental parameters during drying and storage is essential to develop appropriate practices to maintain healthy and safe food products.

Acknowledgments

Work carried out in the framework of the project “Food Digital Monitoring - FDM” (Framework Program MIUR – Piedmont Region, Action 3 “Smart Factory”, Call “Smart Factory Technological Platform”).

Author Contributions

Silvia Valente: Conceptualization, Investigation, Formal Analysis, Writing – original draft; Giovanna Roberta Meloni: Conceptualization, Investigation, Formal Analysis, Writing – original draft; Simona Prencipe: Conceptualization, Formal Analysis, Writing – review and editing; Nicola Spigolon: Conceptualization, Writing – review and editing; Marco Somenzi: Conceptualization, Writing – review and editing; Mauro Fontana: Funding acquisition, Writing—review and editing; Maria Lodovica Gullino: Funding acquisition, Writing—review and editing; Davide Spadaro: Conceptualization, Formal Analysis, Writing—review and editing.

Conflicts of Interest

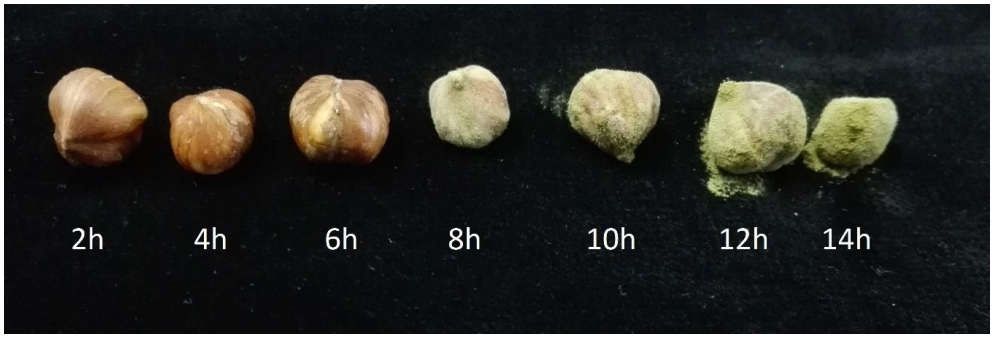
The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Supplementary material

Table S1. Moisture content (%) of hazelnuts soaked for 2-14 hours. Values are expressed as mean values ± SD (3 replicates of 15 nuts per experiment, 2 repetitions of the experiment).

Hours of Soaking	Moisture content (%)
2	19.92 ± 0.47
4	20.69 ± 0.19
6	21.29 ± 0.12
8	26.04 ± 0.26
10	34.23 ± 0.66
14	35.51 ± 0.64

Figure S1. Fungal development on hazelnuts, after fourteen days of storage at 25°C. Fruits were soaked for 2-14 hours and inoculated with *A. flavus* spore suspension.



Chapter 5

General discussion and outlooks

Two *Penicillium* and one *Aspergillus* species able to produce harmful mycotoxins, through the secondary metabolism pathway, were investigated in this work. The biosynthesis of selected mycotoxins produced by these fungi were studied using chemical, molecular, bioinformatic approaches. Gene clusters, transcription factors and genes involved were identified and their role in biosynthesis was elucidated by traditional knockout methods as well as genome editing techniques.

Bioinformatic and genetic tools provide a valuable information about SMs biosynthesis

Bioinformatic software to mine gene clusters, together with the increased number of fungal genomes available, provide a massive amount of information regarding fungal secondary metabolism (Keller, 2019). However, the genetic information available needs to be linked with chemical and molecular mechanisms by empirical means. For example, in the third chapter, the verrucosidin gene cluster was searched in two deposited genomes of *P. polonicum*, considering both strains potentially able to produce verrucosidin. The involved BGC identified to be responsible for verrucosidin biosynthesis was found only in the genome of one *P. polonicum* strain. Therefore, taking into account only the presence of BGCs in both genomes, the real BGC would be excluded from the analysis. Instead, the presence and the expression of these genes was confirmed in *P. aurantiogriseum* and *P. polonicum* X6 which were able to produce this compound *in vitro*. Therefore, the validation of chemical

and molecular analyses is essential in order to avoid possible misinterpretation based only on genomic data.

Additionally, all the predicted BGCs need to be confirmed through genetic approaches, such as deletion of the functional genes and TFs involved. As a matter of fact, borders of gene cluster may contain genes which are not involved in the biosynthesis of a certain compound (Weber and Kim, 2016). In this regard, the presence of biosynthetic genes does not mean they are actively transcribed. Mutation of backbone genes, for example, can lead to inactivate gene clusters, that become silent.

CRISPR-Cas9 represents a promising and efficient tool to induce specific mutations in fungal genomes. Among CRISPR-Cas9 systems, the *in vitro* approach does not require the expression of *Cas9* or the transcription of gRNA, and there is an immediate cleavage activity when the RNP is targeted into the cells. Furthermore, the RNP is degraded by the host cell within a short period, reducing the possibility of off-target activity (Wang and Coleman, 2019). The integration efficiency is quite high even in fungal genomes with functional NHEJ repair system, and could reach 74% by adjusting the length of microhomology sequence (Al Abdallah et al., 2017). In Chapter 3, *P. polonicum* mutants with target integration were easily obtained with high efficiency.

The availability of genomic information reveals the vast SMs potential of fungi

The increased availability of fungal genomes and advanced bioinformatic tools to mine BGC, a massive amount of information is currently available. These BGC represent a source of novel bioactive compounds, potentially important for medical, agricultural and industrial applications. Nevertheless, many of these compounds can have toxic effect for humans or animals. The potential ability of fungi to produce mycotoxins represents a risk for human safety when contaminating foodstuff. For example, Prencipe et al. (2018a, 2018b) isolated more than 180 strains of

Aspergillus spp. and *Penicillium* spp. from chestnuts. Among them, *Aspergillus* section *Flavi* represent the main concern, due to their ability to produce carcinogenic aflatoxins. However, other fungal species were able to produce other harmful mycotoxins on chestnuts such as mycophenolic acid, meleagrin, andrastin A, roquefortine C, patulin, chaetoglobosin A, cyclopenin, cyclopenol, viridicatin, viridicatol, penitrem A, cyclopiazonic acid, verrucosidin and penicillic acid (Prencipe et al., 2018b). A legal threshold for these mycotoxins on nuts does not exist, as they are not considered a significant contributor to human and animal exposure (European Commission, 2010b). Similarly, *P. griseofulvum* was able to produce griseofulvin and roquefortine C in addition to patulin on apples (Banani et al., 2016), but only the presence of patulin is regulated by the authorities on apples and its derived products (European Commission, 2006). Taking into account that most fungi are able to produce more than one mycotoxin, and that several fungi can be found on the same commodities, co-occurrence of mycotoxins in food represents a high risk for human safety (Rodrigues and Naehrer, 2012; Smith et al., 2016). The current legislation, however, does not consider the cumulative effects of many mycotoxins which is not easy to predict. The risk, however, needs to be further investigated, as it could lead to synergistic toxic effects (Smith et al., 2016).

Challenging prediction of mycotoxin and other SM production *in vivo*

The regulation of SM biosynthesis is extremely complex and involves an interconnection of different factors, including pathway-specific TF (Brakhage, 2013; Keller, 2019). For example, in the griseofulvin BGC two putative transcription factors were identified. However, in *P. griseofulvum* only *gsfR1* seems to play a role in the regulation of this compound. In *Xylaria flabelliformis* (Mead et al., 2019), on the other hand, it is expected that *gsfR2* could act as griseofulvin regulator, as the *gsfR1* gene is absent.

The regulation of SMs biosynthesis involves also global regulators, that respond to external stimuli. In general, fungi usually regulate their secondary metabolism based on availability of nutrients, primary metabolites and water. Additionally, environmental stressors, such as temperature and humidity, play also an important role in SMs biosynthesis. Furthermore, SMs can also serve plant pathogens as pathogenicity and virulence factors (Macheleidt et al., 2016; Scharf et al., 2014).

Many global regulators were found to be putatively involved in the regulation of griseofulvin biosynthesis, however, this genetic information is difficult to interpret without the support of experimental assays performed *in vitro* and *in planta*. In our work, the regulation of griseofulvin biosynthesis was confirmed to be dependent on carbon and nitrogen availability. Similarly, Núñez and collaborators (2000) reported that the verrucosidin biosynthesis was found to be dependent on the concentration of these nutrients. Although, the main factors influencing verrucosidin production, in *in vitro* studies, were found to be temperature and water activity. These two factors are considered by several researchers to be the most important for mycotoxin production (Sanzani et al., 2016; Schmidt-Heydt et al., 2008).

Several predictive models of fungal growth and mycotoxin production have been developed taking into account the influence of temperature and water activity in different food matrices such as maize (Battilani et al., 2013), rice (Mousa et al., 2011) and other relevant crops (Garcia et al., 2009; Magan et al., 2011). These models are useful to modify harvest and postharvest practices.

In summary, the regulation of mycotoxin biosynthesis is complex and difficult to predict, but the most important stimuli affecting the biosynthesis are temperature and water activity. Therefore, it is important to monitor these two parameters in order to limit the presence of mycotoxins in food. This is the reason why nuts are immediately dried after harvest, to prevent growth of mycotoxigenic fungi and therefore mycotoxin production (CAC-Codex Alimentarius Commission, 2005). As seen in hazelnuts, processing conditions, especially drying temperature and time,

largely influence both *A. flavus* viability and aflatoxin production. In addition to that, meteorological conditions in field and storage conditions need to be considered because they highly influence the mycotoxin production (Ojiambo et al., 2018).

Outlooks in the study of mycotoxin and other SMs in plant pathogens

Secondary metabolism in plant pathogens represents an interesting field of investigation. Studying the complex system involved in mycotoxin biosynthesis and regulation, would lead to the development of possible approaches to prevent the accumulation of these harmful compounds in food. Moreover, plant pathogens can use SMs to infect crops and therefore the study of virulence factors represent a good starting point to conceive practices for reducing food losses.

As presented in chapters 2, 3 and 4, several approaches can be used to investigate fungal secondary metabolism, using chemical, bioinformatic and genetic analyses. These approaches could lead to misinterpretations of the importance of certain BGCs, genes or TFs. Therefore, a multidisciplinary approach is required to fully explore and understand fungal secondary metabolism. The information obtained in the current work can lead to develop practices to reduce the development of fungal pathogens, the biosynthesis of mycotoxins, reducing food losses and improving food safety.

References

Abdel-Hadi, A., Schmidt-Heydt, M., Parra, R., Geisen, R., and Magan, N. (2012). A systems approach to model the relationship between aflatoxin gene cluster expression, environmental factors, growth and toxin production by *Aspergillus flavus*. *J. R. Soc.* 9, 757–767. doi:10.1098/rsif.2011.0482.

Abrunhosa, L., Morales, H., Soares, C., Calado, T., Vila-Chã, A. S., Pereira, M., et al. (2016). A Review of Mycotoxins in Food and Feed Products in Portugal and Estimation of Probable Daily Intakes. *Crit. Rev. Food Sci. Nutr.* 56, 249–265. doi:10.1080/10408398.2012.720619.

Al Abdallah, Q., Ge, W., and Fortwendel, J. R. (2017). A Simple and Universal System for Gene Manipulation in *Aspergillus fumigatus* : *In Vitro* -Assembled Cas9-Guide RNA Ribonucleoproteins Coupled with Microhomology Repair Templates. *mSphere* 2, e00446-17. doi:10.1128/msphere.00446-17.

Aldars-García, L., Ramos, A. J., Sanchis, V., and Marín, S. (2015). An attempt to model the probability of growth and aflatoxin B1 production of *Aspergillus flavus* under non-isothermal conditions inpistachio nuts. *Food Microbiol.* 51, 117–129. doi:10.1016/j.fm.2015.05.013.

Andersen, B., and Thrane, U. (2006). Food-borne fungi in fruit and cereals and their production of mycotoxins. *Adv. Exp. Med. Biol.* 571, 137–152. doi:10.1007/0-387-28391-9_8.

Andrianopoulos, A., and Hynes, M. J. (1990). Sequence and functional analysis of the positively acting regulatory gene *amdR* from *Aspergillus nidulans*. *Mol. Cell. Biol.* 10, 3194–203. doi:10.1128/mcb.10.6.3194.

Aranda, E., Rodríguez, M., Benito, M. J., Asensio, M. A., and Córdoba, J. J. (2002). Molecular cloning of verrucosidin-producing *Penicillium polonicum* genes by differential screening to obtain a DNA probe. *Int. J. Food Microbiol.* 76, 55–61. doi:10.1016/S0168-1605(02)00008-9.

Asano, Y., Hagiwara, D., Yamashino, T., and Mizuno, T. (2007). Characterization of the bZip-type transcription factor NapA with reference to oxidative stress response in *Aspergillus nidulans*. *Biosci. Biotechnol. Biochem.* 71, 1800–1803. doi:10.1271/bbb.70133.

Aycicek, H., Aksoy, A., and Saygi, S. (2005). Determination of aflatoxin levels in some dairy and food products which consumed in Ankara, Turkey. *Food Control* 16, 263–266. doi:10.1016/j.foodcont.2004.03.004.

Bae, S. H., Seong, J., and Paik, Y. K. (2001). Cholesterol biosynthesis from lanosterol: Molecular cloning, chromosomal localization, functional expression and liver-specific gene regulation of rat sterol Δ8-isomerase, a cholesterogenic enzyme with multiple functions. *Biochem. J.* 353, 689–699. doi:10.1042/0264-6021:3530689.

Bai, Y. Bin, Gao, Y. Q., Nie, X. Di, Tuong, T. M. L., Li, D., and Gao, J. M. (2019). Antifungal Activity of Griseofulvin Derivatives against Phytopathogenic Fungi *in*

Vitro and *in Vivo* and Three-Dimensional Quantitative Structure-Activity Relationship Analysis. *J. Agric. Food Chem.* 67, 6125–6132. doi:10.1021/acs.jafc.9b00606.

Ballester, A.-R., Marcet-Houben, M., Levin, E., Sela, N., Selma-Lázaro, C., Carmona, L., et al. (2015). Genome, Transcriptome, and Functional Analyses of *Penicillium expansum* Provide New Insights Into Secondary Metabolism and Pathogenicity. *Mol. Plant-Microbe Interact.* 28, 232–248. doi:10.1094/MPMI-09-14-0261-FI.

Baltaci, C., Ilyasoğlu, H., and Cavrar, S. (2012). Aflatoxin levels in raw and processed hazelnuts in Turkey. *Food Addit. Contam. Part B Surveill.* 5, 83–86. doi:10.1080/19393210.2012.656146.

Banani, H., Marcet-Houben, M., Ballester, A. R., Abbruscato, P., González-Candelas, L., Gabaldón, T., et al. (2016). Genome sequencing and secondary metabolism of the postharvest pathogen *Penicillium griseofulvum*. *BMC Genomics* 17, 19. doi:10.1186/s12864-015-2347-x.

Barad, S., Espeso, E. A., Sherman, A., and Prusky, D. (2016a). Ammonia activates *pacC* and patulin accumulation in an acidic environment during apple colonization by *Penicillium expansum*. *Mol. Plant Pathol.* 17, 727–740. doi:10.1111/mpp.12327.

Barad, S., Horowitz, S. B., Kobiler, I., Sherman, A., and Prusky, D. (2013). Accumulation of the Mycotoxin Patulin in the Presence of Gluconic Acid Contributes to Pathogenicity of *Penicillium expansum*. *Mol. Plant-Microbe Interact.* 27, 66–77. doi:10.1094/mpmi-05-13-0138-r.

Barad, S., Sionov, E., and Prusky, D. (2016b). Role of patulin in post-harvest diseases. *Fungal Biol. Rev.* 30, 24–32. doi:10.1016/j.fbr.2016.02.001.

Battilani, P., Barbano, C., and Logrieco, A. (2008). "Risk Assessment and Safety Evaluation of Mycotoxins in Fruits," in *Mycotoxins in Fruits and Vegetables* (Academic Press), 1–26. doi:10.1016/B978-0-12-374126-4.00001-2.

Battilani, P., Camardo Leggieri, M., Rossi, V., and Giorni, P. (2013). AFLA-maize, a mechanistic model for *Aspergillus flavus* infection and aflatoxin B1 contamination in maize. *Comput. Electron. Agric.* 94, 38–46. doi:10.1016/j.compag.2013.03.005.

Bergmann, S., Funk, A. N., Scherlach, K., Schroeckh, V., Shelest, E., Horn, U., et al. (2010). Activation of a Silent Fungal Polyketide Biosynthesis Pathway through Regulatory Cross Talk with a Cryptic Nonribosomal Peptide Synthetase Gene Cluster. *Appl. Environ. Microbiol.* 76, 8143–8149. doi:10.1128/AEM.00683-10.

Bergmann, S., Schümann, J., Scherlach, K., Lange, C., Brakhage, A. A., and Hertweck, C. (2007). Genomics-driven discovery of PKS-NRPS hybrid metabolites from *Aspergillus nidulans*. *Nat. Chem. Biol.* 3, 213–217. doi:10.1038/nchembio869.

Bibbins, M., Crepin, V., Cummings, N., Mizote, T., Baker, K., Mellits, K., et al. (2002). A regulator gene for acetate utilisation from *Neurospora crassa*. *Mol. Genet. Genomics* 267, 498–505. doi:10.1007/s00438-002-0682-5.

Brakhage, A. A. (2013). Regulation of fungal secondary metabolism. *Nat. Rev. Microbiol.* 11, 21–32. doi:10.1038/nrmicro2916.

Brown, D. W., Butchko, R. A. E., Busman, M., and Proctor, R. H. (2007). The *Fusarium*

verticillioides FUM gene cluster encodes a Zn(II)2Cys6 protein that affects FUM gene expression and fumonisin production. *Eukaryot. Cell* 6, 1210–8. doi:10.1128/EC.00400-06.

Burger, G., Strauss, J., Scazzocchio, C., and Lang, B. F. (1991). nirA, the pathway-specific regulatory gene of nitrate assimilation in *Aspergillus nidulans*, encodes a putative GAL4-type zinc finger protein and contains four introns in highly conserved regions. *Mol. Cell. Biol.* 11, 5746–55. doi:10.1128/mcb.11.11.5746.

Burka, L. T., Ganguli, M., and Wilson, B. J. (1983). Verrucosidin, a Tremorgen from *Penicillium verrucosum* var. *cyclopium*. *J. Chem. Soc. Chem. Commun.* 1670, 544–545. doi:10.1039/C39830000544.

Buron-Moles, G., López-Pérez, M., González-Candelas, L., Viñas, I., Teixidó, N., Usall, J., et al. (2012). Use of GFP-tagged strains of *Penicillium digitatum* and *Penicillium expansum* to study host-pathogen interactions in oranges and apples. *Int. J. Food Microbiol.* 160, 162–170. doi:10.1016/j.ijfoodmicro.2012.10.005.

CAC-Codex Alimentarius Commission (2005). *Code of practice for the prevention and reduction of aflatoxin contamination in tree nuts (CAC/RCP 59-2005)*. Joint FAO. , ed. FAO Rome: WHO Food Standards Program doi:10.1016/j.ijpvp.2006.08.011.

Cacho, R. A., Chooi, Y. H., Zhou, H., and Tang, Y. (2013). Complexity generation in fungal polyketide biosynthesis: A spirocycle-forming P450 in the concise pathway to the antifungal drug griseofulvin. *ACS Chem. Biol.* 8, 2322–2330. doi:10.1021/cb400541z.

Cacho, R. A., Tang, Y., and Chooi, Y. H. (2015). Next-generation sequencing approach for connecting secondary metabolites to biosynthetic gene clusters in fungi. *Front. Microbiol.* 5, 774. doi:10.3389/fmicb.2014.00774.

Calvo, A. M., Wilson, R. A., Bok, J. W., and Keller, N. P. (2002). Relationship between Secondary Metabolism and Fungal Development. *Microbiol. Mol. Biol. Rev.* 66, 447–459. doi:10.1128/mmbr.66.3.447-459.2002.

Cao, C., Liu, W., and Li, R. (2009). *Penicillium marneffei* SKN7, a Novel Gene, could Complement the Hypersensitivity of *S. cerevisiae skn7* Disruptant Strain to Oxidative Stress. *Mycopathologia* 168, 23–30. doi:10.1007/s11046-009-9192-x.

Ceasar, S. A., Rajan, V., Prykhozhij, S. V., Berman, J. N., and Ignacimuthu, S. (2016). Insert, remove or replace: A highly advanced genome editing system using CRISPR/Cas9. *Biochim. Biophys. Acta - Mol. Cell Res.* 1863, 2333–2344. doi:10.1016/j.bbamcr.2016.06.009.

Chang, P. K., Cary, J. W., Bhatnagar, D., Cleveland, T. E., Bennett, J. W., Linz, J. E., et al. (1993). Cloning of the *Aspergillus parasiticus apa-2* gene associated with the regulation of aflatoxin biosynthesis. *Appl. Environ. Microbiol.* 59, 3273–3279. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8250554.

Chooi, Y. H., Cacho, R., and Tang, Y. (2010). Identification of the Viridicatumtoxin and Griseofulvin Gene Clusters from *Penicillium aethiopicum*. *Chem. Biol.* 17, 483–494. doi:10.1016/j.chembiol.2010.03.015.

Cramer, R. A., Perfect, B. Z., Pinchai, N., Park, S., Perlin, D. S., Asfaw, Y. G., et al. (2008).

Calcineurin Target CrzA Regulates Conidial Germination, Hyphal Growth, and Pathogenesis of *Aspergillus fumigatus*. *Eukaryot. Cell* 7, 1085–1097. doi:10.1128/EC.00086-08.

Damveld, R. A., Arentshorst, M., Franken, A., VanKuyk, P. A., Klis, F. M., Van Den Hondel, C. A. M. J. J., et al. (2005). The *Aspergillus niger* MADS-box transcription factor RlmA is required for cell wall reinforcement in response to cell wall stress. *Mol. Microbiol.* 58, 305–319. doi:10.1111/j.1365-2958.2005.04827.x.

Deng, H., Gao, R., Liao, X., and Cai, Y. (2017). CRISPR system in filamentous fungi: Current achievements and future directions. *Gene* 627, 212–221. doi:10.1016/j.gene.2017.06.019.

Dinamarco, T. M., Almeida, R. S., Alves de Castro, P., Brown, N. A., dos Reis, T. F., Zambelli Ramalho, L. N., et al. (2012). Molecular Characterization of the Putative Transcription Factor SebA Involved in Virulence in *Aspergillus fumigatus*. *Eukaryot. Cell* 11, 518–531. doi:10.1128/EC.00016-12.

Dubois, E., Bercy, J., and Messenguy, F. (1987). Characterization of two genes, ARGRI and ARGRIII required for specific regulation of arginine metabolism in yeast. *MGG Mol. Gen. Genet.* 207, 142–148. doi:10.1007/BF00331501.

Dunn, B. M., Goodenow, M. M., Gustchina, A., and Wlodawer, A. (2002). Retroviral proteases. *Genome Biol.* 3, reviews3006-1. doi:10.1007/978-1-349-11907-3.

Erwig, L. P., and Gow, N. A. R. (2016). Interactions of fungal pathogens with phagocytes. *Nat. Rev. Microbiol.* 14, 163–176. doi:10.1038/nrmicro.2015.21.

European Commission (2002). Commission Regulation (EU) No 1284/2002 of 15 July 2002 laying down the marketing standard for hazelnuts in shell. *Off. J. Eur. Union* 187, 14–20.

European Commission (2006). Commission Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuff. *Off. J. Eur. Union* 364, 5–24.

European Commission (2010a). Commission Regulation (EU) No 165/2010 of 26 February 2010 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. *Off. J. Eur. Union* 50, 8–12.

European Commission (2010b). Commission Regulation (EU) No 178/2010 of 2 March 2010 amending Regulation (EC) No 401/2006 as regards groundnuts (peanuts), other oilseeds, tree nuts, apricot kernels, liquorice and vegetable oil. *Off. J. Eur. Union* 52, 32–43.

Fang, Y., and Tyler, B. M. (2016). Efficient disruption and replacement of an effector gene in the oomycete *Phytophthora sojae* using CRISPR/Cas9. *Mol. Plant Pathol.* 17, 127–139. doi:10.1111/mpp.12318.

Feng, G. H., Chu, F. S., and Leonard', T. J. (1992). Molecular Cloning of Genes Related to Aflatoxin Biosynthesis by Differential Screening. *Appl. Environ. Microbiol.* 58, 455–460. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC195269/.

Fierro, F., Laich, F., García-Rico, R. O., and Martín, J. F. (2004). High efficiency transformation of *Penicillium nalgiovense* with integrative and autonomously

replicating plasmids. *Int. J. Food Microbiol.* 90, 237–248. doi:10.1016/S0168-1605(03)00306-4.

Fink-Gremmels, J., Henning, J., and Leistner, A. (1991). Quantitative determination of verrucosidin producing *Penicillium aurantiogriseum*. Microbiologie, Aliments. *Nutr. Microbiol. Foods Feed. Nutr.* 5, 155–160.

Fischer, J., Schroeckh, V., and Brakhage, A. A. (2016). "Awakening of Fungal Secondary Metabolite Gene Clusters," in *Gene Expression Systems in Fungi: Advancements and Applications* (Cham: Springer), 253–273. doi:10.1007/978-3-319-27951-0_11.

Flaherty, J. E., and Woloshuk, C. P. (2004). Regulation of fumonisin biosynthesis in *Fusarium verticillioides* by a zinc binuclear cluster-type gene, ZFR1. *Appl. Environ. Microbiol.* 70, 2653–9. doi:10.1128/aem.70.5.2653-2659.2004.

Fontana, M., Somenzi, M., and Tesio, A. (2014). Cultivation, harvest and postharvest aspects that influence quality and organoleptic properties of hazelnut production and related final products. *Acta Hortic.* 1052, 311–314. doi:10.17660/ActaHortic.2014.1052.43.

Food and Agriculture Organization of the United Nations (FAO) (2020). FAOSTAT, FAO's food and agriculture database. Available at: http://www.fao.org/faostat/en/#data/QC [Accessed February 4, 2020].

Fox, E. M., and Howlett, B. J. (2008). Secondary metabolism: regulation and role in fungal biology. *Curr. Opin. Microbiol.* 11, 481–487. doi:10.1016/j.mib.2008.10.007.

Frandsen, R. J. N., Andersson, J. A., Kristensen, M. B., and Giese, H. (2008). Efficient four fragment cloning for the construction of vectors for targeted gene replacement in filamentous fungi. *BMC Mol. Biol.* 9, 1–11. doi:10.1186/1471-2199-9-70.

Frisvad, J. C. (2015). Taxonomy, chemodiversity and chemoconsistency of *Aspergillus*, *Penicillium* and *Talaromyces* species. *Front. Microbiol.* 5, 773. doi:10.3389/fmicb.2014.00773.

Frisvad, J. C., Smedsgaard, J., Larsen, T. O., and Samson, R. A. (2004). Mycotoxins, drugs and other extrolites produced by species in *Penicillium* subgenus *Penicillium*. *Stud. Mycol.* 49, 201–241.
Available at: http://www.wi.knaw.nl/publications/Sim49/Sim49Article3.pdf.

Gallo, A., Solfrizzo, M., Epifani, F., Panzarini, G., and Perrone, G. (2016). Effect of temperature and water activity on gene expression and aflatoxin biosynthesis in *Aspergillus flavus* on almond medium. *Int. J. Food Microbiol.* 217, 162–169. doi:10.1016/j.ijfoodmicro.2015.10.026.

Garcia, D., Ramos, A. J., Sanchis, V., and Marín, S. (2009). Predicting mycotoxins in foods: A review. *Food Microbiol.* 26, 757–769. doi:10.1016/j.fm.2009.05.014.

Giorni, P., Magan, N., Pietri, A., and Battilani, P. (2011). Growth and aflatoxin production of an Italian strain of *Aspergillus flavus*: Influence of ecological factors and nutritional substrates. *World Mycotoxin J.* 4, 425–432. doi:10.3920/WMJ2011.1300.

Gioti, A., Pradier, J. M., Fournier, E., Le Pêcheur, P., Giraud, C., Debieu, D., et al. (2008). A *Botrytis cinerea* emopamil binding domain protein, required for full virulence,

belongs to a eukaryotic superfamily which has expanded in euascomycetes. *Eukaryot. Cell* 7, 368–378. doi:10.1128/EC.00159-07.

Golge, O., Hepsag, F., and Kabak, B. (2016). Determination of aflatoxins in walnut sujuk and Turkish delight by HPLC-FLD method. *Food Control* 59, 731–736. doi:10.1016/j.foodcont.2015.06.035.

Grebenok, R. J., Ohnmeiss, T. E., Yamamoto, A., Huntley, E. D., Galbraith, D. W., and Della Penna, D. (1998). Isolation and characterization of an *Arabidopsis thaliana* C-8,7 sterol isomerase: Functional and structural similarities to mammalian C-8,7 sterol isomerase/emopamil-binding protein. *Plant Mol. Biol.* 38, 807–815. doi:10.1023/A:1006028623875.

Grigoriev, I. V., Nikitin, R., Haridas, S., Kuo, A., Ohm, R., Otillar, R., et al. (2014). MycoCosm portal: Gearing up for 1000 fungal genomes. *Nucleic Acids Res.* 42, D699–D704. doi:10.1093/nar/gkt1183.

Gürses, M. (2006). Mycoflora and aflatoxin content of hazelnuts, walnuts, peanuts, almonds and roasted chickpeas (LEBLEBI) sold in Turkey. *Int. J. Food Prop.* 9, 395–399. doi:10.1080/10942910600596597.

Guzmán-Chávez, F., Zwahlen, R. D., Bovenberg, R. A. L., and Driessen, A. J. M. (2018). Engineering of the filamentous fungus *Penicillium chrysogenumas* cell factory for natural products. *Front. Microbiol.* 9, 2768. doi:10.3389/fmicb.2018.02768.

Haas, H., Angermayr, K., and Stöffler, G. (1997). Molecular analysis of a *Penicillium chrysogenum* GATA factor encoding gene (*sreP*) exhibiting significant homology to the *Ustilago maydis urbs1* gene. *Gene* 184, 33–7. doi:10.1016/s0378-1119(96)00570-7.

Han, K.-H., Han, K.-Y., Yu, J.-H., Chae, K.-S., Jahng, K.-Y., and Han, D.-M. (2001). The *nsdD* gene encodes a putative GATA-type transcription factor necessary for sexual development of *Aspergillus nidulans*. *Mol. Microbiol.* 41, 299–309. doi:10.1046/j.1365-2958.2001.02472.x.

He, L., Feng, J., Lu, S., Chen, Z., Chen, C., He, Y., et al. (2017). Genetic transformation of fungi. *Int. J. Dev. Biol.* 61, 375–381. doi:10.1387/ijdb.160026lh.

Hendrickson, L., Davis, C. R., Roach, C., Nguyen, D. K., Aldrich, T., McAda, P. C., et al. (1999). Lovastatin biosynthesis in *Aspergillus terreus*: Characterization of blocked mutants, enzyme activities and a multifunctional polyketide synthase gene. *Chem. Biol.* 6, 429–439. doi:10.1016/S1074-5521(99)80061-1.

Hodge, R. P., Harris, C. M., and Harris, T. M. (1988). Verrucofortine, a major metabolite of *Penicillium verrucosum* var. *cyclopium*, the fungus that produces the mycotoxin verrucosidin. *J. Nat. Prod.* 51, 66–73. doi:10.1021/np50055a008.

Hoffmeister, D., and Keller, N. P. (2007). Natural products of filamentous fungi: Enzymes, genes, and their regulation. *Nat. Prod. Rep.* 24, 393–416. doi:10.1039/b603084j.

Hogues, H., Lavoie, H., Sellam, A., Mangos, M., Roemer, T., Purisima, E., et al. (2008). Transcription Factor Substitution during the Evolution of Fungal Ribosome Regulation. *Mol. Cell* 29, 552–562. doi:10.1016/j.molcel.2008.02.006.

Hoshijima, K., Jurynec, M. J., and Grunwald, D. J. (2016). “Precise genome editing by homologous recombination,” in *Methods in Cell Biology* (Academic Press), 121–147. doi:10.1016/bs.mcb.2016.04.008.

Hynes, M. J., Szewczyk, E., Murray, S. L., Suzuki, Y., Davis, M. A., and Sealy-Lewis, H. M. (2007). Transcriptional control of gluconeogenesis in *Aspergillus nidulans*. *Genetics* 176, 139–50. doi:10.1534/genetics.107.070904.

Igbalajobi, O., Yu, Z., and Fischer, R. (2019). Red- and Blue-Light Sensing in the Plant Pathogen *Alternaria alternata* Depends on Phytochrome and the White-Collar Protein LreA. *MBio* 10, e00371-19. doi:10.1128/MBIO.00371-19.

Ikner, A., and Shiozaki, K. (2005). Yeast signaling pathways in the oxidative stress response. *Mutat. Res. Mol. Mech. Mutagen.* 569, 13–27. doi:10.1016/j.mrfmmm.2004.09.006.

IpCho, S. V. S., Tan, K.-C., Koh, G., Gummer, J., Oliver, R. P., Trengove, R. D., et al. (2010). The Transcription Factor *StuA* Regulates Central Carbon Metabolism, Mycotoxin Production, and Effector Gene Expression in the Wheat Pathogen *Stagonospora nodorum*. *Eukaryot. Cell* 9, 1100–1108. doi:10.1128/EC.00064-10.

Ismaiel, A., and Papenbrock, J. (2015). Mycotoxins: Producing Fungi and Mechanisms of Phytotoxicity. *Agriculture* 5, 492–537. doi:10.3390/agriculture5030492.

Jackson, L. S., and Al-Taher, F. (2008). “Factors Affecting Mycotoxin Production in Fruits,” in *Mycotoxins in Fruits and Vegetables* (Academic Press), 75–104. doi:10.1016/B978-0-12-374126-4.00004-8.

Jensen, B., Knudsen, I. M. B., Andersen, B., Nielsen, K. F., Thrane, U., Jensen, D. F., et al. (2013). Characterization of microbial communities and fungal metabolites on field grown strawberries from organic and conventional production. *Int. J. Food Microbiol.* 160, 313–322. doi:10.1016/j.ijfoodmicro.2012.11.005.

Kabak, B. (2016). Aflatoxins in hazelnuts and dried figs: Occurrence and exposure assessment. *Food Chem.* 211, 8–16. doi:10.1016/j.foodchem.2016.04.141.

Kader, A. A. (2013). “Impact of nut postharvest handling, de-shelling, drying and storage on quality,” in *Improving the Safety and Quality of Nuts* (Woodhead Publishing), 22–34. doi:10.1533/9780857097484.1.22.

Katz, L., and Baltz, R. H. (2016). Natural product discovery: past, present, and future. *J. Ind. Microbiol. Biotechnol.* 43, 155–176. doi:10.1007/s10295-015-1723-5.

Katz, M. E., Bernardo, S. M., and Cheetham, B. F. (2008). The interaction of induction, repression and starvation in the regulation of extracellular proteases in *Aspergillus nidulans*: evidence for a role for CreA in the response to carbon starvation. *Curr. Genet.* 54, 47–55. doi:10.1007/s00294-008-0198-6.

Keller, N. P. (2015). Translating biosynthetic gene clusters into fungal armor and weaponry. *Nat. Chem. Biol.* 11, 671–677. doi:10.1038/nchembio.1897.

Keller, N. P. (2019). Fungal secondary metabolism: regulation, function and drug discovery. *Nat. Rev. Microbiol.* 17, 167–180. doi:10.1038/s41579-018-0121-1.

Kellner, N., Heimel, K., Obhof, T., Finkernagel, F., and Kämper, J. (2014). The SPF27

Homologue Num1 Connects Splicing and Kinesin 1-Dependent Cytoplasmic Trafficking in Ustilago maydis. *PLoS Genet.* 10, e1004046. doi:10.1371/journal.pgen.1004046.

Khosravi, A. R., Shokri, H., and Ziglari, T. (2007). Evaluation of Fungal Flora in Some Important Nut Products (Pistachio, Peanut, Hazelnut and Almond) in Tehran, Iran. *Pakistan J. Nutr.* 6, 460–462. Available at: http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.576.9456&rep=rep1&type=pdf.

Kim, H. Y., Heo, D. Y., Park, H. M., Singh, D., and Lee, C. H. (2016). Metabolomic and transcriptomic comparison of solid-state and submerged fermentation of *Penicillium expansum* KACC 40815. *PLoS One* 11. doi:10.1371/journal.pone.0149012.

Kjærbølling, I., Mortensen, U. H., Vesth, T., and Andersen, M. R. (2019). Strategies to establish the link between biosynthetic gene clusters and secondary metabolites. *Fungal Genet. Biol.* 130, 107–121. doi:10.1016/j.fgb.2019.06.001.

Köksal, Y. A. (2000). *Inventory of hazelnut research, germplasm and references. Interregional (REU/RNE) Cooperative Research Network on Nuts*. FAO.

Kovács, Z., Szarka, M., Kovács, S., Boczonádi, I., Emri, T., Abe, K., et al. (2013). Effect of cell wall integrity stress and RlmA transcription factor on asexual development and autolysis in *Aspergillus nidulans*. *Fungal Genet. Biol.* 54, 1–14. doi:10.1016/j.fgb.2013.02.004.

Krappmann, S. (2017). CRISPR-Cas9, the new kid on the block of fungal molecular biology. *Med. Mycol.* 55, 16–23. doi:10.1093/mmy/myw097.

Kumar, P., Mahato, D. K., Kamle, M., Mohanta, T. K., and Kang, S. G. (2017). Aflatoxins: A global concern for food safety, human health and their management. *Front. Microbiol.* 7, 1–10. doi:10.3389/fmicb.2016.02170.

Kumar, V., Basu, M. S., and Rajendran, T. P. (2008). Mycotoxin research and mycoflora in some commercially important agricultural commodities. *Crop Prot.* 27, 891–905. doi:10.1016/j.cropro.2007.12.011.

Kwon, N.-J., Garzia, A., Espeso, E. A., Ugalde, U., and Yu, J.-H. (2010). FlbC is a putative nuclear C2H2 transcription factor regulating development in *Aspergillus nidulans*. *Mol. Microbiol.* 77, 1203–1219. doi:10.1111/j.1365-2958.2010.07282.x.

Lahouar, A., Marin, S., Crespo-Sempere, A., Saïd, S., and Sanchis, V. (2016). Effects of temperature, water activity and incubation time on fungal growth and aflatoxin B1 production by toxinogenic *Aspergillus flavus* isolates on sorghum seeds. *Rev. Argent. Microbiol.* 48, 78–85. doi:10.1016/j.ram.2015.10.001.

Lee, K., Sung, C., Kim, B.-G., and Hahn, J.-S. (2013). Activation of Aro80 transcription factor by heat-induced aromatic amino acid influx in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* 438, 43–47. doi:10.1016/j.bbrc.2013.07.019.

Li, B., Zong, Y., Du, Z., Chen, Y., Zhang, Z., Qin, G., et al. (2015). Genomic Characterization Reveals Insights Into Patulin Biosynthesis and Pathogenicity in *Penicillium* Species. *Mol. Plant-Microbe Interact.* 28, 635–647. doi:10.1094/MPMI-

12-14-0398-FI.

Li, D., Tang, Y., Lin, J., and Cai, W. (2017). Methods for genetic transformation of filamentous fungi. *Microb. Cell Fact.* 16, 1–13. doi:10.1186/s12934-017-0785-7.

Li, W., Ma, Z., Chen, L., and Yin, W. B. (2018). Synthesis and production of the antitumor polyketide aurovertins and structurally related compounds. *Appl. Microbiol. Biotechnol.* 102, 6373–6381. doi:10.1007/s00253-018-9123-1.

Liebmann, B., Müller, M., Braun, A., and Brakhage, A. A. (2004). The cyclic AMP-dependent protein kinase A network regulates development and virulence in *Aspergillus fumigatus*. *Infect. Immun.* 72, 5193–5203. doi:10.1128/IAI.72.9.5193-5203.2004.

Lin, T. S., Chen, B., Chiang, Y. M., and Wang, C. C. C. (2019). Discovery and Elucidation of the Biosynthesis of Aspernidgulenes: Novel Polyenes from *Aspergillus nidulans* by Using Serial Promoter Replacement. *ChemBioChem* 20, 329–334. doi:10.1002/cbic.201800486.

Lin, T. S., Chiang, Y. M., and Wang, C. C. C. (2016). Biosynthetic Pathway of the Reduced Polyketide Product Citreoviridin in *Aspergillus terreus* var. *aureus* Revealed by Heterologous Expression in *Aspergillus nidulans*. *Org. Lett.* 18, 1366–1369. doi:10.1021/acs.orglett.6b00299.

Lints, R., Davis, M. A., and Hynes, M. J. (1995). The positively acting amdA gene of *Aspergillus nidulans* encodes a protein with two C2H2 zinc-finger motifs. *Mol. Microbiol.* 15, 965–975. doi:10.1111/j.1365-2958.1995.tb02365.x.

Liu, C., Tagami, K., Minami, A., Matsumoto, T., Frisvad, J. C., Suzuki, H., et al. (2015). Reconstitution of biosynthetic machinery for the synthesis of the highly elaborated indole diterpene penitrem. *Angew. Chemie - Int. Ed.* 54, 5748–5752. doi:10.1002/anie.201501072.

Liu, Q., Ying, S.-H., Li, J.-G., Tian, C.-G., and Feng, M.-G. (2013). Insight into the transcriptional regulation of Msn2 required for conidiation, multi-stress responses and virulence of two entomopathogenic fungi. *Fungal Genet. Biol.* 54, 42–51. doi:10.1016/j.fgb.2013.02.008.

Liu, X., Guan, X., Xing, F., Lv, C., Dai, X., and Liu, Y. (2017). Effect of water activity and temperature on the growth of *Aspergillus flavus*, the expression of aflatoxin biosynthetic genes and aflatoxin production in shelled peanuts. *Food Control* 82, 325–332. doi:10.1016/j.foodcont.2017.07.012.

Löpez, A., Piqué, A., and Romero, M. T. (1998). Simulation of deep bed drying of hazelnuts. *Dry. Technol.* 16, 651–665. doi:10.1080/07373939808917428.

López, A., Piqué, M. T., Boatella, J., Parcerisa, J., Romero, A., Ferrá, A., et al. (1997a). Influence of Drying Conditions on the Hazelnut Quality. I. Lipid Oxidation. *Dry. Technol.* 15, 965–977. doi:10.1080/07373939708917271.

López, A., Piqué, M. T., Boatella, J., Parcerisa, J., Romero, A., Ferrán, A., et al. (1997b). Influence of Drying Conditions on the Hazelnut Quality. III. Browning. *Dry. Technol.* 15, 989–1002. doi:10.1080/07373939708917273.

López, A., Piqué, M. T., Ferrán, A., Romero, A., Boatella, J., and Garcí, J. (1997c). Influence of drying conditions on the hazelnut quality: II. Enzymatic activity. *Dry. Technol.* 15, 979–988. doi:10.1080/07373939708917272.

Macheleidt, J., Mattern, D. J., Fischer, J., Netzker, T., Weber, J., Schroeckh, V., et al. (2016). Regulation and Role of Fungal Secondary Metabolites. *Annu. Rev. Genet.* 50, 371–392. doi:10.1146/annurev-genet-120215-035203.

Madden, T. (2013). "The BLAST sequence analysis tool," in *In The NCBI Handbook [Internet]. 2nd edition* (National Center for Biotechnology Information (US)).

Magan, N., Medina, A., and Aldred, D. (2011). Possible climate-change effects on mycotoxin contamination of food crops pre- and postharvest. *Plant Pathol.* 60, 150–163. doi:10.1111/j.1365-3059.2010.02412.x.

Marín, S., Ramos, A. J., and Sanchis, V. (2012). Modelling *Aspergillus flavus* growth and aflatoxins production in pistachio nuts. *Food Microbiol.* 32, 378–388. doi:10.1016/j.fm.2012.07.018.

Marzluf, G. A. (1997). Genetic regulation of nitrogen metabolism in the fungi. *Microbiol. Mol. Biol. Rev.* 61, 17–32.

Mattern, D. J., Valiante, V., Horn, F., Petzke, L., and Brakhage, A. A. (2017). Rewiring of the Austinoid Biosynthetic Pathway in Filamentous Fungi. *ACS Chem. Biol.* 12, 2927–2933. doi:10.1021/acschembio.7b00814.

Mattern, D. J., Valiante, V., Unkles, S. E., and Brakhage, A. A. (2015). Synthetic biology of fungal natural products. *Front. Microbiol.* 6, 1–7. doi:10.3389/fmicb.2015.00775.

Mauro, V., Carette, D., Pontier-Bres, R., Dompierre, J., Czerucka, D., Segretain, D., et al. (2013). The anti-mitotic drug griseofulvin induces apoptosis of human germ cell tumor cells through a connexin 43-dependent molecular mechanism. *Apoptosis* 18, 480–491. doi:10.1007/s10495-012-0800-8.

McMillan, L. K., Carr, R. L., Young, C. A., Astin, J. W., Lowe, R. G. T., Parker, E. J., et al. (2003). Molecular analysis of two cytochrome P450 monooxygenase genes required for paxilline biosynthesis in *Penicillium paxilli*, and effects of paxilline intermediates on mammalian maxi-K ion channels. *Mol. Genet. Genomics* 270, 9–23. doi:10.1007/s00438-003-0887-2.

Mead, M. E., Raja, H. A., Steenwyk, J. L., Knowles, S. L., Oberlies, N. H., and Rokasa, A. (2019). Draft Genome Sequence of the Griseofulvin-Producing Fungus *Xylaria flabelliformis* Strain G536. *Microbiol. Resour. Announc.* 8, e00890-19. doi:10.1128/MRA.00890-19.

Medina, A., Akbar, A., Baazeem, A., Rodriguez, A., and Magan, N. (2017a). Climate change, food security and mycotoxins: Do we know enough? *Fungal Biol. Rev.* 31, 143–154. doi:10.1016/j.fbr.2017.04.002.

Medina, A., Gilbert, M. K., Mack, B. M., OBrian, G. R., Rodríguez, A., Bhatnagar, D., et al. (2017b). Interactions between water activity and temperature on the *Aspergillus flavus* transcriptome and aflatoxin B1production. *Int. J. Food Microbiol.* 256, 36–44. doi:10.1016/j.ijfoodmicro.2017.05.020.

Medina, A., Schmidt-Heydt, M., Rodríguez, A., Parra, R., Geisen, R., and Magan, N. (2015). Impacts of environmental stress on growth, secondary metabolite biosynthetic gene clusters and metabolite production of xerotolerant/xerophilic fungi. *Curr. Genet.* 61, 325–334. doi:10.1007/s00294-014-0455-9.

Mei, Y. Z., Zhu, Y. L., Huang, P. W., Yang, Q., and Dai, C. C. (2019). Strategies for gene disruption and expression in filamentous fungi. *Appl. Microbiol. Biotechnol.* 103, 6041–6059. doi:10.1007/s00253-019-09953-2.

Miller, K. Y., Wu, J., and Miller, B. L. (1992). StuA is required for cell pattern formation in *Aspergillus*. *Genes Dev.* 6, 1770–1782. doi:10.1101/gad.6.9.1770.

Möller, E. M., Bahnweg, G., Sandermann, H., and Geiger, H. H. (1992). A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. *Nucleic Acids Res.* 20, 6115–6. doi:10.1093/nar/20.22.6115.

Moslem, M. A., Yassin, M. A., El-Samawaty, A. E. R. M. A., Sayed, S. R. M., and Amer, O. E. (2013). Mycotoxin-producing *Penicillium* species involved in apple blue mold. *J. Pure Appl. Microbiol.* 7, 187–193.

Moslem, M., Abd-Elsalam, K., Yassin, M., and Bahkali, A. (2010). First Morphomolecular Identification of Penicillium griseofulvum and *Penicillium aurantiogriseum* Toxicogenic Isolates Associated with Blue Mold on Apple. *Foodborne Pathog. Dis.* 7, 857–861. doi:10.1089/fpd.2009.0507.

Mousa, W., Ghazali, F. M., Jinap, S., Ghazali, H. M., and Radu, S. (2011). Modelling the effect of water activity and temperature on growth rate and aflatoxin production by two isolates of *Aspergillus flavus* on paddy. *J. Appl. Microbiol.* 111, 1262–1274. doi:10.1111/j.1365-2672.2011.05134.x.

Mousadoost, M., Eslami, A., and Yousefi, Z. (2018). Determination of the best drying temperature of hazelnut (*Corylus avellana*) after six months of storage. *J. Nuts* 9, 1–10. Available at: http://ijnrs.damghaniau.ac.ir/article_540861_f64dff2a9f98bbe27625a11231c1a545.pdf.

Nagy, G., Szebenyi, C., Csernetics, Á., Vaz, A. G., Tóth, E. J., Vágvölgyi, C., et al. (2017). Development of a plasmid free CRISPR-Cas9 system for the genetic modification of *Mucor circinelloides*. *Sci. Rep.* 7, 1–10. doi:10.1038/s41598-017-17118-2.

Nicholson, M. J., Eaton, C. J., Stärkel, C., Tapper, B. A., Cox, M. P., and Scott, B. (2015). Molecular cloning and functional analysis of gene clusters for the biosynthesis of indole-diterpenes in *Penicillium crustosum* and *P. janthinellum*. *Toxins (Basel).* 7, 2701–2722. doi:10.3390/toxins7082701.

Nicholson, P. (2004). "Rapid detection of mycotoxigenic fungi in plants," in *Mycotoxins in Food* (Woodhead Publishing), 111–152. doi:10.1533/9781855739086.1.111.

Nielsen, J. C., and Nielsen, J. (2017). Development of fungal cell factories for the production of secondary metabolites: Linking genomics and metabolism. *Synth. Syst. Biotechnol.* 2, 5–12. doi:10.1016/j.synbio.2017.02.002.

Nielsen, M. L., Isbrandt, T., Rasmussen, K. B., Thrane, U., Hoof, J. B., Larsen, T. O., et al. (2017). Genes Linked to production of secondary metabolites in *Talaromyces atroroseus* revealed using CRISPR-Cas9. *PLoS One* 12, 1–9. doi:10.1371/journal.pone.0169712.

Nobile, C. J., and Mitchell, A. P. (2005). Regulation of Cell-Surface Genes and Biofilm Formation by the *C. albicans* Transcription Factor Bcr1p. *Curr. Biol.* 15, 1150–1155. doi:10.1016/j.cub.2005.05.047.

Nødvig, C. S., Nielsen, J. B., Kogle, M. E., and Mortensen, U. H. (2015). A CRISPR-Cas9 system for genetic engineering of filamentous fungi. *PLoS One* 10, 1–18. doi:10.1371/journal.pone.0133085.

Núñez, F., Díaz, M. C., Rodríguez, M., Aranda, E., Martín, A., and Asensio, M. A. (2000). Effects of substrate, water activity, and temperature on growth and verrucosidin production by Penicillium polonicum isolated from dry-cured ham. *J. Food Prot.* 63, 231–236. doi:10.4315/0362-028X-63.2.231.

Ojiambo, P. S., Battilani, P., Cary, J. W., Blum, B. H., and Carbone, I. (2018). Cultural and genetic approaches to manage aflatoxin contamination: Recent insights provide opportunities for improved control. *Phytopathology* 108, 1024–1037. doi:10.1094/PHYTO-04-18-0134-RVW.

Ozay, G., Seyhan, F., Pembeci, C., Saklar, S., and Yilmaz, A. (2008). Factors influencing fungal and aflatoxin levels in Turkish hazelnuts (*Corylus avellana* L.) during growth, harvest, drying and storage: A 3-year study. *Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess.* 25, 209–218. doi:10.1080/02652030701711016.

Özdemir, M., and Devres, O. (1999). Turkish hazelnuts: Properties and effect of microbiological and chemical changes on quality. *Food Rev. Int.* 15, 309–333. doi:10.1080/87559129909541193.

Özilgen, M., and Özdemir, M. (2001). A review on grain and nut deterioration and design of the dryers for safe storage with special reference to Turkish hazelnuts. *Crit. Rev. Food Sci. Nutr.* 41, 95–132. doi:10.1080/20014091091779.

Park, H. R., Ryoo, I. J., Choo, S. J., Hwang, J. H., Kim, J. Y., Cha, M. R., et al. (2007). Glucose-deprived HT-29 human colon carcinoma cells are sensitive to verrucosidin as a GRP78 down-regulator. *Toxicology* 229, 253–261. doi:10.1016/j.tox.2006.11.049.

Perrone, G., Gallo, A., and Logrieco, A. F. (2014). Biodiversity of *Aspergillus* section *Flavi* in Europe in relation to the management of aflatoxin risk. *Front. Microbiol.* 5, 377. doi:10.3389/fmicb.2014.00377.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29, e45. doi:10.1093/nar/29.9.e45.

Pohl, C., Kiel, J. A. K. W., Driessen, A. J. M., Bovenberg, R. A. L., and Nygård, Y. (2016). CRISPR/Cas9 Based Genome Editing of *Penicillium chrysogenum*. *ACS Synth. Biol.* 5, 754–764. doi:10.1021/acssynbio.6b00082.

Pratiwi, C., Rahayu, W. P., Lioe, H. N., Herawati, D., Broto, W., and Ambarwati, S. (2015).

The effect of temperature and relative humidity for *Aspergillus flavus* BIO 2237 growth and aflatoxin production on soybeans. *Int. Food Res. J.* 22, 82. doi:10.1016/j.postharvbio.2013.09.006.

Prelle, A., Spadaro, D., Garibaldi, A., and Gullino, M. L. (2012a). Aflatoxin monitoring in Italian hazelnut products by LC-MS. *Food Addit. Contam. Part B Surveill.* 5, 279–285. doi:10.1080/19393210.2012.711371.

Prencipe, S., Siciliano, I., Contessa, C., Botta, R., Garibaldi, A., Gullino, M. L., et al. (2018a). Characterization of *Aspergillus* section *Flavi* isolated from fresh chestnuts and along the chestnut flour process. *Food Microbiol.* 69, 159–169. doi:10.1016/j.fm.2017.08.004.

Prencipe, S., Siciliano, I., Gatti, C., Garibaldi, A., Gullino, M. L., Botta, R., et al. (2018b). Several species of *Penicillium* isolated from chestnut flour processing are pathogenic on fresh chestnuts and produce mycotoxins. *Food Microbiol.* 76, 396–404. doi:10.1016/j.fm.2018.07.003.

Prencipe, S., Siciliano, I., Gatti, C., Gullino, M. L., Garibaldi, A., and Spadaro, D. (2018c). Chestnut drying is critical in determining *Aspergillus flavus* growth and aflatoxin contamination. *Toxins (Basel).* 10, 530. doi:10.3390/toxins10120530.

Prusky, D., McEvoy, J. L., Saftner, R., Conway, W. S., and Jones, R. (2004). Relationship Between Host Acidification and Virulence of *Penicillium* spp. on Apple and Citrus Fruit. *Phytopathology* 94, 44–51. doi:10.1094/phyto.2004.94.1.44.

Quevillon, E., Silventoinen, V., Pillai, S., Harte, N., Mulder, N., Apweiler, R., et al. (2005). InterProScan: Protein domains identifier. *Nucleic Acids Res.* 33, W116–W120. doi:10.1093/nar/gki442.

Randhawa, A., Ogunyewo, O. A., Eqbal, D., Gupta, M., and Yazdani, S. S. (2018). Disruption of zinc finger DNA binding domain in catabolite repressor Mig1 increases growth rate, hyphal branching, and cellulase expression in hypercellulolytic fungus *Penicillium funiculosum* NCIM1228. *Biotechnol. Biofuels* 11, 15. doi:10.1186/s13068-018-1011-5.

Rathinasamy, K., Jindal, B., Asthana, J., Singh, P., Balaji, P. V., and Panda, D. (2010). Griseofulvin stabilizes microtubule dynamics, activates p53 and inhibits the proliferation of MCF-7 cells synergistically with vinblastine. *BMC Cancer* 10, 213. doi:10.1186/1471-2407-10-213.

Reddy, K. R. N., Spadaro, D., Lore, A., Gullino, M. L., and Garibaldi, A. (2010). Potential of patulin production by *Penicillium expansum* strains on various fruits. *Mycotoxin Res.* 26, 257–265. doi:10.1007/s12550-010-0064-5.

Ribeiro, A. I., Costa, E. S., Thomasi, S. S., Brandão, D. F. R., Vieira, P. C., Fernandes, J. B., et al. (2018). Biological and Chemical Control of *Sclerotinia sclerotiorum* using *Stachybotrys levispora* and Its Secondary Metabolite Griseofulvin. *J. Agric. Food Chem.* 66, 7627–7632. doi:10.1021/acs.jafc.7b04197.

Richard, J. L. (2007). Some major mycotoxins and their mycotoxicoses-An overview. *Int. J. Food Microbiol.* 119, 3–10. doi:10.1016/j.ijfoodmicro.2007.07.019.

Riley, M. I., Hopper, J. E., Johnston, S. A., and Dickson, R. C. (1987). GAL4 of *Saccharomyces cerevisiae* activates the lactose-galactose regulon of *Kluyveromyces lactis* and creates a new phenotype: glucose repression of the regulon. *Mol. Cell. Biol.* 7, 780–6. doi:10.1128/mcb.7.2.780.

Rodrigues, I., and Naehrer, K. (2012). A three-year survey on the worldwide occurrence of mycotoxins in feedstuffs and feed. *Toxins (Basel).* 4, 663–675. doi:10.3390/toxins4090663.

Rodrigues, P., Venâncio, A., and Lima, N. (2012). Mycobiota and mycotoxins of almonds and chestnuts with special reference to aflatoxins. *Food Res. Int.* 48, 76–90. doi:10.1016/J.FOODRES.2012.02.007.

Rodríguez, A., Córdoba, J. J., Werning, M. L., Andrade, M. J., and Rodríguez, M. (2012). Duplex real-time PCR method with internal amplification control for quantification of verrucosidin producing molds in dry-ripened foods. *Int. J. Food Microbiol.* 153, 85–91. doi:10.1016/j.ijfoodmicro.2011.10.020.

Sabater-Vilar, M., Nijmeijer, S., and Fink-Gremmels, J. (2003). Genotoxicity assessment of five tremorgenic mycotoxins (fumitremorgen B, paxilline, penitrem A, verruculogen, and verrucosidin) produced by molds isolated from fermented meats. *J. Food Prot.* 66, 2123–2129. doi:10.4315/0362-028X-66.11.2123.

Samson, R. A., Visagie, C. M., Houbraken, J., Hong, S.-B., Hubka, V., Klaassen, C. H. W., et al. (2014). Phylogeny, identification and nomenclature of the genus *Aspergillus*. *Stud. Mycol.* 78, 141–73. doi:10.1016/j.simyco.2014.07.004.

Sanchis, V., and Magan, N. (2004). "Environmental conditions affecting mycotoxins," in *Mycotoxins in Food: Detection and Control*, eds. N. Magan and M. Olsen (Woodhead Publishing), 174–189.

Santini, A., Mikušová, P., Sulyok, M., Krska, R., Labuda, R., and Šrobárová, A. (2014). *Penicillium* strains isolated from Slovak grape berries taxonomy assessment by secondary metabolite profile. *Mycotoxin Res.* 30, 213–220. doi:10.1007/s12550-014-0205-3.

Sanzani, S. M., Reverberi, M., and Geisen, R. (2016). Mycotoxins in harvested fruits and vegetables: Insights in producing fungi, biological role, conducive conditions, and tools to manage postharvest contamination. *Postharvest Biol. Technol.* 122, 95–105. doi:10.1016/j.postharvbio.2016.07.003.

Schäberle, T. F. (2016). Biosynthesis of α-pyrones. *Beilstein J. Org. Chem.* 12, 571–588. doi:10.3762/bjoc.12.56.

Scharf, D. H., Heinekamp, T., and Brakhage, A. A. (2014). Human and Plant Fungal Pathogens: The Role of Secondary Metabolites. *PLoS Pathog.* 10, e1003859. doi:10.1371/JOURNAL.PPAT.1003859.

Schmidt-Heydt, M., Abdel-Hadi, A., Magan, N., and Geisen, R. (2009). Complex regulation of the aflatoxin biosynthesis gene cluster of *Aspergillus flavus* in relation to various combinations of water activity and temperature. *Int. J. Food Microbiol.* 135, 231–237. doi:10.1016/J.IJFOODMICRO.2009.07.026.

Schmidt-Heydt, M., Magan, N., and Geisen, R. (2008). Stress induction of mycotoxin biosynthesis genes by abiotic factors. *FEMS Microbiol. Lett.* 284, 142–149. doi:10.1111/j.1574-6968.2008.01182.x.

Schuster, M., and Kahmann, R. (2019). CRISPR-Cas9 genome editing approaches in filamentous fungi and oomycetes. *Fungal Genet. Biol.* 130, 43–53. doi:10.1016/j.fgb.2019.04.016.

Schwenk, D., Brandt, P., Blanchette, R. A., Nett, M., and Hoffmeister, D. (2016). Unexpected Metabolic Versatility in a Combined Fungal Fomannoxin/Vibralactone Biosynthesis. *J. Nat. Prod.* 79, 1407–1414. doi:10.1021/acs.jnatprod.6b00147.

Seol, J. H., Shim, E. Y., and Lee, S. E. (2018). Microhomology-mediated end joining: Good, bad and ugly. *Mutat. Res. - Fundam. Mol. Mech. Mutagen.* 809, 81–87. doi:10.1016/j.mrfmmm.2017.07.002.

Shapira, R., and Paster, N. (2004). "Control of mycotoxins in storage and techniques for their decontamination," in *Mycotoxins in Food: Detection and Control*, eds. N. Magan and M. Olsen (Woodhead Publishing), 190–223. doi:10.1533/9781855739086.

Shi, T. Q., Liu, G. N., Ji, R. Y., Shi, K., Song, P., Ren, L. J., et al. (2017). CRISPR/Cas9-based genome editing of the filamentous fungi: the state of the art. *Appl. Microbiol. Biotechnol.* 101, 7435–7443. doi:10.1007/s00253-017-8497-9.

Siahmoshteh, F., Siciliano, I., Banani, H., Hamidi-Esfahani, Z., Razzaghi-Abyaneh, M., Gullino, M. L., et al. (2017). Efficacy of *Bacillus subtilis* and *Bacillus amyloliquefaciens* in the control of *Aspergillus parasiticus* growth and aflatoxins production on pistachio. *Int. J. Food Microbiol.* 254, 47–53. doi:10.1016/j.ijfoodmicro.2017.05.011.

Siciliano, I., Dal Bello, B., Zeppa, G., Spadaro, D., and Gullino, M. L. (2017). Static hot air and infrared rays roasting are efficient methods for aflatoxin decontamination on hazelnuts. *Toxins (Basel).* 9, 72. doi:10.3390/toxins9020072.

Siciliano, I., Spadaro, D., Prelle, A., Vallauri, D., Cavallero, M. C., Garibaldi, A., et al. (2016). Use of cold atmospheric plasma to detoxify hazelnuts from aflatoxins. *Toxins (Basel).* 8, 125. doi:10.3390/toxins8050125.

Sigl, C., Haas, H., Specht, T., Pfaller, K., Kürnsteiner, H., and Zadra, I. (2011). Among Developmental Regulators, StuA but Not BrlA Is Essential for Penicillin V Production in *Penicillium chrysogenum*. *Appl. Environ. Microbiol.* 77, 972–982. doi:10.1128/AEM.01557-10.

Silve, S., Dupuy, P. H., Labit-Lebouteiller, C., Kaghad, M., Chalon, P., Rahier, A., et al. (1996). Emopamil-binding protein, a mammalian protein that binds a series of structurally diverse neuroprotective agents, exhibits Δ8-Δ7 sterol isomerase activity in yeast. *J. Biol. Chem.* 271, 22434–22440. doi:10.1074/jbc.271.37.22434.

Şimşek, O., Arici, M., and Demir, C. (2002). Mycoflora of hazelnut (*Corylus avellana* L.) and aflatoxin content in hazelnut kernels artificially infected with *Aspergillus parasiticus*. *Nahrung - Food* 46, 194–196. doi:10.1002/1521-3803(20020501)46:3<194::AID-FOOD194>3.0.CO;2-D.

Smith, M. C., Madec, S., Coton, E., and Hymery, N. (2016). Natural Co-occurrence of mycotoxins in foods and feeds and their *in vitro* combined toxicological effects. *Toxins (Basel).* 8, 94. doi:10.3390/toxins8040094.

Snini, S. P., Tannous, J., Heuillard, P., Bailly, S., Lippi, Y., Zehraoui, E., et al. (2016). Patulin is a cultivar-dependent aggressiveness factor favouring the colonization of apples by *Penicillium expansum*. *Mol. Plant Pathol.* 17, 920–930. doi:10.1111/mpp.12338.

Song, R., Zhai, Q., Sun, L., Huang, E., Zhang, Y., Zhu, Y., et al. (2019). CRISPR/Cas9 genome editing technology in filamentous fungi: progress and perspective. *Appl. Microbiol. Biotechnol.* 103, 6919–6932. doi:10.1007/s00253-019-10007-w.

Song, Z., Yang, J., Xin, C., Xing, X., Yuan, Q., Yin, Y., et al. (2018). A transcription factor, MrMsn2, in the dimorphic fungus *Metarhizium rileyi* is essential for dimorphism transition, aggravated pigmentation, conidiation and microsclerotia formation. *Microb. Biotechnol.* 11, 1157–1169. doi:10.1111/1751-7915.13302.

Sonjak, S., Frisvad, J. C., and Gunde-Cimerman, N. (2006). *Penicillium* mycobiota in Arctic subglacial ice. *Microb. Ecol.* 52, 207–216. doi:10.1007/s00248-006-9086-0.

Spadaro, D., Garibaldi, A., and Gullino, M. L. (2008). Occurrence of patulin and its dietary intake through pear, peach, and apricot juices in Italy. *Food Addit. Contam. Part B Surveill.* 1, 134–139. doi:10.1080/02652030802363790.

Spadaro, D., Lorè, A., Amatulli, M. T., Garibaldi, A., and Gullino, M. L. (2011). First Report of *Penicillium griseofulvum* Causing Blue Mold on Stored Apples in Italy (Piedmont). *Plant Dis.* 95, 76–76. doi:10.1094/PDIS-08-10-0568.

Spadaro, D., Lorè, A., Garibaldi, A., and Gullino, M. L. (2013). A new strain of *Metschnikowia fructicola* for postharvest control of *Penicillium expansum* and patulin accumulation on four cultivars of apple. *Postharvest Biol. Technol.* 75, 1–8. doi:10.1016/j.postharvbio.2012.08.001.

Stierle, A. A., and Stierle, D. B. (2015). Bioactive Secondary Metabolites Produced by the Fungal Endophytes of Conifers. *Nat. Prod. Commun.* 10, 1671–82. doi:10.1177/1934578X1501001012.

Stroe, M. C., Netzker, T., Scherlach, K., Krüger, T., Hertweck, C., Valiante, V., et al. (2020). Targeted induction of a silent fungal gene cluster encoding the bacteria-specific germination inhibitor fumigermin. *Elife* 9. doi:10.7554/eLife.52541.

Sunesen, L. O., and Stahnke, L. H. (2003). Mould starter cultures for dry sausages - Selection, application and effects. *Meat Sci.* 65, 935–948. doi:10.1016/S0309-1740(02)00281-4.

Taevernier, L., Wynendaele, E., De Vreese, L., Burvenich, C., and De Spiegeleer, B. (2016). The mycotoxin definition reconsidered towards fungal cyclic depsipeptides. *J. Environ. Sci. Heal. - Part C Environ. Carcinog. Ecotoxicol. Rev.* 34, 114–135. doi:10.1080/10590501.2016.1164561.

Tang, H.-Y., Zhang, Q., Li, H., and Gao, J.-M. (2015). Antimicrobial and allelopathic metabolites produced by *Penicillium brasilianum*. *Nat. Prod. Res.* 29, 345–348.

doi:10.1080/14786419.2014.940347.

Thomas, S., Sharma, N., Gonzalez, R., Pao, P. W., Hofman, F. M., Chen, T. C., et al. (2013). Repositioning of Verrucosidin, a Purported Inhibitor of Chaperone Protein GRP78, as an Inhibitor of Mitochondrial Electron Transport Chain Complex I. *PLoS One* 8, 1–14. doi:10.1371/journal.pone.0065695.

Tian, L., Yu, J., Wang, Y., and Tian, C. (2017). The C 2 H 2 transcription factor VdMsn2 controls hyphal growth, microsclerotia formation, and virulence of *Verticillium dahliae*. *Fungal Biol.* 121, 1001–1010. doi:10.1016/j.funbio.2017.08.005.

Todd, R. B., Andrianopoulos, A., Davis, M. A., and Hynes, M. J. (1998). FacB, the *Aspergillus nidulans* activator of acetate utilization genes, binds dissimilar DNA sequences. *EMBO J.* 17, 2042–54. doi:10.1093/emboj/17.7.2042.

Tong, Y., Weber, T., and Lee, S. Y. (2019). CRISPR/Cas-based genome engineering in natural product discovery. *Nat. Prod. Rep.* 36, 1225–1372. doi:10.1039/c8np00089a.

Tudzynski, B. (2014). Nitrogen regulation of fungal secondary metabolism in fungi. *Front. Microbiol.* 5, 656. doi:10.3389/fmicb.2014.00656.

Turan, A. (2018). Effect of drying methods on fatty acid profile and oil oxidation of hazelnut oil during storage. *Eur. Food Res. Technol.* 244, 2181–2190. doi:10.1007/s00217-018-3128-y.

Turan, A. (2019). Effect of drying on the chemical composition of Çakıldak (cv) hazelnuts during storage. *Grasas y Aceites* 70, 296. doi:10.3989/gya.0693181.

Turan, A., and İslam, A. (2018). Effect of Drying Methods on Some Chemical Characteristics of Hazelnuts (Corylus avellana L.) During Storage. *J. Inst. Sci. Technol.* 8, 11–19. doi:10.21597/jist.458541.

Van Egmond, H. P., and Jonker, M. A. (2008). "Regulations and Limits for Mycotoxins in Fruits and Vegetables," in *Mycotoxins in Fruits and Vegetables* (Academic Press), 45–74. doi:10.1016/B978-0-12-374126-4.00003-6.

Vienken, K., Scherer, M., and Fischer, R. (2005). The Zn(II)2Cys6 putative Aspergillus nidulans transcription factor repressor of sexual development inhibits sexual development under low-carbon conditions and in submersed culture. *Genetics* 169, 619–30. doi:10.1534/genetics.104.030767.

Visagie, C. M., Houbraken, J., Frisvad, J. C., Hong, S. B., Klaassen, C. H. W., Perrone, G., et al. (2014). Identification and nomenclature of the genus *Penicillium*. *Stud. Mycol.* 78, 343–371. doi:10.1016/j.simyco.2014.09.001.

Wang, H. L., Kim, S. H., Siu, H., and Breuil, C. (1999). Transformation of sapstaining fungi with hygromycin B resistance plasmids pAN7-1 and pCB1004. *Mycol. Res.* 103, 77–80. doi:10.1017/S0953756298006698.

Wang, Q., and Coleman, J. J. (2019). Progress and Challenges: Development and Implementation of CRISPR/Cas9 Technology in Filamentous Fungi. *Comput. Struct. Biotechnol. J.* 17, 761–769. doi:10.1016/j.csbj.2019.06.007.

Wang, S., Chen, H., Tang, X., Zhang, H., Chen, W., and Chen, Y. Q. (2017). Molecular tools for gene manipulation in filamentous fungi. *Appl. Microbiol. Biotechnol.* 101,

8063–8075. doi:10.1007/s00253-017-8486-z.

Wang, W., Jung, J., McGorrin, R. J., Traber, M. G., Leonard, S. W., Cherian, G., et al. (2018a). Investigation of drying conditions on bioactive compounds, lipid oxidation, and enzyme activity of Oregon hazelnuts (*Corylus avellana* L.). *LWT* 90, 526–534. doi:10.1016/J.LWT.2018.01.002.

Wang, W., Jung, J., McGorrin, R. J., and Zhao, Y. (2018b). Investigation of the mechanisms and strategies for reducing shell cracks of hazelnut (*Corylus avellana* L.) in hot-air drying. *LWT* 98, 252–259. doi:10.1016/j.lwt.2018.08.053.

Weber, J., Valiante, V., Nødvig, C. S., Mattern, D. J., Slotkowski, R. A., Mortensen, U. H., et al. (2017). Functional reconstitution of a fungal natural product gene cluster by advanced genome editing. *ACS Synth. Biol.* 6, 62–68. doi:10.1021/acssynbio.6b00203.

Weber, T., Blin, K., Duddela, S., Krug, D., Kim, H. U., Bruccoleri, R., et al. (2015). AntiSMASH 3.0-A comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic Acids Res.* 43, W237–W243. doi:10.1093/nar/gkv437.

Weber, T., and Kim, H. U. (2016). The secondary metabolite bioinformatics portal: Computational tools to facilitate synthetic biology of secondary metabolite production. *Synth. Syst. Biotechnol.* 1, 69–79. doi:10.1016/j.synbio.2015.12.002.

Weidner, G., D'Enfert, C., Koch, A., Mol, P. C., and Brakhage, A. A. (1998). Development of a homologous transformation system for the human pathogenic fungus *Aspergillus fumigatus* based on the *pyrG* gene encoding orotidine 5'-monophosphate decarboxylase. *Curr. Genet.* 33, 378–385. doi:10.1007/s002940050350.

Wheeler, K. A., Lamb, H. K., and Hawkins, A. R. (1996). Control of metabolic flux through the quinate pathway in *Aspergillus nidulans*. *Biochem. J.* 315, 195–205. doi:10.1042/bj3150195.

Wiemann, P., and Keller, N. P. (2014). Strategies for mining fungal natural products. *J. Ind. Microbiol. Biotechnol.* 41, 301–313. doi:10.1007/s10295-013-1366-3.

Wigmann, É. F., Jahn, R. C., Scherer, C. D., Saccomori, F., Alcano-González, M. de J., and Copetti, M. V. (2018). Detection and identification of *Penicillium* spp. in a frozen chicken nuggets production facility. *Food Microbiol.* 70, 42–48. doi:10.1016/j.fm.2017.09.002.

Wilkinson, H. H., Ramaswamy, A., Sung, C. S., and Keller, N. P. (2004). Increased conidiation associated with progression along the sterigmatocystin biosynthetic pathway. *Mycologia* 96, 1190–1198. doi:10.1080/15572536.2005.11832867.

Wilson, R. A., and Arst, H. N. (1998). Mutational analysis of AREA, a transcriptional activator mediating nitrogen metabolite repression in *Aspergillus nidulans* and a member of the "streetwise" GATA family of transcription factors. *Microbiol. Mol. Biol. Rev.* 62, 586–96. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9729601.

Wolfers, S., Kamerewerd, J., Nowrousian, M., Sigl, C., Zadra, I., Kürnsteiner, H., et al. (2015). Microarray hybridization analysis of light-dependent gene expression in *Penicillium chrysogenum* identifies bZIP transcription factor PcAtfA. *J. Basic*

Microbiol. 55, 480–489. doi:10.1002/jobm.201400588.

World Health Organization, I. A. for R. on C. (2001). Some thyrotropic agents. *IARC Monogr. Eval. Carcinog. RISKS TO HUMANS - SOME THYROTROPIC AGENTS* 79, 291–315.

Wu, G., Jurick Ii, W. M., Lichtner, F. J., Peng, H., Yin, G., Gaskins, V. L., et al. (2019). Whole-genome comparisons of *Penicillium* spp. reveals secondary metabolic gene clusters and candidate genes associated with fungal aggressiveness during apple fruit decay. *PeerJ* 7, e6170. doi:10.7717/peerj.6170.

Yaegashi, J., Oakley, B. R., and Wang, C. C. C. (2014). Recent advances in genome mining of secondary metabolite biosynthetic gene clusters and the development of heterologous expression systems in *Aspergillus nidulans*. *J. Ind. Microbiol. Biotechnol.* 41, 433–442. doi:10.1007/s10295-013-1386-z.

Yang, W., Wu, H., Wang, Z., Sun, Q., Qiao, L., and Huang, B. (2018). The APSES Gene *MrStuA* Regulates Sporulation in *Metarhizium robertsii*. *Front. Microbiol.* 9, 1208. doi:10.3389/fmicb.2018.01208.

Yao, G., Yue, Y., Fu, Y., Fang, Z., Xu, Z., Ma, G., et al. (2018). Exploration of the regulatory mechanism of secondary metabolism by comparative transcriptomics in *Aspergillus flavus*. *Front. Microbiol.* 9, 1568. doi:10.3389/fmicb.2018.01568.

Young, C., Itoh, Y., Johnson, R., Garthwaite, I., Miles, C. O., Munday-Finch, S. C., et al. (1998). Paxilline-negative mutants of *Penicillium paxilli* generated by heterologous and homologous plasmid integration. *Curr. Genet.* 33, 368–377. doi:10.1007/s002940050349.

Yu, J. H., and Leonard, T. J. (1995). Sterigmatocystin biosynthesis in *Aspergillus nidulans* requires a novel type I polyketide synthase. *J. Bacteriol.* 177, 4792–4800. doi:10.1128/jb.177.16.4792-4800.1995.

Yu, J., Jurick, W. M., and Bennett, J. W. (2015). Current Status of Genomics Research on Mycotoxigenic Fungi. *Int. J. plant Biol. Res.* 3, 1035. Available at: https://pdfs.semanticscholar.org/f340/059f66a794edced2abaadf0653e0a8544f81.pdf.

Zhou, X., Liu, W., Wang, C., Xu, Q., Wang, Y., Ding, S., et al. (2011). A MADS-box transcription factor MoMcm1 is required for male fertility, microconidium production and virulence in *Magnaporthe oryzae*. *Mol. Microbiol.* 80, 33–53. doi:10.1111/j.1365-2958.2011.07556.x.

Zhu, Y., Takeda, T., Whitehall, S., Peat, N., and Jones, N. (1997). Functional characterization of the fission yeast Start-specific transcription factor Res2. *EMBO J.* 16, 1023–1034. doi:10.1093/emboj/16.5.1023.

Zhuang, Z., Lohmar, J., Satterlee, T., Cary, J., and Calvo, A. (2016). The Master Transcription Factor mtfA Governs Aflatoxin Production, Morphological Development and Pathogenicity in the Fungus *Aspergillus flavus*. *Toxins (Basel).* 8, 29. doi:10.3390/toxins8010029.

Acknowledgments

I would like to gratefully thank:

The institutions that founded the grants that I received:

- Fondazione Cassa di Risparmio di Cuneo (CRC) e Fondazione Cassa di Risparmio di Torino (CRT) which funded the research grant "Molecular characterization of phytopathogenic fungi isolated from plant matrices" coordinated by Prof. Davide Spadaro
- Piedmont Region (project Food Digital Monitoring, FDM) funding the research grant "Development of diagnostic and detection techniques for pathogens and contaminants of the agri-food industry" coordinated by Prof. Maria Lodovica Gullino
- the German Academic Exchange Service (DAAD) with a scholarship "Activation of fungal silent gene clusters and impact of produced compounds on microorganisms" in the program Research Grants - Short-Term Grants, 2019 and the Dept. Molecular and Applied Microbiology, Hans Knöll Institute (HKI) for hosting me
- Fondazione CRC funding the research grant "Isolation and characterization of phytopatogenic agents from cultivar of apple from Piedmont region (SMART APPLE project)" coordinated by Prof. Davide Spadaro

My PhD tutor, Professor Davide Spadaro, who gave me this interesting topic and the opportunity to be funded. I am honoured to have had this opportunity and thanks to his support I guess I grew up as a person and a scientist.

Professor Angelo Garibaldi the President of the Centre Agroinnova, where I performed most of my research activity, and Professor Maria Lodovica Gullino, the Director. With her leadership, she pushed everyone, including me, to do more and do our best.

Professor Axel Brakhage, who had always time to discuss about the project and to give me his wise advices. I am proud that I could spent the period abroad at the HKI in Jena under his supervision.

All the international scientists which helped me with the experiments: Luis González-Candelas, Ana-Rosa Ballester and Toni Gabaldón in Spain, Volker Schroeckh and Thorsten Heinekamp in Germany.

Edoardo Piombo, without your moral support (and without the chocolate and sweets daily provided) it would have been really difficult to overcome the stressful moments. All the colleagues in Agroinnova and DISAFA, it was a pleasure to collaborate, eat cakes and homemade “pasty”! Thank you Slavica Matic, Simona Prencipe, Fabiano Sillo for helpful suggestions and moral and scientific support. Thank you Alessio Bellini, Alexandra Cucu, Athina Vasileiadou, Andrea China Gallo, Davide Farina, Federico Facchinetti, Giovanna Roberta Meloni, Giulia Tabone, Guglielmo Lione, Karin Santoro, Luca Miola, Luana Giordano, Martina Pellicciaro, Riccardo Dipoppa, Silvio Broglia for sharing joyful moments. Master’s students who performed part of the experiments and allow me to improve my communication skills, Agnese Cometto, Martina Occhionegrelli, Luca Laboranti, Davide Bello.

The technicians Renata Luongo, Domenico Bertetti and Guido Martano in Italy and Christina Täumer in Germany for their wisdom.

All the colleagues in Germany, it was a pleasure to find such a welcoming place. Just to mention a few, thank you Daniela Wagner, Yi Enn Cheong, Mario Krespach, Flora Rivieccio, Jakob Sprague, Muhammad Rafiq, Abdulrahman Kelani, Maria Stroe, Michal Flak, Sylke Fricke, Katherine Gonzalez Rojas, Leijie Jia, Matthew Blango, Lukáš Radosa and Tina Netzker. I still miss the labs, your company and the coffee machine!

My friends which are a real treasure: Claudia, Alessia, Silvia, Ola, Federico, Liam, Matte, Dani, Adri.

My family which represent my roots and my strength. I thank with all my heart my mum Daniela and my dad Luciano, my sister Sabrina, a real inspiration for me. My second family, Ruggiero, Altomare, Maurizio and Lucia. Michele, with your love you support me, you make me happy and safe, you have been, and you are, really important for my growth as a person.

Curriculum Vitae

Silvia Valente was born on August 4th, 1988 in Schio (VI). She performed there the studies and in July 2007 she attended the high school diploma in social sciences.

She moved in Torino in 2007 to study Biotechnology and later Plant Biotechnology.

During the Bachelor she investigated the topic "Use of nanosensor for the identifications of plant pathogens" and she graduated in July 2013 with 103/110.

During the Master she mainly focused her attention on "Hydrolytic enzymes from microbial antagonists involved in the biological control of postharvest pathogens" and this work led to two scientific publications. She attended the Master's Degree in Plant Biotechnologies in April 2016 with 110 *cum laude*/110.

In October 2016 she started the PhD in Biology and Applied Biotechnology following the project "Elucidation of the production of secondary metabolites, including mycotoxins, in fungal pathogens" with Professor Davide Spadaro. She performed the experiments in Grugliasco at the Centre of Competence for the innovation in the agro-environmental field (Agroinnova) and she was founded by several research grants.

From March 2019 to August 2019 she spent six months as visiting student at Dept. Molecular and Applied Microbiology, Leibniz Institute for Natural Product Research and Infection Biology – Hans Knöll Institute (HKI) in Jena working on CRISPR-Cas9 on filamentous fungi, under the supervision of Prof. Brakhage.

Publications

International Journals (ISI)

Valente, S., Meloni, G. R., Prencipe, S., Spigolon, N., Somenzi, M., Fontana, M., Gullino, M. L., Spadaro, D. (2020). Effect of drying temperature and exposure times on *Aspergillus flavus* growth and aflatoxin production on artificially inoculated hazelnuts. Journal of Food Protection, In print.

Zhang, D., Spadaro, D., Valente, S., Garibaldi, A., Gullino, M. L. (2011) Cloning, characterization and expression of an exo-1, 3-β-glucanase gene from the antagonistic yeast *Pichia guilliermondii* strain M8 against grey mold on apples. Biological Control, 59 (2), 284-293.

Zhang, D., Spadaro, D., Valente, S., Garibaldi, A., Gullino, M. L. (2012) Cloning, characterization, expression and antifungal activity of an alkaline serine protease of *Aureobasidium pullulans* PL5 involved in the biological control of postharvest pathogens. International Journal of Food Microbiology, 153 (3), 453-464.

Papers and abstracts presented at Congresses

Spadaro D., Piombo E., Valente S., Banani H., Prencipe S., Marcet-Houben M., Gullino M.L., Gabaldòn T. (2019) Secondary metabolites produced by *Penicillium* spp.: scanning the genomes to explore the association with postharvest diseases of fruit. Book of Abstracts, 5th International Symposium on Postharvest Pathology, 19-24 May 2019, Liegi, Belgium, 49.

Spadaro D., Prencipe S., Valente S., Piombo E., Garibaldi A., Gullino M.L. (2020) Innovative management strategies for *Aspergillus* spp. and *Penicillium* spp. on nuts. In: Plant Pathology in the 21th century: Contribution to the 11th international ISPP Congress. Postharvest Pathology: Next Generation Solutions To Reducing Losses And Enhancing Safety. (Spadaro D., Droby S., Gullino M.L.), Springer, Dordrecht, The Netherlands, In print.

Valente S., Cometto A., Meloni G.R., Piombo E., Ballester A.R., Gonzalez Candelas L., Gullino M.L., Spadaro D. (2019) Regulation of griseofulvin biosynthesis and role on growth and virulence of *Penicillium griseofulvum*, causal agent of apple blue mold. Journal of Plant Pathology 101 (4), In print.

Valente S., Cometto A., Meloni G.R., Piombo E., Ballester A.R., Gonzalez Candelas L., Gullino M.L., Spadaro D. (2020) Tole of Gsfr1 and global regulators on griseofulvin and other secondary metabolites biosynthesis and on growth and virulence of *Penicillium griseofulvum*. Book of Abstracts of the 15th European Conference on Fungal Genetics, 17-20 February 2020, Rome. In print.

Valente S., Cometto A., Piombo E., Ballester A.-R., Gullino M.L., Gonzalez-Candelas L., Spadaro D. (2019) New insights into griseofulvin biosynthesis by *Penicillium griseofulvum*, an agent of blue mould on apples. Book of Abstracts 5th International Symposium on Postharvest Pathology, 19-24 May 2019, Liegi, Belgium. P015, 61.

Zhang D., Spadaro D., Valente S., Garibaldi A., Gullino M.L. (2011) Chitinases, glucanases and proteases are involved in the mechanism of action of yeast antagonists of postharvest rots. Journal of Plant Pathology, 93 (S4), 22.

Zhang D., Valente S., Spadaro D., Garibaldi A., Gullino M.L. (2011) Cloning, characterization and expression of the alkaline serine protease genes from the postharvest biocontrol agent *Aureobasidium pullulans* PL5. Book of Abstracts of the International Congress of Postharvest Pathology, 11-14 April 2011, Lleida, Spain, 110.

Non-ISI Journals

Meloni G. R., Valente S., Garibaldi A., Spadaro D., Gullino M. L. (2019) Effetto delle condizioni di essiccazione delle nocciole sullo sviluppo di *Aspergillus flavus* e sulla produzione di aflatossine. Protezione delle Colture, 12 (2), 64-65.

Spadaro D., Prencipe S., Valente S., Piombo E., Garibaldi A., Gullino M. L. (2019) Presenza di funghi micotossigeni e gestione del rischio di contaminazione da micotossine nella frutta secca. Protezione delle Colture, 12 (2), 17-25.

Valente S., Cometto A., Gullino M. L., Garibaldi A., Spadaro D. (2019) Studio della biosintesi della griseofulvina, micotossina prodotta dal patogeno *Penicillium griseofulvum*. Protezione delle Colture, 12 (2), 81-82.

Valente S., Gullino M. L., Garibaldi A. Spadaro D. (2018) Attività antifungina della griseofulvina, un composto prodotto da *Penicillium griseofulvum*. Protezione delle Colture, 11 (2), 52-53.

Valente S., Meloni G. R., Gullino M. L., Garibaldi A., Spadaro D. (2019) Ritrovamento di *Aspergillus parasiticus* forti produttori di alfatossine su nocciole turche. Protezione delle Colture, 12 (2), 82-83.

Valente S., Piombo E., Prencipe S., Meloni G. R., Gullino M. L., Garibaldi A., Gabaldòn T., Spadaro D. (2019) Genomica e HPLC-MS/MS per esplorare il potenziale micotossigeno di *Penicillium* spp.. Protezione delle Colture, 12 (2), 84-85.