Effect of agronomic programmes with different susceptibility to deoxynivalenol risk on emerging contamination in winter wheat

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Title: Effect of agronomic programmes with different susceptibility to deoxynivalenol risk on emerging contamination in winter wheat.

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Abstract

Deoxynivalenol (DON) is the most prevalent mycotoxin in small cereal crops throughout the world, and its occurrence is closely linked to the presence of Fusarium Head Blight (FHB) disease.

In order to minimize the sanitary risk, wheat cropping systems are commonly designed to control DON contamination, as this represents the main target contaminant. However, several other mycotoxins and secondary metabolites produced by *Fusarium* and other fungal species have been detected in wheat. The objective of this study was to evaluate whether the application of agronomic programmes with different susceptibility to DON contamination could also affect the occurrence of emerging mycotoxins in wheat kernels.

Field experiments have been conducted in North Italy, under naturally-infected conditions, over a period of 3 growing seasons, by comparing 4 field programmes, which were constituted by the combination of wheat cultivars (a durum wheat variety that is susceptible to DON contamination and a common moderately resistant one) and 2 fungicide applications at heading (untreated control compared to an azole application at heading).

Grain samples have been analyzed by means of a dilute-and-shoot multi-mycotoxin LC-MS/MS method, and 43 fungal metabolites were detected. In addition to DON, the most abundant compounds were aurofusarin, culmorin and deoxynivalenol-3-glucoside, which were detected in all the growing seasons and agronomic strategies. Other trichothecenes and zearalenone derivatives were also found, but in clearly lower concentrations.

Contamination by enniatins and moniliformin, produced by other *Fusarium* species e.g. *Fusarium avenaceum*, alternariol, alternariol methyl ether and tentoxin, produced by *Alternaria* species, has been observed for all the compared growing seasons. The presence of other mycotoxins and secondary metabolites was clearly affected by the

46 climatic conditions: fumonisin, beauvaricin, bikaverin, fusaric acid and butenolid were
47 detected in the warmer growing seasons, while chrysogine, infectopyrone, secalonic acid
48 and ergot alkaloids (sum of 13 toxins) were only found in the more rainy and cool seasons.
49 Equisetin, decalonectrin, toxin T-2 and HT-2 were only found in traces.
50 The application of the field programmes clearly affected DON contamination in each
51 growing season: a significant increase in this toxin has been observed moving from the
52 lowest risk agronomic strategy to the highest one. The application of the most favourable
53 DON control field programme (a moderately resistant variety combined with fungicide
54 application at heading) reduced the content of this mycotoxin by 89%, compared to the
55 worst programme (untreated susceptible variety).
56 The application of the less risky agronomic strategy for DON contamination led to a
57 significant reduction (>84%) of all the other mycotoxins produced by the DON producing
58 fungal species. Moreover, although the considered agronomic factors (variety susceptibility
59 and fungicide application) resulted in a control efficacy that varied in function of the
60 environmental conditions and the type of mycotoxin, the results show a clear reduction
61 trend, after the application of agronomic strategies that are able to minimize the DON
62 content, for almost all the other *Fusarium*, *Alternaria*, *Claviceps* and *Penicillium*
63 metabolites.
64 The results summarized in this work, which have been obtained under different
65 environmental and agronomical conditions, allow a first assessment to be made of the
66 agronomic strategies that could be applied to control emerging mycotoxins in wheat.
67

KEYWORDS: common wheat, durum wheat, fungicide, fusarium head blight, emerging mycotoxins.

ABBREVIATIONS

3-ADON, 3-acetyldeoxynivalenol; 15-ADON, 15-acetyldeoxynivalenol; AME, alternariol methyl ether; AOH, alternariol; ANOVA, Analysis of variance; AUR, aurofusarin; BEA, beauvericin; BIK, bikaverin; BUT, butenolide; CHRY, chrysogine; CULM, culmorin; DEC, decalonectrin; DON, deoxynivalenol; DON-3-G, deoxynivalenol-3-glucoside; EC, European Commission; EFSA, European Food Safety Authority; ENNs, Enniatins A, A_1, B, B_1, B_2; EQU, equisetin; EAs, ergot alkaloids; FA, fusaric acid; FB, fumonisins; FUS, fusaproliferin; GDD, Accumulated growing degree days; GS, Growth stage; INF, infectopyrone; LC-MS/MS, Liquid chromatography coupled with tandem mass spectrometry detection; LOD, limit of detection; LOQ, limit of quantification; MON, moniliformin; MR, moderately resistant; MS, mass spectrometry detection; NIV, nivalenol; S, susceptible; TENT, tentoxin; TW, test weight; SAD, secalonic acid; ZEA, zearalenone; ZEA-4-S, zearalenone-4-sulphate.

1. INTRODUCTION

Mycotoxins are secondary metabolites that are produced by several fungal species, which could have a range of toxic properties, including carcinogenicity and neurotoxicity, as well as developmental and reproductive toxicity for humans and reared animals and could result in illnesses and economic losses (Pestka and Smolinski, 2005).

Among the various agricultural commodities, cereals are most likely to be contaminated, and deoxynivalenol (DON), a type-B trichothecene produced by *Fusarium* spp., is the most prevalent toxin in small cereal crops throughout the world (Larsen et al., 2004). Regulatory limits have been set by the European Commission (EC) to protect humans from this mycotoxin exposure through cereal consumption (EC No. 1881/2006 and EC No. 1126/2007, with a limit of 1250 and 1750 μg kg^{-1} in unprocessed common and durum wheat, respectively). The occurrence of DON in wheat and other small cereals is closely linked to the presence of Fusarium Head Blight (FHB) disease, which causes total or partial premature ear senescence and consequent negative impacts on both crop yields and grain quality. Different *Fusarium* species are involved in promoting this disease, although *F. graminearum sensu stricto* and *F. culmorum,* are the most important FHB agents and the main causes of DON accumulation in grains in temperate areas (Yli-Mattila, 2010; Somma et al., 2014).

Although DON contamination in wheat grains depends on the meteorological conditions, particularly at flowering (van der Fels-Klerx *et al.*, 2013), an important role is played by agronomic factors, such as crop rotation, debris management, variety susceptibility and fungicide applications (Pirgozliev et al., 2003; Koch et al., 2006). At present, the most effective approaches adopted to minimize DON occurrence in wheat are the use of preventive agronomic practices to reduce the pathogen inocula in the field, the cultivation

109 of varieties that are less susceptible to FHB and the application of fungicides that are
110 effective in controlling *Fusarium* spp, according to an integrated approach that addresses
111 all of the possible risk factors (Blandino et al., 2012). Thus, in order to ensure low sanitary
112 risks, wheat cropping systems in temperate areas are generally designed to control DON
113 contamination, as this is the main target contaminant. However, to date, about 400
114 different mycotoxins have been identified in different commodities, and several of these
115 have been found in cereals (Berthiller et al., 2013). These other fungal metabolites, some
116 of which have been referred to as “emerging” (Streit et al., 2013), have not yet received
117 detailed scientific attention. The European Food Safety Authority (EFSA) is currently
118 working on establishing a scientific opinion on the risks to public health related to the
119 presence of emerging mycotoxins in feeds and food (EFSA, 2010; 2014). Nowadays, it is
120 necessary to collect occurrence data on these mycotoxins in the most important cereal
121 areas in the EU, in order to correctly consider the risk of exposure and to make risk
122 assessments. In addition, there is also a greater interest in verifying the effect of the Good
123 Agricultural Practices (GAP) that are normally applied to control FHB and DON, which is
124 the reference mycotoxin for wheat in temperate areas, on the content of emerging
125 mycotoxins in this crop. Since these compounds could be produced by other *Fusarium*
126 species that are not directly involved in FHB disease, as well as by other fungal species
127 belonging to the *Alternaria*, *Penicillium* and *Claviceps* families, more detailed knowledge
128 on the environmental and agronomic conditions that promote their occurrence is essential
129 in order to set up field programmes that will be able to minimize the overall sanitary risk for
130 grain.
131 The aim of this study was to investigate the effect of agronomic strategies, with different
132 susceptibility to DON, on the occurrence of emerging mycotoxins and fungal metabolites in
133 wheat in different production situations.

2. MATERIALS AND METHODS

2.1. Chemicals

Methanol and acetonitrile (both LC gradient grade) were purchased from J.T. Baker (Deventer, The Netherlands); ammonium acetate (MS grade) and glacial acetic acid (p.a.) were obtained from Sigma–Aldrich (Vienna, Austria). Water was purified successively by means of reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France).

All the fungal metabolite standard solutions were stored at −20°C and were brought to room temperature before use.

2.2. Field Experimental Design and Samples

The effect of agronomic strategies with different susceptibility to deoxynivalenol contamination on emerging mycotoxin occurrence in wheat was studied in North-West Italy in the 2010-11 growing season at Cigliano (45° 18’ N, 8° 01’ E; altitude 237 m), in a sandy-loam soil (Typic Hapludalfs) and in the 2011-12 and 2012-13 growing seasons at Carmagnola (44° 50’ N, 7° 40’ E; altitude 245 m) in a loam soil (Typic Udifluvents).

In each growing season, 4 field programmes, resulting from a factorial combination of 2 wheat cultivars (cv), with different susceptibility to DON contamination, and 2 fungicide applications, were compared in naturally-infected field conditions:

- a common wheat cv., classified as moderately resistant (MR) to FHB infection and DON contamination, combined with an azole fungicide application at heading [growth stage (GS) 59] (Zadoks et al., 1974),
- an untreated common wheat MR cv.,
- a durum wheat cv., classified as susceptible (S) to FHB infection and DON contamination, combined with an azole fungicide application at heading (GS59),
- an untreated durum wheat S cv.

159 The common wheat MR cv. was Generale (Consorzio nazionale sementi, Conselice, RA,
160 Italy), while the durum wheat S cv. was Saragolla (Produttori Sementi Bologna S.p.A.,
161 Argelato, BO, Italy). In temperate areas, the durum wheat is characterized by a generally
162 higher susceptibility to FHB than common one, limiting their potential cultivation in these
163 areas.
164 The applied azole fungicide was prothioconazole [Proline®, Bayer, Italy, emulsifiable
165 concentrate formulation (EC), applied at 0.250 kg of active ingredient (AI) ha^{-1}] and it was
166 sprayed at heading (GS59). The fungicides were applied at the manufacturer's
167 recommended field rates using a 4 nozzle precision sprayer (T-Jeet 110/04) with a fine
168 mist at a slow walk to ensure effective coverage. The delivery pressure at the nozzle head
169 was 324 KPa. No other fungicide was applied to any other GS to control foliar diseases.
170 The commonly adopted agronomic growing area technique was applied. Briefly, the
171 previous crop was maize, the field was ploughed each year, incorporating the debris in the
172 soil, and this was followed by disk harrowing to prepare a proper seedbed. Planting was
173 conducted in 12 cm wide rows at a seeding rate of 450 seeds m^{-2} in the last decade of
174 October. The weed control was conducted with isoproturon and diflufenican at wheat
175 tillering (GS 23). A total of 170 kg N ha^{-1} was applied as a granular ammonium nitrate
176 fertilizer, split into 50 kg N ha^{-1} at wheat tillering (GS 23), 80 kg N ha^{-1} at stem elongation
177 (GS 32) and 40 kg N ha^{-1} at booting (GS 46). The sowing and harvest dates, and the date
178 of fungicide application at heading are reported in Table 1 for each growing season.
179 Each field condition treatment was assigned to an experimental unit using a completely
180 randomised block design with three replicates. The plot size was 7 x 2 m.
181 The grain yields were obtained by harvesting the whole plot using a Walter Wintersteiger
182 cereal plot combine-harvester. A subsample was taken from each plot to determine the
183 grain moisture and test weight (TW). The TW was determined using a Dickey-John

184 GAC2000 grain analysis meter, according to the supplied programme. The grain yield
185 results were adjusted to a 13 % moisture content.
186 The harvested grains were mixed thoroughly and 2 kg grain samples were taken from
187 each plot to analyze the mycotoxin content.
188

189 ## *2.3. FHB symptoms*

190 FHB incidence and severity were recorded for each plot by carrying out visual evaluations
191 of the disease at the soft dough stage (GS 85). FHB head blight incidence was calculated
192 as the percentage of 200 ears per plot with symptoms.
193 FHB severity was calculated as the percentage of kernels per ear with symptoms. A scale
194 of 1 to 7 was used in which each numerical value corresponded to a percentage interval of
195 surfaces exhibiting visible symptoms of the disease, according to the following schedule: 1
196 = 0-5%, 2 = 5-15 %, 3 = 15-30%; 4 = 30-50 %, 5 = 50-75%, 6 = 75-90%, 7 = 90-100%
197 (Parry et al., 1995). The FHB severity scores were converted into percentages of ears
198 exhibiting symptoms, and each score was replaced with the mid-point of the interval.
199

200 ## *2.4. Multi-mycotoxin LC-MS/MS analysis*

201 A 2 kg representative sample of grain from each plot was ground using a ZM 200 Ultra
202 Centrifugal Mill (Retsch GmbH, Haan, Germany) fitted with a 1 mm aperture sieve, and the
203 resulting whole meal was used directly for the extraction. Five g representative sub-
204 samples of the milled material were extracted using 20 mL of a mixture of
205 acetonitrile/water/acetic acid 79:20:1 (v/v/v). After extraction, the samples were
206 centrifuged, diluted 1:1 and injected, as described in detail by Sulyok et al. (2006).
207 Detection and quantification were performed using a QTrap 5500 LC–MS/MS System
208 (Applied Biosystems, Foster City, CA), equipped with a TurboIonSpray electrospray

ionization (ESI) source and a 1290 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic separation was performed at 25 °C in a Gemini® C18-column, 150×4.6 mm i.d., 5 µm particle size, equipped with a C18 security guard cartridge, 4×3 mm i.d. (all from Phenomenex, Torrance, CA, US). The chromatographic and mass spectrometric parameters of the investigated analytes were described by Malachova et al. in 2014. The results of the mycotoxin concentrations were corrected on the basis of the recovery rate.

2.5. Statistical analysis

The normal distribution and homogeneity of variances were verified by performing the Kolmogorov–Smirnov normality test and the Levene test, respectively.

An analysis of variance (ANOVA) was conducted separately for each experiment, to verify the assumption of homogeneity, in order to evaluate the effect of the application of the agronomic strategy on grain yield, test weight, FHB incidence and severity, DON contamination and emerging mycotoxin occurrence using a completely randomized block design, in which the agronomic strategy was the independent variable. The incidence and the severity values of fungal ear rot incidence and severity were previously transformed using $y'=\arcsin\sqrt{x}*180/\pi$ as percentage data derived from counting. The fungal metabolite concentrations were transformed using the $y'=\ln(x+1)$ equation to normalize the residuals.

Simple correlation coefficients were obtained for all the detected fungal metabolites, relative to each another, by joining the data sets that referred to the three growing seasons.

SPSS for Windows, Version 22.0 statistical package (SPSS Inc., Chicago), was used for the statistical analysis.

3. RESULTS

3.1. Meteorological data

The three growing seasons were subject to different meteorological trends, as far as both rainfall and temperature (expressed as growing degree days, GDDs) from the end of tillering to the harvest are concerned (Table 2). Frequent rainfall occurred in 2011, at the tillering stages (March) and at the end of ripening (June). Furthermore, the rainfall in this growing season at the Cigliano site was very low close to the anthesis stage (May) and the GDDs during this period were higher than in the other years. Instead, in 2012 and 2013, rainfall was frequent and regular from stem elongation (April) to the end of flowering. In 2013, the month of May was characterized by a higher incidence of rainy days and lower GDDs than in the previous years.

3.2. FHB symptoms

The rapid canopy senescing process that occurred in the 2010-11 growing season prevented visual measurements of the disease at the dough stage.

In the 2011-12 and 2012-13 growing seasons, ANOVA showed a significant effect of the fungicide treatments on FHB symptoms (Table 3). The application of prothioconazole at heading led to an average reduction of 27% and 13% for FHB incidence and severity, respectively, compared to the untreated control. In the untreated conditions, significant differences were only observed between common and durum wheat for FHB incidence in the 2011-12 growing season.

256 ### *3.3. Yield parameters*

257 ANOVA only showed a significant effect of field programmes on grain yield in the 2011-12
258 growing season (Table 3): the fungicide application significantly increased the durum
259 wheat yield by 22%, compared to the untreated control, while no difference was observed
260 for common wheat. In all the growing seasons, common wheat resulted in a significantly
261 higher TW than durum wheat (+11%). Although the fungicide application did not lead to a
262 significantly increase in TW, the application of this disease control strategy on average
263 increased this parameter by 1.2 kg hl^{-1}. Thus, an increasing trend of TW can be observed
264 moving from the strategy with the highest risk of DON contamination (untreated durum
265 wheat) to the one with the least risk (common wheat combined with a fungicide
266 application).

267

268 ### *3.4. Mycotoxin occurrence*

269 DON was detected in all of the investigated production situations (environment X field
270 programme) and the content of this mycotoxin was clearly related to the meteorological
271 conditions, particularly close to anthesis, in each growing season. DON contamination was
272 low and fell between 42 and 997 µg kg^{-1} in the 2010-11 growing season, while it ranged
273 between 119 and 4271 µg kg^{-1} and 446 - 2774 µg kg^{-1} in the 2011-12 and 2012-13
274 growing seasons, respectively.

275 Moreover, considering the 3 growing seasons and the different agronomic strategies, 43
276 other mycotoxins and secondary metabolites were detected: deoxynivalenol-3-glucoside
277 (DON-3-G), 3-acetyldeoxynivalenol (3-ADON), nivalenol (NIV), culmorin (CULM),
278 zearalenone (ZEA), zearalenone-4-sulphate (ZEA-4-S), butenolide (BUT), aurofusarin
279 (AUR), enniatins (ENNs, sum of ENN A, ENN A_1, ENN B, ENN B_1, ENN B_2), moniliformin
280 (MON), chrysogine (CHRY), fumonisins (FB, sum of FB_1, FB_2, FB_3), beauvericin (BEA),

281 bikaverin (BIK), fusaric acid (FA), decalonectrin (DEC), T-2 toxin, HT-2 toxin, equisetin
282 (EQU), alternariol (AOH), alternariol methyl ether (AME), tentoxin (TENT), infectopyrone
283 (INF), secalonic acid D (SAD) and ergot alkaloids (EAs, sum of ergometrine,
284 ergometrinine, ergocristine, ergocristinine, ergocornine, ergocorninine, ergocryptine,
285 ergocryptinine, ergosine, ergotamine, ergovaline, agroclavine and chanoclavine).
286 In addition to DON (average contamination of 1067 μg kg^{-1}), the most abundant
287 metabolites were AUR, CULM and DON-3-G, which were detected in all of the samples,
288 with an average contamination of 701 μg kg^{-1}, 689 μg kg^{-1} and 250 μg kg^{-1}, respectively.
289 Other mycotoxins produced by *F. graminearum* and *F. culmorum,* such as 3A-DON, NIV,
290 ZEA, ZEA-4S and BUT, were found in generally lower concentrations (< 28 μg kg^{-1}) and
291 not in all of the compared environmental and agronomical situations.
292 Table 4 reports the correlation coefficients and the significance between all of the recorded
293 mycotoxins and metabolites. Among the mycotoxins produced by *Fusarium graminearum*
294 and *culmorum*, all the previously reported ones, with the exception of NIV (r=0.30), result
295 to be significantly correlated to DON: the closest relationship was found for CULM
296 (r=0.94), and this was followed by DON-3G (r=0.74), 3A-DON (r=0.66), ZEA (r=0.63), BUT
297 (r=0.48) and ZEA-4S (r=0.44).
298 As far as the modified mycotoxins are concerned, the ratio between 3-ADON, a phase I
299 plant metabolite, and DON was always lower than 1% in all of the compared production
300 situations. On the other hand, the DON-3G/DON ratio was 54% 31% and 8%, in the 2010-
301 11, 2011-12 and 2012-13 growing seasons, respectively, while the ratio between the
302 different field programmes was more consistent within the growing seasons. The ZEA-
303 4S/ZEA ratio also clearly varied within the growing seasons and was 59%, 22% and 136%
304 in 2010-11, 2011-12 and 2012-13, respectively. Furthermore, the occurrence of this phase
305 II plant metabolite derived from sulfatation seem to be influenced above all by the

306 genotype, since the ZEA-4S/ZEA ratio on average was 134% and 11% for the common
307 and durum wheat cv., respectively.
308 Although AUR could be produced mainly by *F. graminearum* and *culmorum* species, it was
309 here found not to be significantly correlated to DON (r = 0.20), while it was significantly
310 related to ZEA-4S (0.70), ZEA (0.54), BUT (0.51), NIV (0.49) and CULM (0.45).
311 Conversely, this metabolite, which was found in all of the compared production situations
312 above the LOQ, showed a clear and more significant relationship with the other
313 mycotoxins produced by *F. avenaceum* (a probable AUR producer), such as ENNs
314 (r=0.85), MON (r=0.71) and BEA (r=0.68). The highest average content of all these
315 mycotoxins was found in the 2010-11 growing season, which was also characterized by
316 the lowest DON occurrence.
317 ENNs ranging from 7 to 698 μg kg^{-1} were found for all of the growing seasons and field
318 programmes. The most abundant compounds were enniatin B (48% of the cases) and B_1
319 (41% of the cases), while the other forms (A, B_2, B_3) were only found in traces. The ratio
320 between the different ENN forms was extremely stable within the different growing
321 seasons and field programmes. Unlike the AUR and ENNs, MON was found to be
322 correlated to the DON content (r=0.42), although the average content of this toxin was
323 higher in the 2010-11 growing season (80 μg kg^{-1}). CHRY, was only found in the 2012-13
324 growing season (average content in the positive samples of 16 μg kg^{-1}).
325 Among the other *Fusarium* toxins and metabolites, FB, BEA, BIK and FA, which are mainly
326 produced by the *Fusarium* species section *Liseola*, were found in traces in the 2010-11
327 and 2011-12 growing seasons, with an average concentration in positive samples of 12,
328 14, 5 and 44 μg kg^{-1}, respectively.
329 A significant correlation was found between DON and DEC, a metabolite produced by both
330 *F. graminearum* and *F. sporotrichioides*. This metabolite was found in all of the growing

seasons, but in half of the compared production situations, with an average concentration of 2 μg kg^{-1}. Among the mycotoxins produced by other *Fusarium* species, T-2 and HT-2 toxins (*F. sprorotrichioides*, *F. langsaethiae*, *F. poae*) were infrequent and often at very low contamination levels in all of the compared production situations. Conversely, EQU, produced by *F. equiseti*, was found in traces in the 2011-12 and 2012-13 growing seasons, but reached up to a maximum of 74 μg kg^{-1} in the 2010-11 season.

Among the toxins produced by the *Alternaria* species, AOH, AME and TENT were found in all the growing seasons. The occurrence of all of these toxins was higher in the 2010-11 growing season, with average contamination levels of 9, 6 and 3 μg kg^{-1}, respectively. AOH and AME did not result to be correlated to DON, while a significant relationship was observed for TENT, which, however, was not correlated to the other *Alternaria* toxins.

INF, produced by *A. infectoria*, was only found in samples collected in the 2012-13 growing season (average content in the positive samples of 70 μg kg^{-1}). SAD (8 μg kg^{-1}), produced by *Penicillium* and *Aspergillum spp.* and *Claviceps purpurea*, and EAs (21 μg kg^{-1}), produced by *Claviceps purpurea*, were only found in this growing season. Although produced by fungal species from different genera, the occurrence of INF, SAD and EAs was here found to be significantly correlated to each other ($r>0.70$). The main ergot alkaloids were ergometrinine (25%) and ergocristine (22%), followed by ergocornine (16%), ergocryptine (13%) and ergometrine (11%), while the other metabolites were only found in traces. Common wheat was characterized by a higher relative occurrence of ergocristine and ergocornine, while durum wheat was characterized more by erogometrinine and ergocryptine.

3.5. Effect of the agronomic strategies on mycotoxin contamination

In all the trials, the DON contamination was significantly affected by the application of the tested field programmes (Figure 1): the efficacy of the fungicides in reducing DON was on average 58% and 45% for common and durum wheat, respectively. Moreover, a higher susceptibility to the accumulation of this mycotoxin was confirmed for the durum wheat (+ 6 times), compared to the common wheat.

With the exception of the comparison of the untreated common wheat and the durum wheat combined with a fungicide application in the 2011-12 and 2012-13 growing seasons, which resulted to be similar, a clear and significant increase in DON content was reported moving from the lowest risk strategy for this mycotoxin contamination to the highest one. Therefore, the most favourable strategy for DON contamination control (MR variety and fungicide application at heading) reduced this mycotoxin content by 94%, 95% and 78%, compared to the worst one (untreated S variety), in the 2010-11, 2011-12 and 2012-13 growing seasons, respectively.

Moreover, ANOVA also showed a significant effect of the compared agronomic strategies in almost all of the growing seasons for DON-3G, 3A-DON, NIV, CULM, ZEA, ZEA-4S and BUT (Table 5). The application of the less risky agronomic strategy for DON contamination led to a clear reduction in all of these mycotoxins, compared to the worst case. The best DON contamination control strategy reduced the DON-3G, 3A-DON, NIV, CULM, ZEA, ZEA-4S and BUT contents by 89%, 95%, 89%, 98%, 84% and 98%, respectively, compared to the worst one.

A similar trend was also observed within the agronomic strategies for AUR and ENNs (Table 6), which were significantly reduced by the cultivation of the common MR wheat compared to the S durum one. The application of fungicide led to a reduction in the content of both these mycotoxins, which was significant in several cases, in particular for

AUR. The MON content was affected less clearly by the application of the field programmes, although the average content of this mycotoxin was also reduced, moving from the worst to the best strategy for DON control. The adoption of the most careful cropping system on average minimized the AUR, ENN and MON contents by 99%, 90% and 94%, respectively.

CHRY was not affected by variety susceptibility in the 2012-13 growing season, while it was significantly reduced, by 74%, in both cvs. for the fungicide application at heading.

The FB, BEA, BIK and FA occurrences were significantly higher in the durum than in the common wheat in the 2010-11 growing season, while no significant differences were observed for the fungicide application (Table 7). A significant effect was only observed for cv susceptibility for BEA in the 2011-12 period, while this mycotoxin was not found in the next year. As far as the other *Fusarium* toxins are concerned, the highest occurrences of DEC, HT-2 toxin and EQU were always found for the worst agronomic strategy for DON contamination (Table 8), which often resulted in a significantly higher content than for the other field programmes.

Moreover, the adoption of the agronomic strategies in all the growing seasons also affected the contamination by *Alternaria* toxins, in a similar way to that of DON: in all the growing seasons, with the exception of AME in 2011-12, the AOH, AME, TENT and INF contents were significantly higher in the durum S cv. than in the common MR one (Table 9). In addition, the application of a fungicide significantly reduced the AME content in the durum wheat in the 2010-11 season and TENT in common wheat in the 2011-12 period. The application of the more effective GAP for DON control led to average reductions of 96%, 96%, 86% and 42% of AOH, AME, TENT and INF, respectively, compared to the riskiest strategy.

405 EAs and SAD were found only in the 2012-13 growing seasons. The EAs content in the
406 common wheat was < 1 μg kg^{-1} for the wheat treated with fungicide and 8 μg kg^{-1} in the
407 untreated control (data not shown). The durum wheat content was significantly higher
408 ($P<0.01$) and reached 43 and 33 μg kg^{-1} with and without the fungicide application,
409 respectively. No significant differences were observed for SAD, although its occurrence
410 was 2, 7, 13 and 10 μg kg^{-1} in the treated common wheat, the untreated common wheat,
411 the treated durum wheat and the untreated durum wheat, respectively. The most
412 favourable strategy for DON control reduced the EA and SAD contents by 98% and 84%,
413 respectively, compared to the worst case.
414

4. DISCUSSION

The data collected in 3 growing seasons with different climatic conditions give clear information on the presence of different fungal metabolites in wheat kernel at harvest, also considering the so called “emerging mycotoxins”, which till now have not received detailed scientific attention, particularly in raw materials.

Data confirm that DON is the most prevalent mycotoxin in winter wheat in temperate growing areas (Larsen et al., 2004), while CULM was present in similar concentrations as DON and is closely associated with this toxin (Ghebremeskel and Langseth, 2000). Considering the 3 growing seasons, the sum of DON and CULM on average represents more than 60% of the total mycotoxins that were encountered.

In addition to DON, the most abundant metabolites were AUR, CULM and DON-3-G, while other mycotoxins produced by *F. graminearum* and *F. culmorum,* such as 3A-DON, NIV, ZEA, ZEA-4S and BUT, were found in generally lower concentrations. These results are in agreement with the results of a another survey conducted on common and durum wheat in North Italy (Bertuzzi et al., 2014). In literature, the co-presence in wheat of DON, other type B trichothecens and ZEA has been variable, since it depends to a great extent on the predominant species or chemotype strains. The low rate of NIV and DON occurrence observed in the present work (average NIV/DON=0.5%), confirms that the *F. graminearum* DON chemotypes in Italy are predominate over the chemotype strains that are able to produce NIV (Somma et al., 2014). Conversely, in South America (Del Ponte et al., 2012) and Asia (Tanaka et al., 2010), the co-contamination of DON and NIV has been reported at a similar level. The correlation between DON and ZEA in wheat has been found to be very low in Poland (Chelkowsky et al., 2012), compared to the present experiment.

Moreover, the data reported on the occurrence of modified mycotoxins (Rychlik et al., 2014) confirm the results of previous studies (Berthiller et al., 2009; De Boevre et al., 2012; Scarpino et al., 2015) in which the relationship between DON and its phase II plant metabolite changed in relation to the environmental conditions. The rate between DON-3-G/DON was lower in growing seasons with frequent rainfall and low temperature from stem elongation to the end of flowering, while this ration was higher in the drier and warmer season.

Overall, the levels of ENNS, MON and BEA are comparable with those reported in wheat in the North (Lindblad et al., 2013, Uhlig et al., 2013) and South of Europe (Jestoi et al., 2004 and 2008, Scarpino et al., 2015). As far as the occurrence of AUR is concerned, the higher correlation found between this metabolite and ENNS, MON and BEA suggest that, in the compared conditions, the occurrence of AUR is related more to *F. avenaceum* (Uhlig et al., 2006). CHRY, another metabolite produced by *F. avenaceum* (Uhlig et al., 2006), was found only in the cooler year.

T-2 and HT-2 toxins (*F. sprorotrichioides*, *F. langsaethiae*, *F. poae*) were infrequent and often at very low contamination levels in all of the compared production situations, thus highlighting a low incidence of these compounds in winter wheat compared to other small cereals, such as oats (van der Fels-Klerx and Stratakou, 2010). Otherwise, the occurrence of metabolite produced by other *Fusarium* species (*F. verticillioides*, *F. equiseti*) is more frequent, but only in traces.

The occurrence of *Alternaria* metabolites is lower than those reported in wheat cultivated in North Europe (Uhlig et al., 2013), but confirm previous findings for Southern European growing areas (Scarpino et al., 2015). The occurrence of AOH and AME was higher in 2010-11 growing season, with drier conditions before and during flowering, less favourable to *Fusarium* ear infection. Otherwise INF, produced by *A. infectoria* (Larsen et al., 2003),

463 SAD produced by *Penicillium* and *Aspergillum spp.* and *Claviceps purpurea* (Wang and
464 Polya, 1996), and EAs, produced by *Claviceps purpurea* (Malysheva et al., 2014), seem to
465 be related to more rainy environmental conditions.
466 Overall, the results obtained in the current study point out the crucial role of the
467 environmental conditions on the diffusion of different mycotoxins, both the common and
468 emerging ones, in winter wheat (Doohan et al., 2003). Moreover, the collected data
469 highlight the important role that cultural practices can play in determining their level of
470 contamination in the grains. The hypothesis on the different susceptibilities of the
471 compared agronomic field programmes to DON occurrence was confirmed for all the
472 growing seasons. This finding is in agreement with previous findings pertaining to the
473 same environments confirms previous data obtained in the same (Blandino et al., 2006;
474 2009a; Blandino et al., 2012) and in other environments (Beyer et al., 2006; Koch et al.,
475 2006). Above all, conditions such as preceding host crops, especially maize and sorghum,
476 which leave high amount of infected residues in the field, and the cultivation of a
477 susceptible variety contribute to heavy DON contamination of wheat crops (Blandino et al.,
478 2012). In addition, our results confirm that the efficacy of fungicide in reducing DON
479 content is related to the environmental and agronomic conditions (Blandino et al., 2011).
480 Thus, mycotoxin control in wheat should first focus on the agronomic factors that influence
481 the occurrence of inoculum (preceding crop and management of crop residues) and the
482 variety susceptibility to fungal infection, according to a integrate multiple strategy approach
483 (Beyer et al., 2006).
484 Moreover, the data collected allow a first holistic evaluation of the effect of the combination
485 of crop practices in wheat on several fungal metabolites, produced by different species.
486 Although the considered agronomic factors (variety susceptibility and fungicide application)
487 resulted in a variable control efficacy, in function of the environmental conditions and type

488 of mycotoxin, the results show a clear and similar reduction trend after the application of
489 agronomic strategies for DON control for almost all of the mycotoxins present at different
490 contamination levels. This effect was observed for toxins produced by *Fusarium* species
491 involved in FHB infection in temperate areas, but also for metabolites related to the
492 development of other *Fusarium* species that are not involved directly in this disease, such
493 as *F.* section *Liseola* or *F. equisetum*, or other species that can cause other diseases,
494 such as *Claviceps* spp. or saprophytic fungi such as *Alternaria* spp.
495 ~~Thus, in the considered production situations, no evidence has emerged of an increase in~~
496 ~~any mycotoxin as a consequence of a modification in the fungal community, related to the~~
497 ~~different control capacities of the applied strategies on fungal species characterized by~~
498 ~~different ecologies~~. ~~In other words~~, Thus, in the considered production situations the
499 application of field programmes that are able to reduce the predominant FHB pathogens,
500 which are responsible for most of the contamination of winter wheat (DON, CULM), did not
501 lead to an increase in any other metabolite, as a consequence of the development of other
502 species that occupy their ecological niche, and they even resulted to be reduced.
503 The change in the relative competition capacity among fungal species with the application
504 of a control factor, which results in a reduction of certain mycotoxins and the simultaneous
505 increase of others, has been named the “flora inversion” phenomenon and it has been
506 observed for *Fusarium* toxins in maize by Folcher et al. (2010). A change in the relative
507 competition capacity between FB and trichothecens producers was observed for this crop,
508 in the same growing areas as those of the present study, as a consequence of the control
509 of the insects that are responsible for *Fusarium* infection (Blandino et al., 2009b; Blandino
510 et al., 2015). The co-occurrence of fungal species in maize seems to be more complex to
511 manage in temperate areas than that of small cereals. The ecology of the maize infecting
512 fungal species (*Fusarium* section *Liseola* and *Discolor*, *Aspergillus flavus* and *Penicillium*

513 spp.) is slightly different and their relative occurrence is clearly influenced by the climatic
514 conditions during the crop cycle (Doohan et al., 2003). Moreover, the importance of
515 pathway infection (seed, silk and through kernel damage caused by insects) changes
516 according to the fungal species (Munkvold et al., 1997) and this could lead to a different
517 control with the adoption of agronomic control factors, which would make it more
518 complicated to minimize the overall mycotoxin contamination of this crop.
519 Conversely, floral infection seems to be the main pathway for all of the different fungal
520 species in winter wheat (Xu and Nicholson, 2009), but there is a clear predominance of
521 *Fusarium* species, which are responsible for FHB, compared to the other *Fusarium* or
522 genus species. Thus, the negative interaction between different fungal species and
523 mycotoxins in wheat, as a consequence of the application of control strategies, could be
524 more infrequent than in maize, and could thus allow an easier setup of GAP for the overall
525 control of mycotoxins.
526 However, the presence of fungal competitive interaction phenomenon on small cereals
527 has also been documented in literature. In fact, it is well known that the application of
528 fungicides (such as strobilurins) at heading is less effective against *F. graminearum* and
529 *culmorum*, but is able to significantly reduce the non-toxigenic *Microdochium nivale*, and it
530 could therefore increase DON contamination in wheat (Pirgozliev et al., 2003, Blandino et
531 al., 2006). Both the active ingredients and the timing of fungicide application could impact
532 on the composition of the Fusarium Head Blight disease complex (Audenaert et al., 2011).
533 Moreover, from a comparison of *Fusarium* and *Alternaria* species, it has emerged that the
534 different fungicide classes show a different growth reduction capacity (Müllenborn et al.,
535 2007). A field survey conducted in France on barley reported that DON contamination is
536 negatively correlated to that of T-2 and HT-2 toxin (Orlando et al., 2010), thus suggesting
537 the need for a different risk management for these mycotoxins in the considered

538 agronomic conditions. The main agronomic factors identified to increase the risk of T-2
539 and HT-2 toxins on barley were late sowing times, small grain cereals as previous crop
540 and minimum or no tillage (Orlando et al., 2010).
541 Thus, although, in the present study, the single and combined application of a genetic and
542 a chemical control of DON never led to an increase in the contents of any other mycotoxin
543 or fungal metabolite, it remains necessary to verify, whether the agronomic factors that
544 have been identified to reduce the risk of DON in winter wheat are different from those that
545 are able to control the other emerging mycotoxins in different production situations.

5. CONCLUSION

The results of these experiments, obtained under naturally-infected field conditions and conducted over three different growing seasons, underline that the agronomic strategies generally applied in temperate areas to control DON contamination also contribute to minimizing the risk of contamination of the other mycotoxins that could occur in winter wheat grains. Thus, wheat protection programmes, based on the combination of MR varieties and a fungicide application at heading, in order to control FHB, could clearly contribute to improving the global sanity of this crop, by reducing the mycotoxins produced by different *Fusarium* section species, but also other fungal genera, such as *Alternaria*, *Claviceps* and *Penicillium*.

These results, which need to be confirmed in other environments and considering other important risk factors, such as crop rotation and soil tillage or the application of other fungicide active substances with different mechanisms of action, suggest that the “flora inversion” phenomenon rarely occurs in winter wheat, thus making it easier to find and apply integrated management strategies for the overall control of mycotoxins in this crop.

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738

Table. 1

Main trial information on the field experiments conducted on winter wheat in North West Italy in the 2010 - 2013 period.

Growing season	Site	Sowing date	Fungicide application	Harvest date
2010 - 2011	Cigliano	30 October 2010	13 May 2011	01 July 2011
2011 - 2012	Carmagnola	21 October 2011	23 May 2012	11 July 2012
2012 - 2013	Carmagnola	24 October 2012	21 May 2013	12 July 2013

743 **Table. 2**

744 Total rainfall, rainy days, relative humidity and growing degree days (GDDs) from March to June 2010-2013 in the research sites.

Year	Site	Month	Rainfall (mm)	Rainy days	GDDs[a] (°C d^{-1})
2011	Cigliano	March	169	9	268
		April	59	5	474
		May	30	5	578
		June	198	14	627
		July	54	8	670
2012	Carmagnola	March	20	1	347
		April	148	13	365
		May	147	6	539
		June	19	2	674
		July	37	5	725
2013	Carmagnola	March	96	10	241
		April	144	11	406
		May	147	11	499
		June	35	3	623
		July	137	7	740

745 [a] Accumulated growing degree days for each month using a 0°C base.

746

747 **Table. 3**

748 Effect of agronomic strategies with different susceptibility to deoxynivalenol contamination on Fusarium head blight (FHB) incidence
749 and severity, grain yield and test weight of winter wheat; field experiments conducted in North West Italy in the 2010 - 2013 period.

Growing season	Agronomic strategies[a]	FHB incidence[b]		FHB severity[c]		Grain yield	Test weight
		T	N (%)	T	N (%)	t ha^{-1}	kg hl^{-1}
2010 - 2011	common wheat - fungicide	nd[d]		nd		5.4 a	79.1 a
	common wheat - untreated	nd		nd		5.2 a	78.4 a
	durum wheat - fungicide	nd		nd		4.7 a	66.4 b
	durum wheat - untreated	nd		nd		4.1 a	66.9 b
	P (F)[e]					0.109	< 0.001
	sem[f]					1.0	1.9
2011- 2012	common wheat - fungicide	37 C	37	8 c	2	6.2 b	83.6 a
	common wheat - untreated	62 Ab	78	28 a	11	6.0 b	82.9 a
	durum wheat - fungicide	52 B	62	13 bc	12	6.7 a	78.0 b
	durum wheat - untreated	65 A	81	19 b	23	5.5 c	74.3 b
	P (F)	0.001		0.001		< 0.001	0.001
	Sem	8.1		6.3		0.3	3.0
2012- 2013	common wheat - fungicide	38 C	50	10 b	10	6.6 a	83.8 a
	common wheat - untreated	62 B	77	30 a	26	6.4 a	82.7 a
	durum wheat - fungicide	45 C	75	18 b	19	7.5 a	79.9 b
	durum wheat - untreated	78 A	95	35 a	33	6.6 a	78.7 b
	P (F)	< 0.001		< 0.001		0.413	0.001
	Sem	10.5		7.7		1.3	1.6

750 [a] The used cultivars were Generale (common wheat) and Saragolla (durum wheat), which are characterized by a medium and a high susceptibility to FHB, respectively. The
751 fungicide treatment at heading was carried out using the prothioconazole active ingredient (formulation: EC, 0.250 kg active ingredient ha^{-1}).

752 [b] FHB incidence was calculated as the percentage of ears with FHB damage, considering 100 ears per sample.

753 [c] FHB severity was calculated as the percentage of kernels per ear with FHB damage, considering 100 ears per sample.

754 The reported FHB incidence and severity means are transformed (T; y'=arcsin√x*180/π) and not transformed (N) values.

755 [d] nd: not detected, the rapid canopy senescing process that occurred in the 20120-11 growing season did not allow visual measurements to be carried out.

756 [e] Means followed by different letters within each growing season are significantly different (the level of significance is shown in the table). The reported values are based on 3

757 replications.

758 [f] sem: standard error of the means.

759

760 **Table. 4**

761 Correlation matrix for mycotoxin contamination in winter wheat kernel.

	DON	DON3G	3ADON	NIV	CULM	ZEA	ZEA-4S	BUT	AUR	ENNs	MON	CHRY	FB	BEA	BIK	FA	DEC	T-2	HT-2	EQU	AOH	AME	TENT	INF	SAD
DON3G	0.74**																								
3ADON	0.66**	0.22																							
NIV	0.30	0.44**	0.10																						
CULM	0.94**	0.86**	0.51**	0.43**																					
ZEA	0.63**	0.33*	0.32	0.34*	0.60**																				
ZEA-4S	0.44**	0.09	0.27	0.27	0.50**	0.766**																			
BUT	0.48**	0.89**	-0.03	0.57**	0.69**	0.26	0.09																		
AUR	0.20	0.32	-0.02	0.49**	0.45**	0.54**	0.70**	0.51**																	
ENNs	0.14	0.54**	-0.20	0.45**	0.45**	0.22	0.34*	0.76**	0.85**																
MON	0.42*	0.78**	-0.05	0.50**	0.68**	0.35*	0.31	0.86**	0.71**	0.89**															
CHRY	0.34*	-0.26	0.50**	0.00	0.17	0.49**	0.64**	-0.36*	0.10	-0.28	-0.27														
FBs	-0.02	0.29	-0.05	0.44**	0.10	-0.04	-0.15	0.50**	0.20	0.32	0.28	-0.35*													
BEA	-0.14	0.20	-0.26	0.34*	0.11	0.06	0.19	0.48**	0.68**	0.72**	0.61**	-0.20	0.25												
BIK	-0.16	0.21	-0.30	0.64**	0.13	0.09	0.29	0.51**	0.78**	0.81**	0.63**	-0.22	0.32	0.70**											
FA	-0.12	0.25	-0.31	0.27	0.16	0.07	0.30	0.46**	0.78**	0.82**	0.70**	-0.24	0.24	0.79**	0.83**										
DEC	0.76**	0.51**	0.41*	0.44**	0.71**	0.60**	0.49**	0.31	0.26	0.08	0.32	0.36*	-0.14	-0.07	0.03	-0.06									
T-2	0.45**	0.67**	0.30	0.18	0.47**	-0.04	-0.19	0.51**	0.06	0.25	0.40*	-0.32	0.16	0.04	0.04	0.04	0.33*								
HT-2	0.24	0.44**	-0.16	-0.04	0.36*	0.14	0.21	0.35*	0.33	0.49**	0.58**	-0.20	0.10	0.14	0.18	0.51**	0.06	0.22							
EQU	-0.15	0.19	-0.25	0.81**	0.09	0.11	0.21	0.47**	0.64**	0.67**	0.56**	-0.18	0.36*	0.645**	0.89**	0.59**	0.08	0.03	0.06						
AOH	0.15	0.21	-0.03	0.34*	0.37*	0.48**	0.72**	0.33*	0.90**	0.74**	0.61**	0.07	0.13	0.48**	0.68**	0.75**	0.15	-0.02	0.55**	0.51**					
AME	0.04	0.32	-0.28	0.18	0.30	0.16	0.42*	0.44**	0.71**	0.79**	0.71**	-0.22	0.20	0.49**	0.63**	0.83**	-0.01	0.06	0.82**	0.44**	0.84**				
TENT	0.82**	0.86**	0.29	0.25	0.81**	0.46**	0.14	0.64**	0.13	0.25	0.56**	-0.08	0.21	-0.08	-0.10	0.03	0.64**	0.49**	0.50**	-0.11	0.13	0.22			
INF	0.24	-0.29	0.44**	-0.05	0.12	0.21	0.370*	-0.41*	-0.05	-0.34*	-0.33*	0.48**	-0.40*	-0.23	-0.25	-0.28	0.25	-0.36*	-0.23	-0.21	0.05	-0.20	-0.09		
SAD	0.23	-0.21	0.36*	0.26	0.16	0.34*	0.44**	-0.25	0.13	-0.17	-0.17	0.44**	-0.23	-0.13	0.00	-0.18	0.34*	-0.30	-0.22	0.13	0.20	-0.12	-0.08	0.71**	
EAs	0.29	-0.20	0.46**	0.01	0.19	0.35*	0.42*	-0.32	0.06	-0.25	-0.21	0.40*	-0.31	-0.17	-0.19	-0.21	0.27	-0.28	-0.18	-0.16	0.20	-0.14	-0.01	0.85**	0.86**

762 (*) = correlation significant at P ≤ 0.05; (**) correlation significant at P ≤ 0.01. The data reported in the table are Pearson product-moment correlation coefficients.

763 DON = deoxynivalenol, DON-3-G = deoxynivalenol-3-glucoside, 3-ADON = 3-acetyldeoxynivalenol, NIV = nivalenol, CULM = culmorin, ZEA = zearalenone, ZEA-4-S =

764 zearalenone-4-sulphate, BUT = butenolide, AUR = aurofusarin, ENNs = Enniatins A, A_1, B, B_1, B_2, MON = moniliformin, CHRY = chrysogine, FB = fumonisins, BEA = beauvericin,

765 BIK = bikaverin, FA = fusaric acid, DEC = decalonectrin, T-2 toxin, HT-2 toxin, EQU = equisetin, AOH = alternariol, AME = alternariol methyl ether, TENT = tentoxin, INF =
766 infectopyrone, SAD = secalonic acid, EAs = ergot alkaloids, sum of ergometrine, ergometrinine, ergocristine, ergocristinine, ergocornine, ergocorninine, ergocryptine,
767 ergocryptinine, ergosine, ergotamine, ergovaline, agroclavine and chanoclavine.
768

Table. 5

Effect of agronomic strategies with different susceptibility to deoxynivalenol contamination on the occurrence[a] of other fungal metabolites mainly produced by *F. graminearum* and *culmorum*; field experiments conducted in North West Italy in the 2010 - 2013 period.

Growing season	Agronomic strategies[b]	DON-3G		3A-DON		NIV		CULM		ZEA		ZEA-4S		BUT	
		T	N	T	N	T	N	T	N	T	N	T	N	T	N
			µg kg^{-1}		µg kg^{-1}		µg kg^{-1}		µg kg^{-1}		µg kg^{-1}		µg kg^{-1}		µg kg^{-1}
2010 - 11	common wheat - fungicide	3.0 c	19	< LOQ		<LOQ b	<LOQ	4.0 d	54	<LOQ c	<LOQ	<LOQ b	<LOQ	1.0 b	2
	common wheat - untreated	3.8 b	46	< LOQ		0.4 b	1	4.4 c	84	0.8 b	1	0.2 b	0	1.2 b	3
	durum wheat - fungicide	6.0 a	386	< LOQ		1.1 b	3	6.6 b	749	2.2 a	8	0.7 a	1	4.1 a	62
	durum wheat - untreated	6.0 a	395	< LOQ		2.9 a	21	6.9 a	1038	2.2 a	8	1.0 a	2	3.9 a	50
	P (F)[c]	< 0.001				0.003		< 0.001		< 0.001		< 0.001		< 0.001	
	sem[d]	0.13				1.0		0.23		0.43		0.19		0.94	
2011-12	common wheat - fungicide	4.0 d	52	<LOQ a	<LOQ	<LOQ b	<LOQ	4.6 c	99	<LOQ b	<LOQ	<LOQ a	<LOQ	0.4 b	1
	common wheat - untreated	5.5 c	264	1.6 a	7	0.5 ab	1	6.3 b	579	0.8 b	2	<LOQ a	<LOQ	1.2 b	5
	durum wheat - fungicide	6.1 b	440	1.4 a	5	0.9 ab	4	6.7 b	791	1.8 ab	8	<LOQ a	<LOQ	3.8 a	45
	durum wheat - untreated	6.9 a	1003	1.0 a	6	2.4 a	11	7.6 a	1988	2.6 a	14	0.5 a	1	4.4 a	84
	P (F)	< 0.001		0.468		0.020		< 0.001		0.014		0.058		< 0.001	
	Sem	0.31		2.0		1.2		0.42		1.22		0.31		1.15	
2012-13	common wheat - fungicide	3.6 c	38	0.2 b	0	0.6 b	1	5.6 c	275	0.2 c	0	<LOQ b	<LOQ		<LOQ
	common wheat - untreated	4.3 b	75	2.1 a	8	1.2 ab	2	6.3 b	587	0.5 c	1	0.6 b	1		<LOQ
	durum wheat - fungicide	4.8 a	121	2.3 a	9	1.5 a	4	6.7 b	818	1.7 b	5	0.6 b	1		<LOQ
	durum wheat - untreated	5.1 a	158	2.3 a	10	2.0 a	6	7.1 a	1203	3.3 a	28	1.3 a	3		<LOQ
	P (F)	< 0.001		< 0.001		0.009		< 0.001		< 0.001		< 0.001			
	Sem	0.31		0.60		0.6		0.27		0.40		0.20			

773 [a] The reported mycotoxin contamination means are transformed [T; y'= ln (x + 1)] and not transformed (N) values.

774 DON-3-G = deoxynivalenol-3-glucoside (LOQ = limit of quantification = 0.2 µg kg^{-1}), 3-ADON = 3-acetyldeoxynivalenol (LOQ = 0.2 µg kg^{-1}), NIV = nivalenol (LOQ = 0.2 µg kg^{-1}),

775 ZEA = zearalenone (LOQ = 0.2 µg kg^{-1}), ZEA-4-S = zearalenone-4-sulphate (LOQ = 0.1 µg kg^{-1}), BUT = butenolide (LOQ = 1.10 µg kg^{-1}).

776 15-ADON = 15-acetyldeoxynivalenol was also detected, but always under the LOQ (0.4 µg kg^{-1}).

777 [b] The used cultivars were Generale (common wheat) and Saragolla (durum wheat), which are characterized by a medium and a high susceptibility to FHB, respectively. The

778 fungicide treatment at heading was carried out using the prothioconazole active ingredient (formulation: EC, 0.250 kg active ingredient ha^{-1}).

779 [c] Means followed by different letters within each growing season are significantly different (the level of significance is shown in the table). The reported values are based on 3

780 replications.

781 [d] sem: standard error of the means.

782

Table. 6

Effect of agronomic strategies with different susceptibility to deoxynivalenol contamination on the occurrence[a] of aurofusarin (AUR), enniatins (ENNs), moniliformin (MON) and chrysogine (CHRY); field experiments conducted in North West Italy in the 2010 - 2013 period.

Growing Season	Agronomic strategies[b]	AUR		ENNs		MON		CHRY	
		T	N (ppb)	T	N (ppb)	T	N (ppb)	T	N (ppb)
2010 – 2011	common wheat - fungicide	3.6 c	36	2.9 c	18	<LOQ b	<LOQ		<LOQ
	common wheat - untreated	4.4 b	86	4.3 b	72	1.7 b	6		<LOQ
	durum wheat - fungicide	7.8 a	2488	6.2 a	501	4.8 a	117		<LOQ
	durum wheat - untreated	7.9 a	2701	6.4 a	620	4.8 a	117		<LOQ
	P (F)[c]	< 0.001		< 0.001		< 0.001			
	sem[d]	0.47		0.49		0.8			
2011- 2012	common wheat - fungicide	1.8 d	5	3.6 b	41	2.4 a	19		<LOQ
	common wheat - untreated	3.6 c	39	4.3 ab	97	3.1 a	31		<LOQ
	durum wheat - fungicide	5.6 b	270	4.9 ab	147	4.0 a	63		<LOQ
	durum wheat - untreated	6.4 a	616	5.6 a	265	4.7 a	112		<LOQ
	P (F)	< 0.001		0.019		0.093			
	Sem	0.52		1.0		1.6			
2012- 2013	common wheat - fungicide	2.5 c	11	2.4 c	10	<LOQ b	<LOQ	2.2 b	9
	common wheat - untreated	3.0 c	22	3.2 bc	27	1.5 ab	9	3.0 a	22
	durum wheat - fungicide	5.9 b	380	3.6 ab	38	2.9 a	18	1.5 b	4
	durum wheat - untreated	7.4 a	1757	4.5 a	87	3.4 a	31	3.3 a	28
	P (F)	< 0.001		< 0.001		0.007		0.001	
	Sem	0.51		0.65		1.3		0.55	

[a] The mycotoxin contamination means reported are transformed [T; y'= ln (x + 1)] and not transformed (N) values.

AUR = aurofusarin (LOQ = 0.1 µg kg^{-1}), ENNs = Enniatins A, A_1, B, B_1, B_2, (LOQ = 0.01 µg kg^{-1}), MON = moniliformin (LOQ = 0.3 µg kg^{-1}), CHRY = chrysogine (LOQ = 0.09 µg kg^{-1}).

[b] The used cultivars were Generale (common wheat) and Saragolla (durum wheat), which are characterized by a medium and a high susceptibility to FHB, respectively. The fungicide treatment at heading was carried out using the prothioconazole active ingredient (formulation: EC, 0.250 kg active ingredient ha^{-1}).

[c] Means followed by different letters within each growing season are significantly different (the level of significance is shown in the table). The reported values are based on 3 replications.

[d] sem: standard error of the means.

Table. 7

Effect of agronomic strategies with different susceptibility to deoxynivalenol contamination on the occurrence of mycotoxins[a] mainly produced by *Fusarium* section Liseola; field experiments conducted in North West Italy in the 2010 - 2013 period.

Growing Season	Agronomic strategies[b]	FB		BEA		BIK		FA	
		T	N (ppb)	T	N (ppb)	T	N (ppb)	T	N (ppb)
2010 - 2011	common wheat - fungicide	2.2 bc	8	<LOQ b	<LOQ	0.37 b	0.48	<LOQ b	<LOQ
	common wheat - untreated	1.9 c	6	<LOQ b	<LOQ	0.48 b	0.63	<LOQ b	<LOQ
	durum wheat – fungicide	2.9 a	19	3.7 a	39.8	3.21 a	27.58	4.5 a	100
	durum wheat – untreated	2.6 ab	13	3.3 a	26.4	2.48 a	11.00	4.6 a	97
	P (F)[c]	0.002		< 0.001		< 0.001		< 0.001	
	sem[d]	0.4		0.6		0.4		0.5	
2011- 2012	common wheat - fungicide	2.3 a	9	<LOQ c	<LOQ	<LOQ a	<LOQ	<LOQ a	<LOQ
	common wheat - untreated	1.8 a	6	0.1 bc	0.1	0.11 a	0.11	1.1 a	5
	durum wheat – fungicide	2.8 a	27	0.5 a	0.8	0.54 a	0.77	1.5 a	14
	durum wheat – untreated	2.2 a	9	0.5 ab	0.7	0.50 a	0.68	1.0 a	3
	P (F)	0.46		0.018		0.441		0.768	
	Sem	1.16		0.3		0.2		2.22	
2012- 2013	common wheat - fungicide		<LOQ		<LOQ		<LOQ		<LOQ
	common wheat - untreated		<LOQ		<LOQ		<LOQ		<LOQ
	durum wheat – fungicide		<LOQ		<LOQ		<LOQ		<LOQ
	durum wheat – untreated		<LOQ		<LOQ		<LOQ		<LOQ
	P (F)								
	Sem								

[a] The reported mycotoxin contamination means are transformed [T; y'= ln (x + 1)] and not transformed (N) values.

FB = fumonisins (LOQ = limit of quantification = 0.70 µg kg^{-1}), BEA = beauvericin (LOQ = 0.006 µg kg^{-1}), BIK = bikaverin (LOQ = 0.06 µg kg^{-1}), FA = fusaric acid (LOQ = 0.70 µg kg^{-1}).

804 [b] The used cultivars were Generale (common wheat) and Saragolla (durum wheat), which are characterized by a medium and a high susceptibility to FHB, respectively. The
805 fungicide treatment at heading was carried out using the prothioconazole active ingredient (formulation: EC, 0.250 kg active ingredient ha^{-1}).
806 [c] Means followed by different letters within each growing season are significantly different (the level of significance is shown in the table). The reported values are based on 3
807 replications.
808 [d] sem: standard error of the means.
809

810

Table. 8

Effect of agronomic strategies with different susceptibility to deoxynivalenol contamination on the occurrence[a] of decalonectrin (DEC), T-2 and HT-2 toxins and equisetin (EQU); field experiments conducted in North West Italy in the 2010 - 2013 period.

Growing season	Agronomic strategies[b]	DEC		T-2 toxin		HT-2 toxin		EQU	
		T	N (ppb)	T	N (ppb)	T	N (ppb)	T	N (ppb)
2010 - 2011	common wheat – fungicide	<LOQ b	<LOQ		<LOQ	<LOQ b	<LOQ	<LOQ b	<LOQ
	common wheat - untreated	<LOQ b	<LOQ		<LOQ	<LOQ b	<LOQ	0.5 b	1.1
	durum wheat – fungicide	<LOQ b	<LOQ		<LOQ	<LOQ b	<LOQ	4.1 a	68.3
	durum wheat – untreated	0.9 a	1.8		<LOQ	1.8 a	8.3	3.2 a	22.8
	P (F)[c]	0.053				0.042		< 0.001	
	sem[d]	1.3				1.1		0.8	
2011- 2012	common wheat – fungicide	<LOQ b	<LOQ	<LOQ a	<LOQ	<LOQ a	<LOQ	0.1 a	0.1
	common wheat - untreated	<LOQ b	<LOQ	<LOQ a	<LOQ	<LOQ a	<LOQ	0.5 a	0.7
	durum wheat – fungicide	0.6 b	1.0	<LOQ a	<LOQ	0.8 a	3.2	0.3 a	0.4
	durum wheat – untreated	1.6 a	4.2	0.3 a	0.3	1.6 a	4.2	0.4 a	0.5
	P (F)	0.001		0.441		0.077		0.406	
	Sem	1.4		0.24		1.1		0.5	
2012- 2013	common wheat – fungicide	0.3 b	0.4		<LOQ		<LOQ	0.1 b	0.1
	common wheat - untreated	0.8 b	1.2		<LOQ		<LOQ	0.2 b	0.2
	durum wheat – fungicide	0.7 b	1.1		<LOQ		<LOQ	0.2 b	0.2
	durum wheat – untreated	1.7 a	4.6		<LOQ		<LOQ	0.4 a	0.5
	P (F)	0.011						0.040	
	Sem	2.0						0.2	

[a] The reported mycotoxin contamination means are transformed [T; y'= ln (x + 1)] and not transformed (N) values.

DEC = decalonectrin (LOQ, limit of quantification = 0.06 µg/kg), T-2 toxin (LOQ = 0.2 µg/kg); HT-2 toxin (LOQ = 0.2 µg/kg), EQU = equisetin (LOQ = 0.09 µg/kg).

[b] The used cultivars were Generale (common wheat) and Saragolla (durum wheat), which are characterized by a medium and an high susceptibility to FHB, respectively. The fungicide treatment at heading was carried out using the prothioconazole active ingredient (formulation: EC, 0.250 kg active ingredient ha^{-1}).

[c] Means followed by different letters within each growing season are significantly different (the level of significance is shown in the table). The reported values are based on 3 replications.

820 [d] sem: standard error of the means.

821

822

Table. 9

Effect of agronomic strategies with different susceptibility to deoxynivalenol contamination on the occurrence[a] of mycotoxins produced by *Alternaria* species; field experiments conducted in North West Italy in the 2010 - 2013 period.

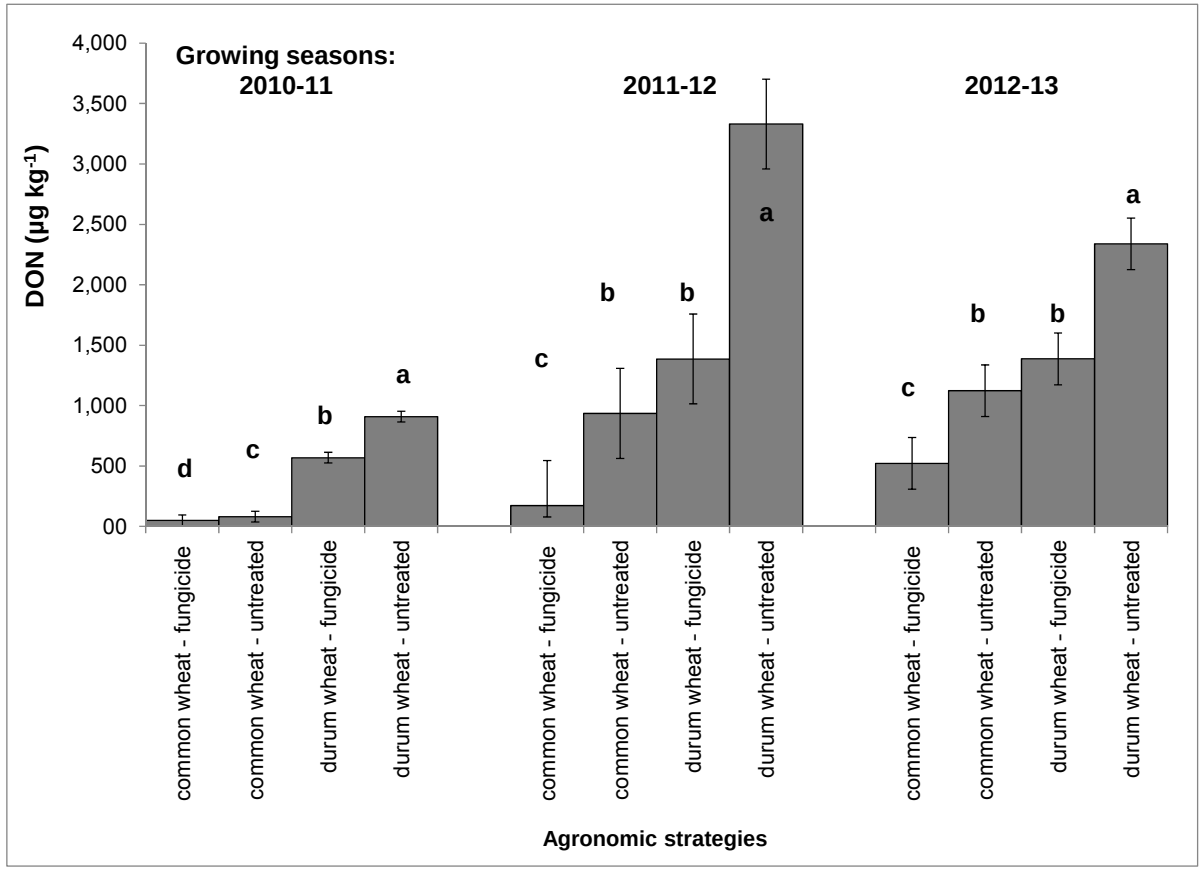
Growing season	Agronomic strategies[b]	AOH		AME		TENT		INF	
		T	N (ppb)	T	N (ppb)	T	N (ppb)	T	N (ppb)
2010 - 2011	common wheat - fungicide	0.9 b	1.5	0.1 c	0.1	0.7 c	0.9		<LOQ
	common wheat - untreated	1.3 b	2.7	0.4 c	0.5	1.1 bc	2.1		<LOQ
	durum wheat - fungicide	2.5 a	11.8	2.1 b	7.2	1.3 ab	2.7		<LOQ
	durum wheat - untreated	3.0 a	19.9	2.9 a	17.5	1.7 a	4.9		<LOQ
	P (F)[c]	< 0.001		< 0.001		0.004			
	sem[d]	0.4		0.4		0.4			
2011- 2012	common wheat - fungicide	<LOQ b	<LOQ	<LOQ a	<LOQ	0.9 c	1.5		<LOQ
	common wheat - untreated	0.2 b	0.3	<LOQ a	<LOQ	1.5 b	3.5		<LOQ
	durum wheat - fungicide	0.9 ab	2.1	0.9 a	3.5	2.4 a	10.2		<LOQ
	durum wheat - untreated	1.4 a	2.9	1.3 a	3.1	2.8 a	15.0		<LOQ
	P (F)	0.026		0.139		< 0.001			
	sem	0.7		1.1		0.4			
2012- 2013	common wheat - fungicide	<LOQ b	<LOQ	<LOQ b	<LOQ	0.6 b	1.0	3.6 b	42
	common wheat - untreated	<LOQ b	<LOQ	<LOQ b	<LOQ	1.0 b	1.8	3.7 b	39
	durum wheat - fungicide	2.0 a	6.3	0.9 a	1.6	1.6 a	3.8	4.9 a	127
	durum wheat - untreated	2.3 a	10.4	0.8 a	1.2	2.0 a	6.6	4.3 ab	73
	P (F)	< 0.001		< 0.001		< 0.001		0.011	
	sem	0.4		0.2		0.4		0.60	

[a] The reported mycotoxin contamination means are transformed [T; y'= ln (x + 1)] and not transformed (N) values.

AOH = alternariol (LOQ = limit of quantification = 0.10 µg kg^{-1}), AME = alternariol methyl ether (LOQ = 0.10 µg kg^{-1}), TENT = tentoxin (LOQ = 0.10 µg kg^{-1}), INF = infectopyrone (LOQ = 0.10 µg kg^{-1}).

829 [b] The used cultivars were Generale (common wheat) and Saragolla (durum wheat), which are characterized by a medium and an high susceptibility to FHB, respectively. The
830 fungicide treatment at heading was carried out using the prothioconazole active ingredient (formulation: EC, 0.250 kg active ingredient ha^{-1}).
831 [c] Means followed by different letters within each growing season are significantly different (the level of significance is shown in the table). The reported values are based on 3
832 replications.
833 [d] sem: standard error of the means.
834

Figure 1. Effect of agronomic strategies[a] on deoxynivalenol (DON) contamination; field experiments conducted in North West Italy in the 2010 - 2013 period.



Means followed by different letters within each growing season are significantly different ($P < 0.05$). The reported values are based on 3 replications. The bars report the standard error of the means for each growing season.

[a] The used cultivars were Generale (common wheat) and Saragolla (durum wheat), which are characterized by a medium and a high susceptibility to FHB, respectively. The fungicide treatment at heading was carried out using the prothioconazole active ingredient (formulation: EC, 0.250 kg active ingredient ha^{-1}).