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The complex metabolic interplay between cereals and mycotoxins: an insight between open field and *in vitro* cultures

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ABSTRACT

Over the course of evolution, plants have developed multiple defensive strategies, including both a manifold arsenal of secondary metabolites and detoxification pathways, involving the biotransformation of xenobiotics and their compartmentalization or segregation. These responses intervene in most relationships between plants and biotic stress and they come into play in the perennial chemical warfare between mycotoxigenic fungi and cereals, the results of which impact food safety and security at a global level. The defensive metabolic responses of plants can be intended as the combination of multiple processes to many different exogenous stimuli, including the simultaneous environmental contamination by several mycotoxins, but the details of the interplay between this exposure and secondary metabolism are still to be fully elucidated in particular in the whole range of cereal crops. Therefore, four separate studies were conducted on a variety of cereals with the aim to better understand plant responses from a phytochemical perspective. Both open-field (study 1 and study 2) and *in vitro* (study 3 and study 4) studies were performed. *In vitro* studies, unlike those in the open field, allowed the evaluation of the reciprocal relationship between mycotoxins and the defensive mechanisms of plants, in a controlled environment.

Study 1. Twelve *Triticum aestivum* L. spp. *aestivum* varieties with pigmented grain, namely one red, six purple, three blue and two black, were grown in open fields over two consecutive years and screened to investigate their risk to the accumulation of multiple *Fusarium*-related mycotoxins. Deoxynivalenol (DON) and its modified forms DON3Glc, 3Ac-DON, 15Ac-DON, and T-2, HT-2, ZEN, Enniatin B were quantified by means of UHPLC-MS/MS, along with 14 different cyanidin, petunidin, delphinidin, pelargonidin, peonidin, malvidin glycosides. A significant strong influence effect of the harvesting year (p = 0.0002) was noticed for DON content, which was more than doubled between harvesting years growing

seasons (mean of 3746 µg kg^{-1} vs. 1463 µg kg^{-1}). In addition, a striking influence of varieties with different grain colour on DON content ($p < 0.0001$) emerged in combination with the harvesting year (year*colour, $p = 0.0091$), with blue grains being more contaminated (mean of 5352 µg kg^{-1}) and red grain the less contaminated (mean of 715 µg kg^{-1}). The trend was maintained between the two harvesting years despite the highly variable absolute mycotoxin content. Varieties accumulating anthocyanins in the pericarp (purple coloration) had significantly lower DON content if compared to those in which aleurone is involved (blue coloration).

Study 2. Forty cultivars of different cereal species or hybrids, namely common and durum wheat, triticale, tritordeum, rye, barley, emmer and spelt, grown over two consecutive years in open field, were screened for multiple *Fusarium*-related mycotoxins in kernel and straw. Deoxynivalenol and its modified forms DON3Glc, 3Ac-DON, 15Ac-DON, T-2, HT-2, ZEN and Enniatin B were quantified by means of UHPLC-MS/MS. Solely DON end ENN B were detected in all the cultivars tested, with the first as the most frequently found between the tested samples. A strong influence of the harvesting year ($p = 0.000$), the cultivar ($p = 0.000$) and their interaction ($p = 0.000$) on DON occurrence were noticed both for kernel and straw, with a DON occurrence higher of about an order of magnitude for the harvesting year 2020 (mean of 1985 µg kg^{-1} for kernel and 3795 µg kg^{-1} for straw) compared to 2021 (mean of 145 µg kg^{-1} for kernel and 490 µg kg^{-1} for straw) and, on average, a higher contamination of straw compared to kernel. At this regard, the average contamination level for 2020 was higher than the legal limits for unprocessed cereals in UE countries according to the European Commission Regulation 1881/2006 (set as 1750 µg kg^{-1} for unprocessed durum wheat and 1250 µg kg^{-1} for the other unprocessed cereals). In addition, a positive correlation between harvesting years ($r = 0.693$, $p = 0.000$) was found for DON occurrence in kernels, depicting a similar trend between cultivars despite the great difference in absolute content. By grouping

cultivars according to the species or hybrids to which they belong, the higher DON contamination was found for tritordeum, which significantly differs from all other groups except from durum wheat when kernels from 2020 and 2021 were considered. In addition, a significant negative correlation was found between DON and DON3Glc/TDON for straw of common wheat cultivars in both harvesting years (2020: r = -0.503, p = 0.000; 2021: r = -0.421, p = 0.023), not previously reported in the literature. Finally, tritordeum is confirmed as one of the most contaminated entities on average also when ENN B from kernels is considered. Further research should be focused on evaluation of the possible susceptibility of tritordeum to mycotoxin accumulation in a wider range of cultivars.

Study 3. An *in vitro* bioassay for the evaluation of a potential effect of aflatoxin B_1 (AFB_1) on anthocyanin biosynthesis, evaluated as total anthocyanin content (TAC), was developed using maize (*Zea mays* L.) roots obtained from etiolated plantlets grown *in vitro*. Two different set of experiments were established. In the first experimental set we induced the biosynthesis of anthocyanins in roots by adding to the culture medium the plant growth regulator 6-benzylaminopurine (6-BAP) together with AFB_1 under continuous light exposure for one week. The second set of experiments involved three days of pre-treatment of maize roots with AFB_1 in the dark, before the induction of anthocyanin biosynthesis performed as described before. Despite differences were noted between the experimental conditions in both experimental sets, such as to suggest a possible positive effect of AFB_1 on anthocyanin biosynthesis, these differences did not reach statistical significance.

Study 4. Two tritordeum cultivars, namely “Aucan” and “HT-460”, showing significantly different ability to biotransform the *Fusarium*-related mycotoxin deoxynivalenol (DON) in its glycosylated form deoxynivalenol-3-glucoside (DON3Glc) in open field, were evaluated for their biotransformation capacity using an *in vitro* approach. Under such controlled conditions, both cultivars have shown the ability to uptake DON from the culture medium by roots and

then translocate it to the aerial parts of the plantlets. Moreover, both cultivars provided a very high biotransformation capacity compared to data from the field, but no significative differences were found between the cultivars tested. Under the conditions tested for *in vitro* experiments, the genotype does not seem to determine differences at a biotransformative level, however further investigations are needed to confirm or reject this hypothesis.

Overall results of the four studies confirmed that, within the complex interplay between mycotoxins and plant metabolism, cause-effect relationships are often difficult to describe. As a consequence of multiple biotic and abiotic factors triggering manifold metabolic effects, the intrinsic ability of plants to modulate ever-changing environment makes the unequivocal evaluation and interpretation of these phenomena particularly challenging.

CHAPTER 1 General introduction

1.1 Mycotoxins

1.1.1 General overview

Mycotoxins are defined as "poisonous, low molecular weight secondary metabolites of moulds" (Berthiller et al., 2005). Their biosynthesis is performed by a variety of fungal species, mainly belonging to *Fusarium*, *Aspergillus* and *Penicillum* genera affecting cereals, legumes, nuts, coffee, and other crops, often determining the contamination of their derived plant-based foodstuffs. Mycotoxins may also affect animal feeds and, as a result, enter the food chain via food products of animal origin (Alshannaq et al., 2017).

Mycotoxins are produced under different conditions both during field growth and storage of plant materials. Their occurrence in the field is influenced by multiple factors including climatic conditions (e.g., temperature, moisture, rainfalls), agronomic practices (e.g., tillage, use of fertilizers or fungicides), genetic background of host plants and a variety of biotic factors such as plant organ damage due to insect pests and the co-occurrence of fungal and bacterial species in the rhizosphere and phyllosphere of plants (Scarpino et al., 2021; Tola et al., 2016). The impact of the latter is particularly complex as it involves ecological interactions between mycotoxigenic fungi and other bacterial and fungal species sharing the same habitat (Venkatesh et al., 2019). Interactions between host plants and mycotoxigenic fungi further complicate the framework, because they involve a multitude of different resistance-related metabolites and resistance quantitative trait loci (Bollina et al., 2010). The risk of stored grain spoilage and mycotoxin contamination by seedborne fungi is dependent on eventual damage due to insect boring or mechanical damage, but mostly to the water activity of grain, that represent the main factor regulating fungal spore germination and mycelial development. During storage, both grain moisture and temperature must be

compatible with the expected period the grain will be stored for the intended use. In developed countries fungi are the major cause of losses on long term storage when these parameters are not adequate (Fleurat-Lessard, 2017). While post-harvest management of crops may therefore represent a critical step towards a reduction of mycotoxin exposure in food, and being this a multifactorial process a proper knowledge of the interplay between plants and mycotoxicogenic fungi yet during growth is crucial.

Mycotoxins can cause a variety of adverse health effects and pose a serious threat to both humans and livestock. Currently, more than 450 different mycotoxins and their metabolites have been associated with health effects, spanning from gastrointestinal symptoms to increased cancer risk. Moreover, mycotoxins affect animal welfare and decrease animal productivity (Benkerroum, 2020).

The toxicity of many mycotoxins, associated with their ubiquitous presence, represents a major challenge to the health and well-being of humans and animals. To meet this challenge, European Commission (EC) issued regulations on the maximal tolerable concentration for many of them in different food and feed commodities and raw materials. Regarding the regulated mycotoxins involved in the studies described in this thesis, European Union limits for foodstuffs and feeds adopted from the European Commission Regulation No. 1881/2006 and the European Commission recommendations No. 2013/165/EU and No. 2006/576/EC are shown in **Table 1**.

Mycotoxins often contaminate foodstuffs and feed as a mixture of multiple noxious compounds. Therefore, the challenge they pose must be addressed with a holistic, comprehensive approach which includes the availability of analytical methods capable to detect native, modified and co-occurring mycotoxins, as well as considering ecology and plant host-fungi interaction, ADME (Absorption, Distribution, Metabolism and Excretion)

and toxicology studies, biomarkers of mycotoxin intake and modelling approaches (Battilani et al., 2020). In an already complex scenario, the effects of climate change have a significant impact on toxigenic fungi development and modify conditions for mycotoxin production, finally influencing also host-resistance and host-pathogen interactions (Moretti et al., 2019). Such effect increases the relevance of this topic, as wider geographical range for pathogenic fungi, more contaminated crops, and ultimately an increased human exposure to mycotoxins are to be expected in the near future.

Table 1: European Union limits for foodstuffs and feeds adopted from the European Commission Regulation No. 1881/2006 and the European Commission recommendations No. 2013/165/EU and No. 2006/576/EC.

Mycotoxin	Foodstuffs for human consumption and animal feed	Maximum levels (µg kg^{-1})
Deoxynivalenol (DON)	**Food**	
	unprocessed cereals other than durum wheat, oats and maize	1250
	unprocessed durum wheat and oats	1750
	pasta (dry)	750
	bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals	500
	Feed	
	cereals and cereal products with the exception of maize by-products	8000
	maize by-products	12000
T-2 and HT-2 (sum)	**Food – unprocessed cereals**	
	barley (including malting barley) and maize	200
	wheat, rye and other cereals	100
	Food - cereal grains for direct human consumption	
	maize	100
	other cereals	50
	Food – cereal product for human consumption	
	cereal bran except oat bran, oat milling products other than oat bran and flaked oats, and maize milling products	100
	other cereal milling products	50
	breakfast cereals including formed cereal flakes	75
	bread (including small bakery wares), pastries, biscuits, cereal snacks, pasta	25
	Feed	
	cereal products other than oats milling products	500
Zearalenone (ZEN)	**Food**	
	unprocessed cereals other than maize	100
	unprocessed maize	200
	bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals, excluding maize snacks and maize-based	50
	Feed	
	cereals and cereal products with the exception of maize by-products	2000
	maize by-products	3000
Aflatoxin B1 (AFB1)	**Food**	
	All cereals and all products derived from cereals, including processed cereals products with exception listen in the EC Regulation No. 1881/2006 L 364/15-16	2

1.1.2 Fusarium mycotoxins

Fusarium genus includes more than 1,500 species estimated, widespread throughout the world and ubiquitous in the environment, in which several strains are considered pathogenic and produce mycotoxins. These strains can infect crops that underpin the world's food supply like common and durum wheat, maize, rice, barley, rye and oats (Arie, 2019).

Fusarium root rot (FRR) and Fusarium head blight (FHB) are the major devastating diseases affecting wheat worldwide. The symptoms of FRR are time-dependent and range from mortality of young seedlings accompanied by browning of the coleoptiles to severe attacks showing blighted heads that appears shrivelled or without grains. Wheat plants affected by FHB show instead bleached lesions on the glumes and rachis, while kernels appear damaged and contaminated with mycotoxins (Rojas et al., 2020). Moreover, it has been reported that, upon infection, mycotoxins are also transported from the roots up to the aerial part of the plant, suggesting FRR contributing to the contamination level also in organs considered edible for humans and animals (Covarelli et al., 2012).

Fusarium graminearum, *F. culmorum* and *F. pseudograminearum* are the most relevant causal agents of FHB and FRR worldwide and can produce a variety of mycotoxins, mainly trichothecenes and zearalenone (Balmas et al., 2015; Lindblad et al., 2013). Trichothecenes are a class of sesquiterpene mycotoxins divided into groups based on their structure. They share a tetracyclic sesquiterpenoid 12,13-epoxytrichothec-9-ene ring, in which the 12,13-epoxy ring is responsible for the toxicological activity (Y. Li et al., 2011).

Type A trichothecenes includes, but are not limited to, T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS), harzanium A, neosolaniol (NEO) and trichodermin. Type B includes, among others, deoxynivalenol (DON) and its acetylated forms 3-acetyl-deoxynivalenol (3Ac-DON) and 15-acetyl-deoxynivalenol (15Ac-DON), nivalenol (NIV),

trichothecin and fusarenon X (FUS-X). Type C (e.g., crotocin) and type D (e.g., satratoxins, roridin A and verrucatin S) are also reported in literature, however *Fusarium* species do not produce types C and D trichothecenes. Moreover, several other genera of fungi, including *Cephalosporium*, *Cyclindrocarpon, Myrothecium, Phomopsis, Stachybotrys*, *Trichoderma*, *Trichotecium*, and *Verticimonosporium* species can also produce trichothecenes (Mahato et al., 2022). The more relevant *Fusarium* mycotoxins commonly found in cereals are described below.

Deoxynivalenol (DON) (**Figure 1/A**), is a polar organic compound classified as a type B trichothecene produced by *Fusarium* species (as shown in **Table 2**), also known as vomitoxin due to his strong emetic effect after consumption; it is a well-known and common contaminant of cereals and cereal-derived products. An important physicochemical property of DON is its heat stability, with no reduction of its concentration after 30 minutes at 170°C. This property is of great relevance regarding its presence in food products even subject to high-temperature processing and hinders the possibility of its breakdown by thermic means (Sobrova et al., 2010). DON was actually frequently found in food commodities like bakery products (bread, biscuits etc.), beer, infant foods, noodles and pasta (Mishra et al., 2020). DON can cause acute and chronic health effects and its toxicity affect the gastrointestinal tract, immune and reproductive systems. At cellular level DON binds the ribosomes, impairing the physiological protein biosynthesis and can interfere with mitogen-activated protein kinase (MAPK) signalling pathway leading to cytotoxicity (Yao et al., 2020). Although DON is not considered a carcinogen for humans, its regulation of reactive oxygen species (ROS) production in cancer cells might indirectly assist the progression of tumours (Habrowska-Górczyńska et al., 2019).

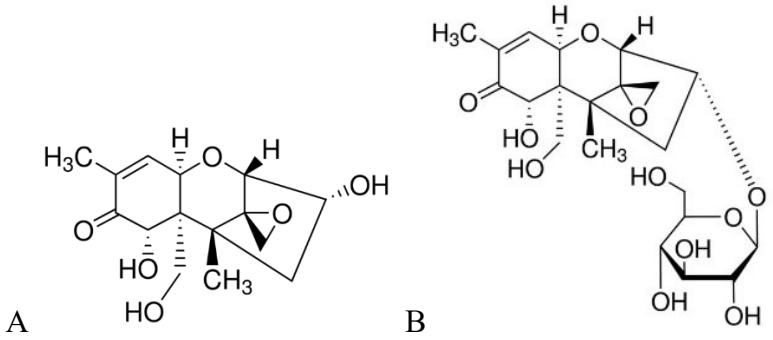

Figure 1: Deoxynivalenol (A) and Deoxynivalenol-3-Glucoside (B).

As previously mentioned, DON is also produced in acetylated forms by *Fusarium*, namely 3Ac-DON and 15Ac-DON. These forms maintain *in vivo* almost the same toxicity of parent compound, because of their enzymatic deacetylation in mammals (Liu et al., 2016). In addition, the presence of deoxynivalenol-3-glucoside (DON3Glc) (**Figure 1/B**), the most representative modified mycotoxin of DON in food commodities, has been extensively addressed in literature. This glycosylated form of DON is due to an important glycosylation reaction that generally occurs in phase II metabolism of plants as a detoxification mechanism to defend against *Fusarium*-related diseases. Some evidence has indicated that colonic microbiota and intestinal lactic acid bacteria can partially hydrolyze DON3Glc, thereby leading to health hazards for humans and animals (Berthiller et al., 2009; Dall'Erta et al., 2013; Knutsen et al., 2017).

Zearalenone (ZEN) (**Figure 2**) is a non-steroidal estrogenic mycotoxin produced by *Fusarium* spp. that is frequently found in cereals such as wheat, rice, corn and many others. Similarly to DON, ZEN exhibits stability during storage and does not degrade when exposed to high temperatures. Because of its widespread occurrence and to its physicochemical properties, ZEN occurs in many food products of plant origin such as bakery products, pasta and breakfast cereals and it can also be present in products of animal origin such milk, eggs or

meat of animals fed by contaminated feed (Rogowska et al., 2019). Regarding its biological effects, ZEN is reported as an immunotoxic and hepatotoxic substance with also hematotoxic effects (Ropejko et al., 2021). ZEN is currently classified by the International Agency for Research on Cancer (IARC) into group 3 (not classifiable as to its carcinogenicity to humans). However, because of its structural similarity to natural occurring oestrogens, ZEN is able to affect metabolic pathways of oestrogens causing damage in reproductive organs and it is reported to also favour the development of otherwise generated cancer diseases (Buszewska-Forajta, 2020).

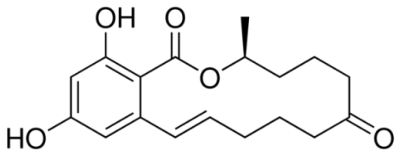

Figure 2: Zearalenone

T-2 (**Figure 3**) and HT-2 toxins are type A trichothecenes, produced by various *Fusarium* species both in the field and during storage. In addition to being a natural contaminant, HT-2 toxin is also a metabolite of T-2 toxin formed by hydroxylation at C-4. Both T-2 and HT-2 toxin are only partially degraded during thermal food processing. The degradation rates are influenced by the food composition and processing parameters (Kuchenbuch et al., 2018). Regarding biological activity, T-2 toxin inhibits protein, DNA and RNA biosynthesis, induce apoptosis and necrosis and disrupt the membrane integrity by lipid peroxidation. In animals, exposure to T-2 toxin leads to reduction of feed intake and weight gain, in particular, the toxin shows immunotoxicity, neurotoxicity, hematotoxicity and reproductive toxicity. Moreover, T-2 toxin can induce gastrointestinal damages, neuroendocrine and hepatological changes (Bertero et al., 2018; Q. Wu et al., 2020). Overall, as reported in literature, both T-2 and HT-2

toxins are able to cause acute or chronic intoxication of humans and animals (Nathanail et al., 2015), thus indicative levels for the sum of these two toxins were set up by EFSA (**Table 1**).

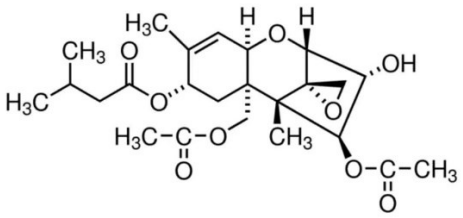

Figure 3: T-2 toxin

Fusarium fungi are also able to produce the so-called emerging mycotoxins, namely mycotoxins whose presence in food and feed are neither routinely monitored nor subjected to a regulation by Authorities. Among these mycotoxins there are fusaproliferin (FUS), beauvericin (BEA), moniliformin (MON), enniatins (ENNs) and many others. Although there is a partial overlap regarding some of the main *Fusarium* species producing DON and ZEN, several different species are involved in emerging mycotoxin production. It is well established that *F. graminearum* and *F. culmorum*, two of the most widespread mycotoxigenic fungi, are able to produce both DON and ZEN, whereas *F. avenaceum* is not known to produce the previously mentioned mycotoxins, but it is able to produce emerging mycotoxins BEA, MON and ENNs (Jestoi, 2008; Lindblad et al., 2013). **Table 2** summarises the main mycotoxins involved in the field studies discussed in this thesis and the most common *Fusarium* species involved in their production.

Table 2: Mycotoxins and most common *Fusarium* species involved in their production, as reported in literature (Bertero et al., 2018; Chakroun et al., 2022; Golge et al., 2020; Shi et al., 2017; Gautier et al., 2020).

Mycotoxin	***Fusarium* species**
DON	*F. graminearum, F. culmorum, F. cerealis, F. pseudograminearum*
T-2, HT-2	*F. sporotrichioides, F. langsethiae, F. poe*
ZEN	*F. graminearum, F. culmorum, F. cerealis, F. equiseti, F. semitectum*
ENNs	*F. tricinctum, F. avenaceum, F. acuminatum, F. poe, F. sporotrichioides*

Enniatins (ENNs) are cyclohexadepsipeptides composed of alternating residues of three N-methyl amino acids, commonly valine, leucine, and isoleucine, and three hydroxy acids, typically hydroxyisovaleric acid. ENNs are only partially degraded during thermal food processing, as reported for pasta (Serrano et al., 2016). The lipophilic nature of ENNs allows them to be incorporated into lipid bilayers, giving them ionophoric properties. The increased membrane permeability to cations determines impairing of the physiological distribution of ions between inside and outside the cells. This mechanism seems to be related to their wide range of biological targets which includes insecticidal, antifungal, antibacterial, and anthelmintic properties. Several ENNs have been identified, namely A, A1, B, B1, B2, B3, B4, D, E, F and G. The currently most studied is ENN B (**Figure 4**) since it has been the most-often detected in unprocessed and processed grains from European countries (Prosperini et al., 2017).

ENNs exerted a potent cytotoxic effect in several human and animal cell lines *in vitro* (Prosperini et al., 2017), moreover EFSA external scientific report in 2018 stated that acute exposure to ENNB poses a genotoxic hazard *in vivo* (Maranghi et al., 2018). Health hazards induced by a chronic exposure to ENNs and their association with other mycotoxins represent a risk that cannot be excluded given the current toxicological data (Gautier et al., 2020).

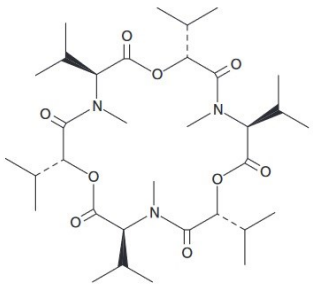

Figure 4: Enniatin B

Enniatin B, such as other ENNs, have been found as contaminants of many cereals such as wheat, rye, oats, barley, rice, triticale, maize and derived products (Gautier et al., 2020).

Given their combined nature and their presence in a wide variety of foods, an increasing attention is paid to the possible risks related to the exposure to multiple mycotoxins and to antagonistic, additive or even synergistic effects that also involve emerging mycotoxins. For example, synergistic cytotoxic effects have been observed due to the exposure to ENNs mixtures *in vitro*, and, overall, a wide range of effects have been described for many mixtures of mycotoxins at different concentrations using *in vitro* or *in vivo* models (Fu et al., 2022; Karsauliya et al., 2022).

1.1.3 Aflatoxins

Aflatoxins are a class of mycotoxins produced by *Aspergillus* fungi, primarily *Aspergillus flavus* and *A. parasiticus*, even though many other *Aspergillus* species are known to produce these mycotoxins (Valencia-Quintana et al., 2020). Structurally, aflatoxins are difurocoumarocyclopentenones (aflatoxins B series and derivatives) or difurocoumarolactones (aflatoxins G series and derivatives). Various types of Aflatoxins have been identified and, among them, aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2) are considered the four main aflatoxins (Benkerroum, 2020). The most frequently found aflatoxin in contaminated food samples is AFB_1 while the three others main types are generally not found in the absence of AFB_1. As other mycotoxins previously described, also aflatoxins are not completely degraded during common food processing such as baking or roasting and no abatement is possible once raw materials are contaminated (Schrenk et al., 2020).

It is reported that AFB_1 can occurs in cereal grains, especially corn, but also rice, wheat, barley, sorghum and oats (Khaneghah et al., 2018). AFB_1 was also frequently found in legumes, nuts, oil seeds, spices, meats, dairy products and eggs (Benkerroum, 2020; Maringe et al., 2017).

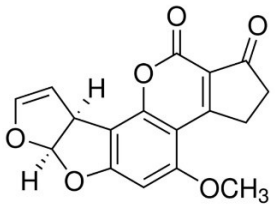

Figure 5: Aflatoxin B1

In animals, AFB_1 is metabolized into several products through the cytochrome P450 system; these includes aflatoxin M_1 (AFM_1), P1 (AFP_1), aflatoxicol (AFL), aflatoxicol H_1 (AFH_1) and

aflatoxin B_{2a} (AFB_{2a}) (Rushing et al., 2019). AFM_1 is a major metabolite commonly detected in human and animals as a result of cytochrome-mediated metabolism (especially through CYP450 1A2) of AFB_1. AFM_1 is commonly found in the milk of dairy cattle exposed to AFB_1 because of liver phase-I biotransformation, but it was also found as a plant metabolite (Righetti et al., 2021).

According to International Agency for Research on Cancer (IARC) classification, aflatoxins belong to the group I as carcinogenic to humans. AFB_1 is the most genotoxic aflatoxin according to most studies and it can induce hepatocellular carcinoma as well as alteration of the immune system and growth suppression. AFB_1 also induces oxidative stress including modulation of antioxidant defence systems (Schrenk et al., 2020).

1.1.4 Distribution of mycotoxins in planta

During the perennial chemical warfare between plants and fungi, the evolution has driven the development of multiple defensive strategies, also including the capability to cope, manage and minimize the noxious effects of mycotoxins. For instance, plants have detoxification systems able to counter a variety of non-natural and natural phytotoxic compounds, among which mycotoxins. Detoxification mechanisms are mostly based on chemical modification, compartmentation and segregation both at cytological and histological level. Chemical modification take place by phase I reactions that frequently involve hydrolysis by the action of amidases and esterases or oxidation through the cytochrome P-450 system. Phase II reactions are characterised by conjugation to hydrophilic molecules such as glucose, malonic acid and glutathione catalysed by transferases. Phase I reactions lead to molecules with different degrees of phytotoxicity compared to the parent compound, whereas phase II reactions lead to non-toxic or less toxic compounds with different chemical properties that make xenobiotics more water soluble. This enhanced water solubility enables their access to the membrane transport system by which xenobiotics are excluded from the cytosol and translocated into the tonoplastic or the apoplastic space. Phase III detoxification reactions are also present in plants and involve sequestration of compounds conjugated to glucose or GSH into the vacuole or their irreversible binding to the cell wall in a permanent way, in organs that can be disposable upon necessity such as leaves and fruits (Berthiller et al., 2013).

Modified forms of many mycotoxins were identified using naturally or artificially infected plants, and in seedlings treated with the parent mycotoxin or *in vitro* models as well. Several DON biotransformation products in different wheat lines were identified and similarly modified forms of T-2 and HT-2 toxins were reported in artificially contaminated wheat (Bryła et al., 2018). ZEN metabolites were for instance found in barley seedlings and in suspension cultures of wheat cells (Freire & Sant'Ana, 2018).

In addition to biotransformation, translocation of mycotoxins within entire plants have also been reviewed in the literature. Mycotoxins can be present in soil, mainly due to plant residues remaining on the field after harvest, but also runoff from infected crops after heavy rains events and contamination input from application of manure. In the soil environment, processes such as adsorption by minerals, leaching to groundwater or degradation by microorganisms contribute to reduce the potential contamination level but, in some occasions, such natural processes are not exhaustive (Juraschek et al., 2022). It has been well established that plants are able to absorb mycotoxins through the root system, as demonstrated by *in vitro* experiments for AFB_1 in maize and ZEN in durum wheat. *In vitro* studies also demonstrated translocation of mycotoxin from the root to the aerial parts of maize plants treated with AFB_1 as well as the presence of several modified forms both in roots and leaves suggesting an extensive phase I metabolism (Righetti, Rolli, et al., 2021; Rolli et al., 2018). Soil contamination might therefore contribute to the risk associated with feed and food contamination or somehow facilitate plant infection by altering its metabolism and physiology, albeit no quantitative studies have been conducted on the matter so far.

Data collected from the field show that mycotoxin contamination of straw and glumes of *Fusarium* infected wheat can occur, even at higher concentration than grains (Ji et al., 2015). Moreover, as recently reported, *Fusarium* emerging, modified and regulated mycotoxins frequently occur in straw of barley. Among them ENNs, DON and DON3Glc as well as ZEN, T-2 and HT-2 toxins are reported (Drakopoulos et al., 2021). While wheat and maize are undoubtedly the most investigated cereal crops on this regard, the growing interest of agricultural firms and food industries for less common or innovative cereals makes relevant the performing of further research on minor crops. This is particularly important when new cultivars or the detection of more or less susceptible or resistant lines or species are involved.

1.2 Plant secondary metabolites

1.2.1 General overview

Plants produce a wide range of different chemical compounds that can be classified as primary or secondary metabolites. Primary metabolites are ubiquitous in plants and possess essential metabolic roles, while secondary metabolites play a large spectrum of physiological roles essential for the adaptation of plants to the surrounding environment. These roles include defence response signalling, response to environmental stresses, defence against pathogens, pests and herbivores and regulation of ecological interactions. Secondary metabolites also play a role in plant growth and reproduction and their classification is not always univocal as in the case of carotenoids which may be considered as primary metabolites among lipids for their key involvement in photosynthesis but also included in secondary metabolites for their pigmenting and antioxidant properties (Caretto et al., 2015; Pang et al., 2021).

Plant secondary metabolites can be classified according to their biosynthetic pathway, according to their main precursor or ultimately according to their structure, leading to three main groups: phenolic compounds terpenoids and alkaloids (Chomel et al., 2016). Other classifications are proposed in literature, for instance, alkaloids are sometimes included in the larger group of nitrogen-containing compounds, and sulfur-containing secondary metabolites are also reported in literature as a separate group (Jan et al., 2021). Secondary metabolites are synthesized in plants through different metabolic pathways and their precursors are derived from the primary metabolism.

Shikimic acid, the precursor of the shikimate pathway, is produced starting from phosphoenolpyruvate derived from glycolytic pathway and erythrose-4-phosphate derived from the pentose phosphate pathway. Shikimate pathway produce aromatic amino acids that

are involved in the biosynthesis of phenolic compounds through the phenylpropanoid pathway. Aromatic amino acids are also precursors of metabolites belonging to the alkaloid group that are also biosynthesised starting from nitrogen-containing metabolites derived from aliphatic amino acids (Jan et al., 2021).

Regarding terpene biosynthesis, the condensation of three units of acetyl CoA leads to the synthesis of 3-hydroxy-3-methylglutaryl-CoA, which later on produces mevalonic acid that is subsequently converted into isopentenyl diphosphate (IPP), the common precursor of terpenes (Singh et al., 2015).

Phenolics are compounds that include at minimum one phenol ring and are divided into groups and subgroups according to their chemical structure, namely phenolic acids (hydroxybenzoic and hydroxycinnamic acids), flanovoids (flavonols, flavones, flavanols, flavanones, isoflavones and anthocyanins), tannins, stilbenes and lignans (Albuquerque et al., 2021). Phenolics are ubiquitous in plants, in soluble or bound forms, and play various biological roles. They contribute to the pigmentation of plant organs, act as protective agents against biotic (e.g. bacteria, fungi, herbivores) and abiotic (e.g. UV light, drought, low temperature) stressors, function as signal molecules and allopathic compounds (Jan et al., 2021; Oufensou et al., 2020; Panche et al., 2016). Phenolics are also involved in developmental processes like cell division, hormonal regulation, photosynthetic activity, nutrient mineralization and reproduction. Moreover, phenolics act as ROS (reactive oxygen species) scavengers thus modulating cellular redox homeostasis (Sharma et al., 2019).

Terpenes compound are classified according to the number of isoprene units of which are made of, that is hemiterpenes, monoterpenes, sesquiterpenes, diterpenes, triterpenes, tetraterpenes and polyterpenes. Some terpenes act as pollinator attractants, herbivore deterrents or are able to attract the natural enemies of herbivores, acting as an indirect mode

of defence action because of their aroma. Other terpenes act as phytoalexins, that are antimicrobial compounds induced during infection. More in detail, monoterpenes can be toxic to insect and potentially involved in growth regulation and heat tolerance as well as contribute to the specific smell of flowers and fruits. Sesquiterpens are known for their property of repelling feeding of mammals and insect, and acting as phytoalexins (Schmelz et al., 2014). Diterpenes are precursor of essential metabolites such as phytol as well as plant growth regulators named gibberellins and includes molecules of significant biological activity used in medicine. Triterpenes show a large spectrum of biological activities and are recognized as signal molecules and capable of acting against pathogens and insect pests. Terpenes of higher molecular weight spanning from tetraterpenes, which includes carotenoid pigments, to polyterpenes such as rubber (Jan et al., 2021; Meyer et al., 2016; Ninkuu et al., 2021; Singh et al., 2015).

Alkaloids belong to the larger group of nitrogen-containing secondary metabolites together with cyanogenic glycosides, glicosinolates and non-protein amino acids (Jan et al., 2021). Alkaloids are subdivided in several subgroups and possess many biological activities. Their biological role in plants is linked to protection against herbivores and pathogens and many of them are wide studied for their pharmacologic properties (Thawabteh et al., 2019).

Among the properties of secondary metabolites, their compartmentation, inducibility and their extreme plasticity must be mentioned. These compounds are selectively segregated in specialized tissues or organelles and may be synthetized on demand according to a variety of environmental stimuli. As a result, their presence, abundance, and combination are extremely changeable in consequence of a multitude of biotic and abiotic factors. Finally, their remarkable complexity is epitomized by their appearance in intricate mixture of hundreds of different chemical entities, whose complete separation, quantification and description represents a challenging phytochemical task. It must be also highlighted their multiple nature

in most occasions, as many secondary metabolites may be involved at the same time as a response to different stimuli and may act with synergistic effects, often making the efficacy of the whole larger than the sum of its parts or not directly relatable to a single cause-effect explanation.

1.2.2 Secondary metabolites and plant defence system

As mentioned above, a wide spectrum of secondary metabolites is involved in defensive mechanisms against a variety of abiotic and biotic stresses. Some of these stressors are called elicitors, that can be defined as chemical compounds from abiotic and biotic sources capable to stimulate stress responses in plants, leading to the enhanced synthesis and accumulation of secondary metabolites associated with the adaption of plants to stressful conditions (Jan et al., 2021).

It was reported that many plants show increased synthesis of phenolics under abiotic stress conditions such as salinity, UV radiations, high/low temperatures, drought and heavy metals. Under such conditions, for instance, phenols are accumulated helping the plants to cope with environmental constraints which are furthermore expected to increase due to climate change (Sharma et al., 2019; Yang et al., 2018). Furthermore, a presence in soil of soluble salts in amounts capable to induce osmotic stress results in increased levels of flavonoids, tannic acid and gossypolin as reported in literature for cotton plants. Moreover, increased levels of flavonoids were reported for safflower, tomato and mung bean exposed to different levels of salinity. Willow, quinoa and common snapdragon show a variety of responses related to drought stress tolerance, including enhanced flavonoids, including anthocyanins, and phenolic acid contents. Additionally, it was reported that heavy metals exposure led to accumulation of terpene and phenolic compounds in some plants. Furthermore, UV stress is reported to

increase stilbenes in grapevines callus culture (Khare et al., 2020). Finally, salinity, high/low temperatures, light irradiance and drought are shown to be able to influence anthocyanin accumulation in pigmented wheat (X. Li et al., 2018).

Secondary metabolites regulate defence systems also in their interactions with insect herbivores, especially as toxins or repellents. When plants are exposed to herbivore associated elicitors, they produce and release a blend of volatiles, mainly terpenes, nitrogen compound and indoles, that can attract predators, parasites, and other natural enemies (Aljbory & Chen, 2018).

As previously mentioned, *Fusarium* fungi are responsible for major diseases that affect many cereals. Resistance to *Fusarium* includes the ability of plants to prevent initial infection and its spread, but also to counteract the accumulation of mycotoxins. In general, a variety of types of plant resistance and their respective classification have been described in literature (Chrpová et al., 2021). Regarding resistance to mycotoxin accumulation, it depends on the ability of plant both to biotransform mycotoxins and to inhibit their fungal biosynthesis. The last mechanism involves host metabolites able to interfere with mycotoxin biosynthesis that can be both constitutively synthetized, belonging to the group of phytoanticipins, and induced in response to a pathogen infection, which, as mentioned previously, are called phytoalexins (Gauthier et al., 2015).

Different methodological approaches have attempted to elucidate the involvement of primary and secondary metabolites as part of *Fusarium* resistance in cereals. Metabolic profiling offers the possibility to identify resistance-related metabolites (RR) that are commonly designated as metabolites whose levels were significantly higher in resistant than in susceptible genotypes. These RR metabolites can be both constitutive (RRC) or induced (RRI) following *Fusarium* infection (Bollina et al., 2011). Targeted and untargeted

metabolomic approaches have identified a very large set of RR metabolites derived from primary and secondary metabolism, some of which are listed in **Table 3**.

Other studies aimed at identifying metabolites involved in *Fusarium*-resistance were focused on the inhibition properties of some secondary metabolites exerted on mycotoxin biosynthesis or their ability to modulate fungal development *in vitro*. However, for metabolites such as phenolics, the exact mechanisms by which fungal growth and mycotoxin production are inhibited remain unclear. Among these compounds, cinnamic acid derivatives such as caffeic, *p*-coumaric, chlorogenic and ferulic acids are efficient inhibitors of type B trichothecenes in *F. graminearum* and *F. culmorum* and contribute to the inhibition of their growth. Moreover, some flavonoids such as naringenin, quercetin and kaempferol were reported to partially inhibit growth of *F. graminearum* (Atanasova-Penichon et al., 2016).

For some metabolites an increased concentration was observed after treatment of wheat plants with DON, leading to suggest a possible correlation. Among these compounds there are amino acids such as tyrosine, tryptophan and phenylalanine, but also carbohydrates such as sucrose, confirming the dual involvement of primary and secondary metabolism in this type of stress response (Warth et al., 2015). At the same time, however, the presence of multiple environmental stressors capable to activate and modulate these biosynthetic pathways may make difficult the establishment of precise cause-effect relationships in particular under field conditions.

Table 3: Metabolites proposed as resistance-related (RR) in cereals.

Metabolite	Plant host	Reference
Amino acids		
Phenylalanine	*Hordeum vulgare* L.	Kumaraswamy et al., 2011
Isoleucine	*Hordeum vulgare* L.	Bollina et al. 2011
Methionine	*Hordeum vulgare* L.	Bollina et al. 2011
Hydroxycinnamic acid amides		
Caffeoylserotonin	*Triticum aestivum* L.	Gunnaiah et al., 2012
Feruloylserotonin	*Triticum aestivum* L.	Gunnaiah et al., 2012
Phenolic acids		
Ferulic acid	*Triticum aestivum* L.	Dhokane et al., 2016
trans-cinnamic acid	*Triticum aestivum* L	Gunnaiah et al., 2012
p-coumaric acid	*Hordeum vulgare* L.	Bollina et al. 2011
Sinapic acid	*Hordeum vulgare* L.	Bollina et al. 2011
Flavonoids		
Quercitrin	*Triticum aestivum* L	Dhokane et al., 2016
Naringenin	*Hordeum vulgare* L.	Bollina et al. 2011
Pelargonidin-3-*O*-rutinoside	*Hordeum vulgare* L.	Kumaraswamy et al., 2012
Cyanidin 3-*O*-glucoside	*Hordeum vulgare* L.	Bollina et al., 2011
Malvidin 3-*O*-glucoside	*Triticum aestivum* L.	Gunnaiah et al., 2014
Terpenoids		
Brusatol	*Triticum aestivum* L.	Dhokane et al., 2016
Atractyloside A	*Triticum aestivum* L.	Dhokane et al., 2016
Phytuberin	*Triticum aestivum* L.	Dhokane et al., 2016
Fatty acids		
Heptadecatrienoic acid	*Hordeum vulgare* L.	Kumaraswamy et al., 2012
Linolenic acid	*Hordeum vulgare* L.	Kumaraswamy et al., 2012
Linoleic acid	*Hordeum vulgare* L.	Kumaraswamy et al., 2012

Secondary metabolites with antioxidant activity such as phenolic acids and flavonoids are frequently reported in literature as metabolites potential involved in resistance to *Fusarium* and mycotoxin accumulation (Atanasova-Penichon et al., 2016; Etzerodt et al., 2016). Oxidative burst, that is the generation of ROS in plant cells in response to a pathogen attack, is one of the earliest plant defence responses to *Fusaria*. Accumulation of ROS inhibits the growth of biotrophic pathogens by their antifungal properties and by mechanism that involve cell death with the aim to restrict pathogen infection to the site of attempted ingress. In the latter case, cell death can help the infection process of necrotrophic pathogens.

Moreover, ROS act as second messengers leading to activation of defence-related genes and interfaces with signalling pathways and defence compounds including phytohormones, nitric oxide, lignin and callose. Since pathogens such as *F. graminearum* are hemibiotrophic, uncontrolled ROS accumulation leading to cell death can enhance plant susceptibility to the necrotrophic phase of the life cycle of the pathogen. On the other side, *F. graminearum* respond to oxidative burst by generating mycotoxins stimulating programmed host cell death with the aim to accelerate its own development through the necrotrophic phase (Taheri, 2018). In addition to their key role as plant defence mediators, phenolics can also display antifungal properties and, in particular, protect plant cell wall integrity upon fungal infection by inhibiting the activity of several degrading enzymes secreted by fungal pathogens to weaken plant cell wall and penetrate plant tissues (Gauthier et al., 2015).

1.2.3 Anthocyanins

Anthocyanins are water-soluble phenolic pigments responsible for red, purple, blue or black colours in fruits, vegetables, grains, flowers and other pigmented plant tissues and organs. Structurally anthocyanin share a common core structure consisting in two aromatic rings linked by a three-carbon heterocyclic ring that contain oxygens, this structure is called aglycone form. The addition of a sugar side chain give rise to glycosidic form of the anthocyanidin named anthocyanin. The most common sugars residues found in anthocyanin are glucose, xylose, rhamnose, arabinose, galactose, but di- and tri- saccharide groups and acyl glycosidic forms are also reported. Over 23 anthocyanidins and 500 different anthocyanins have been isolated from plants. This wide diversity is due both to the different anthocyanidin core structure and the number and type of sugars forming the side chain. (Francavilla & Joye, 2020; Gupta et al., 2021).

In the context of their wide distribution, anthocyanins are also present in the grains of several cereal varieties, where they simultaneously intervene to cope with a large variety of biotic and abiotic stressors, including for instance salinity, UV exposure due to altitude and exposure to pathogens. Chemical structure of cyanidin-3-glucoside, one of the most common anthocyanin found in pigmented wheat, is shown in **Figure 6.**

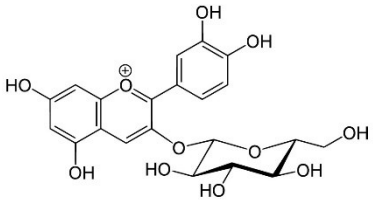

Figure 6: Cyanidin-3-glucoside

In pigmented wheat (*Triticum aestivum* L.) anthocyanin are accumulated in the aleurone or pericarp layer and give purple, blue or black colour to the grain. In purple wheat, anthocyanins are localised in the pericarp, whereas in blue wheat they are found in the aleurone layer. In black wheat, anthocyanins can be found in both the pericarp and the aleurone layer. It was reported that cyanidin 3-glucoside, cyanidin-3-rutinoside, and cyanidin 3-(6" - succinyl-glucoside) are the main anthocyanidin-3-O-glycosides in purple wheat grains, while the major anthocyanins for blue wheat are delphinidin 3-rutinoside and delphinidin 3-glucoside, however the anthocyanin profile of pigmented wheat is complex and diverges between varieties (Francavilla & Joye, 2020; Gupta et al., 2021; Lachman et al., 2017). Anthocyanins such as cyanidin-3-glucoside, pelargonidin-3-glucoside, and peonidin-3-glucoside with their malonyl derivatives have been found in Peruvian purple corn (Gálvez

Ranilla et al., 2017). It was also reported anthocyanin accumulation in roots of non-pigmented maize exposed to continuous light (Tselas et al., 1979).

Anthocyanins play various key roles in plants as they assist attracting pollinators and seed disperser while participating at the same time in plant defence against abiotic and biotic stress factors. Anthocyanins were reported to have a photoprotective role in plants under stress condition by shielding of the chloroplast from excess of light and scavenging ROS. They are commonly induced in response to various stress conditions, including pathogen infection, wounding, drought, salinity, excess of light, high/low temperature and some nutritional deficiencies (Moustaka et al., 2020). Their involvement in a large variety of stress responses testify for the above-mentioned difficulty in the definition of a precise, direct and univocal involvement in the defensive mechanisms involved in mycotoxin management and resistance.

For being natural, non-toxic and water-soluble pigments, anthocyanins have been widely studied as alternatives to the mostly used artificial food colorants, also considering consumers increasing awareness of synthetic colorants issue. Along with a great colouring capacity, these compounds have recognized bioactive properties, acting as antioxidants and helping in the prevention of cardiovascular and neurological diseases, cancer and diabetes, which make them even more suitable for food application (Chen et al., 2021; Khoo et al., 2017).

1.3 *In vitro* culture of plants

In vitro culture of plants, of their organs and cells is characterized by aseptic conditions in a defined physical and chemical environment. *In vitro* culture is an important tool in both basic and applied studies as well as in commercial application as bioreactors. *In vitro* plant cell and tissue culture techniques can be used for the selection of stress-tolerant plants, in this case, selection is based on the induction of genetic variation among cells, tissues or organs in cultured and regenerated plants. The genetic variation can be induced by genetic engineering techniques or it can be based on the somaclonal variations (Rai et al., 2011).

Owing to the constraints faced by natural plant extraction or of occasionally complex chemical syntheses, bioreactors based on plant cell or tissue culture has emerged as an alternative platform for the *in vitro* production of bioactive secondary metabolites of high commercial value. This includes drugs (e.g. taxol), food colourants (e.g. anthocyanins), dyes, plant bioactives and other secondary metabolites (Wilson & Roberts, 2012). To enhance their production, elicitation represent a technique commonly adopted. It involves the exogenous addition of biotic or abiotic compounds in the growth medium, which consequently triggers a stress response with concomitant enhancement in secondary metabolite production (Narayani & Srivastava, 2017).

Speaking of mycotoxins, *in vitro* models represent a useful tool to elucidate the plant biosynthetic potential for modified mycotoxins as well as to study the absorption and translocation of mycotoxins and their metabolites that can be further investigated and validated in field crops. Their use in investigating the interplay between plants and mycotoxins may reduce the number of confounding variables involved the field, thus helping in establishing a clearer scenario in terms of cause-effect relationship. Such approach was

already successful applied by our group to maize and durum wheat (Righetti et al., 2021b; Rolli et al., 2018).

CHAPTER 2 Aims of the Doctoral Thesis

Plants, in order to meet the constraints posed by the surrounding environment, have developed, during their evolutionary path, a chemical arsenal, represented by secondary metabolites, which they use for many purposes, including defense against potentially harmful events. The defensive metabolic responses of plants, which also include the ability of plants to biotransform xenobiotics, can be intended a combination, often with synergistic elements, of multiple simultaneous responses to many different exogenous stimuli to which plants are constantly exposed.

The studies carried out during this PhD course are divided into two complementary groups: open field studies (study 1 and study 2) and *in vitro* studies (study 3 and study 4). On one hand, through open field studies it was possible to make a comparison between multiple cultivars of different cereal crops in their natural growing conditions, to obtain indispensable data regards to their susceptibility/resistance to the accumulation of mycotoxins, biotransformation capacity and response of their secondary metabolism. On the other hand, *in vitro* studies allowed to address the issue of the metabolic responses, that plants implement as defensive mechanism, "cleaning" the surrounding environment of most unselected stressors.

Since the metabolic response of plants in open fields is intended as an overall response to multiple events, such complementary approach could be useful to shed light on a complex interpretative framework.

CHAPTER 3 Experimental studies

3.1 Study 1: Anthocyanin content and *Fusarium* mycotoxins in pigmented wheat (*Triticum aestivum* L. spp. *aestivum*): an open field evaluation.

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Anthocyanin content and Fusarium mycotoxins in pigmented wheat (Triticum aestivum L. spp. aestivum): an open field evaluation.

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***G.M. contribution**: Investigation and writing/review of the original draft.*

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3.1.1 Introduction

The *Triticum* species went through a long domestication process and subsequent intensive breeding (Gill et al., 2004). The resulting large intraspecific variability of cultivated wheat, along with the contribution of wild relatives, guarantees the availability of multiple traits of agronomic, dietary, and even gastronomic relevance (Mourad et al., 2019). Such biodiversity is constantly exploited to breed new cultivars needed to provide adequate yields, innovative technological or nutritional properties, and to improve wheat resistance to biotic and abiotic stress.

Within the available germplasm, pigmented *Triticum aestivum* L. spp. *aestivum* lines recently emerged for multiple reasons. These varieties are reputed as good sources of bioactive compounds, mainly carotenoids, phlobaphenes, phenolic acids, anthocyanins, and other polyphenols valuable for their contribution to human health (Gupta et al., 2021). In agricultural practice, varieties with red grain controlled by dominant *R-1* alleles located on the long arm of hexaploid wheat chromosomes 3A, 3B, and 3D (*R-A1*, *R-B1*, and *R-D1*, respectively) are widely used. This grain colour is determined by catechin and proanthocyanidin (Himi et al., 2011). Recessive alleles cause white tinging of the grain. In addition to varieties with red and white grain, there are forms with the presence of anthocyanins. Belong to this group, wheat genotypes with a purple pericarp, which is conditioned by *Pp* genes. According to Tereshchenko et al., (2012), a dark purple pericarp results from complementary action of the dominant alleles, *Pp-D1* (7D) and *Pp3* (2A). Presence of the dominant alleles, *Pp-A1* and *Pp3*, results in light purple colour, and the grain will remain uncoloured in the presence of the recessive *Pp3* allele even if two other alleles (*Pp-A1* and *Pp-D1*) are in the dominant state (E. I. Gordeeva et al., 2015). The complementary action of *Pp3* and *Pp1* genes was explained by interaction of their products resulting in a functional regulatory complex for anthocyanin biosynthesis (Shoeva et al.,

2014). In addition to the purple pericarp, there is a blue aleurone conditioned by *Ba* genes (*Ba1*, *Ba2* and *Ba3*) located on the fourth group of homologous chromosomes (4B, 4A and 4D, respectively) (Jeewani et al., 2021)(Qualset et al., 1983; Watanabe & Martinek, 2022). Since the genes can be jointed each other, black wheat genotype can be created by combining genes for blue aleurone and purple pericarp. In caryopses of pigmented wheat, more than forty different anthocyanins are selectively located in the pericarp or in the outer aleurone layers, giving the grain characteristic colours (purple, blue, black or red) according also to their quality and quantity (Lachman et al., 2017; Saini et al., 2020).

Overall, the accumulation of anthocyanins is considered a recently evolved trait in wild relatives of wheat, resulting from environmental adaptation to various forms of stress (Jia et al., 2020). Their presence in common wheat is instead the consequence of multiple and independent gene transfers occurred during breeding from, among others, diploid *T. boeoticum* Boiss., tetraploid *T. turgidum* L. subsp. *abyssinicum* Vavilov/now *T. aethiopicum* Jakubz. var. *arraseita* (Hochst. & Körn.) Philat./, decaploid *Thinopyrum ponticum* (Podp.) (Lachman et al., 2017). In planta, these secondary metabolites are involved in multiple roles, serving as a protection from frost, excessive UV exposure, osmotic disbalance and drought. Their contribution is mostly related to their antioxidant properties and to the regulation of ROS-induced signalling cascades (Santos-Buelga et al., 2014). More recently, a high anthocyanin content was also linked to a reduced susceptibility to pathogen attacks in various crops and in fruits, both in pre- and postharvest conditions, in agreement with the great flexibility of flavonoids (Lin et al., 2021). Regarding phlobaphenes, but not yet for anthocyanins, a correlation between antifungal defence and accumulation of these secondary metabolites has been recently suggested in maize with regard to mycotoxin accumulation related to *Fusarium* infection (Landoni et al., 2020). A larger body of evidence is instead available for sorghum, in which anthocyanins are actively involved in phytoalexin response

against invading fungi (Chandrashekar & Satyanarayana, 2006). Furthermore, since the consumption of wheat rich in anthocyanins require the use of whole grain flour, maintaining after milling the pericarp and/or the aleurone layer, it is crucial to verify the possible sanitary risk associate to the accumulation of contaminants, such as mycotoxins, which are generally located in the external kernel layers (Spaggiari et al., 2019). Fusarium head blight (FHB) is a major fungal disease in wheat, determining significant losses in yield and crop quality. FHB is caused mainly by *Fusarium graminearum* and *F. culmorum*, able to produce a variety of mycotoxins that accumulate in kernels leading to harmful consequences after food and feed consumption (Simsek et al., 2012). *Fusarium* mycotoxins include, among others, trichothecenes like deoxynivalenol (DON), T-2, HT-2, and other co-occurring toxins like enniatins (ENNs) and zearalenone (ZEN). Cereal crops can also biotransform many of the above-mentioned compounds to modified forms (McCormick et al., 2015). For instance, DON is transformed by adding a glucose moiety giving rise to DON3Glc, the most common and abundant masked mycotoxin (Berthiller et al., 2013)

As reported by Atanasova-Penichon et al., (2016) and Etzerodt et al., (2016), polar secondary metabolites with antioxidant properties may counteract toxigenic *Fusaria* and mycotoxin accumulation in wheat, although anthocyanin have not been evaluated in depth on this regard. In further metabolomics studies, anthocyanins were deemed as resistance-related metabolites in barley and in wheat cultivar Sumai-3 (Bollina et al., 2011; Gunnaiah & Kushalappa, 2014). Landoni et al., 2020 found that the accumulation of anthocyanins and phlobaphenes is related to a lower contamination by fumonisin B_1 in maize kernels. Finally, Choo et al., 2015 demonstrated that black barley is more resistant to DON accumulation than yellow barley.

As reported for other secondary metabolites, anthocyanin biosynthesis and accumulation are strongly influenced by environmental factors including climate, water availability, temperature, and ultimately growing seasons. This highlights the need for evaluations

considering multiple harvests in the same location and a wider range of pigments (Yang et al., 2018).

An exact assessment of the effect of anthocyanins in wheat grain on mycotoxin accumulation would be possible if we compared genotypes imprinting each other only with individual genes responsible for individual grain colours. Near-isogenic lines derived from common spring wheat seeded Novosibirskaya 67 will be available in the future (Watanabe, 2003), with the possibility to develop also lines with different content of anthocyanins.

This study is therefore aimed at investigating the susceptibility of pigmented wheat genotypes to *Fusarium*-related mycotoxins. To obtain reliable results, the investigation was carried out in open fields over a period of two years, by comparing the anthocyanin profile and the accumulation of multiple mycotoxins in twelve wheat varieties of different colour and genetic background.

3.1.2 Materials and methods

3.1.2.1 Chemicals

Analytical standards of DON and its acetylated forms 3Ac-DON and 15Ac-DON (100 mg l^{-1} in acetonitrile), DON3Glc (50 mg l^{-1} in acetonitrile), T-2 toxin (100 mg l^{-1} in acetonitrile), HT-2 toxin (100 mg l^{-1} in acetonitrile), ZEN (100 mg l^{-1} in acetonitrile) and ENN B (1 g l^{-1} in methanol) were purchased from Romer Labs (Getzersdorf, Austria). UHPLC-grade methanol, acetonitrile, acetic acid and water were purchased from VWR Chemicals (Radnor, USA). Ammonium acetate was purchased from Sigma-Aldrich (St. Louis, USA). Analytical standards of cyanidin 3-O-glucoside chloride, cyanidin 3-O-rutinoside chloride, malvidin 3-O-glucoside chloride, peonidin 3-O-glucoside chloride, pelargonidin 3-O-glucoside chloride, delphinidin 3-O-glucoside chloride and petunidin 3-O-glucoside chloride were purchased from Extrasynthese (Genay Cedex, France).

3.1.2.2 Samples

Samples from twelve varieties of pigmented common wheat (*T. aestivum* L. spp. *aestivum* L.), grown in a sandy-loam soil at Cigliano (NW Italy, 45° 18′ N, 8° 01′ E; altitude 237 m) were collected over two harvesting years (2019 and 2020). These varieties are characterized by four different grain colours (one red, chosen as a conventional control, 6 purple, 3 blue and 2 black varieties) as described in **Table 4**, **Figure S1** and **Figure S2** (supplementary materials).

Table 4 Pigmented wheat varieties compared in the 2-growing season field experiment.

Grain color	Variety	Status	Registration	Responsible of selection
Red (control)	Aubusson	Cultivar	2003	Limagrain Italia S.p.A., Fidenza (PR), Italy
Purple	Anthograin™ CDC	Cultivar	2018	Hetland Seeds Ltd, Naicam, SK, Canada
	Ceraso	Cultivar	2014	Saatzucht Donau GesmbH. & CoKG, Austria
	AF Jumiko	Cultivar	2018	Agrotest Fyto, Ltd., Kroměříž, Czech Rep.
	KM 106-18	Breeding line		Agrotest Fyto, Ltd., Kroměříž, Czech Rep.
	Merlot	Cultivar	2015	Saatzucht Donau GesmbH. & CoKG, Austria
	Rosso	Cultivar	2011	Saatzucht Donau GesmbH. & CoKG, Austria
Blue	Skorpion	Cultivar	2011*	Agrotest Fyto, Ltd., Kroměříž, Czech Rep.
	AF Oxana	Cultivar	2019	Agrotest Fyto, Ltd., Kroměříž, Czech Rep.
	KM 72-18	Breeding line		Agrotest Fyto, Ltd., Kroměříž, Czech Rep.
Black	AF Zora	Cultivar	2021	Agrotest Fyto, Ltd., Kroměříž, Czech Rep.
	KM 98-18	Breeding line		Agrotest Fyto, Ltd., Kroměříž, Czech Rep.

The daily temperatures and precipitation were measured at a meteorological station near the experimental area. According to the wheat development, the daily temperatures were reported as Growing Degree Days (GDD), using a 0 °C base value (**Table 5**). The agronomic technique commonly adopted in the area was applied. Briefly, the previous crop was maize, the field was ploughed each year, incorporating the debris into the soil. Planting was conducted in 12 cm wide rows at a seeding rate of 450 seeds m^{-2} in November. A total of 160 kg N ha^{-1} was applied, split equally at wheat tillering [growth stage (GS) 23] and at the beginning of the stem elongation (GS31). No fungicide was applied to control fungal diseases, in particular at flowering (GS61-65) no fungicide treatment has been carried out to control FHB infection. The sowing and harvest dates, together with the dates of the main GS, for each growing season are described by Scarpino et al., (2021). Treatments were assigned to an experimental unit using a completely randomized block design with three replicates. The plots measured 7 × 1.5 m.

Table 5: Monthly rainfall, rainy days, and growing degree days (GDD 0s) from the sowing (November) to the end of ripening stage (June) in the 2 growing seasons.

Growing season	Month	Rainfall (mm)	Rainy days (days)	GDD[a] (Σ °C d^{-1})
2019-20	November	314	12	249
	December	132	8	193
	January	5	1	168
	February	1	0	229
	March	62	7	285
	April	81	10	414
	May	122	7	579
	June	113	7	624
	November - June	830	52	2741
	November - March	514	28	1124
	April - June	317	24	1617
2018-19	November	124	5	292
	December	11	1	151
	January	6	1	141
	February	43	7	195
	March	17	4	314
	April	116	7	393
	May	178	9	478
	June	40	4	667
	November - June	535	39	2632
	November - March	200	19	1093
	April - June	335	21	1539

The grain yields were obtained by harvesting the whole plot using a Walter Wintersteiger cereal plot combine-harvester. The harvested grains were mixed thoroughly, and 4 kg grain samples were taken from each plot, ground using a ZM 200 Ultra Centrifugal Mill (Retsch GmbH, Haan, Germany) and representative sub-samples of each whole-grain flour were used directly to analyze the anthocyanin and the mycotoxin content. A further subsample of grain (500 g) was taken from each plot to determine the test weight (TW) using a GAC® 2000 Grain Analyzer (Dickens-John Auburn, IL, The USA).

3.1.2.3 Sample preparation for anthocyanin analysis

Samples were extracted according to Zaupa et al., (2015), with some modifications. 1.5 mL of ethanol/water/formic acid (97:2:1, v/v/v) mixture were added to 150 mg of ground wheat. The samples were extracted for 15 min using a platform shaker (Ika Werke, Germany) at a speed of 200 strokes/min and subsequently centrifugated for 10 minutes at 10000 rpm (radius 9.5 cm) at 4°C. 1 mL of clear supernatant was evaporated to dryness using a centrifugal vacuum concentrator (Labconco, USA) and re-dissolved in 100 µL of methanol/water (50:50, v/v) mixture and then transferred into vials prior to be injected into the UHPLC-MS/MS system.

3.1.2.4 UHPLC-MS/MS anthocyanin analysis

UHPLC-MS/MS analysis was carried out on UHPLC Dionex Ultimate 3000 coupled to a triple quadrupole mass spectrometer TSQ Vantage (Thermo Fisher Scientific, Waltham, USA) equipped with an electrospray source (ESI). The chromatographic separation was obtained using a Sunshell column (Chromanik Technologies, Osaka, Japan) 2.1 × 100 mm, 2.6 µm particle size, heated to 40° C. 2 µL of sample extract was injected into the UHPLC system and the flow rate was set up to 0.35 mL min^{-1}. Gradient elution was performed by using water (eluent A) and acetonitrile (eluent B) both acidified with 0.1% v/v formic acid. Initial conditions were set at 99% A and 1% B for 10 min, then eluent B was increased to 80%, after an isocratic step (4 min), the gradient was returned to the initial condition. The total run time was 23.5 min. Mass spectrometric analysis was performed in positive ionization mode in multiple reaction monitoring (MRM), spray voltage 3500 V, capillary temperature 270°C, vaporizer temperature 300°C, sheath gas pressure 50 units, auxiliary gas pressure 10 units.

The following quantifier transitions were measured: cyanidin 3-O-glucoside m/z 449>287 (CE 30eV), cyanidin 3-O-rutinoside m/z 595>287 (CE 30eV), cyanidin O-malonyl-hexoside m/z 535>287 (CE 30eV), delphinidin 3-O-glucoside m/z 465>303 (CE 30eV), delphinidin O-rutinoside m/z 611>303 (CE 30eV), peonidin 3-O-glucoside m/z 463>301 (CE 30eV), peonidin O-rutinoside m/z 609>301 (CE 30eV), peonidin O-malonyl-hexoside m/z 549>301 (CE 30eV), petunidin 3-O-glucoside m/z 479>317 (CE 30eV), petunidin O-rutinoside m/z 625>317 (CE 30eV), malvidin 3-O-glucoside m/z 493>331 (CE 30eV), pelargonidin 3-O-glucoside m/z 433>271 (CE 30eV), pelargonidin O-rutinoside m/z 579>271 (CE 30eV), pelargonidin O-malonyl-hexoside m/z 519 (CE 30eV). Calibration curves were set up using external standards (range 5 µg kg^{-1} – 6500 µg kg^{-1}) for target analyte quantification. The concentration of cyanidin O-malonyl-hexoside was expressed as cyanidin 3-O-glucoside equivalents. The concentration of delphinidin rutinoside was expressed as delphinidin 3-O-glucoside equivalents. The concentration of peonidin O-rutinoside and peonidin O-malonyl-hexoside were expressed as peonidin 3-O-glucoside equivalents. The concentration of petunidin O-rutinoside was expressed as petunidin 3-O-glucoside equivalents. The concentration of pelargonidin O-rutinoside and pelargonidin O-malonyl-hexoside were expressed as pelargonidin 3-O-glucoside equivalents. Data acquisition was performed by Thermo Xcalibur 2.2 software (Thermo Fisher Scientific, Waltham, USA).

3.1.2.5 Sample preparation for mycotoxin analysis

Samples were extracted according to Malachová et al. (2014), with some modifications. Briefly, 8 mL of acetonitrile/water (80:20, v/v) mixture acidified with 0.1% formic acid were added to 2 g of ground wheat. The samples were extracted for 90 min using a platform shaker (Ika Werke, Germany) at a speed of 200 strokes/min and subsequently centrifugated for 2

minutes at 3000 rpm (radius 17.8 cm) at room temperature. 1000 µl of supernatant were transferred into vials and then injected into the UHPLC-MS/MS system. Three biological replicates and three technical replicates for each variety and each year have been analysed (N = 3*3*12*2 = 216).

3.1.2.6 UHPLC-MS/MS mycotoxin analysis

UHPLC-MS/MS analysis was carried out on UHPLC Dionex Ultimate 3000 coupled to a triple quadrupole mass spectrometer TSQ Vantage (Thermo Fisher Scientific, Waltham, USA) equipped with an electrospray source (ESI). The chromatographic separation was obtained using a Sunshell column (Chromanik Technologies, Osaka, Japan) 2.1 × 100 mm, 2.6 µm particle size, heated to 40° C. 2 µl of sample extract was injected into the UHPLC system and the flow rate was set up to 0.35 mL min^{-1}. Gradient elution was performed by using water (eluent A) and methanol (eluent B) both acidified with 0.2% v/v acetic acid. Ammonium acetate was added to the eluent A at the final concentration of 5mM. Initial conditions were set at 98% A and 2% B for 2 min, then eluent B was increased to 20%, after an isocratic step (6 min), eluent B was further increased to 90% and this condition was maintained for 10 min until the return to the initial condition. The total run time was 26.5 min. Mass spectrometric analysis was performed both in positive and negative ionization mode in multiple reaction monitoring (MRM), spray voltage 3000 V, capillary temperature 270°C, vaporizer temperature 200°C, sheath gas pressure 50 units, auxiliary gas pressure 5 units. The following quantifier transitions were measured: DON m/z 355>295 (CE 13eV) and m/z 355>265 (CE 19eV), DON3Glc m/z 517>457 (CE 17eV) and m/z 517>427 (CE 24eV), 3Ac-DON and 15AcDON m/z 397>307 (CE 18eV) and m/z 397>59 (CE 20eV), T-2 m/z 484>215 (CE 19eV) and m/z 484>185 (CE 22eV), HT-2 m/z 442>263 (CE 11eV), ZEN m/z 317>175

(CE 26eV) and m/z 317>131 (CE 32eV), ENN B m/z 640>528 (CE 20eV), m/z 640>314 (CE 31eV), m/z 640>214 (CE 26eV), m/z 640>196 (CE 29eV), m/z 640>186 (CE 37eV) and m/z 640>86 (CE 44eV). Calibration curves were set up using external standards (range 1 μg kg^{-1} – 2500 μg kg^{-1}) for target analyte quantification. Data acquisition was performed by Thermo Xcalibur 2.2 software (Thermo Fisher Scientific, Waltham, USA).

3.1.2.7 Statistical analysis

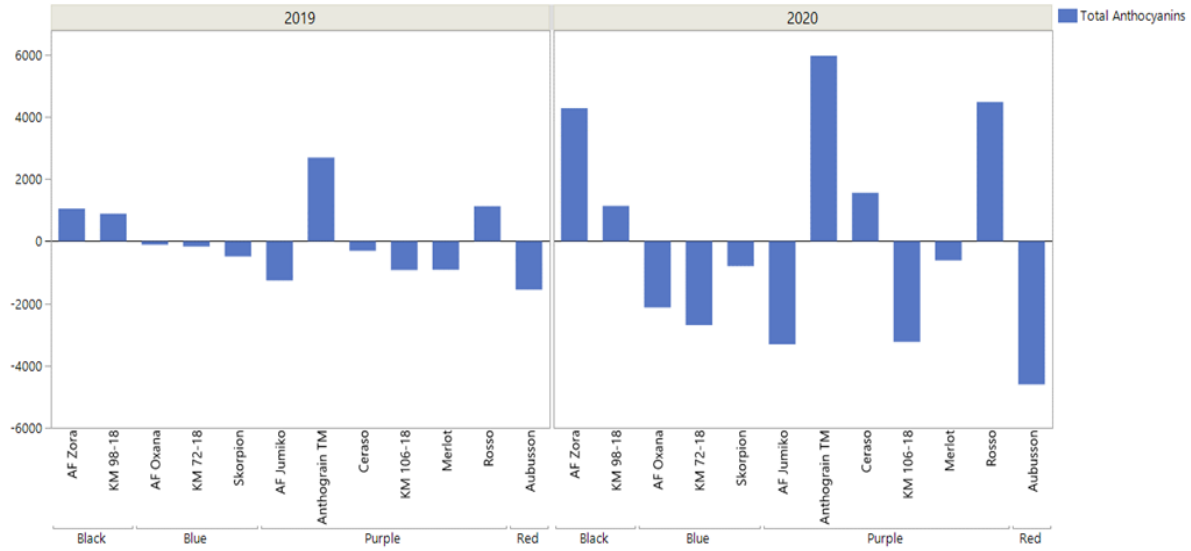
The statistical analysis was performed using IBM SPSS v.25.0 (SPSS Italia, Bologna, Italy). Three independent technical replicates have been considered for each biological replicate, and three biological replicates have been considered for each variety and each year. Data were log-normalised prior to statistical analysis. ANOVA followed by Tukey post-hoc test (α=0.05) and Pearson's correlation test (α=0.01) were run using Statistica 13.5.0.17 (Tibco Software Inc., Palo Alto, CA, USA).

3.1.3 Results and discussion

The two growing seasons showed a similar meteorological trend throughout the wheat crop cycle (**Table 5**), with the rainfall concentrated after sowing (November) and between stem elongation and flowering (April-May). The precipitation during the ripening stage (June) were higher in 2020 than 2019 (113 vs 40 mm), while during flowering (May) the rainfall were similar between the two years. In addition, during flowering, 2020 had higher temperature than 2019 as described by differences in GDD data (**Table 5**).

We opted for a precise quantification and profiling of a wide range of glycosides and aglycones, including delphinidin and malvidin that were seldomly evaluated in previous investigations. As expected, significant differences emerged between blue, purple, red and black varieties both from quantitative and qualitative standpoint (**Table 6**). The total anthocyanins content for each variety is returned in **Figure 7** as the deviation from the overall mean value. The dataset underwent a full factorial ANOVA, returning both the harvest year ($p < 0.0001$) and the variety ($p < 0.0001$) as significant factors, while their interaction was not significant ($p = 0.0672$). Purple variety Anthograin^{TM} provided the best results in both harvest years (11171 and 4451 µg kg^{-1} in 2020 and 2019, respectively), with Ceraso (6676 and 1458 µg kg^{-1}), Rosso (9690 and 2895 µg kg^{-1}), AF Zora (9488 and 2810 µg kg^{-1}) and KM 98-18 (6345 and 2650 µg kg^{-1}) providing above-average contents. Overall, blue and black-grained wheats proved to be more varied in chemical composition, with the black ones showing quantities above LOQ for all 14 anthocyanins tested, while red and purple ones had only 9-10 types.

Figure 7: Deviation from the mean value calculated for total anthocyanins (expressed as the sum of all the metabolites detected within this study) for each variety and for each harvest year. Data are expressed in µg kg^{-1}.



Overall, the pattern obtained was in accordance with previous reports, as purple varieties were richer in peonidin and had limited content in petunidin, while malvidin glucoside was absent in purple varieties. Its presence was consistent in both blue and black ones, which were the sole varieties containing delphinidin glycosides (Landoni et al., 2020). Blue-grained wheats instead had higher amounts of cyanidin, malvidin and petunidin, while red varieties were reported in the literature as the less rich of total anthocyanins and devoid of pelargonidin derivatives (Lachman et al., 2017).

It must be noticed however that anthocyanin distribution within a single colour group was not uniform, and few exceptions were registered. For instance, despite being listed as a purple varieties, Anthograin™ and Ceraso differed for being rich in cyanidin, delphinidin and malvidin derivatives which were absent or very scarce in other purple varieties. Ceraso, despite being registered as a purple variety, interestingly had a phytochemical profile almost superimposable to blue ones, therefore showing a unique pattern. A large phytochemical variability must be then expected even within chromatic groupings. Also, several genes are

responsible for the genetic basis of the purple colour of the grain (E. Gordeeva et al., 2020; E. I. Gordeeva et al., 2015) but detailed evaluation is often lacking in varieties that are under continuous development. The purple varieties used in our experiment have an evidently different genetic determination of the pericarp colour, where Anthograin^{TM} is visually darker and AF Jumiko is lighter, and this is also consistent with **Table 6**. The genetic composition of *Pp* (*Purple pericarp*) alleles in the purple genotypes used in this study is unknown to us.

In absolute terms, the anthocyanin content for all varieties was lower than the literature data for the available varieties and with consistent differences between harvest years, which may be related to distinct agronomic conditions which may be related to distinct environmental conditions, including mycotoxin exposure. However, if we refer to the 2019 and 2020 growing seasons, the agronomic cycle was not so different and, for instance, flowering of wheat took place after the same number of days from sowing. In particular, the significant lower test weight between years, besides contributing to a dilution effect for anthocyanin content due to endosperm/pericarp ratio favouring the former, might be a sign of a different FHB pressure between 2019 and 2020.

Beyond the effects of environmental conditions on anthocyanin profile, also the high accumulation of mycotoxins, likely due to a relevant fungal infection, may have altered biosynthetic pathways for anthocyanins in both harvest years. It has been often reported, in other crops than wheat, that anthocyanins may increase as a consequence of *Fusarium* infection albeit different behaviour was reported for scarcely and heavily infected plants (Chrpová et al., 2021). It remains unclear if variations in anthocyanin profile and content could be a consequence of an alteration in plant biochemistry induced directly by mycotoxin exposure during the active part of the infection, or the result of previous elicitation exerted by biological exposure to *Fusarium*, e.g. by simple contact by wheat and fungal hyphae.

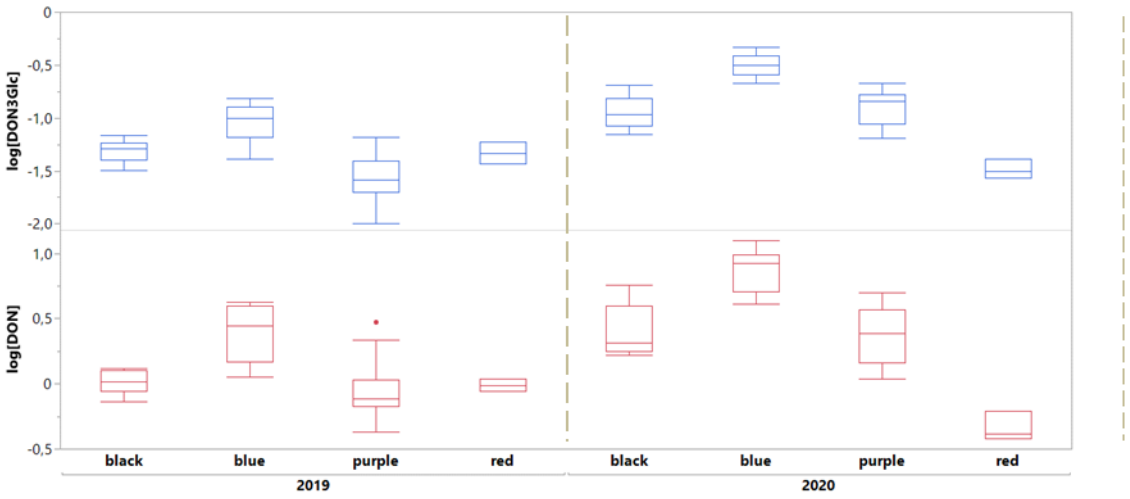
Table 6: Occurrence of anthocyanins in twelve varieties of coloured common wheat. Three biological replicates were considered for each variety. Data are reported as means ± SD. Different letters indicate significant differences ($p > 0.005$) among varieties within the same harvest year.

Year	Grain color	Variety	Cyanidin			Dephinidin		Peonidin			Petunidin		Malvidin	Pelargonidin			Total Anthocyanines
			µg kg-1														
			3-O-Glc	3-O-Rut	O-MalHex	3-O-Glc	3-O-Rut	3-O-Glc	3-O-Rut	O-MalHex	3-O-Glc	3-O-Rut	3-O-Glc	3-O-Glc	3-O-Rut	O-MalHex	
2020	Black	AF Zora	735±36 b	1867±89 a	398±92 c	622±53 a	1041±97 a	644±149 b	1583±345 a	1660±409 b	127±6 a	353±27 a	122±0	86±11 b	99±14 b	34±4 c	9488±930
		KM 98-18	415±84 c	1983±399 ab	225±26 c	419±51 b	1185±18 9a	241±35 c	576±78 c	597±90 d	106±5 a	387±51 a	118±2	27±2 c	30±2 de	<LOD	6345±1012
	Blue	Skorpion	278±48 d	1449±240 b	93±21 d	323±28 c	838±93 b	129±20 d	420±66 c	251±55 e	99±4 ab	293±27 b	113±1	32±4 c	41±7 d	<LOQ	4372±630
		AF Oxana	146±29 d	967±213 c	29±3 e	289±37 c	817±152 b	58±4 d	236±48 d	61±6 f	90±4 b	253±42 b	111±4	<LOQ	25±1 e	25±0 c	3074±551
		KM 72-18	164±45 d	752±273 c	32±8 e	255±40 c	508±123 c	66±11 d	217±50 d	74±30 f	90±5 b	197±45 bc	109±3	<LOD	27±0 e	150±27 b	2453±591
	Purple	Anthograin™ CDC	1362±180 a	1459±154 b	2194±239 a	<LOD	<LOD	1354±185 a	729±81 b	3517±341 a	<LOD	72±0.2 c	111±3	97±9 b	64±5 c	37±2 c	11110±1158
		Ceraso	451±31 c	2077±282 a	281±19c	412±33 b	1211±172 a	287±24 c	593±83 c	758±90 c	104±5 a	379±51 a	118±3	29±1 c	31±2 d	35±2 c	6766±720
		Jumiko	87±9 e	124±6 e	142±16 d	<LOD	118±5 d	273±27 c	262±19 d	735±77 c	<LOD	74±1 c	<LOD	26±0 c	25±0 d	50±4 c	1891±135
		KM 106-18	94±14 e	206±28 e	169±12 d	<LOD	122±6 d	224±24 c	321±41 d	650±54 cd	<LOD	75±2 c	<LOD	30±2 c	30±3 d	174±42 b	1970±185
		Merlot	244±41 d	464±74 d	277±50 c	<LOD	111±0 d	587±122 b	842±157 b	1665±368 b	<LOD	75±1 c	<LOD	77±14 b	79±14 bc	365±16 a	4471±823
		Rosso	571±14 c	1443±94 b	794±34 b	<LOD	<LOD	1071±20 a	1699±82 a	3367±104 a	<LOD	77±1 c	<LOD	135±3 a	167±10 a	49±9 c	9664±348
	Red	Aubusson	28±5 f	59±32 f	25±1 e	118±0 d	125±22 d	52±1 d	57±3 e	66±4 f	<LOQ	80±1 c	<LOD	<LOQ	<LOQ	212±14 ab	437±206
	Mean ± SD		*381±376*	*1071±742*	*388±680*	*348±158*	*608±465*	*416±422*	*628±526*	*1117±1215*	*103±14*	*193±133*	*115±5*	*60±40*	*56±44*	*113±12*	*5170±3495*
			3-O-Glc	**3-O-Rut**	**O-MalHex**	**3-O-Glc**	**3-O-Rut**	**3-O-Glc**	**3-O-Rut**	**O-MalHex**	**3-O-Glc**	**3-O-Rut**	**3-O-Glc**	**3-O-Glc**	**3-O-Rut**	**O-MalHex**	
2019	Black	AF Zora	151±14 b	413±54 a	97±25 c	214±28 a	324±67 ab	209±51 b	509±76 a	509±145 b	78±5	163±35	95±3	13±4	13±2	4±2	2818±161
		KM 98-18	129±7 b	596±52 a	111±34 b	194±18 ab	474±43 a	125±26 c	296±14 bc	321±97 c	75±3	228±14	96±0	<LOQ	<LOQ	<LOQ	2650±86
	Blue	Skorpion	38±6 d	275±57 b	7±2 e	136±5 b	252±34 b	44±4 d	166±38 c	57±14 e	70±1	137±19	92±3	<LOQ	<LOQ	<LOQ	1275±149
		AF Oxana	47±3 d	404±26 a	3±1 e	175±9 b	423±20 a	37±1 d	175±8 c	38±6 e	72±1	185±9	92±1	<LOQ	<LOQ	<LOD	1639±51
		KM 72-18	73±36 c	393±156 ab	3±2 e	186±45 ab	324±96 ab	42±5 d	187±55 c	36±3 e	78±8	176±49	96±6	<LOQ	<LOQ	31±8	1593±461
	Purple	Anthograin™ CDC	436±56 a	381±73 b	711±64 a	<LOD	<LOQ	655±70 a	329±58 b	1789±140 a	63±0,2	<LOD	<LOD	22±3	6±2	65±9	4451±457
		Ceraso	65±13 c	131±21 c	118±24 b	<LOD	88±2 c	195±34 bc	196±39 c	572±84 b	<LOQ	<LOD	<LOD	19±3	11±3	<LOQ	1458±229
		Jumiko	14±6 e	53±3 d	28±13 d	<LOD	89±2 c	79±25 d	56±11 e	186±77 d	<LOQ	<LOD	<LOD	<LOQ	<LOD	9±1	504±133
		KM 106-18	33±5 d	73±4 d	72±9 c	<LOD	86±0 c	123±9 c	97±9 d	345±30 c	<LOQ	<LOD	<LOD	<LOQ	<LOQ	18±2	780±72
		Merlot	33±3 d	77±4 d	37±3 d	<LOD	88±2 c	147±5 c	107±7 d	333±17 c	<LOQ	<LOD	<LOD	5±0.4	<LOQ	83±45	844±41
		Rosso	167±85 b	258±122 bc	222±109 b	<LOD	86±0 c	381±164 b	419±206 ab	1238±565 ab	<LOQ	<LOD	<LOD	23±13	17±12	<LOQ	2866±1276
	Red	Aubusson	<LOQ	49±0 d	<LOQ	<LOD	86±0 c	<LOQ	35±0 e	39±2 e	<LOQ	<LOD	<LOD	<LOQ	<LOD	59±5	82±112
	Mean ± SD		*108±120*	*258±182*	*128±204*	*181±29*	*211±153*	*185±185*	*215±146*	*455±540*	*73±6*	*178±33*	*94±2*	*16±8*	*12±51*	*39±31*	*1747±1240*

The whole potential array of compounds associated with *Fusarium* infection in wheat was screened, namely DON and its major modified form DON3Glc, 3Ac-DON, 15Ac-DON, together with other relevant *Fusarium* mycotoxins such as T-2, HT-2, ZEN and Enniatin B (**Table 7**). The overall mycotoxins occurrence is within 471-9603 µg kg^{-1} for DON, <LOQ-357 µg kg^{-1} for DON3Glc, <LOD-98 µg kg^{-1} for ZEN, 22-951 µg kg^{-1} for Enniatin B. T-2 and HT-2 in all samples were <LOD or <LOQ except for a sample of the variety KM 72-18 (year 2019) in which HT-2 was quantified at 21 µg kg^{-1} (data not shown). Acetylated forms of DON were not detected in any samples. Biotransformation rate, expressed as DON3Glc/TDON (where TDON is the sum of DON and DON3Glc), was in the range of 0.025-0.066 with a mean value of 0.047. Regarding the co-occurrence, solely DON and Enniatin B were detected in all the varieties analysed.

Based on statistical analysis, results show a strong highly significant influence effect of the harvesting year ($p = 0.0002$) on DON content, which is more than doubled for the harvesting year 2020 (mean of 3746 µg kg^{-1} vs. 1463 µg kg^{-1}). In addition, a striking influence of genotype with different grain colour on DON content ($p < 0.0001$) emerged as well, also in combination with the harvesting year (year*colour, $p = 0.0091$) with the blue grain found as the more contaminated (mean of 5352 µg kg^{-1}) and red grain found as the less contaminated (mean of 715 µg kg^{-1}) (**Figure 8**). This trend is also maintained between the two harvesting years, in spite of the highly variable absolute mycotoxin content.

Figure 8: Box plot describing the DON and DON3Glc content in pigmented grains clustered according to the group and the harvest year. Data are log-normalised.



This phenomenon is of particular interest because, at the best of our knowledge, has not been reported so far. Both the effects mentioned above were found as significant according to ANOVA analysis and, particularly, a post-hoc Tuckey test showed a significant difference between blue and the other grain colours. The content of DON3Glc follows the trend described above for DON, with a higher DON3Glc content detected for the harvesting year 2020 (mean of 170 µg kg^{-1}) and for blue grain (mean of 211 µg kg^{-1}), even in that case we have seen an effect of the harvesting year ($p = 0.0021$) and colour ($p = 0.0240$) very similar to what was reported for DON. We have also found a positive correlation between the DON and DON3Glc content, considering all varieties for both years, based on Pearson's correlation coefficient ($r = 0.927$, $p < 0.0001$), consistently with the current literature (Ovando-Martínez et al., 2013). About the DON3Glc/TDON ratio, previously reported as correlated to resistance to FHB in durum and common wheat, the highest value has been found for red grain (0.059) and the lowest for blue grain (0.038) consistently with what was observed regarding the contamination by DON (Amarasinghe et al., 2016). However, this difference has not been found as significant. Regarding the presence of Enniatin B, an emerging mycotoxin, we have found an effect of the harvesting year ($p = 0.001$), with a greater contamination for the year 2020, but an influence of the genotype (grain colour) only for the year 2019. We have also found a

significant positive correlation between DON and Enniatin B content only for 2020 and not for 2019, this is probably due to the different chemical structure, metabolic pathway and main *Fusarium* species involved in biosynthesis (Jestoi, 2008). Finally, we were able to quantify ZEN in 32 out of 72 samples, of which 30 from the harvesting year 2020, in accordance with a greater overall mycotoxin contamination. The maximum concentration detected for ZEN was 98 µg kg^{-1} for the blue grain variety Skorpion, year 2020.

During both years a correlation was observed between the chromatic typology of wheat and the presence of DON and its derivatives (**Figure 8**). Based on Pearson's test, DON was positively correlated with total petunidin ($r = 0.5338$; $p < 0.0001$) and malvidin content ($r = 0.4025$; $p = 0.0005$) in wheat genotype, while no significant correlation was found with other compounds.

Table 7: Occurrence of mycotoxins in twelve varieties of coloured common wheat.

Year	Grain color	Variety	DON	DON-3-Glc	3/15Ac-DON	ZEN	Enn B	T-2	HT-2
						mg kg-1			
2020	Black	AF Zora	2.327±1.021 d	0.101±0.034 d	<LOD	0.012±0.000 d	0.247±0.138 d	<LOQ	<LOD
		KM 98-18	3.280±2.093 c	0.141±0.060 c	<LOD	0.045±0.014 b	0.518±0.388 b	<LOQ	<LOQ
	Blue	Skorpion	7.235±2.594 b	0.289±0.060 b	<LOD	0.098±0.047 a	0.418±0.317 c	<LOQ	<LOQ
		AF Oxana	9.603±2.767 a	0.357±0.092 a	<LOD	0.088±0.037 a	0.681±0.314 b	<LOQ	<LOQ
		KM 72-18	6.769±3.163 b	0.329±0.103 a	<LOD	0.057±0.020 b	0.951±0.366 a	<LOQ	<LOQ
	Purple	Anthograin™ CDC	1.466±0.561 d	0.084±0.010 d	<LOD	0.028±0.013 c	0.281±0.227 d	<LOD	<LOD
		Ceraso	2.333±1.406 d	0.100±0.054 d	<LOD	0.034±0.007 c	0.554±0.509 b	<LOD	<LOQ
		Jumiko	2.562±0.271 d	0.177±0.017 c	<LOD	0.023±0.021 c	0.317±0.192 c	<LOD	<LOD
		KM106-18	3.889±1.008 c	0.174±0.033 c	<LOD	0.040±0.032 b	0.416±0.103 c	<LOQ	<LOQ
		Merlot	3.460±0.494 c	0.152±0.030 c	<LOD	0.032±0.015 c	0.194±0.071 de	<LOD	<LOD
		Rosso	1.555±0.247 d	0.107±0.028 d	<LOD	0.027±0.014 c	0.129±0.066 e	<LOD	<LOD
	Red	Aubusson	0.471±0.128 e	0.033±0.007 e	<LOD	0.01±0.000 d	0.125±0.057 e	<LOD	<LOD
	Mean ± SD		*3.746±2.991*	*0.170±0.109*	*n.d.*	*0.046±0.033*	*0.403±0.326*	*n.d.*	*n.d*
2019	Black	AF Zora	1.058±0.176 cd	0.054±0.013 b	<LOD	<LOD	0.083±0.011 c	<LOD	<LOD
		KM 98-18	1.046±0.299 cd	0.046±0.013 c	<LOD	<LOQ	0.121±0.068 b	<LOD	<LOQ
	Blue	Skorpion	3.212±0.772 a	0.127±0.026 a	<LOD	0.081±0.000	0.130±0.047 ab	<LOD	<LOD
		AF Oxana	3.922±0.266 a	0.105±0.018 a	<LOD	<LOD	0.103±0.012 b	<LOD	<LOQ
		KM 72-18	1.377±0.352 c	0.059±0.021 b	<LOD	<LOQ	0.171±0.043 a	<LOD	0.021±0.000
	Purple	Anthograin™ CDC	0.539±0.121 f	0.030±0.000 d	<LOD	<LOD	0.034±0.012 d	<LOQ	<LOQ
		Ceraso	0.653±0.199 e	<LOQ	<LOD	0.010±0.000	0.026±0.000 e	<LOD	<LOD
		Jumiko	0.752±0.060 e	0.029±0.006 d	<LOD	<LOD	0.044±0.014 d	<LOD	<LOD
		KM106-18	0.960±0.361 d	0.033±0.008 d	<LOD	<LOD	0.039±0.005 d	<LOD	<LOD
		Merlot	2.039±0.986 bc	0.053±0.015 b	<LOD	<LOD	0.022±0.000 e	<LOD	<LOD
		Rosso	1.040±0.449	0.057±0.013 b	<LOD	<LOD	0.038±0.027 d	<LOD	<LOD
	Red	Aubusson	0.961±0.114 d	0.054±0.015 b	<LOD	<LOQ	0.058±0.037	<LOQ	<LOQ
	Mean ± SD		*1.463±1.096*	*0.053±0.034*	*n.d.*	*0.052±0.021*	*0.079±0.011*	*n.d.*	*n.d.*

Three biological replicates were considered for each variety. Data are reported as means ± SD. Different letters indicate significant differences ($p > 0.005$) among varieties within the same harvest year.

While observing the overall contamination, the blue and black varieties were found to be more contaminated, while the red and purple ones showed an overall lower occurrence of mycotoxin. While it would be incorrect to argue of greater or lesser resistance to *Fusarium*, it was nevertheless possible to note a possible histological pattern for such data. In fact, in black and blue-grained wheats, anthocyanins are accumulated in the inner aleurone layer, whereas in purple types these secondary metabolites are concentrated directly in the pericarp that is outer layer directly exposed to the first contact with *Fusarium*. This distinct location could have led to a different dynamic in exposure to the pathogen, or to a possible greater protection during the initial stages of infection in spikelet.

Blue-grained wheats in this study are also characteristically less resistant and for instance the variety Skorpion is considered extremely susceptible to *Fusarium* infection (Martinek et al., 2013). This hypothesis is seemingly reinforced by Tukey's post-hoc test on DON, suggesting that the only really different group is that of blue grains. In our case, the wheats with black grain (AF Zora, KM 98-18) are the result of a combination of the *Ba2* gene and genes for purple pericarp. Black wheat, on the other hand, does not significantly differentiate from purple one. In this case, it seems that in the black grain, the purple pericarp may have a protective role, saving the deeper blue aleurone against *Fusarium* attack. However, this hypothesis needs to be verified with further experiments able to compare near-isogenic lines, which differ only for the genes responsible for the anthocyanin biosynthesis, especially considering that the number of replicates considered in the present study is limited and post-hoc test may suffer from distortion with low sample-size experiments. Furthermore, if the hypothesis is valid, it makes sense to further investigate the *Fusarium*-resistance mechanisms among the current purple-grained wheats grain. This would lead to knowledge to drive breeding in order to create varieties with black-grained wheat with an enhanced resistance to mycotoxins accumulation. This is a point of reflection, given that both blues and blacks have

a similar genetic, but blacks also have anthocyanins in the pericarp and not limited to the inner aleurone layer. The fact that the blue-grained varieties conditioned by the *Ba2* gene in this study were susceptible to mycotoxin accumulation is a problem that may prevent their use in practice. It would be very appropriate to carry out a special study evaluating the importance of various donors carrying all individual *Ba* genes in terms of resistance to FHB or other factors causing plant stress. At the same time, the sole conventional red cultivar tested (Aubusson), despite providing the lowest amount of anthocyanins, was the less affected by mycotoxin contamination. This observation leading to suggest that other defensive compounds than anthocyanins are involved in determining a greater resistance to mycotoxin accumulation for this variety. Since the genes for red, purple and blue colour of the grain lie on different chromosomes, they can be combined with each other in the breeding process. A detailed study should be carried out with common varieties with red grain and with different degrees of resistance to FHB in relation to important phenolic compounds in the grain. For an exact evaluation of the effect of anthocyanins in wheat grain on mycotoxin accumulation, it should be appropriate to compare almost isogenic lines, differing from each other in individual genes for grain colour. This approach would make it possible to eliminate the interfering effects of unknown genes in the genetic background. Some near-isogenic lines for purple grain colouration have been already created (Morgounov et al., 2020), while near-isogenic lines for blue colouration are being also gradually created (Watanabe and Martinek, 2022). It will be important that these lines are derived from the same recipient variety. It is clear that the presence of coloured substances in plant tissues has an evolutionary significance (Khlestkina, 2013) and it is likely that the anthocyanin colouration of the grain affects mycotoxin accumulation in wheat. Further studies will be needed to confirm this hypothesis.

3.1.4 Supplementary material

Figure S1: Appearance of ears of used varieties: A – Aubusson; B – Anthograin; C – Ceraso; D – AF Jumiko; E – KM 106-18 (with elongated glume transferred from Triticum polonicum L.); F – Merlot; G – Rosso; H – Skorpion; I – AF Oxana; J – KM 72-18; K – AF Zora; L – KM 98-18.

Figure S2: Appearance of grains of different colour selected between the varieties considered in this study: Aubusson (top left), Merlot (top right), Skorpion (bottom left) and AF Zora (bottom right).

3.2 Study 2: *Fusarium* mycotoxins in cereals: a cultivar comparison

3.2.1 Introduction

Fusarium head blight (FHB) is a devastating disease of cereals caused by a complex of many species, among which *F. graminearum, F. culmorum, F. avenaceum* and *F. poae* are predominant (Audenaert et al., 2009). These and other fungal species are known to infect various cereal crops, among which durum and common wheat, barley, triticale, rye, emmer, spelt and tritordeum among others (Karlsson et al., 2021; Spaggiari et al., 2019; Zrcková et al., 2019). Pathogenic fungi responsible for FHB also produce a wide range of mycotoxins, such as trichothecenes and ZEN, but also emerging mycotoxins like ENNs. On this regard, it is well known that mycotoxins frequently co-occur because of the ability of a single *Fusarium* strain to produce multiple mycotoxins and to the frequent co-infection by different *Fusarium* species which takes place in the open field. Co-occurrence poses a significant threat to public health, due to the additive or synergistic effects of these compounds, hence, a multi-mycotoxin approach is paramount (Ederli et al., 2021; Lee & Ryu, 2017; Lindblad et al., 2013).

Plant resistance to FHB is classified considering various factors such as the resistance to fungal infection and its spread from the initial infection site, but also the accumulation of mycotoxins. On this regard, cultivar comparison based on the multi-mycotoxin occurrence represent a useful tool to identify resistant cultivars. For this purpose, modified forms of mycotoxins should be also considered. In fact, in literature DON3Glc/DON ratio is reportedly higher in FHB-resistant lines than in susceptible ones, speculating that DON-3-glucoside formation may represent a possible detoxification mechanism in response to *Fusarium* infection (Lemmens et al., 2016).

In addition, it is well known that straw can be also contaminated with mycotoxins, posing serious threat to animal welfare due to its use as feed and also a potential risk for humans due to their persistence in the food chain. Contamination, also considering lipophilic emerging mycotoxins such as enniatin B, must be therefore carefully evaluated due to its possible bioaccumulation in some animal tissues (Křížová et al., 2021).

Overall, to carry out a cultivar comparison, the evaluation of the occurrence of multi-mycotoxins and modified forms such as DON3Glc in kernels and straw at the same time, allows to obtain more detailed contamination data. Furthermore, it allows to evaluate biotransformation capacity of single cultivar or species as well as to estimate their ability to translocate mycotoxins. These are all useful indications to better understand the plant-mycotoxin interaction.

Considering the large variety of factors affecting development and infection of *Fusaria* in open field and to obtain more solid data on resistance/susceptibility to mycotoxin accumulation, kernel and straw samples from two consecutive harvesting years at the same location, were considered for each cultivar. To properly compare cultivars, consistent agronomic conditions were applied, and climate data were recorded.

3.2.2 Materials and methods

3.2.2.1 Chemicals

Analytical standards of DON and its acetylated forms 3Ac-DON and 15Ac-DON (100 mg l^{-1} in acetonitrile), DON3Glc (50 mg l^{-1} in acetonitrile), T-2 toxin (100 mg l^{-1} in acetonitrile), HT-2 toxin (100 mg l^{-1} in acetonitrile), ZEN (100 mg l^{-1} in acetonitrile) and ENN B (1 g l^{-1} in methanol) were purchased from Romer Labs (Getzersdorf, Austria). UHPLC-grade methanol, acetonitrile, acetic acid and water were purchased from VWR Chemicals (Radnor, USA). Ammonium acetate was purchased from Sigma-Aldrich (St. Louis, USA).

3.2.2.2 Samples

Samples from forty cultivars of different cereals, as summarized in **Table 8**, grown in a sandy-loam soil at Cigliano (NW Italy, 45° 18′ N, 8° 01′ E; altitude 237 m) were collected over two harvesting years (2020 and 2021). These cultivars belong to different species or are hybrids among which Triticale is obtained by breeding *Triticum turgidum* subs. *durum* and *S. cereale*, while Tritordeum is obtained by breeding *T. turgidum* subs. *durum* and *Hordeum chilense*. Kernels and straw were collected for each cultivar in three biological replicates/harvesting year.

Table 8: Cultivars considered in this study and corresponding species or hybrids.

Species or hybrid	**Cultivar**
Triticum aestivum L.	456GT13/69; Adriatic; Akim; Altamira; Amburgo; Argone; Arkeos; Artico; Aubusson; Bagou; Bologna; Bramante; Canaletto; Cosmic; Ethic; Hystar; Jaguar; Lancillotto; Santorini; Solehio; SY Alteo; SY Capitano; SY Liam; SY Passion; Verna
×*Triticosecale* Wittm.	Satiro; Sileno; Trica
×*Tritordeum* Asch. & Graebn.	Aucan, Bulel, HT-444
Secale cereale L.	Antoninskie; SU Nastri (hybrid); SU Performer (hybrid)
Triticum turgidum subs. *durum* Desf.	Antalis; Odisseo
Hordeum distichum L.	Cometa
Hordeum polystichum L.	Ketos
Triticum dicoccon Sch.	Luni
Triticum spelta L.	Rossella

The growing area is characterized by a humid subtropical climate according to the Köppen climate classification and by a probable high FHB pressure, due to the environmental and agronomic conditions involving a frequent rotation with maize (Beck et al., 2018). The daily temperatures and precipitation, shown in **Table 9**, were measured at a meteorological station near the experimental area.

Table 9: Monthly rainfall and growing degree days (GDD) for each growing season. [1]Accumulated growing degree days for each month using a 0°C base value.

Month	2019-20		2020-21	
	Rainfall (mm)	GDD [1] (Σ °C d^{-1})	Rainfall (mm)	GDD [1] (Σ °C d^{-1})
November	314	249	4	277
December	132	193	79	144
January	5	168	116	128
February	1	229	29	203
March	62	285	8	286
April	81	414	37	353
May	122	579	69	501
June	113	624	86	674
Nov - June	830	2741	428	2567

The agronomic growing technique commonly adopted in the area for small cereal was applied to all the cultivars. Briefly, the previous crop was maize, the field was ploughed each year, incorporating the debris into the soil, and this was followed by disk harrowing to prepare a suitable seedbed. Planting was conducted in 12 cm wide rows at a seeding rate of 450 seeds m^{-2} in November. A total of 130 kg N ha^{-1} was applied, split 50 at wheat tillering [growth stage (GS) 23] and 80 kg N ha^{-1} at beginning of stem elongation (GS31), as an ammonium nitrate fertilizer.

A mixture of a strobilurin and a carboxamide fungicide (pyraclostrobin 150 g ha^{-1} and fluxapyroxad 75 g ha^{-1}, Priaxor®, BASF Agricultural Solutions,) was applied at the booting stage (GS 45) to control foliar diseases. No fungicide was applied at flowering (GS61-65) to control FHB infection. The sowing and harvest dates are reported in **Table 10**, together with the dates of the main growth stages, for each growing season. Treatments were assigned to an experimental unit using a completely randomized block design with three replicates. The plots

measured 7 x 1.5 m. The grain yields were obtained by harvesting the whole plot using a Walter Wintersteiger cereal plot combine-harvester. The harvested kernels were mixed thoroughly, and 4 kg kernel samples were taken from each plot to analyse the mycotoxin content.

Table 10: Sowing and harvesting date. [1]from the end of stem elongation (GS39) to the booting stage (GS45), according to the different GS of considered genotypes.

Year	Sowing date	N fertilization		Fungicide[1]	Harvest date
		GS 23	GS 31	GS 39-45	
2020	6/11/2019	5/03/2020	3/04/2020	23/04/2020	29/06/2020
2021	3/11/2020	2/03/2021	8/04/2021	21/04/2021	5/07/2021

3.2.2.3 Sample preparation for mycotoxin analysis

Samples were extracted according to Malachová et al., (2014) with some modifications. 8 mL of acetonitrile/water (80:20, v/v) mixture acidified with 0.1% formic acid were added to 2 g of ground kernels (0.5 g of ground straw). The samples were extracted for 90 min using a platform shaker (Ika Werke, Germany) at a speed of 200 strokes/min and subsequently centrifugated for 2 minutes at 3000 rpm (radius 17.8 cm) at room temperature. 1000 µl of supernatant were transferred into vials and then injected into the UHPLC-MS/MS system.

3.2.2.4 UHPLC-MS/MS mycotoxins analysis

UHPLC-MS/MS analysis was carried out on UHPLC Dionex Ultimate 3000 coupled to a triple quadrupole mass spectrometer TSQ Vantage (Thermo Fisher Scientific, Waltham, USA) equipped with an electrospray source (ESI). The chromatographic separation was obtained using a Sunshell column (Chromanik Technologies, Osaka, Japan) 2.1 × 100 mm, 2.6 µm particle size, heated to 40° C. 2 µl of sample extract was injected into the UHPLC system and the flow rate was set up to 0.35 mL min^{-1}. Gradient elution was performed by using water (eluent A) and methanol (eluent B) both acidified with 0.2% v/v acetic acid. Ammonium acetate was added to the eluent A at the final concentration of 5mM. Initial conditions were set at 98% A and 2% B for 2 min, then eluent B was increased to 20%, after an isocratic step (6 min), eluent B was further increased to 90% and this condition was maintained for 10 min until the return to the initial condition. The total run time was 26.5 min. Mass spectrometric analysis was performed both in positive and negative ionization mode in multiple reaction monitoring (MRM), spray voltage 3000 V, capillary temperature 270°C, vaporizer temperature 200°C, sheath gas pressure 50 units, auxiliary gas pressure 5 units. The following quantifier transitions were measured: DON m/z 355>295 (CE 13eV) and m/z 355>265 (CE 19eV), DON3Glc m/z 517>457 (CE 17eV) and m/z 517>427 (CE 24eV), 3Ac-DON and 15AcDON m/z 397>307 (CE 18eV) and m/z 397>59 (CE 20eV), T-2 m/z 484>215 (CE 19eV) and m/z 484>185 (CE 22eV), HT-2 m/z 442>263 (CE 11eV), ZEN m/z 317>175 (CE 26eV) and m/z 317>131 (CE 32eV), ENN B m/z 640>528 (CE 20eV), m/z 640>314 (CE 31eV), m/z 640>214 (CE 26eV), m/z 640>196 (CE 29eV), m/z 640>186 (CE 37eV) and m/z 640>86 (CE 44eV). Calibration curves were set up using external standards (range 1 µg kg^{-1} – 2500 µg kg^{-1}) for target analyte quantification. Data acquisition was performed by Thermo Xcalibur 2.2 software (Thermo Fisher Scientific, Waltham, USA).

3.2.2.5 Statistical analysis

Statistical analysis was performed using IBM SPSS v.25.0 (SPSS Italia, Bologna, Italy). Data were log-normalised prior to statistical analysis.

3.2.3 Results and discussion

3.2.3.1 General overview

The two growing seasons showed different meteorological trends as far as both rainfall and temperature (expressed as growing degree days, GDDs) are concerned (**Table 9**). During the year 2020 the rainfall was much higher after sowing (November) and between stem elongation and flowering (April-May) (**Table 9**). Overall, rainfall was almost double in 2020 compared to 2021 (839 vs 428 mm). 2020 had also higher temperature than 2019, as described by differences in GDD data (2741 vs 2567) (**Table 9**).

Some of the most relevant *Fusarium* mycotoxins were screened and quantified by means of HPLC-MS/MS, namely DON and its major modified form DON3Glc, 3Ac-DON, 15Ac-DON, T-2, HT-2, ZEN together with the emerging mycotoxin ENN B. The overall range of occurrence for each mycotoxin is shown in **Table 11**.

Table 11: Overall range of concentration of mycotoxins detected in kernel and straw samples collected from the harvesting years 2020 and 2021. Data are expressed in μg kg^{-1}. Acetylated forms of DON were found as <LOD (Limit od Detection) or <LOQ (Limit of Quantification) for all the samples.

Mycotoxin	**Kernel**		**Straw**	
	2020	**2021**	**2020**	**2021**
DON	160-8029	<LOD-733	240-15730	<LOD-2430
DON3Glc	<LOD-1049	<LOD/<LOQ	<LOD-4640	<LOD-824
T-2	<LOD/<LOQ	<LOD-28	<LOD-143	<LOD-126
HT-2	<LOD-41	<LOD-36	<LOD/<LOQ	<LOD/<LOQ
ZEN	<LOD-187	<LOD/<LOQ	<LOD-762	<LOD-30
ENN B	14-8585	<LOQ-222	26-3606	<LOD-1064

Biotransformation rate expressed according to Cirlini et al., (2013) as DON3Glc/TDON (where TDON is the sum of DON and DON3Glc), was in the range of 0-0.321 for kernel (year 2020), 0-0.522 for straw harvested in 2020, 0-0.500 for straw harvested in 2021, while DON3Glc was found as <LOD/LOQ for all kernel samples collected from the harvesting year

2021. Regarding co-occurrence, solely DON and ENN B were detected in all the cultivars tested, but solely for the harvesting year 2020 in both kernel and straw. Moreover, DON was the most frequently found mycotoxin for all the samples overall tested.

Regarding DON contamination, straw samples from the harvesting year 2020 show the highest mean value (3795 μg kg^{-1}) followed by the corresponding kernel samples (1985 μg kg^{-1}). Samples from the year 2021 are less contaminated by about an order of magnitude, with a mean of 145 μg kg^{-1} for kernel samples and a mean of 490 μg kg^{-1} for straw samples. Overall, the average DON contamination of straw was lower than the legal limits adopted in the EU set at 8000 μg kg^{-1} according to the European Commission Recommendation 2006/576/EC for feed other than maize intended for adult ruminants. This great difference is not surprising if we consider different meteorological conditions, especially during the spring period, as shown in **Table 9**. In fact, conditions such as those present in 2020, which is characterized by higher temperature and rainfall, especially during anthesis, are often associated with higher levels of contamination by DON and other mycotoxins (Scarpino et al., 2021).

Moreover, the difference observed between kernel and straw for DON, with the latter being more contaminated, was expected in consideration of what was reported in the literature. Such significant occurrence of mycotoxins in straw poses a serious threat to the wellness of livestock, especially swine and other non-ruminants that are sensitive to mycotoxins such as DON and who may exhibit symptoms including feed refusal and poor weight gain (Bissonnette et al., 2018; Ji et al., 2015). Moreover, starting from the occurrence in feed, mycotoxins can enter the food chain through products of animal origin exposing consumers to health risks (Alshannaq & Yu, 2017). Mycotoxins in straw may also contribute to the contamination of the soil, potentially determining an environmental exposure from which significative health risks may arise, since it was demonstrated the ability of various crops to

uptake mycotoxins from contaminated soil (Hariprasad et al., 2015; Snigdha et al., 2015). Finally, the role of mycotoxins as environmental pollutants and currently far from being full understood (Juraschek et al., 2022).

A full factorial ANOVA on DON occurrence returned both the harvest year (p = 0.000), the cultivar (p = 0.000) and their interaction (p = 0.000) as significant factors in both kernel and straw. Considering the kernel samples for the harvesting year 2020, the cultivar Santorini shown the highest DON contamination (6253±2185 μg kg^{-1}) and Satiro shown the lowest one (243±92 μg kg^{-1}). For the harvesting year 2021, Bagou was found as the most contaminated cultivar (470±250 μg kg^{-1}), while Antoninskie and Satiro were the only cultivars in which DON occurrence was found below LOD. Regarding straw samples, the cultivar Amburgo showed the highest DON contamination (12333±2036 μg kg^{-1}) and Satiro (313±76 μg kg^{-1}) the lowest one for the harvesting year 2020. Canaletto was found as the most contaminated cultivar (1644±936 μg kg^{-1}) and Rossella was found as the only cultivar with a contamination below LOD for the harvesting year 2021. Overall, the average DON contamination of kernels, intended as unprocessed cereals for food purpose, and straw, intended as feed for adult ruminants, was lower than the legal limits adopted by EU countries when the harvesting year 2021 was considered for each cultivar tested. Regarding the harvesting year 2020, twenty-three out of forty cultivars shown DON occurrence above the legal limit for kernels and only four out of forty cultivars for straw.

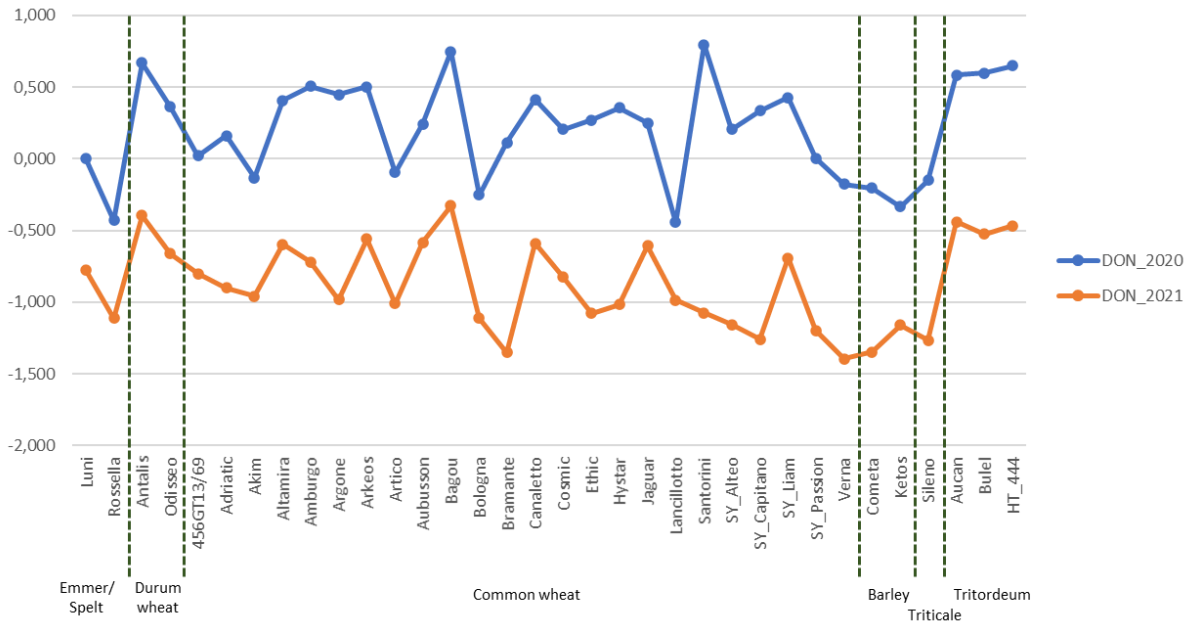
As shown in **Figure 9**, the occurrence of DON in kernel samples for the two harvesting years followed a similar trend between the different cultivars considered in this study, despite the wide differences in absolute terms. Moreover, according to Pearson's correlation test, a positive correlation was found between DON occurrence in 2020 and 2021 for kernel samples (r = 0.693, p = 0.000), but not for straw, when all the cultivars tested were considered. This

positive correlation found for the kernels highlights the importance of varietal choice even in a context of very different meteorological trends and load of mycotoxins.

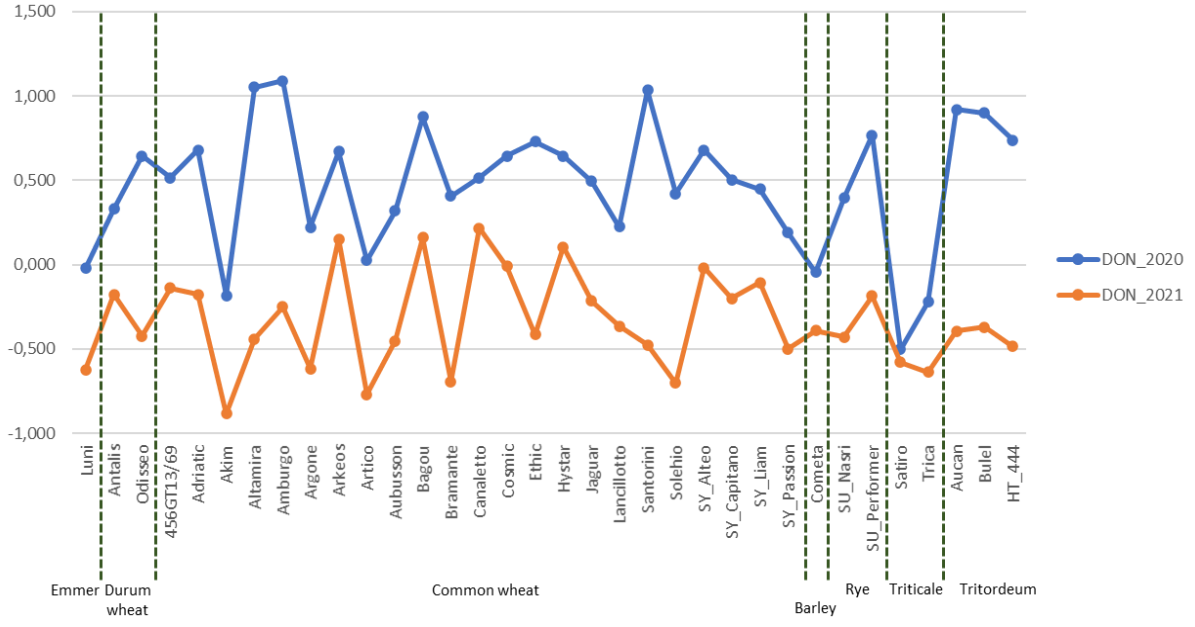
Finally, according to Pearson's correlation test, a positive correlation was found between DON occurrence in kernels and straw for the harvesting year 2020 ($r = 0.694$, $p = 0.000$) and 2021 ($r = 0.443$, $p = 0.004$) when all cultivars were considered, consistently with what was reported in literature for wheat (Bissonnette et al., 2018b).

Figure 9: Occurrence of DON in kernel (**A**) and straw (**B**) for both the harvesting years for all the cultivars considered in this study. Cultivars were all the biological replicates were found below LOQ or LOD are not shown. Data were log-normalized.

A



B

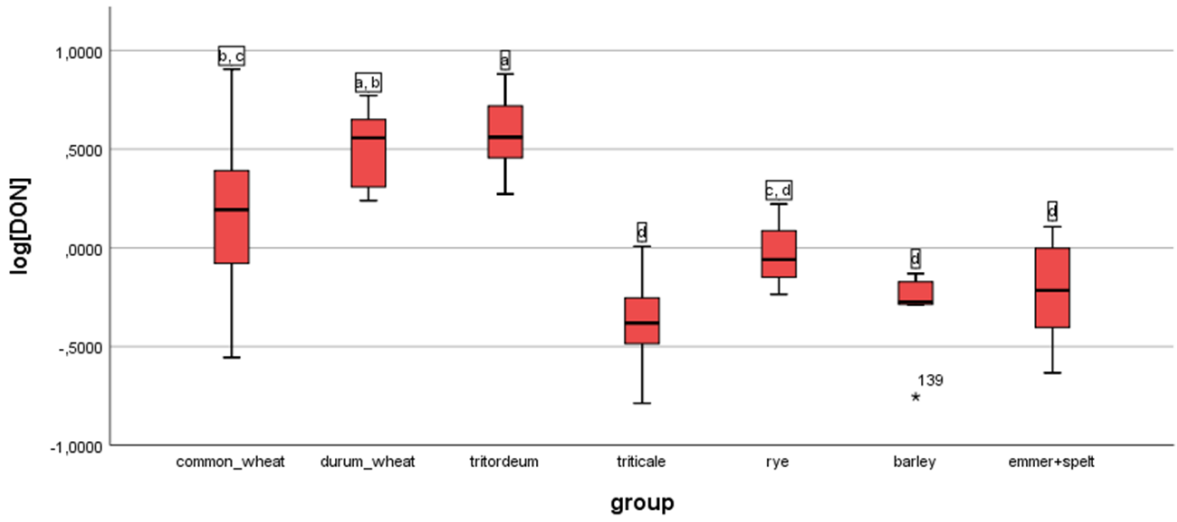


If cultivars are grouped on the basis of the species or hybrids to which they belong, namely common wheat, durum wheat, tritordeum, triticale, rye, barley and emmer/spelt, significative differences in DON contamination can be observed. Emmer and spelt were considered in the same group as they are old *Triticum* forms, between the early cereals that were domesticated (Arzani & Ashraf, 2017). In Italy, togheter with *Triticum monococcum* L. they are collectively referred to as farro.

3.2.3.2 Occurrence of DON and DON3Glc

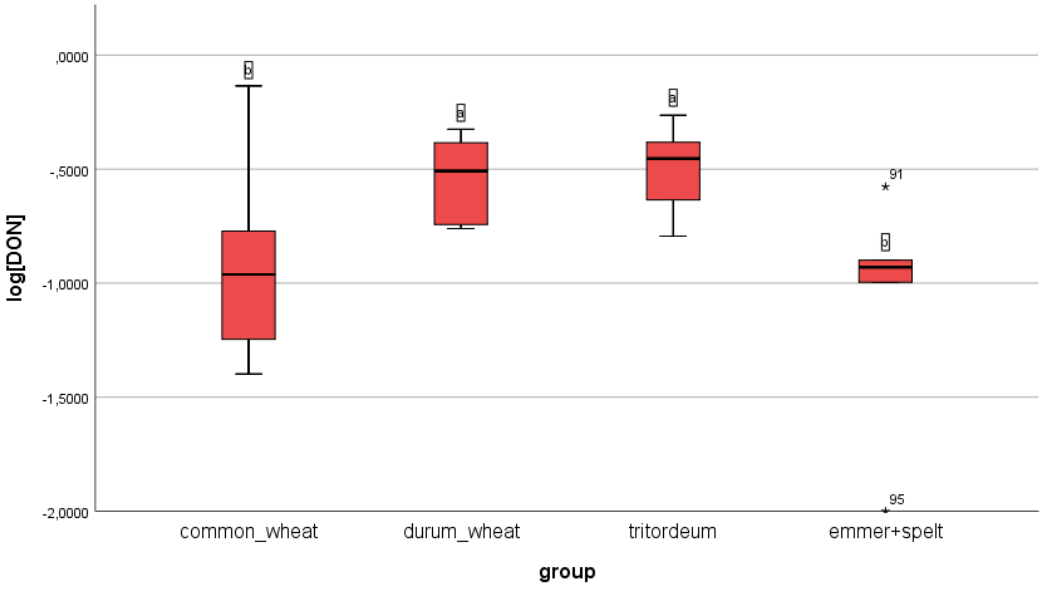
Regarding DON occurrence in kernels from 2020, tritordeum was the most contaminated group (mean of 4098±1792 µg kg^{-1}) and triticale the less contaminated one (mean of 462±254 µg kg^{-1}). One-way ANOVA analysis followed by post-hoc Tukey test (α=0.05) showed significant differences between groups as shown in **Figure 10**.

Figure 10: Boxplot describing occurrence of DON in kernels from the harvesting year 2020, according to the group of different species/hybrids. Data were log-normalized.



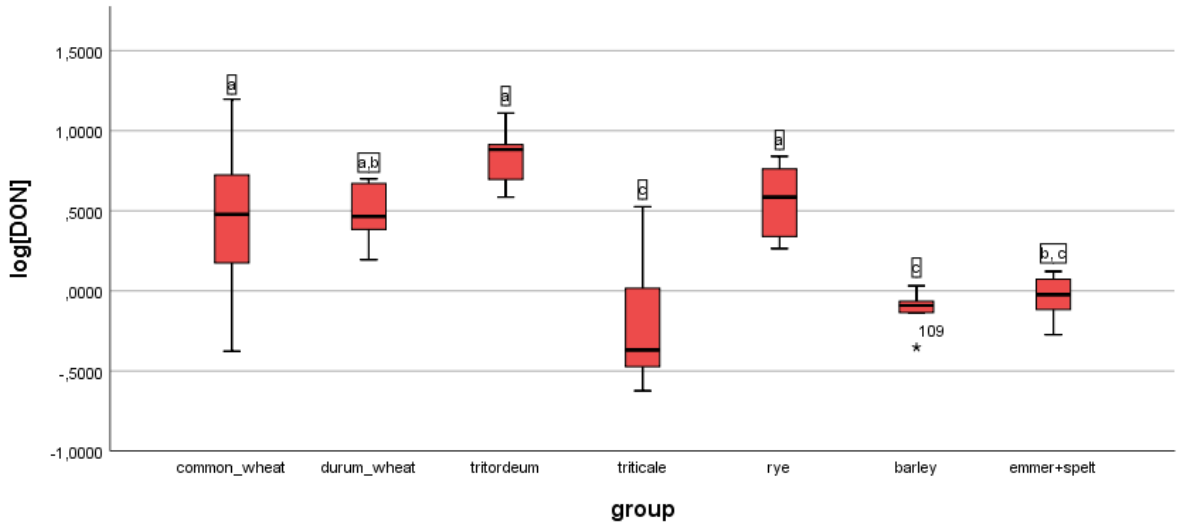
Regarding DON occurrence in kernels from 2021, tritordeum was confirmed as the most contaminated group (mean of 335±127 µg kg^{-1}). Only three out of six barley samples were found above LOQ, within the range of 115-53 µg kg^{-1} (data not shown). All triticale and rye samples were found below LOD/LOQ, except for a single sample of triticale (cultivar "Sileno", 83 µg kg^{-1}). One-way ANOVA analysis followed by post-hoc Tukey test (α=0.05) showed significant differences between groups as shown in **Figure 11**.

Figure 11: Boxplot describing occurrence of DON in kernels from the harvesting year 2021, according to the group of different species/hybrids. Outliers belong to the cultivar "Luni" (sample "91", 265 µg kg^{-1}) and "Rossella" (sample "95", 10 µg kg^{-1}). Data were log-normalized.



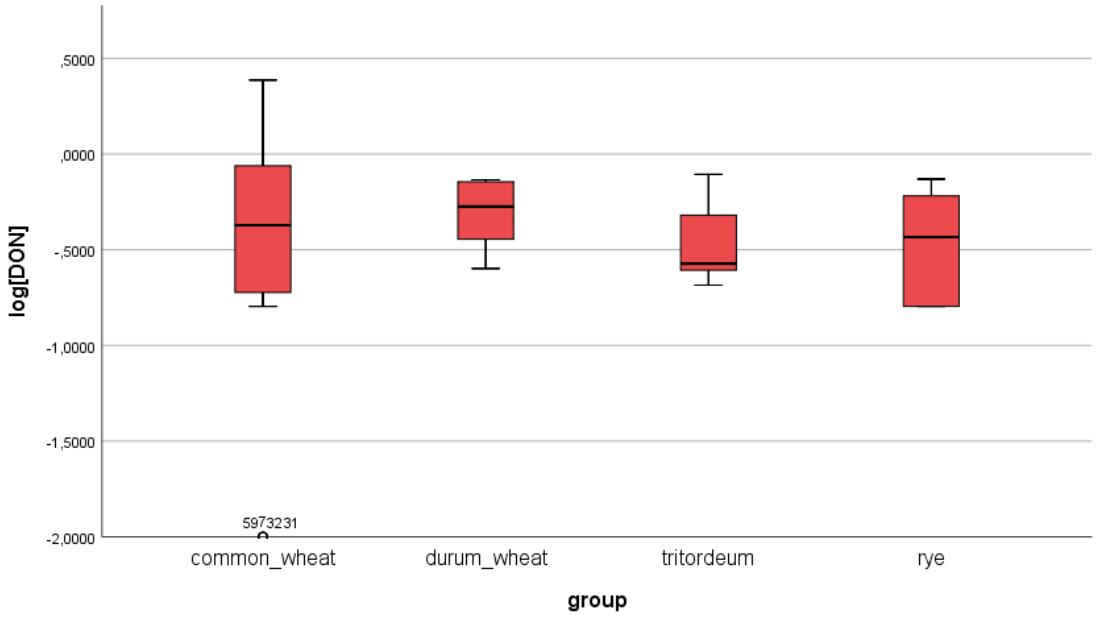
Regarding DON occurrence in straw samples from the harvesting year 2020, consistently with the kernel contamination, tritordeum was the most contaminated group (mean of 7232±3080 µg kg^{-1}), while barley was found as the less contaminated one (mean of 785±229 µg kg^{-1}). One-way ANOVA analysis followed by post-hoc Tukey test (α=0.05) showed significant differences between groups as shown in **Figure 12**.

Figure 12: Boxplot describing occurrence of DON in straw from the harvesting year 2020, according to the group of different species/hybrids. Outlier belong to the cultivar "Ketos" (samples "109", 445 µg kg^{-1}). Data were log-normalized.



Regarding DON occurrence in straw from the harvesting year 2021, common wheat is the most contaminated group on the average (590±537 µg kg^{-1}), but an high intra-group variability must be noted. Considering the group of emmer/spelt, only one sample results above LOQ, in particular from the cultivar "Luni" (0,54 µg kg^{-1}). Moreover between barley samples, only two out of six samples are above LOQ, both belonging to the cultivar "Cometa" (mean of 535 µg kg^{-1}). About triticale, only three out of nine samples are above LOQ, of which two belong to the cultivar "Satiro" (mean of 315 µg kg^{-1}) and one belong to the cultivar "Trica" (370 µg kg^{-1}). One-way ANOVA analysis followed by post-hoc Tukey test (α=0.05) did not show significant differences between groups (**Figure 13**).

Figure 13: Boxplot describing occurrence of DON in straw from the harvesting year 2021, according to the group of different species/hybrids. Outliers belong to the cultivars "Akim", "Bologna" and "Solehio" (10 µg kg^{-1}). Data were log-normalized.



Considering the data on DON occurrence within the whole set, it appears that tritordeum is the group that presents a greater susceptibility in particular for the kernels. Such susceptibility also extends to the tested durum wheat cultivars, even if for the year 2020 durum wheat cultivars does not differ significantly from the common wheat ones. The latter are affected by high variability, but it should be considered that this is at least in part due to the presence of a wider genetic base and thus different degrees of susceptibility.

About the DON3Glc/TDON ratio, reported as correlated to resistance to FHB in durum and common wheat by Amarasinghe et al., (2016), a possible negative correlation between DON and DON3Glc/TDON was investigated for all the cultivars using Pearson's correlation test. No correlation was found in kernel samples from 2020, not even considering common wheat cultivars only, however, when straw samples were considered, a significant negative correlation was found (r = 0.462; p = 0.000), even considering only common wheat (r = -

0.503; p = 0.000). Moreover, a significative negative correlation between DON and DON3Glc/TDON was found for straw samples of common wheat for the harvesting year 2021 (r = -0.421; p = 0.023). This correlation in naturally infected common wheat cultivars was not reported so far in literature and could be useful as an indicator of susceptibility/resistance to accumulation of DON in common wheat varieties, at the same time represent a useful data to evaluate the possible exposure of livestock to DON and its modified form DON3Glc.

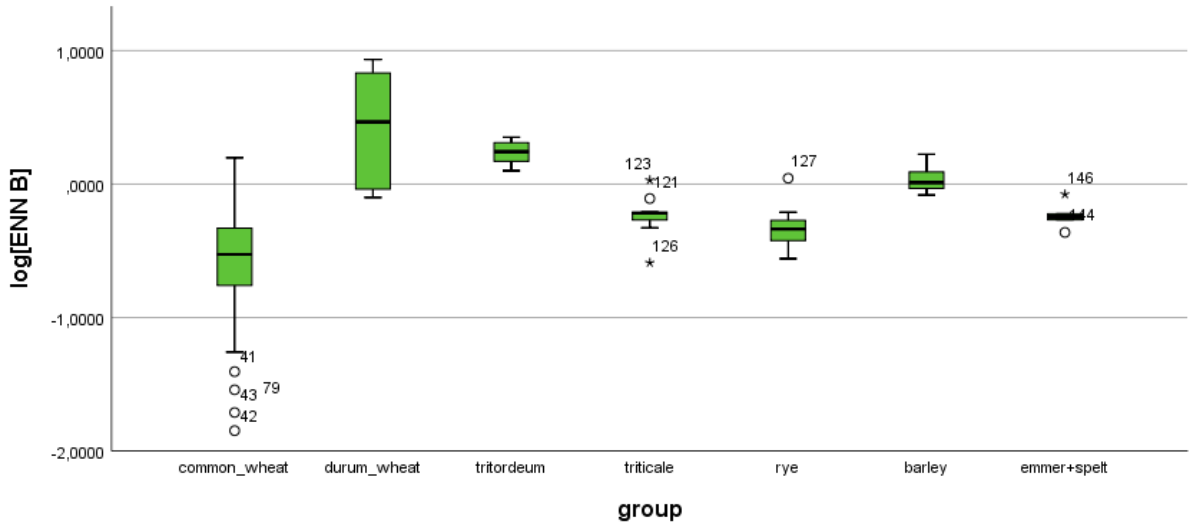
In addition, a significative positive correlation was found between DON and DON3Glc considering both kernel (r = 0.682; p = 0.000) and straw (r = 0.593; p = 0.000) for 2020 for all cultivars and even considering solely common wheat cultivars (r = 0.750 and p = 0.000 for kernel; r = 0.654 and p = 0.000 for straw). This significant positive correlation was found also for straw of common wheat from 2021 (p = 0.000). These findings are consistent with evidence for kernels in the previous study presented in this thesis for pigmented wheat and with the current literature for wheat (Ovando-Martínez et al., 2013). No further correlation analysis can be performed due to the frequent detection of DON3Glc below LOD or LOQ for 2021.

3.2.3.3 Occurrence of ENN B

Regarding occurrence of ENN B in kernel collected from the harvesting year 2020, as shown in **Figure 14**, durum wheat is the more contaminated on average, but there is a large difference between the cultivars “Antalis” (mean of 7063±1166 µg kg^{-1}) and “Odisseo” (mean of 1011±276 µg kg^{-1}). On the contrary, common wheat is the less contaminated (mean of 365±283 µg kg^{-1}) overall, with the lowest contamination found for the cultivar “Bologna” (mean of 559±97 µg kg^{-1}) of which three out of four biological replicates are shown as

outliers. According to one-way ANOVA followed by post-hoc Tukey test (α=0.05), durum wheat differs statistically significantly from rye, triticale, emmer/spelt, but not from tritordeum and barley. Common wheat is significantly different from durum wheat, tritordeum and barley but not from the other groups. In addition, according to Pearson's correlation test, a significative positive correlation was found between DON and ENN B for the kernels considering all cultivars (r = 0.393; p = 0.000) or solely common wheat (r = 0.418; p = 0.000).

Figure 14: Boxplot showing occurrence of ENN B in kernel samples from the harvesting year 2020, based on groups of different species/hybrids. Significant differences are described in the text. Data were log-normalized.

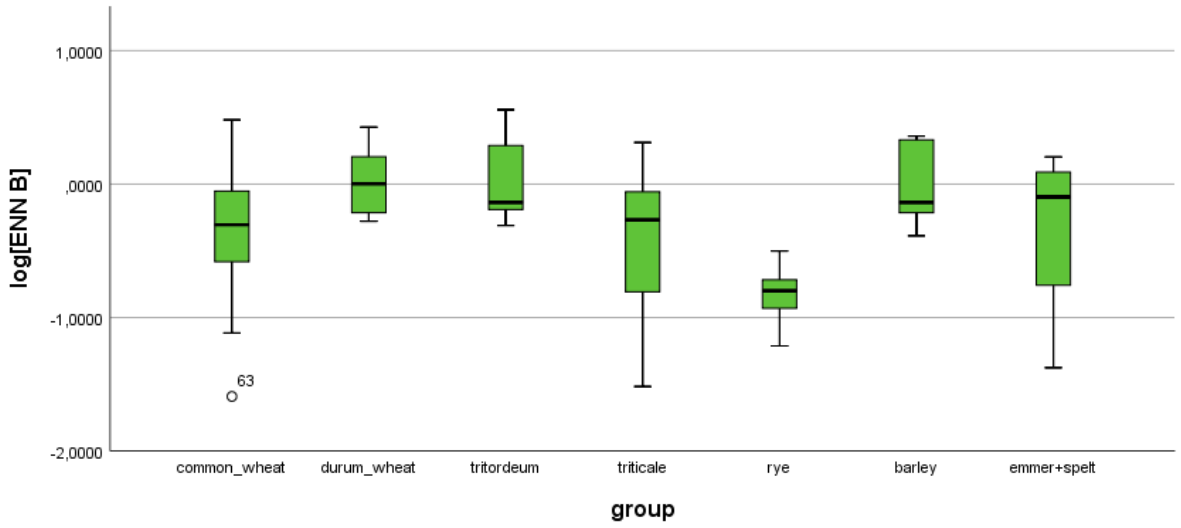


Regarding occurrence of ENN B in straw collected from the harvesting year 2020 (**Figure 15**), tritordeum is the more contaminated on average (mean of 1415±1120 µg kg^{-1}), but with a high variability, both intra-group and between biological replicates. Moreover, tritordeum significantly differ only from rye (mean of 423±81 µg kg^{-1}) (p = 0.000), which are the less

contaminated group. Durum wheat and barley show almost the same level of contamination and are significantly different only for rye (p = 0.003 and *p* = 0.01 respectively). However, it must be noted that, regarding barley, there is a high intra-group variability, in which the cultivar "Ketos" is the second most contaminated cultivar overall (mean of 1720±861 µg kg^{-1}) after the tritordeum cultivar "Aucan". Common wheat significantly differs only from rye (p = 0.018). In addition, no correlation was found between DON and ENN B content in straw samples from 2020 when all cultivars or even only common wheat cultivars were considered.

Finally, ENN B was found below LOD/LOQ for the majority of samples of kernel and straw for the harvesting year 2021, therefore no comparison can be made. As reported in the literature for wheat, ENNs positively correlate with the rainfall in May and June. Hence, based on the meteorological trend shown in **Table 9**, the lower rainfall for the year 2019-2020 might have helped to mitigate the contamination by ENN B (Decleer et al., 2019).

Figure 15: Boxplot showing occurrence of ENN B in straw samples from the harvesting year 2020, based on groups of different species/hybrids. Data were log-normalized.



Contrarily to what observed for the occurrence of DON, the differences in ENN B occurrence constitute a more complex scenario even if tritordeum is confirmed as particularly susceptible to mycotoxin accumulation. In addition, barley appears to be more susceptible to ENN B accumulation in kernels compared to its susceptibility to DON accumulation, between the considered groups. Although no statistical significance emerged, this observation deserves further investigation in the light of what was recently reported in the literature (Orlando et al., 2019).

The significative correlation found between DON and ENN B occurrence in kernel (year 2020) for all the cultivars tested was found also in the previous study of this thesis for the same year and location. However, it is not possible to confirm this behavior for 2021 due to the frequent detection of ENN B below LOQ or LOD. The frequent co-presence of DON and ENN B suggests a large co-infection by different *Fusarium* species and a possible common susceptibility/resistance to the accumulation of the two mycotoxins for the tested cultivars.

3.2.3.4 Occurrence of T-2 and HT-2

When focusing on the least frequently detected mycotoxins, T-2 toxin was found below LOQ in all the samples of kernel from the harvesting year 2020 and HT-2 was found in only two samples of the cultivar "Antalis" (41 and 20 µg kg^{-1}) in the same dataset. Regarding straw samples from 2020, HT-2 was found below LOQ in all the samples, while T-2 toxin was found in 12 out of 117 samples belonging to different cultivars and groups, but it must be noted that all three biological replicates of the cultivar "Ketos" are contaminated above LOQ (mean of 88±56 µg kg^{-1}) and two out of three samples of the cultivar "Bulel" are also found as contaminated above LOQ (23 and 38 µg kg^{-1}). Regarding kernel from 2021, HT-2 was found in only one sample of the cultivar "Hystar" (36 µg kg^{-1}) together with T-2 (28 µg kg^{-1}) that

was also found in other 7 samples, of which two belonging to the cultivar “Aucan” (both 5 µg kg^{-1}) and two belonging to the cultivar “Bulel” (6 µg kg^{-1} and 12 µg kg^{-1}) that are both tritordeum. Moreover, for straw samples of the harvesting year 2021, HT-2 was found below LOQ for all the samples, while T-2 was found in 8 out of 118 samples of different cultivars and groups. Overall, T-2 and HT-2 toxins were found for each cultivar on average below the legal limits adopted by EU countries according to the European Commission Recommendation 2013/165/EU.

3.2.3.5 Occurrence of ZEN

ZEN was found above LOQ in only two straw samples from the harvesting year 2021, from the cultivars “Bagou” (30 µg kg^{-1}) and “Canaletto” (24 µg kg^{-1}). Regarding kernel samples from 2020, ZEN was found above LOQ in the majority of samples of common wheat (mean of 35±24 µg kg^{-1}), of which the most contaminated is the cultivar “Amburgo” (mean of 90±68 µg kg^{-1}) that is also the most contaminated cultivar overall by ZEN. ZEN was also found below LOQ in samples from emmer, spelt, rye and triticale. Only one sample of barley (15 µg kg^{-1}) and three sample of different tritordeum cultivar were found as contaminated by ZEN above LOQ (range 6-60 µg kg^{-1}). Regarding durum wheat, all biological replicates of the cultivar “Antalis” were found as contaminated by ZEN above LOQ (mean of 70±35 µg kg^{-1}) and only one biological replicate of the variety “Odisseo”.

Regarding straw samples from 2020, ZEN was found in all samples from durum wheat (mean of 233±144 µg kg^{-1}), two out of three samples from emmer (67±20 µg kg^{-1}), three out of five samples of barley and, in particular, the cultivar “Ketos” shown high contamination (mean of 324±40 µg kg^{-1}). ZEN was also found in the majority of samples of rye, in particular the cultivar “SU Performer” shows the highest contamination (mean of 220±140 µg kg^{-1}) and in

two samples of triticale, cultivar “Sileno” (mean of 100±38 µg kg^{-1}) and all the biological replicates of tritordeum cultivar “Aucan” and “Bulel” (mean of 94±40 µg kg^{-1}, not considering a very high contamination found in one biological replicate from “Aucan” detected as 762 µg kg^{-1}). ZEN was found in most samples from common wheat, of which the most contaminated is the cultivar “Bagou” (mean of 328±248 µg kg^{-1}) followed by the cultivar “Amburgo” (mean of 317±177 µg kg^{-1}). Overall, ZEN occurrence was found on average below the legal limits adopted by UE countries according to European Commission Recommendation 2006/576/EC and European Commission Regulation 1881/2006 for each cultivar tested.

The literature on mycotoxin occurrence in tritordeum cultivars is very scarce and a broad varietal comparison with other cereals, performed under the same agronomic conditions at the same location and for more than one year of harvest, is completely lacking. These elements support the solidity of our investigation, given the large number of factors influencing the occurrence of mycotoxins in samples collected from the field. The collected DON contamination data should focus attention on the importance of better understanding the possible susceptibility of tritordeum to DON and ENN B accumulation due to its use as an alternative cereal to produce food products.

3.3 Study 3: Development of an *in vitro* bioassay on anthocyanin biosynthesis in maize root

3.3.1 Introduction

In vitro culture has widely demonstrated its potential for understanding the metabolism of mycotoxins and their distribution in wheat as well as in maize (Righetti et al., 2020; Righetti et al., 2021b). For the latter, the spatial distribution of aflatoxin B_1 (AFB_1) in maize plantlets at a tissue and organ level as well as the impact of this toxic compound on plant metabolome is also reported. Based on untargeted metabolomics approach, anthocyanin biosynthesis and chlorophyll metabolism in roots are shown to be affected by administration of AFB_1 in *in vitro*-cultured maize plants (Righetti et al., 2021a). Given these results, we decided to perform a more specific experiment to shed light on the potential mechanism involved in AFB_1 management in maize, with a specific focus on anthocyanin biosynthesis and its putative involvement in the interplay between maize and *Fusarium*.

Anthocyanins are flavonoids pigments located in many parts of the plants such as leaves, roots, flowers and fruits and are known to play a role as secondary metabolites in attracting pollinators and seed disperser and protect the plant from biotic (e.g. pathogens) and abiotic (e.g. UV light) stresses (Das et al., 2012). It is well known that maize roots can produce notable amounts of anthocyanins and their biosynthesis can be induced by various factors such as light and sucrose (Kim et al., 2006). The biosynthesis of anthocyanin is also enhanced by cytokinins, as demonstrated for *Arabidopsis thaliana* (L.) and other plants (Deikman et al., 1995).

Investigating a possible effect of AFB_1 on the biosynthesis of anthocyanins could represent a useful element for a better understanding of the pathogenic fungus-host plant interaction and above all, on the dynamics of the chemical warfare in which they are both involved.

To date, an *in vitro* bioassay to evaluate the effect of exogenous compounds on the biosynthesis of colored secondary metabolites is currently available. This bioassay involved *Amaranthus caudatus* (L.) in which the exogenous application of different compounds affected the accumulation of the red pigment amaranthin, that is a red-colored betacyanin, a class of pigments that replace anthocyanins in ten plant families of the order *Caryophyllales*, in which these pigments are involved in stress response against UV light, pathogens and mechanical lesion as well as act as antioxidants (Hayakawa & Agarie, 2010; Romanov et al., 2000). This model, however, proves to be unsuitable for evaluating the possible action of exogenous compounds which requires more time to exert a potential biological activity.

With the aim to investigate a possible effect of AFB_1 on anthocyanin biosynthesis, an *in vitro* bioassay was developed using maize roots cultured under conditions capable to induce anthocyanin accumulation.

3.3.2 Materials and methods

3.3.2.1 Plant material and chemicals

Belgrano maize (*Z. mays*) hybrid (FAO class 300) caryopses were used as starting plant material. Analytical standard of cyanidin-3-*O*-glucoside chloride was purchased from ChromaDex (Los Angeles, USA). Aflatoxin B_1 reference material (5 mg of powder subsequently dissolved in 5 mL of acetonitrile) was purchased from Merck (Darmstadt, Germany) and used for *in vitro* experiments. UHPLC-grade ethanol, formic acid and water were purchased from VWR Chemicals (Radnor, USA). Dimethyl sulfoxide was purchase from Sigma-Aldrich (St. Louis, USA). Murashige and Skoog (MS) medium, sucrose, phyto agar and 6-benzylaminopurine (6-BAP) were purchased from Duchefa Biochemie (Harleem, Netherlands).

3.3.2.2 Plantlets growth conditions and experimental design

Maize caryopses were sterilized as described by Rolli et al. (2018). Briefly, caryopsis were soaked in 70% (v/v) ethanol for 5 min, then surface disinfection was performed with 2.5% v/v sodium hypochlorite for 50 min under vacuum (-15 inch of Hg) followed by three washes with sterile distilled water to remove sodium hypochlorite residues.

The sterilized caryopses were sowed in glass jars (8 seeds per each) containing about 200 mL of 1/4 strength MS medium solidified with 0.8% (w/v) agar (pH 5.8) previously sterilized by autoclaving at 121°C for 20 minutes and kept in the dark for 7 days in a climate chamber at a temperature of 25±1°C. Afterwards, roots portions of approximately 20 mm in length were excised from the etiolated plantlets removing the very apical portion and transferred in 100 mm Petri dishes containing 25 mL of MS liquid medium added with 3% (w/v) sucrose (pH

5.8) and maintained in a growth chamber at 25±1°C under continuous light exposure for 7 days at different conditions, as described below:

1- Control (without the addition of AFB_1 or plant growth regulator to MS medium, named “hormone-free” or “HF”)
2- BAP (with the addition of 6-BAP to MS medium at concentration of 10^{-6}M)
3- AFB_1 (with the addition of AFB_1 to MS medium at final concentration of 2 µg mL^{-1})
4- AFB_1+BAP (both 6-BAP and AFB_1 were added to MS medium at the concentration described above)

For each condition, about twelve root portions from different etiolated plantlets are cultured in the same Petri dishes and culture media were screened for the presence of cultivable organism on specific media to assess the absence of fungal or bacterial contamination. At the end of the experiments, root portions are collected and stored at -20°C until extraction and analysis to determine the total anthocyanin content (TAC).

The removal of the very apical part of the roots was performed for all the experiments in order to limit the impact of endogenous cytokinins that are mainly concentrated at the root cap and can represent a source of non-predictable variability (L. Wu et al., 2021).

6-BAP was used at a previously tested concentration (10^{-6} M) and exposure time (7 days) for all the experimental sets. Such concentration was previously observed to induce a notable increase in anthocyanin biosynthesis in this experimental system.

Pre-treatment with AFB_1 were carried out maintaining the root segments excised from etiolated plantlets in Petri dishes in 25 mL of MS medium for three days in the dark in a growth chamber at a temperature of 25±1°C at the following conditions:

1- Control (without the addition of AFB_1 or plant growth regulator to MS medium, named "hormone-free" or "HF")
2- AFB_1 (with the addition of AFB_1 to MS medium at the final concentration of 2 µg mL^{-1})

After this period, a total removal of the culture medium and replacement with fresh culture medium added with BAP at the concentration of 10^{-6} M was performed for both conditions described above to enhance anthocyanin biosynthesis. After 7 days root portions are collected and stored at -20°C until extraction and analysis to determine total anthocyanin content. The final conditions tested are therefore the following:

1- Control followed by induction with BAP (named "HF>BAP")
2- Treated with AFB_1 followed by induction with BAP (named "AFB_1>BAP")

3.3.2.3 Sample preparation and extraction of anthocyanins

Before extraction, samples were freeze-dried for 12 hours using a laboratory lyophylizator (LIO-5PDGT, 5 Pa s.r.l., Trezzano sul Naviglio, Italy) and then ground using a mortar and pestle in liquid nitrogen. The extraction procedure was performed as described by Barnes et al., (2009) with some modifications and opting for green solvents. Briefly, 1500 µL of ethanol/water (70:30, v/v) mixture acidified with 1% (v/v) formic acid were added to 10 mg of ground sample and extracted for 30 min using a platform shaker (Ika Werke, Germany) at a speed of 200 strokes/min in the dark and subsequently centrifugated for 10 minutes at 10.000

rpm (radius 9.5cm) at 4°C. 1000 µL of supernatant was transferred into disposable cuvettes and analysed.

3.3.2.4 Spectrophotometric determination of total anthocyanin content

The analysis of the total anthocyanin content (TAC) was carried out using a Jasco V-530 spectrophotometer (Jasco, Japan) at the wavelength of 520 nm according to Barnes et al., (2009). The quantification was performed using a calibration curve of cyanidin-3-*O*-glucoside chloride solutions prepared in the extraction solvent within the range of 0.5-50 µg mL^{-1} and expressed as mg g^{-1} of cyanidin-3-*O*-glucoside equivalents (CGE). A good linearity was obtained for all the quantifications performed ($R^2 > 0.99$).

3.3.2.5 Statistical analysis

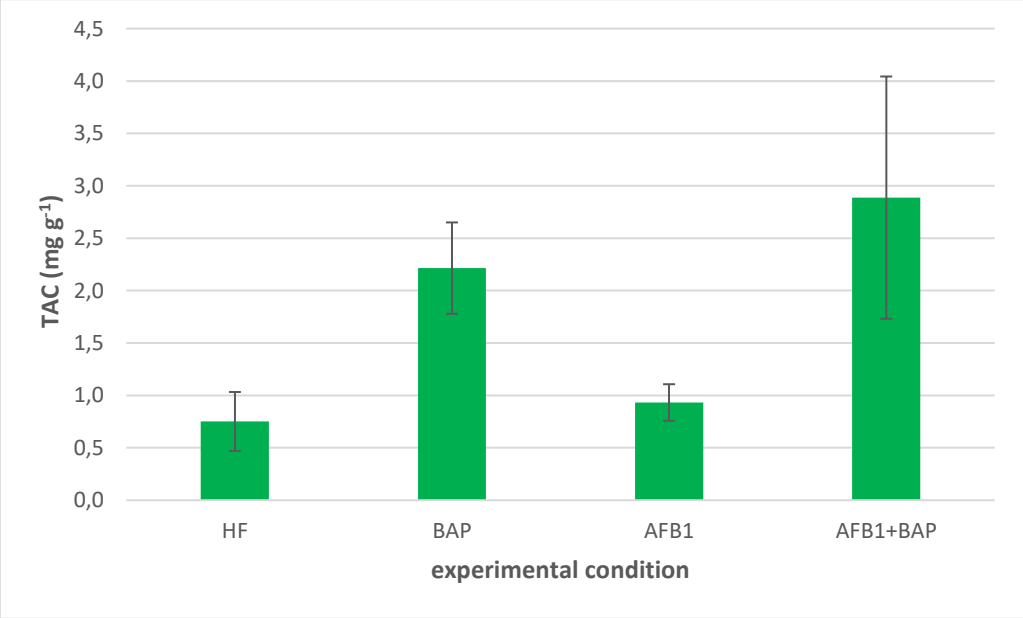
The statistical analysis was performed using IBM SPSS v.25.0 (SPSS Italia, Bologna, Italy).

3.3.4 Results and discussion

The first series of experiments, following referred as "non-pretreated", was carried out by exposing the maize radical portions to AFB_1 during the whole period of induction of anthocyanin biosynthesis (7 days). The experimental conditions tested, controls included, were the following: HF, BAP, AFB_1 and AFB_1+BAP.

The total anthocyanin content (TAC) for non-pretreated experiments is shown in **Figure 16**. The conditions in which 6-BAP was added to the culture medium shown a higher TAC on the average, however, according to one-way ANOVA followed by Tukey post-hoc test ($\alpha = 0.05$), the only significant difference found was between AFB_1+BAP (mean of 2.887±1.156 mg g^{-1}) compared to both HF (mean of 0.751±0.281 mg g^{-1}) ($p = 0.015$) and AFB_1 (mean of 0.932±0.174 mg g^{-1}) ($p = 0.024$).

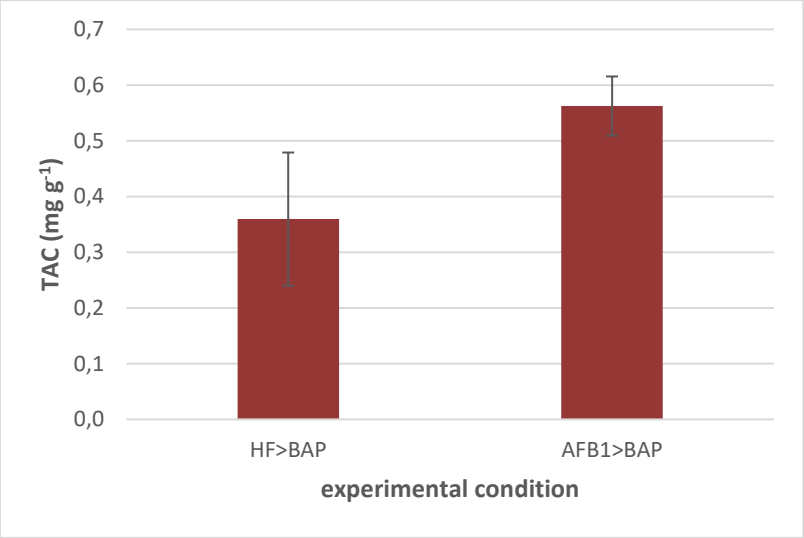
Figure 16: Total anthocyanin content in maize roots for non-pretreated experiments. TAC is expressed in mg g^{-1} of dry weight as a mean±SD CGE of three biological replicates.



Based on of the previously shown results (**Figure 16**), it was hypothesized a possible role of AFB_1 in the enhancement of anthocyanin biosynthesis under the conditions of interest in this study, namely BAP and AFB_1+BAP. However, the adopted experimental design may not have significantly highlighted the observed differences. Therefore, a second set of experiments, involving a pre-treatment with AFB_1 of roots for three days, was carried out to allow AFB_1 to exert a potential biological activity. In fact, although data on the uptake of AFB_1 by isolated portions of roots are not available, it seems reasonable to hypothesize that possible differences in the first experimental set may have not reached a significant level as a consequence of the biosynthesis of anthocyanins starts, while AFB_1 might requires time to exert a potential biological effect once absorbed (Kim et al., 2006).

The second set of experiments, following referred as "pretreated", was carried out by exposing maize radical portions to AFB_1 for three days before the induction of anthocyanin biosynthesis (7 days) under the conditions previously described, namely: HF>BAP and AFB_1>BAP. The total anthocyanin content (TAC) for "pretreated" experiments is shown in **Figure 17**.

Figure 17: Total anthocyanin content in maize roots for "pretreated" experiments. TAC is expressed in mg g^{-1} of dry weight as a mean±SD CGE of two biological replicates.



In this second set of experiments solely the conditions of interest were tested, as the experimental framework was already defined. According to unpaired T-test no significative difference was found between the two experimental conditions tested, even if the TAC of the condition AFB_1>BAP was confirmed higher on average (mean of 0.563±0.053 mg g^{-1}) with respect to HF>BAP (0.360±0.120 mg g^{-1}), as observed for the first experimental set.

In addition, it must be noted that the absolute TAC for the last experimental set was much lower than the previous one. In general, a wide variability has been observed both between biological replicates and, visually, within the context of the same replicate that consists of about twelve radical portions from different plantlets. A contribution to the observed variability could come from endogenous cytokinins, whose impact it is not possible to predict.

3.4 Study 4: *In vitro* exposure of Tritordeum to DON: uptake and biotransformation

3.4.1 Introduction

Mycotoxin-producing fungi can infect several cereal crops determining losses in crop yield and quality as well as contamination by mycotoxins of food and feed. Among the most relevant mycotoxigenic fungi *Fusarium* genus play a central role and several species, such as *F. graminearum* and *F. culmorum*, are responsible for the production of these toxic compounds (Gauthier et al., 2015). Deoxynivalenol (DON) is reported to be one of the most prevalent mycotoxins worldwide and as first reported by Berthiller et al., (2005), many cereals are capable to biotransform DON into deoxynivalenol-3-glucoside (DON3Glc) that is the most representative modified form of DON in food commodities (Mishra et al., 2020). As DON3Glc can be partially hydrolyzed during digestive processes, consumers are exposed to an additional load of DON that can lead to further health hazards both for humans and animals and therefore should be monitored along with its parent form (Berthiller et al., 2009; Dall'Erta et al., 2013).

Tritordeum is an alternative cereal crop obtained by breeding *Triticum turgidum* subs. *durum* Desf. and *Hordeum chilense* Roem. & Schult. Little is known regarding susceptibility of tritordeum to mycotoxin contamination and occurrence data on DON and DON3Glc are almost completely lacking. Spaggiari et al. (2019) reported occurrence of DON, DON3Glc and other mycotoxins for the tritordeum cultivars "Aucan" and "Bulel" and the "Study 2" of this thesis adds some information about mycotoxin contamination for this cereal grown in open field.

DON3Glc/DON ratio can vary in relation to many factors including genotype and environmental conditions and was reported to be higher in resistant wheat cultivars than in

susceptible ones, suggesting that detoxification of DON by conjugating with glucose molecules is a mechanism involved in reducing DON content (Amarasinghe et al., 2016; Cirlini et al., 2013; Lemmens et al., 2016). Despite the availability of data from wheat, this aspect has not been specifically addressed in tritordeum so far.

Since biotransformation also depends on environmental factors, the use of *in vitro* models represents a useful tool to reveal the plant biosynthetic potential for modified mycotoxins as well as to study the potential absorption and translocation of mycotoxins and their metabolites in a controlled environment. Such *in vitro* approach was already successfully applied to maize and durum wheat, for which it was demonstrated in our laboratory both the uptake from the medium and translocation of mycotoxins to the aerial part of the plants as well as the biotransformation potential of the crops tested (Righetti, et al., 2021b; Rolli et al., 2018).

In particular, *in vitro* systems, giving their aseptic and controlled conditions, allows to totally exclude environmental factors, making possible to evaluate differences between cultivars only based on the genotypes with the aim to reach a more complete understanding of the factors at the basis of the differences observed in open field. Moreover, the administration of mycotoxins by root uptake can simulate a potential environmental exposure, since that was demonstrated that uptake from the soil and translocation to above-ground organs take place under field conditions in various crops (Hariprasad et al., 2015; Snigdha et al., 2015).

By administration of DON via root uptake *in vitro,* this study investigated the translocation and biotransformation ability of two selected tritordeum cultivars that showed significantly different DON3Glc/DON ratios when grown in open field, with the aim to better understand the factors underlying this difference.

3.4.2 Materials and methods

3.4.2.1 Plant material and chemicals

Caryopses of tritordeum "Aucan" and "HT-460" cultivars were kindly provided by Prof. Massimo Blandino (University of Turin, Turin, Italy).

Analytical standards of DON (100 mg L^{-1} in acetonitrile) and DON3Glc (50 mg L^{-1} in acetonitrile were purchased from Romer Labs (Getzersdorf, Austria) and used for the quantification of mycotoxins by UHPLC-MS/MS. DON reference material (5 mg of powder subsequently dissolved in 5 mL of acetonitrile) was purchased from Merck (Darmstadt, Germany) and used for *in vitro* experiments. UHPLC-grade methanol, acetonitrile, acetic acid, formic acid and water were purchased from VWR Chemicals (Radnor, USA). Ammonium acetate and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, USA). Murashige and Skoog (MS) medium, sucrose and phyto agar were purchased from Duchefa Biochemie (Harleem, Netherlands).

3.4.2.2 Plantlets growth conditions and experimental design

Plantlets growth conditions and treatment with DON followed what was reported by Righetti et al., (2021b) for maize, with some modifications as described as follows. Caryopses were soaked in 70% (v/v) ethanol for five minutes, subsequently a surface disinfection was performed using 2.5% (v/v) sodium hypochloride for fifty minutes under vacuum (15 mmHg), followed by three washes with sterile distilled water to remove sodium hypochloride residues. The sterilized caryopses were sowed individually in glass tubes containing about 15 mL of 1/4 strength MS medium solidified with 0.8% (w/v) agar (pH 5.8) previously sterilized by autoclaving at 121°C for 20 minutes. Cultures were maintained in a growth chamber at

25±1°C with a 16 hours photoperiod under fluorescent tubes at a light intensity of 27 µmol $m^{-2}s^{-1}$. 14 days after germination, seedlings were screened for the presence of cultivable organism on specific media to assess the absence of fungal or bacterial contamination and then transferred individually in glass jars on 100 mL of MS medium added with 3% (w/v) sucrose (pH 5.8) and maintained for 14 days. Afterwards, the medium was removed and replaced with 100 µg of DON dissolved in 100 mL of MS medium added with 3% (w/v) sucrose (pH 5.8) and maintained for 14 days after which the roots and stem/leaves of each plantlet were harvested separately and stored at -20°C until extraction of target compounds.

3.4.2.3 DON administrations, sampling and sample extraction

DON was dissolved in an adequate amount of DMSO so that the final concentration of the solvent in culture medium did not exceed the one considered toxic (0.2%) with mycotoxin being at the final concentration of 1 µg mL^{-1}.

DON presence in liquid media was determined three times at the following intervals: t = 0, t = 7 day and t = 14 days by sampling 1 mL of liquid culture media.

Before extraction, samples were freeze-dried for 24 hours using a laboratory lyophylizator (LIO-5PDGT, 5 Pa s.r.l., Trezzano sul Naviglio, Italy) and then ground using a mortar and pestle in liquid nitrogen.

Samples were extracted according to Righetti et al., (2020), that is 1.5 mL of extraction solvent mixture of water/methanol/formic acid (79:20:1, v/v/v) was added to 50 mg of plant material and stirred for 90 min on a platform shaker (Ika Werke, Germany) at a speed of 200 strokes/min and subsequently centrifugated for 10 minutes at 14000 rpm (radius 9.5 cm) at room temperature. 1 mL of supernatant was evaporated to dryness under a stream of nitrogen

and then reconstitute in 0.5 mL of water/methanol (80:20, v/v) prior to UHPLC-MS/MS analysis.

All medium samples were diluted with water/methanol (80:20, v/v) to achieve a final ratio of 1:2 (v/v), vortexed for 1 min and then subjected to UHPLC-MS/MS analysis.

3.4.2.4 UHPLC-MS/MS detection and quantification of DON and DON3Glc

UHPLC-MS/MS analysis was carried out on UHPLC Dionex Ultimate 3000 coupled to a triple quadrupole mass spectrometer TSQ Vantage (Thermo Fisher Scientific, Waltham, USA) equipped with an electrospray source (ESI). The chromatographic separation was obtained using a Sunshell column (Chromanik Technologies, Osaka, Japan) 2.1 × 100 mm, 2.6 µm particle size, heated to 40° C. 2 µl of sample extract was injected into the UHPLC system and the flow rate was set up to 0.35 mL min^{-1}. Gradient elution was performed by using water (eluent A) and methanol (eluent B) both acidified with 0.2% v/v acetic acid. Ammonium acetate was added to the eluent A at the final concentration of 5mM. Initial conditions were set at 98% A and 2% B for 2 min, then eluent B was increased to 20%, after an isocratic step (6 min), eluent B was further increased to 90% and this condition was maintained for 2 min until the return to the initial condition. The total run time was 18.4 min. Mass spectrometric analysis was performed in negative ionization mode in multiple reaction monitoring (MRM), spray voltage 3000 V, capillary temperature 270°C, vaporizer temperature 200°C, sheath gas pressure 50 units, auxiliary gas pressure 5 units. The following quantifier transitions were measured: DON m/z 355>295 (CE 13eV) and m/z 355>265 (CE 17eV), DON3Glc m/z 517>457 (CE 16eV) and m/z 517>427 (CE 23eV). Calibration curves were set up using external standards (range 10 µg kg^{-1} – 2500 µg kg^{-1}) for target analyte quantification. A good linearity was obtained for all the quantifications performed (R^2 >

0.99). Data acquisition was performed by Thermo Xcalibur 2.2 software (Thermo Fisher Scientific, Waltham, USA).

3.4.2.5 Statistical analysis

The statistical analysis was performed using IBM SPSS v.25.0 (SPSS Italia, Bologna, Italy).

3.4.3 Results and discussion

3.4.3.1 Background from the field

As reported by the "Study 2" of this thesis, during the harvesting year 2020, in kernels from tritordeum cultivar "Aucan" we detected a mean of 3854±1997 µg kg^{-1} of DON and a mean of 385±225 µg kg^{-1} of DON3Glc. The cultivar "HT-460", grown under the same conditions contained a mean of 1573±540 µg kg^{-1} of DON and a mean of 385±126 µg kg^{-1} of DON3Glc. This cultivar was not included in the previous study, because its occurrence data are limited to the harvesting year 2020.

Regarding the occurrence of DON and DON3Glc in straw samples, as reported by the "Study 2" of this thesis, tritordeum cultivar "Aucan" provided a mean of 8300±4514 µg kg^{-1} of DON and a mean of 595±525 µg kg^{-1} of DON3Glc. The cultivar "HT-460" showed a mean of 1379±583 µg kg^{-1} of DON and a mean of 216±83 µg kg^{-1} of DON3Glc.

Biotransformation ratios expressed as DON3Glc/DON are shown in **Table 12**. Despite the high variability that affect DON occurrence data previously described, according to unpaired T-test, significative differences were found between the two cultivars tested for both kernel ($p = 0.000$) and straw ($p = 0.028$). The two cultivars were therefore selected for their large variability in DON3Glc/DON, to evaluate if such difference might be related to a different genetic lineage or if it is more under control of environmental factors.

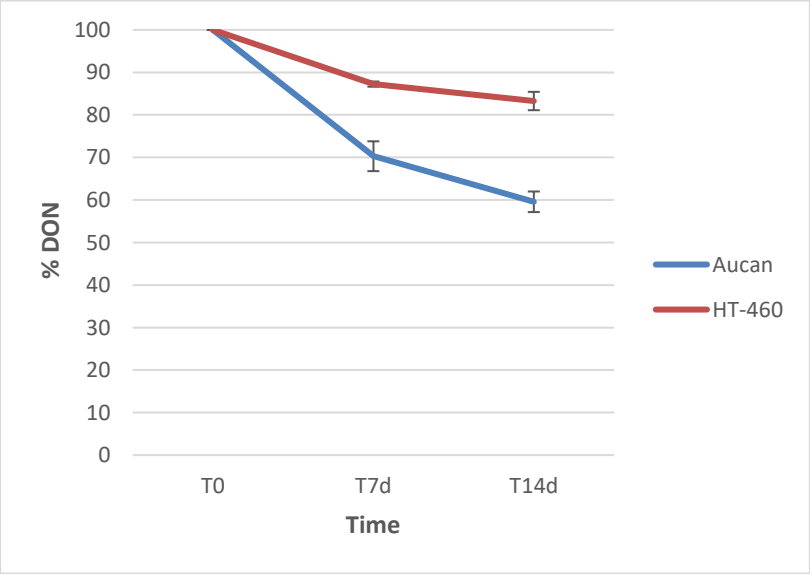
Table 12: Biotransformation rate, expressed as DON3Glc/DON ratio, reported for the cultivars "Aucan" and "HT-460" grown in open field for the harvesting year 2020, at the conditions described for the "Study 2" of this thesis. Data are shown as a mean±SD of three biological replicates.

Cultivar	DON3Glc/DON ratio	
	Kernel	Straw
Aucan	0.097±0.028	0.068±0.030
HT-460	0.247±0.020	0.158±0.007

3.4.3.2 In *vitro* experiments

DON uptake from the medium was evaluated at three time points, namely t = 0 (before the exposure of plantlets to DON), t = 7 days and t = 14 days (end of the experiment). The absorption kinetics is shown in **Figure 18**.

Figure 18: Absorption kinetics for the two tested cultivars. Data are expressed in percentage of the initial concentration of DON, set at 100%, as a mean±SD of two biological replicates.

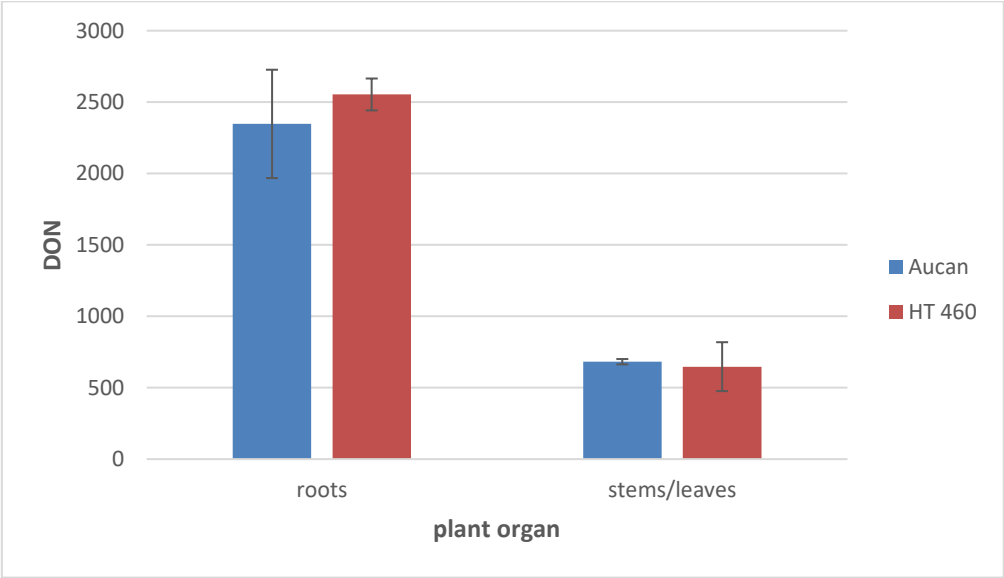


Differences in absorption kinetics between cultivars could be due to the slightly different growth rates observed *in vitro* for the two cultivars. In fact, even though DON exposure took place after an adequate development of all plant organs, the cultivar "Aucan" showed a quicker development and therefore a wider root and leaf surface than "HT-460". This might have led to faster absorption of DON from the medium. Further experiments will be performed to evaluate the absorption kinetics at the same degree of development using seedlings of different ages.

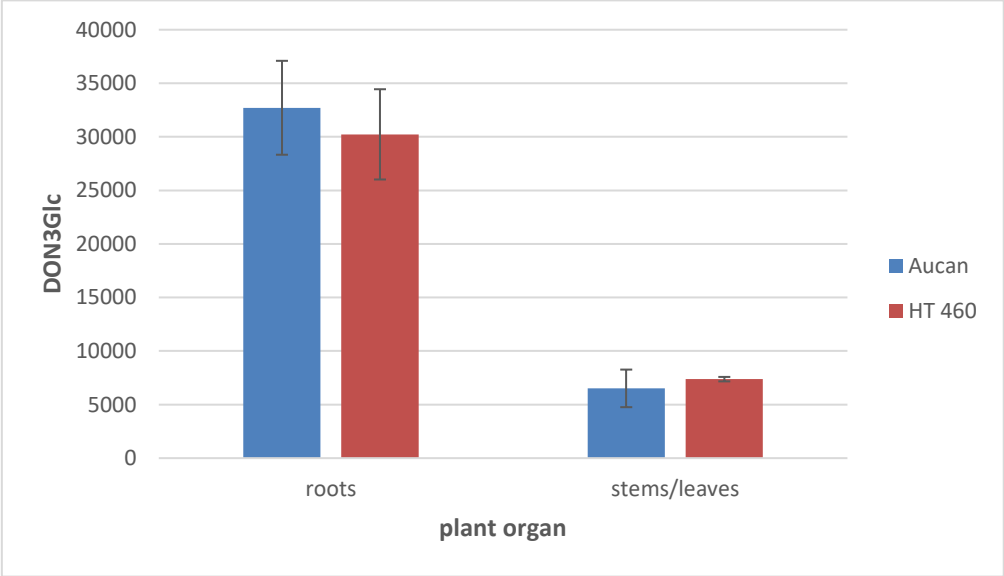
DON and DON3Glc in both roots and stems/leaves were quantified (**Figure 19**) in order to evaluate the biotransformation capacity of the two cultivars cultured *in vitro*.

Figure 19: DON (A) and DON3Glc (B) content for the two tested cultivars divided on the basis of plant organ. Data are expressed in µg kg^{-1} as a mean±SD of two biological replicates.

A



B



Based on the data showed above, both cultivars showed a very high biotransformation capacity when exposed to DON *in vitro* and over 90% of the DON occur in glycosylated

form. Moreover, the majority of DON and DON3Glc found was localized in roots, that is in the organs directly exposed to DON and responsible for its uptake from the medium.

The comparison between *in vitro* and open field data (**Table 12**), highlights a remarkable difference because a possible explanation resides in the hypothesis that in *in vitro* systems plants must cope with only selected constraints - in this case the exposure to DON - and can therefore focus their metabolic energies on detoxification mechanisms. The simultaneous exposure to multiple stressors and elicitors may in fact activate multiple detoxification pathways and diverge the resources allocated to plant defense. While we cannot estimate the extent of this phenomenon nor the enzymes involved, a different environment seemingly induces a different response in tritordeum cultivated *in vitro*, thus leading to suggest prevalence of the phenotypic response over the genotypic profile.

When focusing on overall biotransformation rate (**Table 13**), unpaired T-test did not show significative differences between the two tested cultivars. This preliminary finding supports the hypothesis that other factors than genotypes are involved in determining the differences observed between cultivars grown in open field.

Table 13: Biotransformation rate, expressed as DON3Glc/DON ratio, reported for the cultivars "Aucan" and "HT-460" grown *in vitro*. Data are shown as a mean±SD of two biological replicates.

Cultivar	**DON3Glc/DON ratio**		
	Roots	**Stems/Leaves**	**Overall**
Aucan	13.97±0.39	9.59±2.83	11.78±3.02
HT-460	11.89±2.17	11.85±3.45	11.87±2.36

CHAPTER 4 Conclusions and future perspectives

Data collected in “study 1” highlighted a remarkable susceptibility of blue pigmented wheat varieties to mycotoxin contamination, compared to genotype with different anthocyanin content and histological placement in the kernel. To enhance the food use of wholegrain flour from blue-grained wheat, for both hedonic or health purposes, it will be necessary to substantially improve their resistance to mycotoxin accumulation. Multiple reports have suggested that anthocyanins may have a positive effect on oxidative stress, including resistance against this specific facet of *Fusarium* infection. Scaling up these evaluations to field grown plants proved to be a challenge and these first results did not provide a clear and unequivocal correlation between anthocyanin content and mycotoxin accumulation, although the black-grained wheat varieties, sharing the same genetic lineage of to blue ones, but with anthocyanins also in the pericarp layer, showed a lower mycotoxin content compared to blue ones. A leading result, however, was that varieties accumulating these pigments in the pericarp (purple colouration) had significantly lower DON content if compared to those in which aleurone is involved (blue colouration). This evidence is relevant in term of further experimental design. As histological distribution may affect the interplay between pigmented wheat and mycotoxin presence, further investigations should not look just at the phytochemical profile of pigmented wheat but also at their histological properties and at the composition of individual alleles responsible for anthocyanin biosynthesis. Investigations with techniques capable of unveiling the spatial distribution of mycotoxins and plant defense metabolites in pigmented wheat kernels such as MALDI mass spectrometry imaging, seem to be warranted.

According to the comparison between cultivars carried out in “study 2”, a susceptibility emerges for to DON accumulation in kernels of tritordeum cultivars. Therefore, further studies should put a specific focus on the reasons and on relapses of a likely higher

susceptibility of tritordeum to mycotoxin accumulation. This is of particular relevance given its use as an alternative cereal to produce food products and fulfill the interest of consumers for new products. Moreover, giving the lack of data reported in literature, future research should be focused on evaluating the occurrence of mycotoxins in a wider range of tritordeum cultivars, to hopefully detect lines with increased resistance. In addition, this study highlights the need to routinely evaluate regulated, modified and emerging mycotoxins also in straw, that was confirmed at high risk of contamination and therefore representing a critical issue for food and feed safety. As straw may be left in fields and ploughed into soil, their high content in mycotoxins may also alter the environmental plant exposure to these compounds via root uptake.

The *in vitro* bioassay presented as "study 3", was developed to test the hypothesis, emerged at the end of the PhD course, of a possible effect of AFB_1 on anthocyanin biosynthesis. Based on the preliminary results, a possible positive effect of AFB_1 on anthocyanin biosynthesis has been reported as a speculative hypothesis. Further experiments will be needed to consolidate this *in vitro* model and to confirm or reject the formulated hypothesis. This *in vitro* model, once consolidated, could provide a methodology that can be used in the future as a "long-exposure" alternative to the already reported bioassays to test the possible effect of other compounds on anthocyanin biosynthesis.

The *in vitro* comparison of two tritordeum cultivar presented as "study 4" didn't show any substantial difference in their ability to convert DON into DON-3-glucoside despite a significant difference previously observed in open field. Although other experiments are needed, it is possible to hypothesize that the different biotransformative capacity observed in the open field could be the result of concurrent environmental stimuli. A genetic determinant in this sense may be, at least according to this preliminary investigation, not resolutive. Due to the large number of variables involved in open field studies, this kind of simplified *in vitro*

approach might prove in the future its usefulness for formulating hypotheses regarding the metabolic characteristics of given cultivars. Furthermore, this *in vitro* approach could be valuable in the selection of cultivars or varieties in order to identify metabolic characteristics of interest to be subsequently verify in the open field.

Overall, within the complex interplay between mycotoxins and plant metabolism, cause-effect relationships are often difficult to describe, as a consequence of multiple factors of different origin triggering manifold metabolic effects as well as the intrinsic ability of plants to modulate a surrounding, ever-changing environment that incudes organisms interacting between themselves.

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Studies

Marco Gozzi, after a bachelor's degree in Biology (University of Parma), got the Master's degree in Biology and Biomedical Applications (University of Parma) in July 2017, project titled: "Epigenetic mechanisms involved in chromium tolerance and response to sulfate deprivation in *Scenedesmus acutus* (M.)", under the supervision of Prof. Anna Torelli. Subsequently, November 2019, started his PhD in Food Science under the supervision of Prof. Renato Bruni.

Original papers

Anthocyanin Content and Fusarium Mycotoxins in Pigmented Wheat (Triticum aestivum L. spp. aestivum): An Open Field Evaluation. Marco Gozzi, Massimo Blandino, Chiara Dall'Asta, Petr Martinek, Renato Bruni, Laura Righetti. Plants (2023), 12, 693. https://doi.org/10.3390/plants12040693

Congress attended and communications

Recent Advances in Food Analysis (RAFA 2021) - University of Chemistry and Technology Prague, Wageningen University – (Virtual Event, 3/11/2021 – 4/11/2021).

MycoKey-MycoTWIN International Conference 2021 - MycoKey-MycoTWIN, ISM – Bari (Italy) – (9/11/2021 – 12/11/2021).

The World Mycotoxin Forum - University of Parma – Parma (Italy) – (16/05/2022 - 18/05/2022), poster presentation: "In-depth study of mycotoxin accumulation in relation to anthocyanin composition in pigmented wheat".

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