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Metabolomics Approaches and their Hidden Potential for Explaining the Mycotoxin Contamination Problem

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Abstract

Food is essential for life. On the basis of the previous sentence, consumers have a right to expect that the foods they purchase and consume will be safe, authentic and of high quality. On these premises, target compounds, such as mycotoxins, pesticides or antibiotics, have been commonly investigated on the food chain, and subsequently, were regulated by authorities. This raises the following question: may consumer be prevented to these risk exposures? Probably not, food chain is step-by-step longer and more complex than ever before. Note that food chain is affected by globalized trade, culture, travel and migration, an ageing population, changing consumer trends and habits, new technologies, emergencies, climate change and extreme weather events which are increasing foodborne health risks, especially for mycotoxins. Because of the fact that mycotoxins are natural toxic compounds produced by certain filamentous fungi on many agricultural communities. In fact, these toxins have adverse effects on humans, animals and crops that result in illnesses and economic losses. Nevertheless, so far mycotoxins and their modified forms have been mainly monitored in cereal and cereal-based products, however, may an early detection of mycotoxins be considered a reliable strategy? In this chapter, recent metabolomics approaches have been reviewed in order to answer this question and to understand future strategies in the field of mycotoxin contamination.

Keywords: food metabolomics, mycotoxins, plant metabolome, fungal pathogens

1. Introduction

Mycotoxins are secondary metabolites (300–800 Da) produced by filamentous fungi that colonize crops in field and upon storage, being among them cereals one of the most affected

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commodities [1]. Fungal colonization is strongly dependent on environmental conditions and agricultural practices. Climatic factors such as temperature, humidity, rainfalls, as well as the concomitant presence of other pests or insects may support the fungal infection. Therefore, climate change is significantly affecting the mycotoxin contamination of crops worldwide. As a consequence, fungal infection and related pathogenic diseases can cause significant yield losses, quality reductions and mycotoxins accumulation in crops, particularly grains [2].

Although regulations, adequate quality controls and good agricultural practices have been implemented in many countries, the mycotoxin contamination represents a serious challenge for global trade in terms of animal and human health threat and economical losses. For this reason, the establishment of common standard procedure for fungal biocontrol and mycotoxin mitigation are under investigation.

From a toxicological perspective, mycotoxins can cause both acute and chronic effects for humans and animals. They are responsible for a broad spectrum of toxic activities, ranging from severe adverse effects on the liver, kidney, hematopoetic, immune system, foetal and reproductive systems, as well as significant contribution to carcinogenic and mutagenic developments [3]. In fact, The International Agency for Cancer Research (IARC) has formally classified a number of mycotoxins. For example, four aflatoxins are classified in Group 1 (AFB_1, AFB_2, AFG_1 and AFG_2) while ochratoxin A (OTA) is classified in Group 2B [4, 5].

Among them, those produced by *Fusarium* spp. are often found in cereals, and are related to pathogenic diseases in plants, as well. In particular, *Fusarium* Head Blight (FHB) is recognized as one of the most destructive global diseases of wheat and barley [6]. FHB can cause, indeed, significant reductions in grain yield and quality, and is associated with the accumulation of mycotoxins, such as deoxynivalenol (DON). Thus, besides the severe economic impact, due to losses in productivity, FHB represents a serious health risk for consumers and livestock [3]. In order to reduce the economic and health impact of FHB, several cultural practices have been proposed so far. However, crop rotation, tillage, use of fungicides or other biocontrol agents are generally regarded as insufficient to tackle FHB and mycotoxins contamination alone [3]. This is mainly due to the fact that the breeding of grains for superior technological properties has led to a decrease of the genetic diversity, with a subsequent increase of susceptibility towards pathogenic diseases. Therefore, the study of the plant response to fungal infection is crucial for developing possible strategies to counteract mycotoxin accumulation.

From a biological point of view, the role of mycotoxins in fungal colonization is still to be clarified. Some of them—such as deoxynivalenol (DON) have been proved to be virulence factor for fungal infection [7]. However, the intense cross-talk among plant and pathogen affects the biological cascade, from genes to metabolites, and plays a significant role in mycotoxin accumulation. Fungal infection and mycotoxin contamination are commonly addressed with classical methods, from DNA-based techniques for fungal identification to analytical methodologies for mycotoxin detection. The residual DNA content of fungal pathogens was used to identify unequivocally fungal species, and they were associated with cereals and their mycotoxins [8], basically allowing for a toxigenic fungi monitoring. However, the main disadvantage of this technique is associated to relatively high cost and the fact that

it is time-consuming. In addition, a poor correlation between fungal growth and mycotoxin accumulation has been pointed out.

For this reason, classical chromatographic methods are often used for mycotoxin determination in crops and products thereof [9–11]. Over the last decade, mass spectrometry (MS)-based methods have become the golden standard for mycotoxin analysis, being the multitoxin approach the most promising strategy to control the occurrence of multiple analytes in the same material [12]. As a complementation, rapid diagnostic methods are commonly based on immunochemical assays (i.e., lateral flow devices, dipsticks, etc.) for early detection at pre- and post-harvest [13]. More recently, nondestructive imaging methods have been proposed as well as rapid diagnostic tool [14]. In this context, the untargeted methodologies have started to be applied only recently, and only to meet specific needs. In particular, the omics strategies have been applied to the mycotoxin issue to investigate the interaction between the plant and the pathogen in field, leading to mycotoxin accumulation [3, 15–19].

In a top-down view, genomics and transcriptomics studies have proposed to investigate the biosynthetic pathways for mycotoxin production, and their regulation upon biotic and abiotic stress. Similarly, proteomics has been often proposed for identifying enzymes and proteins responsive to pathogenic diseases, such as FHB [20], or responsible for mycotoxin modification in plant [21, 22]. Over the last decade, however, the field of metabolomics has gained increasing interest across all disciplines, and has found a prominent role in mycotoxin-related studies as well. Metabolomics is an emerging technique that can be considered complementary to the other omics approaches and highlighting unique advantages. A metabolic fingerprint may generate thousands of data points, of which only a handful might be needed to describe the problem adequately [23, 24]. Extracting the most meaningful elements of these data is thus key to generating useful new knowledge with mechanistic or explanatory power.

To date, however, in the vast majority of cases, mycotoxin contamination has been directionally explored. In this way, up to now, the mycotoxin contamination loop has not been properly closed and many issues are still open. One of the main challenges in mycotoxin analysis will be to improve our limited understanding of the roles of plant pathogen cross-talk at the molecular level. In this context, a multiomics global strategy may be able to identify chemical markers at the earliest stage, and to univocally characterize resistant varieties and the early detection of mycotoxins. The early detection of toxigenic fungi or of markers of the interaction between the pathogen and its host can be usefully exploited to limit entering of mycotoxins into the food/feed production chain.

2. Advanced analytical tools merged with chemometrics

The multiomics approach has been poorly compared to classical approaches described in the previous section. Initially, innovative spectral techniques (i.e., imaging analysis, near-infrared, Raman) have been proposed for the early detection of fungal pathogens [25, 26]. Since fungal growth is not strictly related to mycotoxin accumulation, and to the pattern of occurring mycotoxins, these techniques cannot provide a response on mycotoxin occurrence or chemical

markers, mainly linked to the plant-pathogen interactions. In this framework, metabolomics may represent the golden tool for understanding the biological pathways involved in mechanisms of plant resistance. Nowadays, gas chromatography (GC) and liquid chromatography (LC) are commonly used for metabolomics approaches, mainly coupled to mass spectrometry (MS) [3]. In principle, LC-MS and GC-MS provide a high number of scans per peak, allowing peak picking and alignment (feature extraction), and if necessary quantification, as well as a large dynamic range in order to monitor low and high concentration levels of metabolites.

2.1. Liquid chromatography coupled to mass spectrometry (LC-MS)

LC-MS has been the most commonly used metabolic fingerprinting/profiling approach for understanding plant resistance mechanisms and the plant-pathogen cross-talk. For instance, Cajka et al. [27] have recently developed an analytical procedure based on the optimization of a solid-liquid extraction procedure using methanol/water (50:50, v/v), in order to isolate polar/medium-polar barley metabolites followed by ultra high performance liquid chromatography quadrupole-time-of-flight (UHPLC-QTOF) [27]. **Figure 1** shows unique and shared metabolites acquired by UHPLC-QTOF using both positive and negative ionization modes.
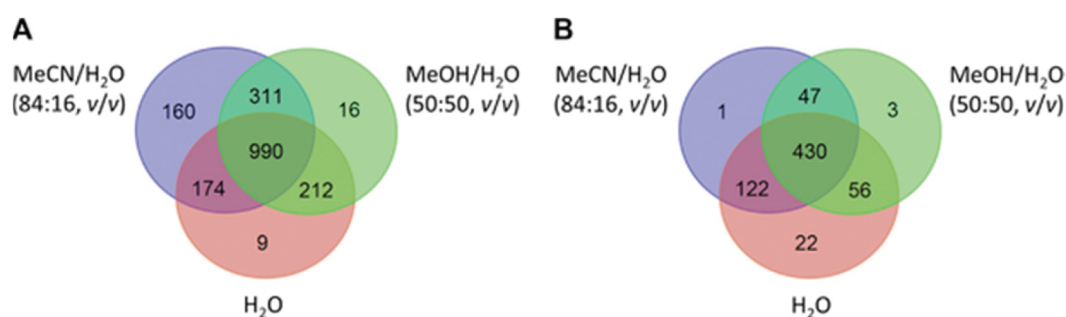

Figure 1. Venn diagrams illustrating shared and unique features in barley extracts prepared under the different extraction procedures and analyzed by both positive (A) and negative (B) ionization modes UHPLC-QTOF.

The authors demonstrated how the carefully in-depth investigation of sample preparation could support the extraction of the broadest spectrum of metabolites isolated from the matrix, in this particular case barley. Obviously, UHPLC-QTOF chemical fingerprints differed significantly depending on the extraction solvent used (see **Figure 2**). For example, when deionized water was used, a lower extraction efficiency of less polar compounds was exhibited. Nevertheless, sample preparation using a mixture of acetonitrile/water (84:16, v/v) or methanol/water (50:50, v/v) enhanced the extraction of less polar and polar compounds were also detected. The authors, as a compromise, chose methanol/water (50:50, v/v), since the extraction mixture permitted isolation of both highly polar and less polar metabolites. So far, various proportion of aqueous methanol has been mainly applied, as it can be seen in **Table 1**. In this way, the changes occurring both in primary carbohydrates and primary nitrogen metabolism upon plant infection have been partially elucidated. On the other hand, lipidomic approaches applying more nonpolar solvent (e.g., hexane, dichloromethane, ethyl acetate) have been exclusively used to investigate the plant-pathogen cross-talk in maize [28–30]. Increasing

evidence indicates, indeed, that lipid signalling is an integrated part of the complex regulatory network in plant pathogen cross-talk.
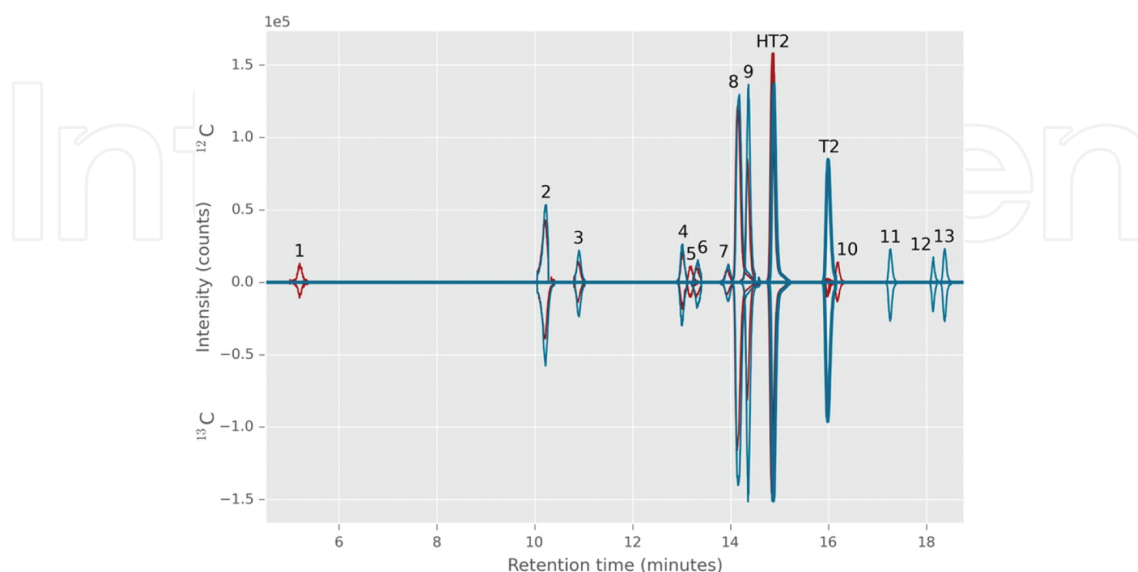

Figure 2. Overlaid extracted ion chromatograms (EICs) based on MetExtract data processing output showing the bio-transformation products of a sample treated with a mixture of $^{12}C/^{13}C$-HT-2 toxin (red trace) and one treated with a mixture of $^{12}C/^{13}C$ T-2 toxin (blue trace). EICs of nonlabeled metabolites were displayed with positive intensities; those of the corresponding labeled metabolites were displayed as negative intensities.

Not only fingerprinting approaches, but also metabolic profiling strategy has been recently performed using a stable isotopic labelling approach in order to understand the metabolic fate of HT-2 toxin and T-2 toxin in wheat [31]. In general, untargeted metabolomics approaches are usually based on generic settings for sample preparation (which usually include a simple extraction without any purification step, or nonsample preparation), separation and detection. In contrast, if a particular group of metabolites is preselected, a metabolic profiling is carried out. Thereby, a more specific extraction procedure and chromatographic separation/detection has to be performed. In this way, this study was focused on Type A trichothecenes, such as HT2 and T2 toxins, and their detoxification pathways.

The stable isotopic labelling approach applied is really innovative since monitoring pairs of corresponding nonlabeled and labeled precursor allowed metabolome to be easily monitored and interpreted, providing further information. Liquid chromatography high-resolution tandem mass spectrometry (LC-HRMS/MS) spectra of the observed metabolites of HT2 and T2 were compared with those obtained in wheat and were shown to be identical. **Figure 2** shows overlaid extracted ion chromatograms of all detected biotransformation products. In this frame, the authors demonstrated that the exposure of wheat to either HT2 or T2 toxins primarily activates biotransformations involving hydroxylation, (de)acetylation and various conjugations. Furthermore, kinetic data revealed that detoxification progressed rapidly, resulting in the almost complete degradation of the toxins, within 1 week, after a single exposure.

Instrumentation	Extraction	Plant	Chemical group	Markers class	References
LC-HRMS	Water/methanol (45:65, v/v)	Barley	Fatty acids, flavonoid phenylpropanoids, amino acids, terpenoids, organic acids	RRC, PRr, PRs, RI	Bollina et al. [16]
	Water/methanol (50:50, v/v)	Barley	Fatty acids, flavonoid phenylpropanoids	RRI, RRC, RI	Bollina et al. [17]
	Water/methanol (50:50, v/v)	Barley	Fatty acids, phenylpropanoids	RR, RI	Cajka et al. [27]
	Water/methanol (40:60, v/v)	Wheat	Fatty acids, phenylpropanoids, terpenoids	RRI, RRC, RI, PRp	Gunnaiah et al. [18]
	Water/methanol (40:60, v/v)	Wheat	Flavonoid phenylpro-panoids, terpenoids, amino acids, carbohydrates	RRC, RRI, RI	Gunnaiah et al. [19]
GC-MS	Water/methanol /formic acid (74:25:1, v/v)	Wheat	Polyamines, amino acids, phenylpropanoids, carbohydrates	RR	Warth et al. [15]
	Water/methanol /formic acid (74:25:1, v/v)	Wheat	Amino acids, amines, carbohydrates	RR	Nussbaumer et al. [36]
	Water/methanol (50:50, v/v)	Wheat	Polyamines, amino acids, phenylpropanoids, carbohydrates	RR	Paranidharan et al. [35]
H NMR	Methanol/water (40:60, v/v)	Wheat	Amines, amino acids, carbohydrates	RR, PR	Browne et al. [33]
	Methanol/water (40:60, v/v)	Wheat	Amines, amino acids, carbohydrates, phenylpropanoids	RR, PR	Cuperlovic-Culf et al. [24]

RRC, resistance-related constitutive; RRI, resistance-related induced; RI, resistance indicator; PR, pathogenesis-related; PRr, pathogenesis-related resistant; PRs, pathogenesis-related susceptible; PRp, pathogenesis-related proteins.

Table 1. Putative metabolites involved in *Fusarium* Head Blight resistance, reported in the literature so far.

2.2. Gas chromatography-mass spectrometry (GC-MS)

Surprisingly, GC coupled to high-resolution mass spectrometry (HRMS) has not been applied to metabolomics strategies. As it was discussed above, the applicability of HRMS permitted metabolic pathways to be clearly described. Nevertheless, GC coupled to a single quadrupole shows selectivity and specificity for metabolomics approaches, since available databases containing mass spectra and retention indexes can tentatively identify metabolites due to the extensive and reproducible fragmentation pattern obtained in full-scan mode using electron ionisation (EI). A recent research work was focused on the applicability of GC-EI-MS in order

to understand deoxynivalenol (DON) accumulation in wheat [15]. In this research, the experimental design was nicely described, and similar to previous research described above. Nevertheless, sample preparation took extra time compared to LC-MS, due to derivatization procedure based on silylation. Many metabolites contain polar functional groups and are thermally labile for separation by GC or present limited volatility, therefore, derivatization often has to be applied. Oximation or silylation has been commonly applied due to their universality and versatility [24].

2.3. Data processing to extract meaningful markers

For processing massive information based on separation techniques and mass spectrometry, effective software tools capable of rapid data mining procedures have to be used. Note that data matrices contain thousands of variables (m/z, RT, intensity), and they have to be converted into more manageable information [24].

Data processing and data pretreatment must be carried out in order to permit the identification of significant metabolites, which capture the bulk of variation between different datasets and may therefore potentially serve as biomarkers. Data processing usually involves four basic steps: deconvolution, alignment, filtering and gap filling. The features, defined by their m/z and retention time, and their intensities in different samples are used for the statistical analysis. Samples would be grouped and it can be observed using scores plots, heatmaps or hierarchical clustering. After data pretreatment, a statistical comparison can be performed using the multivariate data analysis (MVDA). Usually this step involves unsupervised models (PCA) and supervised classification tools, such as PLS-DA and OPLS-DA. These supervised methods are performed to maximize differences between groups and to highlight potential biomarkers. When the experimental design is more complex, the use of t-test or other univariate data analysis (UVDA) tools represents the best choice [32].

3. Metabolomics to decipher pathways involved in *Fusarium* Head Blight resistance

As it was already mentioned in the Introduction section, mycotoxins and fungal pathogens, such as *Fusarium graminearum*, can cause global diseases of wheat (*Triticum aestivum* L.) and barley [6]. Nevertheless, up to now, all preventive techniques used have been pointless, such as fungicides or crop rotation. Breeding strategies for increasing pathogen resistance seem to be the most promising and environmentally safe strategy for controlling mycotoxin accumulation in grains. It is known, indeed, that plant resistance mechanisms may be controlled by several quantitative trait loci (QTLs) that contribute to overall pathogen resistance in three different ways classified as type 1, 2 and 3, and referred as resistance to initial infection of spikelets, spread of pathogen within spikes and accumulation of mycotoxins, respectively. The involved QTLs typically are linked to, or contain, the genes that control the phenotype. Over hundred of QTLs for FHB resistance in wheat have been already identified [3, 7, 15, 32]. However, fully resistant varieties are still to be identified or inbred. Thus, there is an urgent

need to better understand the mechanisms of resistance against *Fusarium* spp. in order to develop novel strategies and resistance varieties.

Nowadays, recent advances in metabolomics offer new opportunities to elucidate complex metabolic pathways involved in *Fusarium* resistance and potential FHB resistance biomarker metabolites in barley and wheat [3, 15–19, 32]. In fact, during the last decade, the applicability of metabolomics has significantly increased in this field. Nevertheless, knowledge remains still partial, and a long way has to be covered towards the development and understanding of the plant-pathogen interactions. This new scenario will provide suitable knowledge related to plant metabolome, which was already explained by a few examples in the previous section.

Different strategies have been applied so far, NMR for polar metabolites [33, 34], LC-QTOF for semipolar metabolites [16–19, 32] and GC-MS for volatile compounds [15, 35, 36]. However, we should keep in mind that a strategy able to simultaneously extract and detect the entire metabolome does not exist. Consequently, the data delivered by metabolomics studies only cover a fraction of the metabolome. In other words, the picture taken exclusively reveals one part of the metabolome. In addition, the resistance mechanism is a result of multi-interactions between biomolecules such as genes, proteins, metabolites and environmental factors. Therefore, a multiomics approach based on proteomics and metabolomics could overcome any limitation in the experimental design. For example, an integrated nontargeted metabolo-proteomics approach was recently published [18, 32]. This strategy demonstrated to be a powerful tool for a more comprehensive analysis in order to elucidate the mechanism, revealing successfully changes in the wheat primary metabolism, in response to *F. graminearum*.

4. Setting up of the experimental plan

Depending on the hypotheses to be tested, different combinations of plants and fungal pathogens can be employed to explore the system relationship. Up to date, the metabolomic approaches have been mainly restricted to study the resistance against *F. graminearum* and *F. culmorum* in wheat and barley [3]. Resistance mechanisms have been elucidated by using wheat/barley genotypes with various levels of resistance, classified as susceptible, intermediate and resistant. However, in most of the studies, unrelated germoplasms are compared, leading to a confusing interpretation of the data delivered, since the differences in the metabolic profiles may actually result from the cultivar background [3]. Thus, the use of near isogenic lines (NILs) that differ in QTL conditioning FHB, is suggested to be the best approach to simplify the complexity, and allow to reach conclusive evidence related to resistance functions [18].

As for the comparison, mock-inoculated versus pathogen-inoculated plants is considered the best approach to highlight differences. Gunnaiah et al. [19] instead designed a different experiment in order to elucidate the host biochemical resistance to FHB spread in response to trichothecene producing and nonproducing isolates of *F. graminearum*. The two *F. graminearum* strains differed in the loss of function of Tri5 gene [19]. In addition to *F. graminearum* inocula-

tion, Warth et al. [15] also used DON injection into the middle florets of spikelets to decipher the mechanism of plant resistance to the toxin. Experiments have been performed in field conditions [27], under greenhouse [16–18, 33, 36] with computer-controlled settings for light, temperature and relative air humidity [15] and more recently, in environmental controlled growth chamber [34]. All these approaches are summarized in **Table 1** together with the extraction and detection methodologies applied, the plants used and the main classes of metabolites identified by the authors so far.

5. Elucidating FHB resistance mechanisms by metabolomics

Plant resistance to *Fusarium* Head Blight and related mycotoxin accumulation has been described through five major types of mechanism, mainly described for wheat and further applied to other cereals. These mechanisms are often host-specific, thus requiring plant-specific elucidation studies. Type I resistance is related to initial infection of the floret in wheat and barley, and of the silk in maize [37]. The spreading of infection is then limited by type II and type III resistance. Type IV resistance is related with tolerance and ability to maintain yields, and type V resistance gathers all mechanisms of resistance to mycotoxin accumulation [38–40]. According to Boutigny et al. [41], type V-1 represents resistance to toxin accumulation operated by metabolic biotransformation [42, 43], while type V-2 corresponds to resistance due to the inhibitory effect of mycotoxin biosynthesis exerted by plant endogenous compounds. Metabolomics has been exploited so far in this field for the comparison of metabolite composition of resistant and susceptible varieties upon *Fusarium* infection, allowing for the definition of a large set of compounds potentially involved in FHB modulation [3, 15–19]. Among those, fatty acids and compounds thereof have been found to be involved in the plant-pathogen signalling system, while terpenoids and phenylpropanoids take part to cell wall reinforcement, show antifungal properties and may interfere with mycotoxin biosynthesis [3]. Generally, the workflow of markers identification comprises the following steps: (1) marker identification based on accurate mass (MS), isotopic pattern and MS/MS pathway, (2) off- or online database searching and (3) data interpretation. These markers can be tentatively identified without analytical standards, or unambiguously identified using analytical standards. The identification of markers usually represents the last step within metabolomics studies. This is crucial in order to understand the metabolite pathway, since they can be interesting intermediates or final secondary metabolites. In this particular topic of mycotoxin contamination, hundreds of metabolites related to FHB resistance have been putatively identified so far by metabolomics strategies [4]. It was already mentioned that the number and chemical structures of metabolites significantly vary according to the experimental design and the applied analytical strategy.

Biomarker metabolites of resistance can be further subclassified according to their function. Those metabolites, whose abundance was increased in both resistant and susceptible cultivars, following pathogen inoculation as compared with those inoculated with water, were referred as pathogenesis-related (PR) metabolites [44]. Accordingly, metabolites that were significantly higher in resistant cultivars than in susceptible one were designated as resistance-related (RR) metabolites.

Among RR metabolites, some of them have been demonstrated as constitutive, while others are induced upon fungal infection [16, 17]. Among them, resistance-indicator metabolites [3, 16, 17] include modified mycotoxins such as DON, DON-3Glc and the other DON-biotransformation products (**Figure 3**). Following wheat inoculation by *Fusarium*, DON is spread within spike, and the host counteracts mycotoxins by conjugating them to endogenous metabolites (i.e., by glycosylation, acylation, conjugation to amino acids and glutathione). Thus, all the modified forms are designed as resistance indicators, since they indicate that the plant is reacting against the infection also by converting mycotoxins into their less toxic forms. According to the literature [19, 32], the chemical defense against fungal pathogens including DON producing *Fusarium* species is linked to three main mechanisms of resistance: cell wall reinforcement through the deposition of lignin, production of antimicrobial compounds and specific induction of defense signalling pathways. As reported by Gunnaiah et al. [18] among the metabolites reported as involved in plant response to FHB in soft wheat, the main chemical groups are phenylpropanoids, and terpenoids, followed by amino acid derivatives. On the other hand, when functional properties are considered, the majority of resistance-related metabolites showed an antimicrobial activity, followed by cell wall strengthening properties.
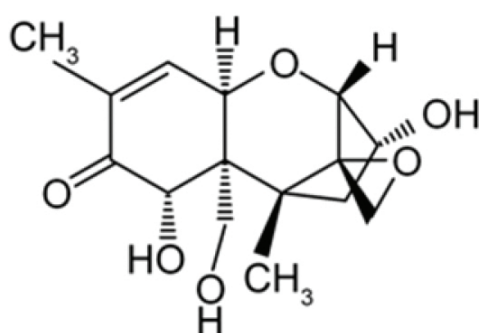

Figure 3. Chemical structure of deoxynivalenol (DON).

Phenylpropanoids such as flavonoids and phenolic acids have been frequently described for their contribution to plant defense mechanisms. Their activity is exerted either through direct interference with the fungus, or through the reinforcement of plant structural components acting as a mechanical barrier [45, 46]. Flavonoids, especially flavones, flavonones and isoflavonoids, lignans and other phenolic compounds were induced in Sumai-3 as antimicrobial agents, following *F. graminearum* inoculation. This is mainly due to their antioxidant activity leading to the neutralization of ROS, produced under biotic stress. A similar profile was identified upon *F. graminearum* inoculation in barley cultivars [16, 47] and in wheat [18]. In addition, phenolic acids have been reported as inhibitory agents towards mycotoxin biosynthesis in vitro [48, 49]. Among phenolic acids, hydroxycinnamic acid (HCA) derivatives, such as ferulic and caffeic acids, have been reported as important contributors to FHB resistance [4], probably on account of the high antioxidant properties [50].

Among HCAs, chlorogenic acid has been reported as a potential resistance factor in different pathosystems [49, 51, 52]. Concerning the cell wall reinforcement, hydroxycinnamic acid

amides (HCAAs) are deposited as cell wall appositions at the inner side of plant cell walls after cross-linking with polysaccharides, lignin and suberin [27]. These HCAAs are synthesized by condensation of hydroxycinnamoyl-CoA thioesters with aromatic amines (e.g., spermidine, spermine, tyramine) originated from aromatic amino acids. Thus, the involvement of amino acids in resistance to *Fusarium* may also be related to their role as a precursor of cell wall-bound HCAAs. Among those identified so far, N-caffeoylputrescine, 4-coumaroyl-3-hydroxyagmatine and feruloyl-serotonin are significantly upregulated upon *F. graminearum* infection in the resistant cultivar Sumai-3 [27]. With regards to the differences in terpenoid profile, Sumai-3 was characterized by a higher amount of syringyl lignin precursors like sinapoyl alcohol and sinapaldehyde, and glucose conjugate of sinapoyl alcohol, syringing [27]. Lignin results from monolignol glucosides' polymerizations and lead to a reinforced cell wall that is more resistant to fungal cell wall degradation enzymes [4].

Moreover, changes in the cell wall polysaccharides following infection were described by Cuperlovic-Culf et al. [24]. Large increase in concentration of sugars and inositols was found in all wheat varieties, particularly for Sumai-3, indicating an attempt at creation of cell wall barrier for *F. graminearum* penetration. In addition, fatty acids were also suggested to participate in resistance as physical barrier to pathogen ingress through their role in cuticle formation [4]. As far as the involvement of resistance related metabolites—mainly lipids—in the plant signalling pathways, significant results are summarized in the last part of this review.

6. The role of lipids in the plant-pathogen cross-talk

Increasing evidence indicates that lipid signalling is an integral part of the complex regulatory network in plant response to stress/infection. Modifications of membrane lipids produce different classes of signalling messengers, such as phosphatidic acid (PA), diacylglycerol (DAG), DAG pyrophosphate (DAGPP), lysophospholipids, free fatty acids (FFAs), oxylipins, phosphoinositides and inositol polyphosphates. Lipidomic approaches were developed to investigate in depth the plant-pathogen cross-talk, demonstrating a close relationship between the modification of the pathogen oxylipin profile with the mycotoxin synthesis [28].

Among metabolites associated with fatty acid metabolic pathways, a number of compounds have been identified for their potential contribution to cereal resistance towards FHB [53]. Fatty acids and their derivatives play significant role in plant defense against pathogens. Among their functions, they contribute to basal immunity, gene-mediated and systemic acquired resistance in plants. In addition, fatty acids are involved in the plant defense signalling pathway, through the formation of important mediators such as oxylipins and jasmonates. The unsaturated C18:1, C18:2 and C18:3 fatty acids, namely oleic, linoleic and linolenic acid, are often described as involved into defense mechanisms against fungal pathogens [47, 54, 55] and able to modulate mycotoxin production [55, 56]. The antimicrobial activity is probably due to their role in modulating ROS production, and in cuticle formation, which constitutes a physical barrier to pathogen infection [57]. In addition, they are precursors of the plant oxylipin pathway, which moves from the enzymatic formation of hydroperoxides, carried out by

lipoxygenase (LOX) [58]. Distinct LOX isoforms, referred as 9-LOX and 13-LOX, preferentially add a hydroxyl moiety at C9 or C13 position of the fatty acid backbone, leading therefore to 9- and 13-hydroperoxides, respectively. These compounds act then as substrates for the two distinct biosynthetic cascades, with the formation of approximately 150 known oxylipins including hydroxy-, oxo- or keto-fatty acids, green leaf volatiles (GLVs) and jasmonic acid (JA) [59]. Jasmonates originate from 13-LOX products, while 9-LOX products lead to less-known metabolites known as defense factors in response to fungal attack [60]. Jasmonic acid and methyl jasmonate are well known for their roles as plant stress hormones. They cause pro-grammed cell death activation, the production of ROS and the deposit of wax layers on plant tissues [61]. Jasmonates play, in addition, an active role in the regulation of the phenylpropa-noids pathway [62], exhibit antimicrobial properties towards toxigenic fungi [47, 60] and modulate mycotoxin accumulation [63, 64].

Besides these functions, jasmonates were proved to activate glucosyltransferase in *Arabidopsis thaliana* and barley [65]. This is a key enzyme activity involved in a DON detoxification pathway that transforms DON into less phytotoxic DON-3-Glc. Several metabolomic studies have highlighted the involvement of jasmonic acid [15–19, 33] in resistance to DON-producing *Fusarium* species. While the physiological function of jasmonates has been well described over the last years, little is known about other 9-LOX-derived compounds. Recent studies demon-strated that 9-oxylipins contribute to maize susceptibility or resistance to fungal pathogens, in a pathosystem-dependent way [61]. Several studies, indeed, suggested that mycotoxin accumulation is modulated by host oxylipins. In particular, linoleic acid and 9-oxylipins seem to be conserved signal molecules modulating mycotoxin biosynthesis, fungal sporulation and other aspects of fungal differentiation processes [54]. The effects of mutation of LOX gene were often studied in maize, observing that inactivation of the 9-LOX gene led to an increased susceptibility of maize to *Aspergillus flavus*, *A. nidulans* and *F. verticillioides* [66–68]. Similarly, modification of LOX genes led to a modulation of fumonisin production in the maize—*F. verticillioides* pathosystem [69, 70]. The deep involvement of oxylipins in the intense cross-talk between host and pathogen has still to be clarified. Endogenous fungal oxylipins are known indeed for supporting host colonization, as well as mycotoxin biosynthesis. Some authors suggest the possible interaction between fungal oxylipins and plant GPCRs, transmembrane-proteins or receptor-like kinases, for host manipulation.

7. Conclusions

A metabolomics approach may support the quick growth of this relatively new field of research, allowing for a better understanding of the changes occurring in the plant and pathogen metabolites upon interaction. In principle, analytical methods developed have demonstrated significant advances in sensitivity, robustness, flexibility and discrimination power in order to build successfully statistical models, and subsequent marker identification. Increasing evidence indicates that lipid signalling is an integral part of the complex regulatory network in plant response to stress/infection. Modifications of membrane lipids produce different classes of signalling messengers, such as phosphatidic acid, diacylglycerol pyro-

phosphate, lysophospholipids, free fatty acids, oxylipins, phosphoinositides and inositol polyphosphates. Lipidomic approaches can be developed to investigate in depth the plant-pathogen cross-talk, demonstrating a close relationship between the modification of the pathogen oxylipin profile with the mycotoxin synthesis. Therefore, metabolomics approaches will provide new solutions to old problems. In fact, the early detection of mycotoxins and smart detoxifications can be performed by metabolomics strategies for the first time, and these approaches can fill the gap in order to answer these questions and go a step further.

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