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The "-omics" contributions to the understanding of mycotoxin production under diverse environmental conditions

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Abstract

Extreme environmental changes and fluctuations mainly driven by climate change will have a profound effect on natural food contaminants. Among these contaminants mycotoxins will be very important due the high adaptability of the producing fungal genera to the forecasted conditions. The availability of modern, high through-put –omic techniques, including genomics, transcriptomics, metagenomics, proteomics and metabolomics has facilitated a rapid expansion of data on the biology of mycotoxigenic fungi. This has facilitated a significant increase in our knowledge of the biological, biochemical and biophysical molecular processes regulating the production of mycotoxins, and the adaptation of these fungi to environmental stresses.

In this paper we highlight recent advances where -omics approaches have been used and where they have contributed to the knowledge on how mycotoxigenic fungi adapt to diverse interacting environmental conditions and their relationship with phenotypic toxin production. We also highlight potential future directions where these approaches can be effectively utilised for the development of minimisation strategies in the context of expected climate change scenarios and the food security agenda.

Introduction

When examining different natural food contaminants, especially under extreme environmental events, is there potential for changes in the levels of contamination of staple commodities with mycotoxins [1]. The key fungal genera responsible for production of toxic secondary metabolites in food and feedstuff are *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria.* These filamentous fungi exhibit tremendous physiological plasticity which provides them with the capacity for adaptation and

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enables them to colonize food commodities over a range of environmental conditions. Because of their ecological ruderal characteristics, they are able to produce the necessary enzymes and secondary metabolites to provide them with advantages, ecophysioloically, to successfully colonize a range of different food matrices and contaminate the edible parts with mycotoxins [2–4]. Thus, a better understanding of the tolerance and adaptation to diverse environmental conditions and the triggers and cues at a genetic and translational level would be beneficial for a better understanding of mycotoxin production would allow the development of more effective minimisation and control strategies.

The availability of modern, holistic and high through-put –omic techniques, including genomics, transcriptomics, proteomics and metabolomics has facilitated a rapid expansion of data on the biology of these mycotoxigenic fungi (Figure 1). By combining these data sets with advanced computational methods including analyses of a range of genome sequencing projects focused on fungal plant pathogens has had a positive impact on the current state of knowledge available [5]. This has facilitated a significant increase in our knowledge of the biological, biochemical and biophysical molecular processes regulating the production of mycotoxins, especially of *Aspergillus flavus* and *Fusarium* species, such as *F.graminearum* and *F.verticillioides* [6,7].There have been some studies examining the relationship between interacting conditions of water availability (water activity, a_w) and temperature on expression of gene clusters involved in type A trichothecenes, fumonisins and aflatoxins using microarray analyses which has demonstrated a correlation between growth, biosynthetic gene cluster expression and environmental conditions (see [8]). In most other cases, -omics related studies have investigated genomics or transcriptomics only under one set of environmental condition, often those optimum for growth. However, such conditions are often not related to optimum conditions for secondary metabolite production or indicative of impacts of environmental stress on gene functioning and transcriptomic changes. Recent studies on the transcriptome of *A. flavus* in relation to a_w x temperature stress has suggested significant impacts on different gene clusters, including those for aflatoxins and cyclopiazonic acid production [8–10].

This paper highlights recent advances where –omics approaches have been used, where this has contributed to the knowledge on how mycotoxigenic fungi adapt to diverse interacting environmental conditions and their relationship with phenotypic toxin production. We also highlight potential future directions where these approaches can be effectively utilised for the development of minimisation strategies in the context of expected climate change scenarios and the food security agenda. Although divided in sections, these different approaches can not be considered in isolation and there are multiple links between each other.

Genomics

The availability of accurate genomic datasets is critical to consider any further transcriptomic level analysis. Regarding mycotoxigenic fungi, since the first genome was published 13 years ago (http://www.aspergillusflavus.org/), more than 30 mycotoxigenic fungal genomes are today openly available (http://www.aspgd.org/, http://genomea.asm.org/). The recent breakthrough involved the genome of *Aspergillus westerdijkiae* [11] to decipher Ochratoxin A biosynthesis. With genomes

being available, epigenomic studies have been increasing in the last few years. Regarding *A. flavus*, methylation regulators such as *RmtA*, *DmtA* are described as epigenetic factors involved in fungal development and mycotoxin production [12,13]. Another example is the impact of chromatin remodelling on *FUM1, FUM21* and *FUM8* expression in fumonisin production by *F. verticillioides* [14].

Transcriptomics

A significant number of studies examining the impact of interacting environmental factors on secondary metabolite production were focused on the use of microarrays. Indeed, for a range of mycotoxins a microarray with different sub-arrays have been exploited to enable a better understanding the environmental related stress responses of the gene clusters involved. Examples are shown in the recent reviews by Medina et al., (2015) [7] and Magan & Medina, (2016) [8]. Complimentary studies using RT-qPCR for a better temporal understanding of the relative gene expression of key regulatory and structural genes involved in mycotoxin production for *A. flavus*, *F. verticillioides* and *P. verrucosum* have been done. This has included studies on genes for aflatoxin production (*aflR/aflS*; *aflD*) [15]; fumonisins (*FUM1*; *FUM21*) [16]; ochratoxin A (*otaPKS*) [17]. However, these studies were focused on a few genes only and did not give a more global understanding of primary and secondary metabolism which is needed to decipher the mechanisms involved. Recently, by using RNAseq it has been possible to provide new insights into the molecular ecology of *A. flavus* and how this might relate to aflatoxins and other secondary metabolite clusters.

The initial transcriptomic studies of *A. flavus* colonisation of maize revealed that the adaptation to temperature conditions (30°C, 37°C) significantly impacted 1,153 out of the 13,487 genes. At 30°C, the most highly up-expressed genes belonged to the aflatoxin biosynthesis cluster [18]. More recently, Medina et al. (2017) added a complementary condition by including the interacting effect of a_w (0.91, 0.99). This showed that 4,307 and 702 genes were affected by a decreasing the water stress at 30°C and 37°C, respectively. Among those, the reduction of a_w led to a down-expression in the aflatoxin cluster of up to 7.8 fold [19]. A recent complementary study examined the effect of forecasted climate change environmental conditions which included temperature (30 vs 37ºC), a_w stress (0.99 vs 0.90) and CO_2 levels (350 vs. 650 ppmv and 1000 ppmv). The increase in CO_2 mediated the response to temperature and a_w allowing for an increase in the aflatoxin B1 accumulation under stress conditions. For example, gene expression was down-regulated for the aflatoxin cluster at 30°C, 0.99 when increasing the CO_2 input [20].

These transcriptomic approaches using RNAseq should provide the foundation starting point for building up datasets to better understand the interaction between primary and secondary metabolism. Further deciphering of the pathways triggered under different interacting environmental factors requires a meta-analysis of transcriptome behaviour by using enhanced bio-informatics tools for the understanding of the different mycotoxin–omes, especially in relation to environmental fluxes.

Metagenomics –Mycobiome

The high-throughput sequencing techniques provide a powerful tool to assess the impact of the microenvironment in crops on the "Mycobiome". Recent publications have highlighted the usefulness of those tools [21–23]. Xing et al. (2016) examined peanuts stored for 90 days at 20-30^{o}C and relative humidities of 70, 75 and 80%. Sequencing analysis revealed an overall decrease of fungal operational taxonomic units (OTU) throughout storage and an increase in *A. flavus* relative abundance throughout the storage period, independent of the conditions tested [24].

Future research on temporal metagenomics and transcriptomic data in 3-dimensional environmental interacting stress factors (temperature, a_w and CO_2) should provide breakthrough data to decipher the fungal interaction in crops and the dominance of different fungal communities. This would be beneficial in understanding these changes and also identifying beneficial microorganisms, for example potential biocontrol candidates in specific ecological niches for mycotoxin control. These approaches will require parallel development of robust bioinformatics tools to gather data and define patterns in response to interacting environmental stress factors.

Proteomics

Fungal proteomics research, especially that related to filamentous fungi, has progressed dramatically over the past 10 years. Similar to genomics and transcriptomics, proteomics has evolved to incorporate high-throughput techniques and protocols that allow a faster analysis of large numbers of proteins [25].
In the study of plant pathogenic fungi, the first studies using a proteomic approach were carried out in 1980s with the aim of a better understanding of plant–fungal pathogen interactions through the search of resistance-related proteins [26]. However, although several research studies have used proteomics in order to study the biology and pathogenesis of fungal plant pathogens (e.g., *Blumeria graminis f.sp. hordei, Botrytis cinerea*, *Leptosphaeria maculans, Magnaporthe grisea, Ustilago maydis, Phytophthora infestans),* including mycotoxigenic fungi (*A. flavus; F. graminearum*), the effect of interacting environmental changes on adaptation and toxin production has not been widely considered [27,28].

Lu et al. (2010) compared the secretome of *Aspergillus niger* when xylose or maltose were supplemented in the medium. Their results showed that although the secretome was strongly affecting the intracellular proteome, this was not significantly changed. Conversely, differences in culture conditions (pH control versus no pH control, aeration versus no aeration and stirring versus shaking) had a profound effect on the intracellular proteome [29].

Sørensen et al. (2009) described the effect of the combined addition of lactate and starch in the medium for the growth of *A. niger*. The results showed that fumonisin B2 production was significantly increased. The proteome of *A. niger* was clearly different during growth on media containing 3% starch, 3% starch + 3% lactate or 3% lactate. The identity of 59 proteins was obtained. Many of these were enzymes involved in primary metabolism and other processes that affect the intracellular level of acetyl-CoA or NADPH [30].

The effect of different light wavelengths on differentially expressed proteins produced by *Penicillium verrucosum* grown either in the dark or under light with a wavelength of 450 nm was analysed (Stoll et al., 2014). They identified 46 significantly differential proteins (light vs. dark) comprising proteins of a broad range of isoelectric points and molecular masses. Most proteins were involved in response to stress (e.g. antioxidative proteins, heat shock proteins) and general metabolic processes (e.g. glycolysis, ATP supply). Furthermore, the results indicated that light of short wavelength led to oxidative stress in the fungal cell and under this condition the mycotoxin biosynthesis revealed a shift from ochratoxin A to citrinin [31].
The effect of a_w on the proteomic profile of *A. flavus* was investigated by Zhang et al. (2015). A total of 3566 proteins were identified, of which 837 were differentially expressed in response to variations in a_w. Among these, 403 were over-expressed at 0.99 a_w, whereas 434 were over-expressed at 0.93 a_w. Two proteins (AFL2G_04330 and KapK) were identified as having a critical role in the induction of aflatoxin biosynthesis [32]. Bai et al. (2015) investigated the changes in transcript and relative protein levels in response to temperature, complementary transcriptomic and proteomic analyses were used to identify changes in *A. flavus* grown at 28°C and 37°C. A total of 3,886 proteins were identified, and 2,832 were reliably quantified. Interestingly the authors pointed out that there was a low correlation between the proteome and transcriptome data, suggesting that post-transcriptional gene regulation may have a very important role on different biological pathways and secondary metabolite gene clusters [33].

Metabolomics

The fungal metabolome represents the collection of all metabolites in a biological organism (including metabolic intermediates, hormones and other signalling molecules, and secondary metabolites), which are the end products of its gene expression [34]. Metabolomics represents a newer complementary technique to functional genomics as it provides integrative information, i.e., a large number of genes may be involved in the production of one metabolite [35]. This is the reason why the metabolomics approach (both targeted and untargeted) has gained popularity in the field of filamentous fungi, and today these techniques are already being used with different purposes.

Focusing on fungi, the metabolite profile has been used as a common tool in studies of fungal taxonomy and physiology [35] as well as for the early detection of food pathogens and food spoilage microorganisms by means of the volatile production patterns [36]. In addition, metabolomics can be an excellent tool for determining and also predicting the function of unknown genes by comparison with the metabolic perturbations caused by a genetic manipulation such as gene deletion or insertion [37,38]. However, the metabolome is also dynamic and is constantly changing in relation with other microorganisms, the host and environmental fluctuations. Luo et al., 2017 [39] recently identified differential metabolites in the interaction zone between two interacting fungi, when compared to those metabolites produced individually in the absence of such interactions. This has provided new information on the molecular ecology of fungal communities on plants. For mycotoxigenic fungi most of the metabolomics studies have been focused on understanding the roles of plant resistance mechanism and pathogen cross-talk at a molecular level [40–43]. It has been demonstrated that the influence of the environment on the synthesis of

secondary metabolites (SM) by fungi is an important factor to consider [44]. However, few studies have examined targeted SM production patterns in relation to interacting environmental conditions [45–47]. Very recently, Garcia-Cela et al., [45], studied the effect of the environment (10-25°C vs 0.90-0.93 a_w) on targeted metabolite production in natural wheat and wheat inoculated with *F. graminearum* after 15 days storage (Fig. 2). Out of a total of 121 metabolites it was noted that approx. 30–50 metabolites were present at 15–25°C regardless of a_w conditions. At 10°C, slower mould colonization occurred resulting in significantly less SMs being produced in both naturally contaminated stored wheat and that inoculated with *F. graminearum*. However, the relative ratio of the compounds produced in significant concentrations in these treatments, were significantly less. Thus, about 20–25 compounds were produced at >50 ng/g and <10 at >500 ng/g. This suggests that, surprisingly, there are a wide range of SM compounds which are produced over a range of interacting environmental stress conditions at similar concentrations. More studies are required on other staple food commodities.
Similar experiments were carried out on natural maize and that inoculated with *A. flavus* under different environment conditions (15-35°C vs 0.80-0.99 a_w) after 11 days (Fig. 3). Approx. 20-95 metabolites were present out of total of 168 metabolites analysed. Interestingly, the number of SMs synthetized decreased at 0.95 a_w when comparing 25 and 35°C showing a correlation with the environmental conditions. As was observed on wheat, the increase in inoculum of the *A. flavus* in maize prior to storage promoted the number of SMs produced (Unpublished data).

Only few authors have developed experiments on maize plants looking at interaction between different environmental conditions. Among them, Vaughan et al. 2016 [48] showed how that maize simultaneously exposed to elevated CO_2 and drought are even more susceptible to *F. verticillioides* proliferation and also prone to higher levels of fumonisin contamination.

Future trends

Although the recent improvemements in bioinformatics tools in the last decade the main challenge of systems biology remains the integration of “omics” information to give a more complete picture of living organisms. The contribution of -omics techniques to our better understanding of a range of fungal pathogens including mycotoxigenic species in the last decade is clear. However, under the pressures of changing climate and the food security agenda mycotoxigenic fungi and their mycotoxins may prove to represent increased risks to human and animal health based on the limited data available at the present time. By combining the utilization and integration of holistic and high throughput molecular approaches and appropriate bioinformatics and modelling contributions, significant improvements in the understanding of these fungi will be possible and more appropriate minimisation/control strategies be developed.

Of course, mycotoxigenic fungi do not exist in the field or post-harvest in isolation, but as part of changing ecological communities which are influenced by environmental pressures. These ecological relationships and interactions together with environmental fluxes determines the type and range of secondary metabolites produced. Thus, in the coming years, microbiome approaches studying the microbial communities of food commodities using genomics and transcriptomic approaches will

help us to better understand mycotoxin production regulation and help to identify the best strategies for the development of better control systems.

The availability of metabolomics techniques to study the production of the full range of secondary metabolites, including toxic ones in food commodities means that a better understanding is necessary of the relationships between these metabolites/toxins and whether their presence individually or in combination represents additive or synergistic impacts on staple foods. Also, we now have better knowledge of the interaction between mycotoxins and their binding by the host plant to for bound or modified mycotoxins. Although there is some data available about the changes in free mycotoxin ratios (Figure 4) we need more data and a better understanding of the impact that changes in environmental stress affect the ratio of free- vs bound mycotoxins, e.g. DON glucosides; FUM related ones etc.). In this area, mycotoxyc-omic studies involving what perhaps could be called the “mycotoxicome” will produce new valuable information in the coming years.

A better understanding of the relationship between environmental stresses and the production of secondary metabolites at a molecular and transcriptome level may be critical in understand the impact of crop protection approaches in sustainable food production systems. In addition, we need more knowledge of the potential for a switching of ratios of mycotoxins produced by the same toxigenic species (e.g. *A. flavus*: aflatoxins vs cyclopiazonic acid) and whether other secondary metabolites may become more important under environmental stresses such as climate change factors. Thus new-omics based approaches are essential for a better understanding of the conditions under which mycotoxin contamination of staple foods can be minimised and identify strategies which will be beneficial in terms of food safety and food security.

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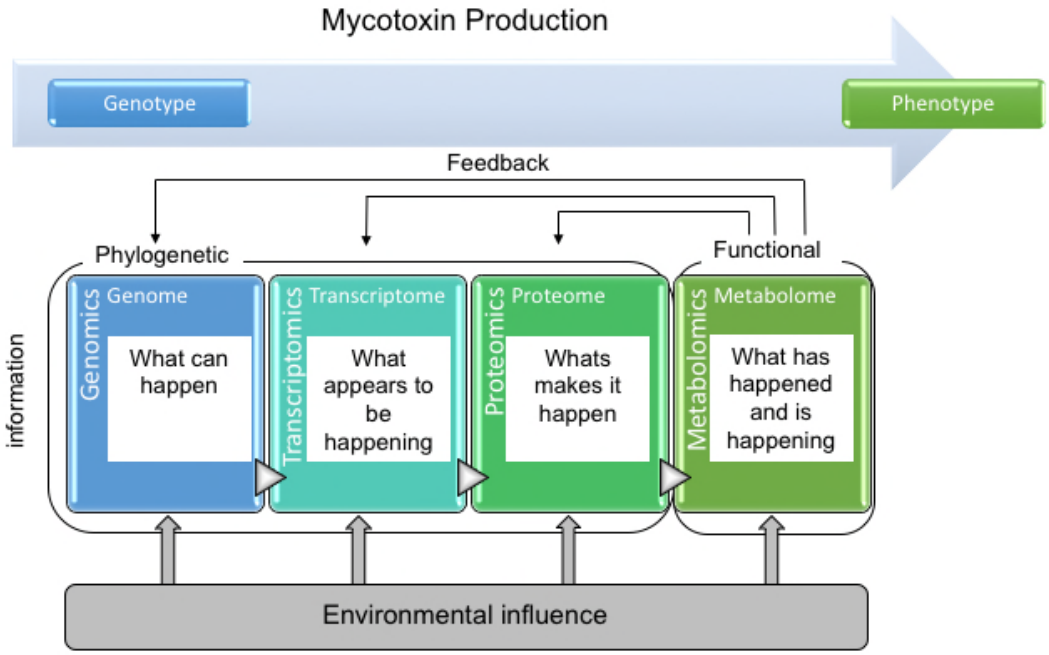


Figure 1

Figure 1. Relationship between mycotoxin production and the relationship between the diferent -OMIC techniques.

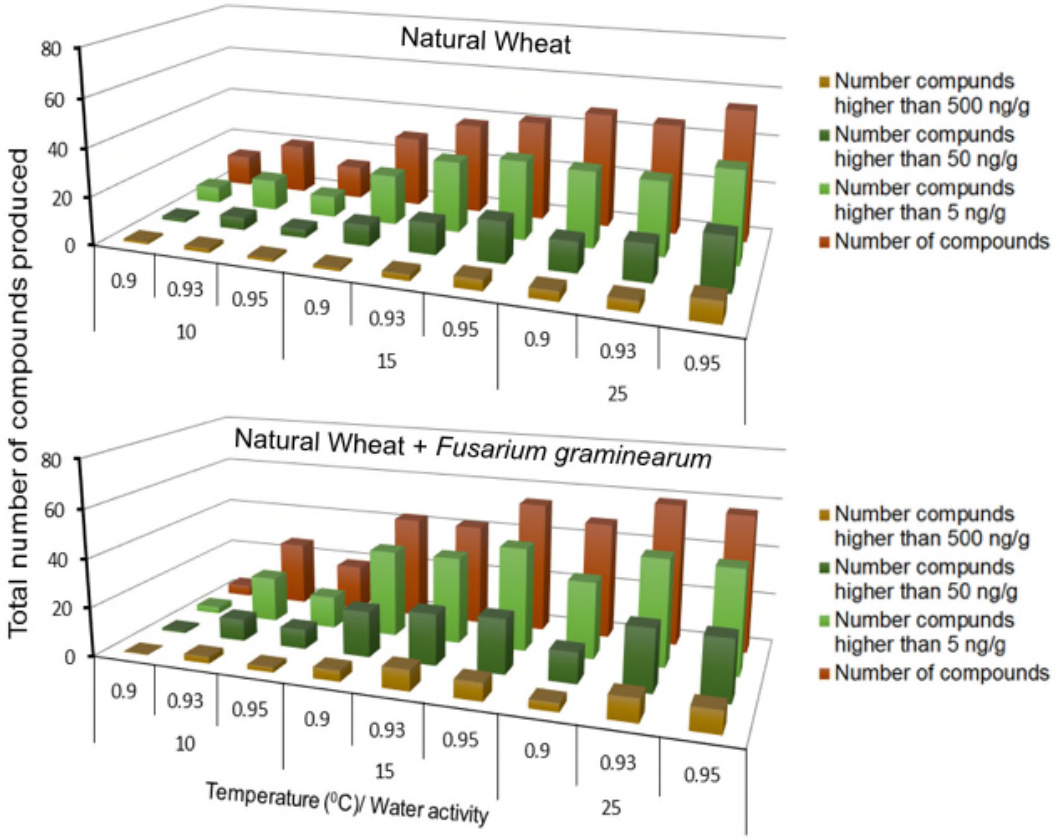


Figure 2. Comparison of the number and amount of metabolites produced between natural wheat and natural wheat contaminated with *Fusarium graminearum* and incubated under different environmental conditions

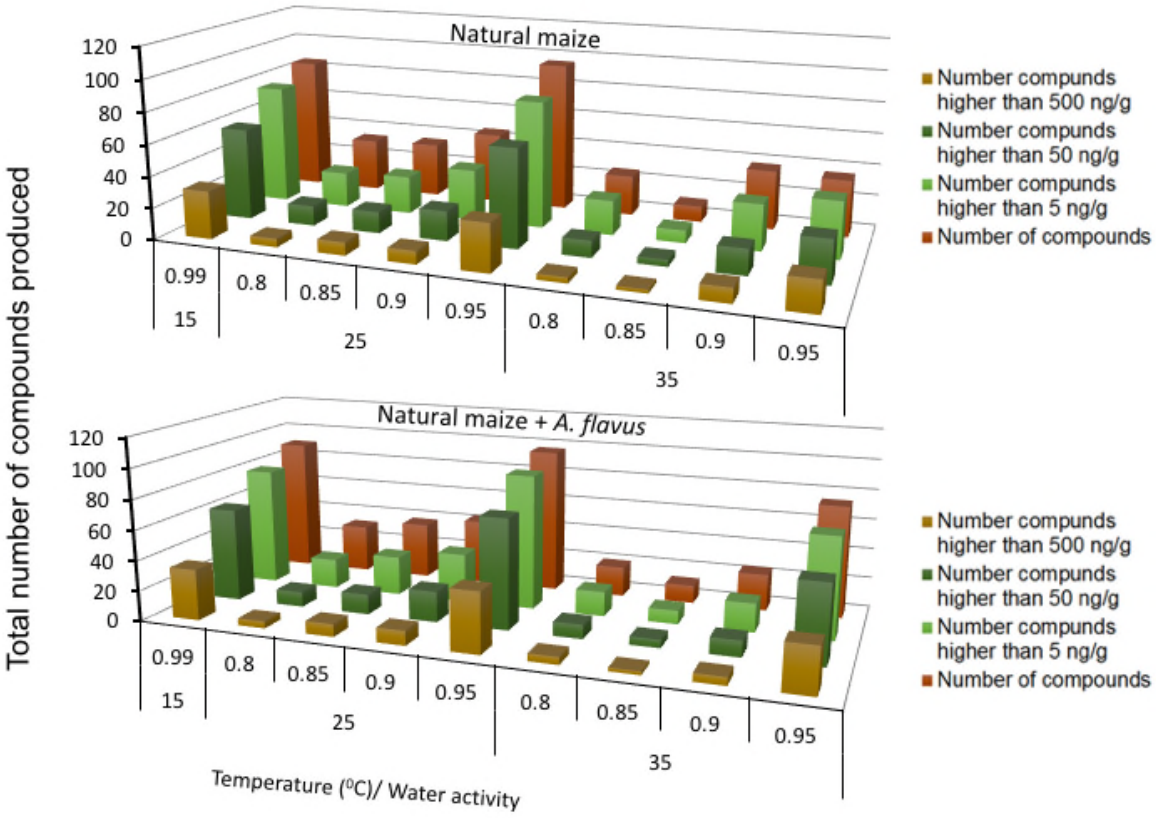


Figure 3. Comparison of the number and amount of metabolites produced between natural maize and natural mize contaminated with *Aspergillus flavus* and incubated under different environmental conditions

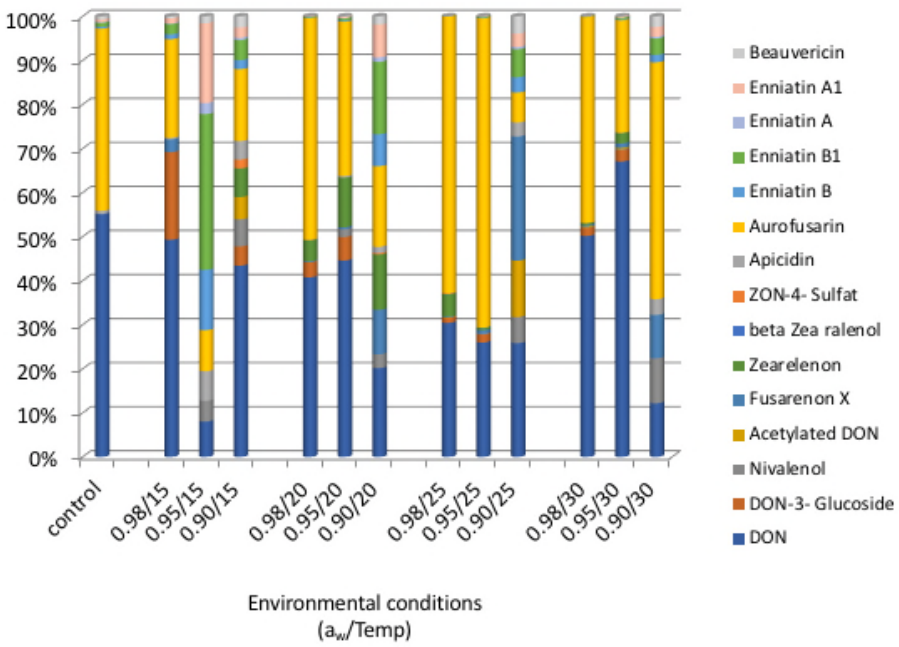


Figure 4 Relative concentrations of different mycotoxins found in natural wheat grain inoculated with *F.graminearum* and stored under different conditions for 25 days



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The "-omics" contributions to the understanding of mycotoxin production under diverse environmental conditions

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