

The current state of mycotoxin biomarker development in humans and animals and the potential for application to plant systems

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Abstract

Filamentous fungi that contaminate livestock feeds and human food supply often produce toxigenic secondary metabolites known as mycotoxins. Among the hundreds of known mycotoxins, aflatoxins, deoxynivalenol, fumonisins, ochratoxin A and zearalenone are considered the most commercially important. Intense research on these mycotoxins, especially aflatoxin, has resulted in the development of 'biomarkers' used to link exposure to disease risk. In the case of aflatoxin this effort has led to the discovery of both exposure and mechanism-based biomarkers, which have proven essential for understanding aflatoxin's potential for causing disease in humans, including subtle effects on growth and immune response. Fumonisin biomarkers have also been used extensively in farm and laboratory animals to study the fumonisin-induced disruption of cellular and systemic physiology which leads to disease. This review summarises the status of mycotoxin biomarker development in humans and animals for the commercially important mycotoxins. Since the fungi responsible for the production of these mycotoxins are often endophytes that infect and colonise living plant tissues, accumulation of mycotoxins in the plant tissues may at times be associated with development of plant disease symptoms. The presence of mycotoxins, even in the absence of disease symptoms, may still have subtle biological effects on the physiology of plants. This review examines the question of whether or not the knowledge gained from mechanistic studies and development of biomarkers in animal and human systems is transferable to the study of mycotoxin effects on plant systems. Thus far, fumonisin has proven amenable to development of mechanism-based biomarkers to study maize seedling disease caused by the fumonisin producer, *Fusarium verticillioides*. Expanding our knowledge of mechanisms of toxicity and the overt and subtle effects on animal, human, and plant systems through the identification and validation of biomarkers will further our ability to monitor and limit the damage and economic impact of mycotoxins.

Keywords: aflatoxin, deoxynivalenol, trichothecenes, fumonisin, ochratoxin, zearalenone

1. Introduction

Mycotoxins are low molecular weight secondary metabolites produced by filamentous fungi that frequently occur on foods and are potentially toxic to humans and animals (CAST, 2003). While several mycotoxins are known or suspected to be involved in human disease, many more are

known or suspected to contribute to disease in farm and laboratory animals. Because of their widespread occurrence on foods and their potential for toxicity, several mycotoxins pose a threat to food safety and security (CAST, 2003). The mycotoxins that are both known causes of disease and occur in significant amounts in commercially important commodities consumed by humans are limited to aflatoxins,

deoxynivalenol (DON), fumonisins (primarily fumonisin B₁ – FB₁), ochratoxin A (OTA) and zearalenone (ZEA). These commercially important mycotoxins will be the focus of this review. While there are other mycotoxins that are known to cause or contribute to animal disease, such as ergot alkaloids, macrocyclic trichothecenes and T-2 toxin, they will not be covered in this review.

Despite their widespread presence in grains and other commodities, little is known about the selective advantages conferred to the fungi that produce mycotoxins *in planta*. Nonetheless, the ubiquitous production of these energetically costly metabolites by naturally occurring fungal strains, within species and across genera, suggests they confer an advantage over non-producing strains. Some have suggested mycotoxins impart an advantage by inhibiting the growth of competing microorganisms for the same niche environment (Fox and Howlett, 2008) while others have suggested that mycotoxins may reduce herbivory by insects (Tanaka *et al.*, 2005) and animals (Clay, 2009). Yet others have suggested that infection and colonisation of the host plant by mycotoxin-producing fungi is enhanced due to phytotoxic effects or an altered physiological state of the plant to better suit the fungus (reviewed in Reverberi *et al.*, 2010). In animal systems, mechanism-based approaches using biomarkers have contributed greatly to the understanding of mycotoxins and their mechanisms of action as potential contributing factors to human and animal disease. Similar approaches may prove useful for studying plant pathogenicity and the broader physiological effects of mycotoxins *in planta*. Compared to the knowledge base in animal systems, the biological effects of most mycotoxins in plants are poorly understood and require more in-depth investigation of mycotoxin-based phytotoxicity and related mechanisms of action.

For this review, the mechanism of action of mycotoxins is defined as the initial underlying biochemical or molecular interaction resulting in the principle downstream effects which may include disruption of metabolic and signalling pathways, altered physiological responses, altered cell proliferation and differentiation, and cell death (reviewed in Riley *et al.*, 2011). These initial interactions could be the direct result of the mycotoxin or a metabolite of the mycotoxin. For any mechanism of action it is critical to identify the proximate cause, which is that event that sets everything downstream into motion. An example of an easy to understand mechanism of action is that of a classical agonist binding to an extracellular receptor, which sets into motion a series of downstream effects ending ultimately in some measurable biological response. These responses can sometimes be monitored through the utilisation of detection tools known as 'biomarkers'.

The Biomarkers Definition Working Group (Atkinson *et al.*, 2001) defined biomarkers as characteristics that objectively measure and indicate the biological state of a pathogenic process or pharmacologic response. In this review we are defining biomarkers as measurable biochemical or molecular indicators of either exposure or biological response to a mycotoxin that can be specifically linked to the proximate cause. Mechanism-based biomarkers often include changes in the level of specific proteins, cellular metabolites, or gene expression resulting from specific alterations in metabolic or signalling pathways, stress responses, cell proliferation, or cell death. In contrast to mechanism-based biomarkers, exposure-based biomarkers are most often the mycotoxin itself or the metabolised mycotoxin by-product (e.g. glutathione or glucuronide conjugates). These compounds can be detected in easily-accessed biological fluids or tissues. Perhaps the best example is in the case of aflatoxin where the exposure biomarkers are also potential indicators of the proximate cause. Specifically, the covalent binding of metabolically activated reactive metabolites to essential macromolecules, most importantly DNA and proteins (Groopman *et al.*, 2005).

Within the context of biomarker utility, many observed down-stream effects may be linked to the proximate cause of toxicity, but their lack of specificity precludes them from being useful for either exposure or effect. An example of this lack of sufficient specificity includes oxidative stress response in both animals and plants. Such broad-based stress responses can be triggered by a number of abiotic and biotic factors.

In this review we briefly summarise the status of mycotoxin biomarker development in humans and animal research for commercially important mycotoxins. We will also address the question of whether or not the accumulated knowledge of biomarkers in animal and human systems is transferable to studies of mycotoxin effects on plant systems using our work examining the cellular and pathological effects of fumonisin on maize seedlings disease as an example. We have characterised both mechanism-based and exposure-based biomarkers associated with seedling disease development, which provides a better understanding of the underlying mechanism of action and provides clues for other potential physiological effects.

2. Assessment of the state of biomarker development in animals and humans

Table 1 summarises the likely mechanism of action for each of the mycotoxins considered in this review and also provides our assessment of the current status of biomarker development for each mycotoxin in animals and humans. The rating assigned to status of biomarker development is based on a generalised scheme (Figure 1) derived from the validation scheme proposed by Groopman *et al.* (2008)

Table 1. Current status of biomarker development in animals and humans. For each mycotoxin the likely mechanism of action is given followed by currently available exposure and mechanism-based biomarkers¹.

Mycotoxin	Likely mechanism of action	Biomarker	Location	Type	Species	Status
AFB ₁	Metabolism of AFB ₁ by cytochrome P450 to intermediates, namely AFB ₁ -8,9-epoxide, which bind to DNA and proteins forming adducts.	AFB ₁ -DNA adducts	urine, tissue	exposure & mechanistic	Farm Lab Human	+++ +++ +++
		AFB ₁ -albumen adducts	blood	exposure	Farm Lab Human	+++ +++ +++
		DNA mutation in p53 tumor suppressor gene	blood, tissue	mechanistic	Farm Lab Human	- + +++
AFM ₁		AFM ₁	milk, urine	exposure	Farm Lab Human	+++ +++ +
		DON and DON-glucuronide	urine, tissue, faeces	exposure	Farm Lab Human	++ +++ +++
		proinflammatory cytokines	blood, tissue	mechanistic	Farm Lab Human	+++ +++ ++
DON	Induces proinflammatory cytokines via MAPK pathways, induces expression of suppressor of cytokine signalling (SOCS), impairs expression of hepatic insulin-like growth factor acid labile subunit (IGFALS) and insulin-like growth factor 1 (IGF1). Ribotoxic stress response caused by DON-induced activation of p38, JNK and ERK via RNA-activated protein kinase (PKR)- and hematopoietic cell kinase (Hck)-dependent mechanisms.	up-regulation of hepatic SOCS and mRNA/protein	tissue	mechanistic	Farm Lab Human	+ +++ +
		decrease of IGFALS and IGF1	blood	mechanistic	Farm Lab Human	+ +++ +
		elevation of sphingoid base and sphingoid base 1-phosphate and depletion of more complex sphingolipids	blood, tissue	mechanistic	Farm Lab Human	+++ +++ +
FB ₁	Disruption of sphingolipid metabolism through inhibition of ceramide synthase.	levels of FB ₁	urine, faeces, hair	exposure	Farm Lab Human	+++ +++ ++

Table 1. Continued.

Mycotoxin	Likely mechanism of action	Biomarker	Location	Type	Species	Status
OTA	OTA disrupts phenylalanine related processes (i.e., phenylalanine metabolism, protein synthesis, etc.). Disrupts signalling pathways and processes regulating cell growth and survival. OTA-DNA adducts.	OTA or OTA metabolites/conjugates OTA-DNA adducts	blood, urine, tissue tissue	exposure mechanistic	Farm Lab Human	+++ +++ ++
		metabonomic assessment of metabolite changes	blood, urine, tissue	mechanistic	Farm Lab Human	+++ ++ +
ZEA	Effects are based on similarities to oestrogen. Conversion to α -ZOL exacerbates oestrogenic effects of ZEA. Both ZEA and α -ZOL disrupt steroid metabolism.	ZEA, ZOL, and ZAL	urine, faeces	exposure	Farm Lab Human	+++ +++ +
		glucuronic acid-conjugates	urine, faeces	exposure	Farm Lab Human	+++ +++ +
		endocrine disruption	tissue	mechanistic	Farm Lab Human	+++ ++ +

¹ AFB₁ = aflatoxin B₁, AFM₁ = aflatoxin M₁, DON = deoxynivalenol, FB₁ = fumonisin B₁, OTA = ochratoxin A, ZEA = zearalenone, ZOL = zearalenol, ZAL = zearalanol. The column labelled 'Location' indicates the biological material in which the biomarker is detected. Tissue is normally liver or kidney, but other organs are also possible. Blood includes serum, plasma and/or whole blood. For simplicity, the column labelled 'Species' includes 'Farm' (animals raised for commercial purposes), 'Lab' (studies in laboratory animals), and 'Human' (development of human biomarkers). Current status is given for 'Species' and indicated as follows: - = not demonstrated, + = theoretically possible but not convincingly demonstrated, ++ = *in vitro* experimentation only or *in vivo* studies without dose-response relationships for the biomarkers, +++ = correlation demonstrated between exposure and/or disease incidence and the biomarker.

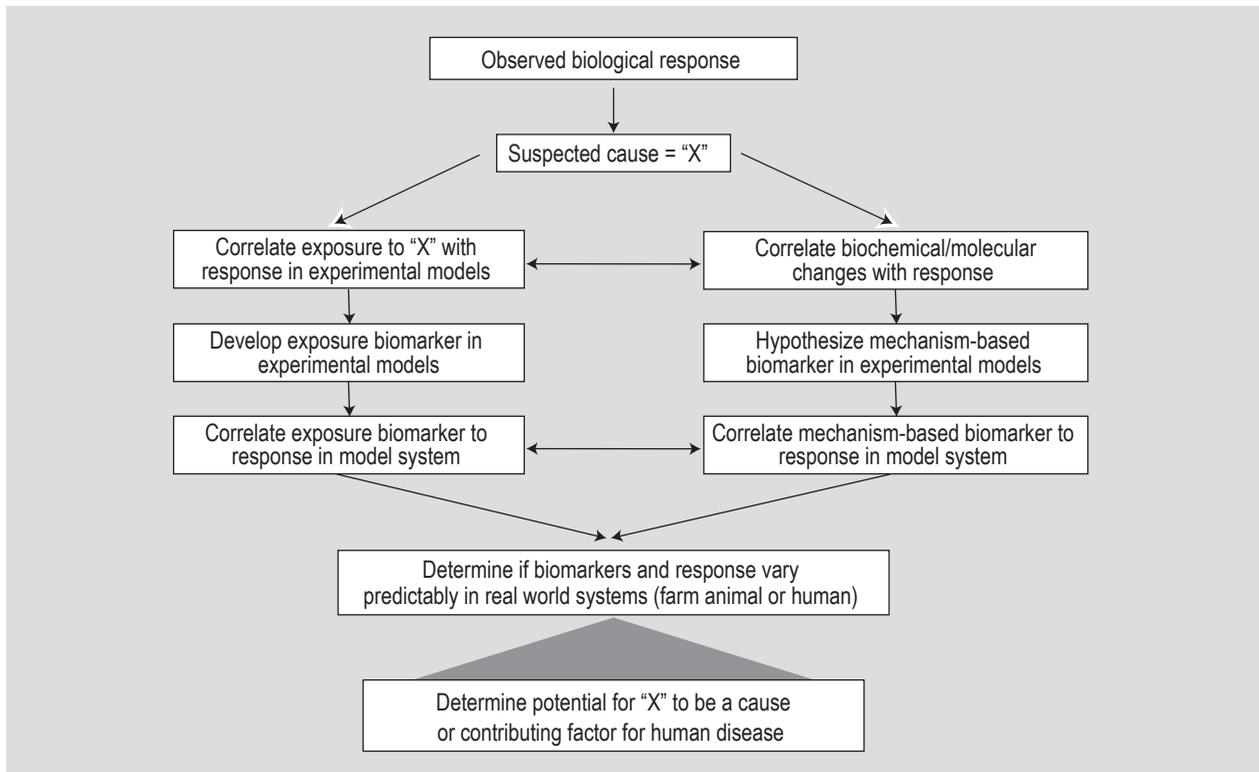


Figure 1. A generalised scheme for ranking the current status of biomarker discovery and validation for aflatoxins, fumonisins, deoxynivalenol, ochratoxin A, and zearalenone. This scheme is based on that of Groopman *et al.* (2008) but focuses in a generic way on discovery, development and validation of both exposure biomarkers and mechanism-based biomarkers. Each box represents a step in the discovery and experimental validation of the biomarkers with the ultimate goal being the demonstration of the potential for the mycotoxin to be a cause or contributing factor to animal or human disease.

for molecular biomarker research and specifically aflatoxin and hepatitis B virus as risk factors for cancer. Currently, aflatoxin B₁ (AFB₁) is the only mycotoxin for which there are validated biomarkers of both exposure and disease risk in humans and animals (Groopman *et al.*, 2005; Wild and Gong, 2010). The AFB₁-N7 guanine DNA adducts and AFB₁-lysine adducts have been used successfully to study the role of AFB₁ as a cause or contributing factor for toxicity and diseases in animals and humans (Wild and Gong, 2010). The G-T transversion mutation in codon 249 in the *p53* tumour suppressor gene has also been used successfully in population based studies in humans (Wild and Gong, 2010). The G-T mutation at the codon homologous to human codon 249 has not been demonstrated in AFB₁ exposed farm animals (Smela *et al.*, 2001). Nonetheless, studies have shown low levels of *p53* mutations in AFB₁ treated ground squirrels (Rivkina *et al.*, 1994) and rats (Lee *et al.*, 1998), and studies in transgenic mice suggest *p53* mutations enhance AFB₁ liver tumorigenicity (Tong *et al.*, 2006). Together, these studies have qualitatively and quantitatively verified exposure, linked exposure to mechanism of action, identified high risk populations, defined dose-response relationships and disease thresholds, tested the efficacy of intervention and decontamination methods, and allowed for the prediction of potential interactions and other factors

contributing to susceptibility. The process of biomarker development with aflatoxin (Kensler *et al.*, 2011) is the 'gold standard' by which the current state of biomarker development for other mycotoxins should be judged.

Exposure and mechanism-based biomarkers are available for use in animal studies with fumonisin, (reviewed in Shephard *et al.*, 2007; Riley *et al.*, 2011; Routledge and Gong, 2011) and while urinary fumonisin has been used successfully in human studies as an exposure biomarker, fumonisin disruption of sphingolipid metabolism has not been demonstrated in humans. Likewise, the blood or urinary levels of DON as exposure biomarkers work well in laboratory animals and humans (reviewed in Pestka, 2010a,b; Riley *et al.*, 2011; Routledge and Gong, 2011; Turner, 2010). Additionally, some non-specific mechanism-based biomarkers have been used in laboratory animal studies, and to a lesser extent in farm animals (reviewed in Pestka, 2010a,b; Riley *et al.*, 2011), but there have been no studies linking the DON exposure biomarkers with the mechanism-based biomarkers in humans. For OTA, exposure biomarkers have been used widely in human and animal studies (reviewed in Riley *et al.*, 2011; Routledge and Gong, 2011; Scott, 2007), but because the mechanism of action of OTA is unclear and controversial (Mally and

Dekant, 2009; Mantle *et al.*, 2010; Marin-Kaun *et al.*, 2008; O'Brien and Dietrich, 2005; Pfohl-Leszkowicz and Manderville, 2007) progress has been slow for development of validated mechanism-based biomarkers for use in either animal models or human studies. The levels of ZEA and its metabolites in excreta have been used successfully as exposure biomarkers in farm and laboratory animal studies (reviewed in Fink-Gremmels and Malekinejad, 2007; Riley *et al.*, 2011) but not in human studies. The easily observable and pathognomonic overt oestrogenic effects of ZEA have been linked to ZEA exposure in animal studies, but this has not been done convincingly in humans.

3. Application of biomarker-based approaches to plant studies

We discussed above how the development of biomarkers has aided the understanding of mycotoxin-associated disease development and identification of underlying proximate causes and mechanisms of action in animals and humans. In this section we will explore the potential translation and application of animal-associated mycotoxin effects and biomarker development to plant systems in order to better characterise the phytopathological interactions between mycotoxigenic fungi and their hosts. In essence,

can we apply what we know regarding the animal toxicity of these mycotoxins and their mechanisms of action to the host plants in which the mycotoxins are produced and accumulated? Are these secondary metabolites toxic to the host plants? Is there measurable biochemical and molecular disruption or alteration of cellular processes within the plants? Does such disruption or alteration have any negative impact on the plants, whether overt (disease development) or subtle (altered physiology and development)?

In practice, development of plant-based biomarkers would follow similar procedures as outlined for biomarker development in animal and human systems (Figure 2). In natural field environments, exposure of a plant to a mycotoxin occurs through either infection of the plant by mycotoxigenic fungi or through environmental exposures such as the presence of the mycotoxin in the soil. Uptake of water and water-soluble mycotoxins from the soil by the roots may result in accumulation in the roots and perhaps transpiration-mediated shootward mobility of the mycotoxins. Whether through this type of environmental exposure and uptake, or from the *in planta* production and accumulation of the mycotoxin by infecting fungi, a mycotoxin could function as an exposure-based biomarker if the compound is easily detected in plant tissues and

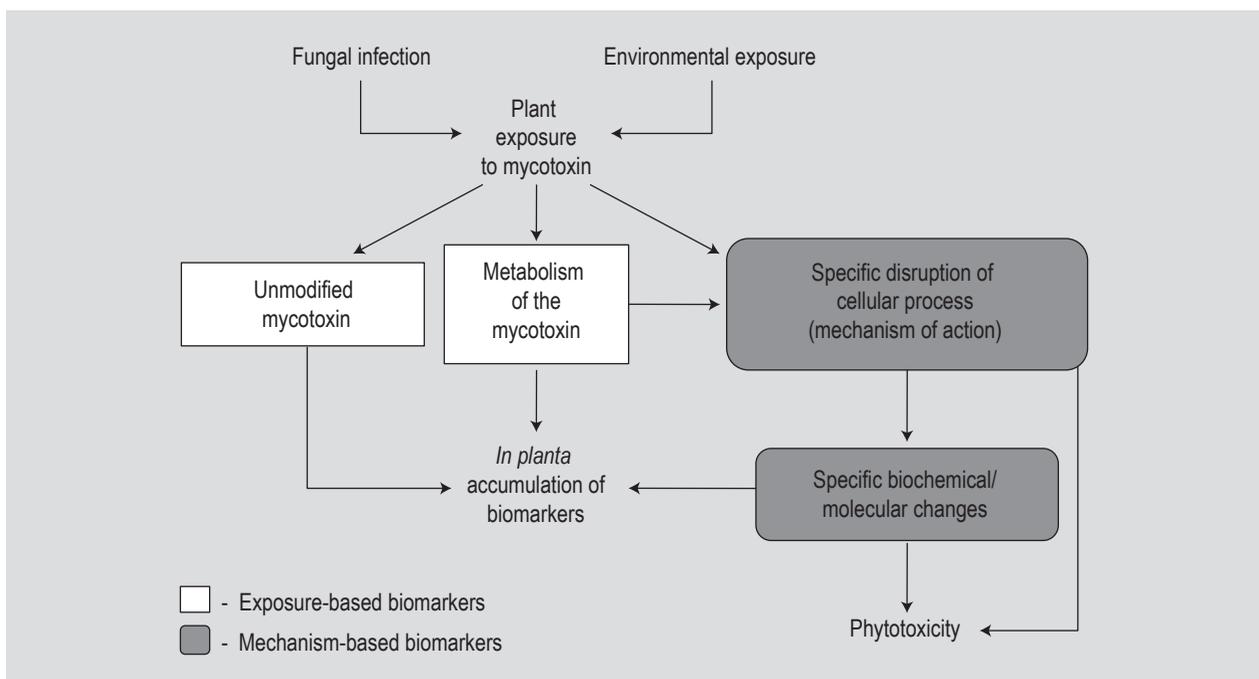


Figure 2. Flow chart for development of *in planta* biomarkers resulting from exposure of plants to mycotoxins. Mycotoxins gain entry to the plant from either fungal infection or environmental exposure (e.g. uptake from the soil). The initial mechanism of action is the first specific interaction that is required for disruption of metabolic and cellular processes. The resulting changes in levels of metabolites and regulatory molecules directly linked to the mechanism of action are the best candidates for mechanism-based biomarkers (shaded boxes). The parent mycotoxin and/or its metabolites are the best candidates for exposure-based biomarkers (white boxes) and should be easily detected in plant tissues. The *in planta* accumulation of the parent mycotoxin, its metabolites, and the resulting evidence of disruption of metabolic and regulatory processes should correlate in a dose-dependent manner in order to be validated as biomarkers.

correlated to exposure (Figure 2). The same may be true for metabolites of the mycotoxin formed by the plants, such as glucoside conjugates.

Given that the proximate causes and mechanisms of action of mycotoxins often involve disruption of universal cellular processes (e.g. protein synthesis or sphingolipid metabolism), individual mycotoxins may have phytotoxic properties in addition to their animal toxicity. Disruption of plant cellular processes and the potential accumulation of associated cellular metabolites could be assessed and quantified for use as mechanism-based biomarkers correlated to specific mechanisms of action and potential adverse effects on plant health and development (Figure 2). Our knowledge of mycotoxin effects on animal systems is therefore a valid and useful starting point for evaluating mechanisms of action in plants. An understanding of the mechanistic basis of phytotoxicity exhibited by fungal metabolites allows for in-depth studies into interactions between the fungi and host plants and increases the potential development of refined strategies to monitor or manage fungal virulence and plant disease.

Mycotoxins and phytotoxins have been defined historically as functionally separate fungal secondary metabolites, and in some cases the distinction is justified, for example the production of the host-selective toxin victorin by *Cochliobolus victoriae* (*Helminthosporium victoriae*) that causes Victoria blight of oats (Wolpert *et al.*, 2002). In other cases the mycotoxin-phytotoxin distinction begins to blur, such as the phytotoxin produced by *Alternaria alternata* f. sp. *lycopersici* (AAL) that causes *Alternaria* stem canker of tomato (Brandwagt *et al.*, 2000; Gilchrist *et al.*, 1992; Wang *et al.*, 1996). The AAL phytotoxin is a sphinganine analogue very similar in structure to the fumonisin mycotoxins. In fact, FB₁ is able to cause disease symptoms on susceptible tomato that are similar to symptoms caused by AAL phytotoxin (Abbas *et al.*, 1994; Brandwagt *et al.*, 2000; Gilchrist *et al.*, 1992). This review does not further address traditional phytotoxins such as victorin, but we suggest that the approach of biomarker development could be applied to these phytopathogenic systems. Use of exposure-based biomarkers may be the most amenable approach since mechanistic biomarkers may be more difficult to validate given the nonspecific plant defence responses that occur in response to phytotoxins, such as formation of reactive oxygen species, ethylene signalling, and *de novo* synthesis of phytoalexins (Wolpert *et al.*, 2002).

Currently, the only intensely studied mechanism-based biomarkers in plant systems are those used in studying fumonisin phytotoxicity. We outline below the *in planta* detection of sphingoid bases and sphingoid base 1-phosphates as mechanism-based biomarkers indicative of sphingolipid metabolism disruption in maize tissues and their correlation to foliar disease development in

maize seedlings. While mechanism-based biomarkers may be applicable for studying phytotoxic effects of other important mycotoxins, such data are currently lacking for the most part. In contrast, numerous studies have quantified the *in planta* accumulation of mycotoxins or mycotoxin metabolites (i.e. glucoside conjugates), so we begin by outlining the utility of such assessments as exposure-based biomarkers.

4. Exposure-based biomarkers for *in planta* mycotoxin accumulation

Many studies have quantified the production and accumulation of mycotoxins in different tissues of plants. This is to be expected since analytical assessment of mycotoxin concentrations in agricultural commodities is the basis for monitoring potential animal or human exposure from consumption of those products. For this review we are focusing on studies that have addressed the effects of mycotoxins on plant disease, growth, and development and suggest the utility of exposure-based biomarkers for such studies as a prelude to future development of mechanism-based biomarkers.

AFB₁ production by *Aspergillus flavus* and *Aspergillus parasiticus* is a major concern due to the toxicity of the mycotoxin and the wide distribution of these fungi on various host plants. Despite near ubiquitous production of AFB₁, there have been few studies into the role of the mycotoxin in the biology of *Aspergillus* species (McLean, 1994b). AFB₁ was shown to have phytotoxic effects on maize embryos and tobacco (*Nicotiana tabacum*) seedlings. The plants exhibited a dose-dependent response to AFB₁ exposure, showing increasing inhibition of root elongation with increasing AFB₁ concentrations (McLean, 1994a; McLean *et al.*, 1995). Such root inhibition has been noted in diseased peanuts infected with *A. flavus* and was deemed 'aflaroot' (Chohan and Gupta, 1968). Since the mode of action for aflatoxin phytotoxicity is unknown, mechanism-based biomarkers are not currently attainable, yet detection of AFB₁ in different tissues of the plant can serve as an exposure-based biomarker for detailed examination of phytotoxicity. Detection of *in planta* derived metabolites may also be possible. For example, metabolism of AFB₁ to aflatoxicol was documented in parsley (Howes *et al.*, 1991). Aflatoxicol is mutagenic and carcinogenic along with AFB₁ and has been detected as a metabolite of AFB₁ exposure in animals and humans (Kussak *et al.*, 1998; Mariën *et al.*, 1987; Wong and Hsieh 1978).

DON is one of the few mycotoxins definitely linked to fungal virulence and plant disease development. *Fusarium graminearum* and *Fusarium culmorum* produce DON and other trichothecenes, including nivalenol and the acetyled DON derivatives, 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol (Kimura *et al.*, 2007). Evaluating

the role of DON production on virulence was enhanced by the use of molecular genetics and creation of mutant strains. Specifically, deletion of *Tri5*, the gene encoding trichodiene synthase, the first committed enzyme in the DON biosynthetic pathway (Kimura *et al.*, 2007), resulted in loss of DON production by *F. graminearum* as well as reduced development of wheat head blight and maize ear rot (Bai *et al.*, 2002; Desjardins *et al.*, 1996; Harris *et al.*, 1999; Proctor *et al.*, 1995). They also were less effective at colonising wheat or barley heads (Boddu *et al.*, 2007; Jansen *et al.*, 2005). The same principles have been demonstrated in other DON-producing *Fusarium* species (Desjardins and Hohn, 1997). Additionally, DON was shown to inhibit root elongation in wheat (Masuda *et al.*, 2007). DON elicited programmed cell death by inducing hydrogen peroxide production and was also linked to increased expression of defence related genes (Desmond *et al.*, 2008). The acute phytotoxic effects of DON and other trichothecenes have been studied in-depth on the non-host *Arabidopsis thaliana* with similar symptoms noted (Masuda *et al.*, 2007). Tolerance to DON exposure has been illuminated by studying the *Fhb1* gene associated with resistance to *Fusarium* head blight in wheat (Liu *et al.*, 2006) and the *DOG1* homologue in *Arabidopsis* (Poppenberger *et al.*, 2003). Over-expression of *DOG1*, a UDP-glucosyltransferase, in *Arabidopsis* resulted in increased tolerance to DON due to detoxification by transformation to deoxynivalenol-3-*O*-glucoside (Poppenberger *et al.*, 2003). Further studies in barley have demonstrated conversion to deoxynivalenol-3-*O*-glucoside even in susceptible barley varieties and a non-enzymatic formation of DON-glutathione adducts that may be suppressing the negative effects of DON (Gardiner *et al.*, 2010). These metabolites as well as DON itself represent the available exposure-based biomarkers.

There has been little work thus far on determining the role, if any, of OTA in the pathogenicity of *Aspergillus ochraceus*, *Aspergillus niger*, and *Aspergillus carbonarius*. A recent study with *A. thaliana* has shown that detached leaves exposed to OTA produce a hypersensitive-like response, suggesting a defence response similar to the effects of DON (Peng *et al.*, 2010). They demonstrated a biphasic oxidative burst resulting from exposure to OTA with co-occurring down-regulation of antioxidant enzymes, suggesting that the phytotoxic effects of OTA may be associated with the generation of reactive oxygen species, as seen in animal systems (Baudrimont *et al.*, 1994). Detection of OTA in plants is the only available exposure-based biomarker thus far.

Unlike the other mycotoxins, ZEA is not necessarily detrimental to plants and even has been suggested as a seed treatment for increased yield and seed viability since it increases the percentage of viable generative winter wheat embryos, reviving them from vernalisation (Biesaga-Kościelniak and Filek, 2010; Biesaga-Kościelniak *et al.*,

2010; Kościelniak *et al.*, 2009). The effects of ZEA resemble the effects of auxins, although auxins and ZEA share little structural similarity, unlike the animal associated similarity of oestrogen and ZEA. The stimulative effect of ZEA was shown on winter wheat, winter rape, soybean, and spring wheat (Biesaga-Kościelniak and Filek, 2010). Methods are available for detection of ZEA *in planta* that could facilitate its use as an exposure-based biomarker for more thoroughly evaluating its phytostimulative activity (Biesaga-Kościelniak and Filek, 2010). In animals, ZEA is a substrate for 3- α and 3- β hydroxysteroid dehydrogenase (reviewed in Fink-Gremmels and Malekinejad, 2007), important enzymes in steroid metabolism. The ability of ZEA to interfere with hydroxysteroid dehydrogenases has not been investigated *in planta*. However, in *Arabidopsis* a putative hydroxysteroid dehydrogenase has been shown to be involved in regulating plant growth and development (Li *et al.*, 2007).

5. Fumonisin exposure and mechanism-based biomarkers in plants

The primary producers of fumonisin mycotoxins are *Fusarium verticillioides* and *Fusarium proliferatum*. The predominant cause of maize ear rot and fumonisin contamination in the United States is *F. verticillioides*, while both species are common causes of ear rot in southern Europe (Logrieco *et al.*, 2002; Munkvold, 2003). *F. proliferatum* is of additional concern due to its broad host range (e.g. banana, maize, fig, mango, pine, sorghum, wheat, and native prairie grasses) (Leslie, 1995; Leslie *et al.*, 2004; O'Donnell *et al.*, 1998) and production of additional mycotoxins (Glenn, 2007). With regard to human and animal exposure to fumonisins through maize and its processed products, *F. verticillioides* is generally regarded as the primary concern since it is the dominant *Fusarium* species commonly infecting maize kernels and stalks (Desjardins *et al.*, 2000; Leslie *et al.*, 1990).

In addition to ear rot, *F. verticillioides* is noted to cause seed rot, seedling blight, root rot, and stalk rot (Kommedahl and Windels, 1981; White, 1999). Development of disease will be dependent on genetic variability of *F. verticillioides* populations, the genetic resistance of maize hybrids, and environmental conditions (Desjardins *et al.*, 1995; Duncan and Howard, 2010; Munkvold, 2003). Additionally, ear rot severity is positively correlated with insect feeding damage (Munkvold, 2003). As a result of such variability, symptomless *F. verticillioides* infections are common, and the fungus is generally considered an endophyte capable of systemic infection of maize (Bacon and Hinton, 1996; Foley, 1962). Also, contamination of symptomless kernels with significant levels of fumonisins can occur (Bush *et al.*, 2004), indicating that visual scoring or sorting of symptomatic kernels may not be sufficient to effectively reduce fumonisin exposure to recommended levels.

The role of fumonisin production in the development of *F. verticillioides*-associated maize diseases has been thoroughly assessed only for ear rot and seedling blight. Detailed studies with fumonisin-nonproducing strains have shown that fumonisin production is not necessary for development of ear rot (Desjardins and Plattner, 2000; Desjardins *et al.*, 1998, 2002). In relation to maize seedling blight, fumonisins were suggested to increase the virulence of *F. verticillioides* but were not necessary or sufficient for disease development (Desjardins *et al.*, 1995). More recently a significant positive correlation was demonstrated between leaf lesion development on maize seedlings and the production of FB₁ by *F. verticillioides* (Williams *et al.*, 2006, 2007). Also shown in these studies was a significant inverse correlation between root weight and stalk height and the amount of FB₁ associated with seedling roots. Most notably, fumonisin-nonproducing strains did not cause leaf lesions and had significantly less effect on root weight and stalk height. Similar to the inoculation studies, watering uninoculated maize seedlings with FB₁ caused leaf lesions and a significant dose-dependent reduction in root weight and stalk height. Glenn *et al.* (2008) provided conclusive evidence supporting the role of fumonisin production on maize seedling disease development. A fumonisin non-producing and non-pathogenic strain possessing a deletion of the fumonisin biosynthetic gene cluster was genetically complemented by transformation with the gene cluster. The complemented transformants were able to produce fumonisins and caused the full suite of seedling disease symptoms. Differences in disease development susceptibility were noted between varieties of maize, with sweet corn genotypes being more susceptible than dent genotypes (Glenn *et al.*, 2008). Desjardins *et al.* (2005) noted differential sensitivity of maize genotypes to germination and growth on various concentrations of FB₁ and postulated that sensitivity is likely the ancestral trait in maize, with insensitivity being a derived and inheritable trait.

These studies have facilitated the development of both exposure and mechanism-based biomarkers for evaluating the role of fumonisin in maize-*Fusarium* interactions. For example, an important initial physiological process leading to foliar disease development, as well as adverse effects on plant growth, is the absorption and preferential accumulation of FB₁ by roots (Williams *et al.*, 2007; Zitomer *et al.*, 2010). Approximately 10 times the amount of FB₁ accumulated in roots compared to fumonisin B₂ when growing seedlings were watered with a combined solution of both fumonisins (Zitomer *et al.*, 2010).

Such accumulation of FB₁ in maize roots was shown to cause disruption of sphingolipid metabolism. Building on the development of fumonisin-related biomarkers in animals and humans as outlined above, elevated concentrations of sphingoid bases (sphinganine and phytosphingosine) and sphingoid base 1-phosphates (sphinganine 1-phosphate

and phytosphingosine 1-phosphate) due to inhibition of ceramide synthase by FB₁ was shown in both roots and leaves of maize seedlings (Williams *et al.*, 2006, 2007; Zitomer *et al.*, 2008, 2010). These analyses provided useful mechanism-based biomarkers for evaluating cellular effects of toxicity and were the first to document fumonisin-related elevation of the 1-phosphates in plants. Elevated concentrations of sphinganine and phytosphingosine were previously observed in *Nicotiana*, *Lemna*, and AAL toxin susceptible varieties of *Lycopersicon* (tomato) species, with the increase in sphingoid bases occurring prior to the onset of disease symptoms (Abbas *et al.*, 1994). Such increases were not observed in AAL toxin resistant varieties of tomato that were exposed to fumonisin. AAL toxin is a structural analogue of FB₁, and as with FB₁, the cellular effects of AAL toxin are associated with disruption of sphingolipid metabolism and accumulation of sphingoid bases in the tissues of susceptible varieties of tomato (Abbas *et al.*, 1994). Pharmacological evidence supports the hypothesis that AAL phytotoxicity initially results from the accumulation of sphingoid bases and not the depletion of complex sphingolipids (Spassieva *et al.*, 2002). Myriocin is an inhibitor of serine palmitoyltransferase, the first enzyme in the *de novo* ceramide biosynthesis pathway that produces sphingoid bases, which are then utilised by ceramide synthase. Co-exposure of susceptible tomato plants to AAL toxin and myriocin provided a protective effect, reducing phytotoxicity by preventing the buildup of sphingoid bases (Spassieva *et al.*, 2002) and their subsequent metabolism to sphingoid base 1-phosphates.

Williams *et al.* (2006, 2007) and Zitomer *et al.* (2008, 2010) have examined the differential effects of FB₁ alone versus *F. verticillioides* infection of maize tissues and the *in planta* production of FB₁. Interesting distinctions were observed and are summarised in Figure 3. Both experimental treatments resulted in accumulation of fumonisin in the roots and inhibition of ceramide synthase as assessed from elevated sphingoid bases and sphingoid base 1-phosphates as mechanistic biomarkers. Yet, only the inoculation experiments detected fumonisin accumulation in the leaves (Zitomer *et al.*, 2008); the fumonisin watering experiments indicated no fumonisin accumulation in the leaves (Zitomer *et al.*, 2010). Furthermore, FB₁ was the predominant fumonisin found in the leaves from the inoculation experiments, with only trace amounts of fumonisin B₂ and fumonisin B₃ detected. Both treatments resulted in accumulation of sphinganine, phytosphingosine, and their 1-phosphates in the leaves. Collectively the data suggest that transpiration mediated shootward movement of fumonisins, sphingoid bases, and sphingoid base 1-phosphates from roots to leaves does not occur as chemically expected. The water-soluble fumonisins have limited movement while the much less soluble sphingoid bases and 1-phosphates appear to be readily translocated from roots to leaves, suggesting the need for some mechanism other than

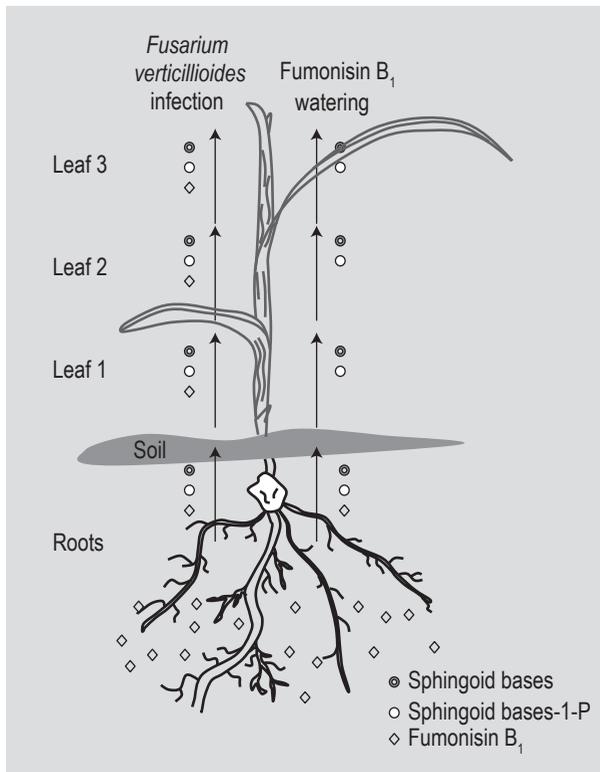


Figure 3. *In planta* biomarker detection in maize seedlings in response to fumonisin exposure. The figure is based on data from Zitomer *et al.* (2008, 2010) and indicates detection of fumonisin B₁, sphingoid bases, and sphingoid base 1-phosphates in seedling tissues. Experiments consisted of either *Fusarium verticillioides* infection of the seedlings (left) or exposure to only water-dissolved fumonisin B₁ (right). Infections resulted from planting seed inoculated with *F. verticillioides* (approx. 2,500 conidia/seed). In the watering experiment, pots of 10 uninoculated seedlings were exposed to a total of 4.71 mg fumonisin B₁. Both treatments resulted in high accumulation of fumonisin B₁ in the soil. Fumonisin B₁ also accumulated in the roots, causing inhibition of ceramide synthase and increased accumulation of sphingoid bases and sphingoid base 1-phosphates. Use of these exposure and mechanistic biomarkers highlights the different effects resulting from infection (presence of fungal hyphae producing fumonisin B₁ *in planta*) compared to presence of fumonisin B₁ solely. *F. verticillioides* infected seedlings accumulated detectable levels of fumonisin B₁ in the 1st, 2nd, and 3rd leaves (and very little fumonisin B₂ or fumonisin B₃), while no fumonisin B₁ was detected in any of the leaves from the fumonisin B₁-watering treatment.

passive translocation through the plant, such as carrier molecules. Furthermore, while fumonisin is required for leaf lesion development in maize seedlings, such disease progression occurs via a mechanism that does not require either endophytic fungal colonisation or fumonisin accumulation in the leaves. Lesion development may occur as a consequence of disrupted sphingolipid metabolism in

the roots and subsequent mobilisation of signal molecules. The sphingoid bases and/or their 1-phosphates may be those molecules, as suggested for AAL toxin (Spassieva *et al.*, 2002), but data are needed to support this hypothesis and the mechanisms leading to leaf lesion formation. Overall these studies demonstrate the information that can be gained using *in planta* biomarkers.

Numerous studies have utilised FB₁ as a research tool for studying the molecular events associated with pathogen-induced programmed cell death in *A. thaliana* (Watanabe and Lam, 2006; Chivasa *et al.*, 2005; Norholm *et al.*, 2006; Kuroyanagi *et al.*, 2005; Bindschedler *et al.*, 2006; Lin *et al.*, 2008; Shi *et al.*, 2007). Collectively, these studies provide additional evidence for the FB₁ inhibition of ceramide synthase and disruption of sphingolipid metabolism as the proximate cause of *F. verticillioides* induced diseases in maize. Interestingly, these studies show that the response to FB₁, a fungal metabolite, mimics the responses of plants to infectious pathogens such as *Pseudomonas syringae* (Norholm *et al.*, 2006). Not surprisingly, many of the affected systems in plants are similar to those in fumonisin-exposed animals, such as induction of oxidative stress (Shi *et al.*, 2007; Harvey *et al.*, 2008; Bindschedler *et al.*, 2006). One of the systems affected by FB₁ in *Arabidopsis* is depletion of extracellular ATP which could be expected given the high levels of sphingoid base 1-phosphates produced in maize plants when ceramide synthase is inhibited and given the fact that sphinganine production is uncoupled from ceramide production in fumonisin-treated cells.

Another potential downstream effect of the fumonisin-induced accumulation of sphingoid bases in maize, particularly phytosphingosine, is disruption of stomatal regulation. As demonstrated in *Arabidopsis*, sphingosine kinase is an important component of abscisic acid induction of stomatal closure by phosphorylating sphingosine and phytosphingosine to their respective 1-phosphates (Coursol *et al.*, 2003, 2005). These 1-phosphates were shown to both induce stomatal closure and inhibit stomatal opening. As outlined above, phytosphingosine and phytosphingosine 1-phosphate significantly increase in concentration in maize leaf tissues when plants are inoculated with fumonisin producing *F. verticillioides* or when uninoculated plants are exposed to fumonisin alone. The potential therefore exists that FB₁-induced elevation in sphingoid base 1-phosphates may affect stomatal regulation as they accumulate in leaves.

6. Conclusions

The economic cost and health risks due to mycotoxin contamination has provided the impetus for research to better understand the dose-response relationships between mycotoxin exposure and increased risk of disease in humans and farm animals. Development and validation of exposure and mechanism-based biomarkers is critical for the accurate

assessment of disease risk. This is best exemplified with aflatoxins, where biomarkers have been successfully used to establish the role of aflatoxins in human disease and to assess the effectiveness of interventions (Kensler *et al.*, 2011). Development and validation of biomarkers for other mycotoxins has progressed considerably, but has not yet reached the same level of practical utility as the biomarkers developed for aflatoxin. For example, the extensive use of elevated sphingoid bases and their metabolites as biomarkers in farm and laboratory animals has validated the dose-response relationships between inhibition of ceramide synthase and the fumonisin-induced disruption of cellular processes leading to the various farm animal diseases and carcinogenicity of FB₁ in rodents. Progress has also been made to assess individual exposure in humans using urinary fumonisin, but definitively linking exposure to disease has been unsuccessful largely due to the lack of a validated mechanism-based biomarker in humans. Extension of this knowledge base to experiments addressing the effects of fumonisin on maize and other plants has demonstrated that inhibition of ceramide synthase is the key event in the mechanism of action resulting in plant disease. This information has resulted in useful biomarkers for studies on disease development and the potential for altered physiological responses in maize that could contribute to susceptibility to other risk factors for diseases. The biomarkers may also prove beneficial to monitoring efforts since their early detection may provide management options for limiting plant disease and accumulation of fumonisin in maize products. Application of similar biomarker development strategies to other plant-fungal systems involving mycotoxigenic fungi could lead to a better understanding of the underlying phytopathology and also the potential of mycotoxins to modulate basic physiological processes that could contribute to the susceptibility of plants to environmental stress and disease.

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