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Integrating gene expression, ecology and mycotoxin production by *Fusarium* and *Aspergillus* species in relation to interacting environmental factors

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Abstract

Environmental factors such as water availability (water activity, a_w), temperature and their interactions, have a significant impact on the life cycle of mycotoxigenic fungi. Growth and mycotoxin production are influenced by these interacting factors resulting in a broader range of a_w x temperature conditions for germination, than growth or mycotoxin production. The biosynthetic genes are mostly clustered together and by using microarrays with sub-arrays for specific mycotoxins such as trichothecenes, fumonisins and aflatoxins it has been possible to examine the relationship between interacting a_w x temperature conditions on growth, toxin gene cluster expression and relate these to phenotypic toxin production. The data for groups of biosynthetic genes (*F. culmorum*/*F. graminearum*; *F. verticillioides*; *A. flavus*) were integrated with data on growth and mycotoxin production under different a_w x temperature conditions using a mixed growth model. This was used to correlate these factors and predict toxin levels which may be produced under different abiotic stress conditions. Indeed, the relative importance of the different genes could be examined using ternary diagrams of the relative expression of 3 genes at a time in relation to a_w, temperature and mycotoxin production to identify the most important relationships. The effect of three-way interacting environmental factors representative of climate change (CC) scenarios (water stress x temperature (+2-4^oC) x elevated CO_2 (350-400 vs 650 and 1000 ppm) on growth and mycotoxin production by *A. flavus* and by species of the *Aspergillus* section *Circumdati* and section *Nigri* have been determined. These studies on maize grain and coffee respectively suggest that while growth may not be significantly affected, mycotoxin production may be stimulated by CC factors. This approach to integrate such data sets and model the relationships could be a powerful tool for predicting the relative toxin production under extreme stress conditions, including CC scenarios.

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1. Introduction

The impact of environmental factors, especially related to climate change (CC) parameters on pests and diseases of staple food crops has received attention because of the impact on food security and quality. The global nature of food chains has also focused attention on the effect that such changes in the environment may have on the resilience of staple food crops. Any enhancement in contamination of such food crops with mycotoxins, both pre- and post-harvest would have significant implications, both from a food security view point and economically. For example, Wu et al. (2011) suggested significant economic impacts in the USA from CC impacts on key food chains. Other studies have suggested significant potential impacts of CC factors on both growth and mycotoxin production (Magan et al., 2011; Medina et al., 2015a). Recent studies in Europe, including the EFSA report, suggest that CC factors will impact pre-harvest on ripening periods and harvest times (earlier than at present) and this would have implications for pests/fungal diseases and perhaps the predominant types of mycotoxins which could become more important, e.g. in maize a switch from fumonisins to aflatoxins (Battilani et al., 2012; Battilani et al., 2016). Indeed, recent reports in the Balkans supports these findings where extremes in temperature and drought stress have resulted in significant contamination of maize with aflatoxins, above the EU legislative limits (Kos et al., 2013; Dobolyi et al., 2013).

A significant amount of knowledge has been gathered over the last two decades on the impact that important environmental factors such as water activity (a_w), temperature, pH, and in some cases modified atmospheres, have on germination, growth and mycotoxin production by mycotoxigenic species from the genera *Aspergillus*, *Alternaria*, *Fusarium* and *Penicillium* (see Sanchis and Magan, 2004; Magan et al., 2010). This has included the development of boundary conditions using contour maps for germination, growth and mycotoxin production. The impact of interacting conditions of a_w x temperature on sporulation has received less attention although the pioneering studies by Ayerst (1969) included such observations but less quantitative data are available on this phase of the life cycle of mycotoxigenic fungi (Parra et al., 2004; Abdel-Hadi & Magan, 2009; Leggieri et al., 2014; Giorni et al., 2011).

Parallel to this, the discovery of the whole genome sequences of mycotoxigenic species such as *Aspergillus flavus* (aflatoxins), *Fusarium verticillioides* (fumonisins) and *F. graminearum* (trichothecenes) has enabled the gene clusters involved in the biosynthetic pathways for mycotoxin production to be studied in more detail. The molecular work has concentrated on elucidating the role of the different genes in these clusters and their associated enzymes to understand the importance in terms of structural or regulatory function. However, to a large extent the molecular work has, up to the last 5-6 years, being done to the exclusion of ecophysiological considerations and its relationship with phenotypic mycotoxin quantification. This is surprising as many of these mycotoxigenic fungi grow and produce more mycotoxins under environmental stress conditions (Jurado et al., 2008; Kim et al., 2005; Kohut et al., 2009; Schmidt-Heydt et al., 2009; Abdel-Hadi et al., 2012; Medina et al., 2013). We have thus examined a more integrated systems approach which includes both molecular and ecological data to develop models which could help to understand the functional basis of mycotoxin production. This would also be beneficial to the development of more targeted prevention strategies. The objective of this review is to examine (a) the relationship between environmental factors, growth and mycotoxin production, (b) the use

of a microarray to examine the gene cluster expression and biosynthetic pathways for mycotoxin production in relation to environmental factors, (c) the use of such data sets to model the relationship between environmental factors, growth of mycotoxigenic species, toxin gene expression and mycotoxin production, and (d) the potential effect of three way interactions between CC factors (drought stress, +4^{o}C above optimum for growth and 2x or 3x existing CO_2 levels) on growth, gene expression of key biosynthetic genes and mycotoxin production.

2. Relationship between environmental factors, growth and mycotoxin production

Studies over the last 2-3 decades have shown the impact that changes in a_w or temperature alone and their interactions can have on germination, lag time prior to growth, growth rates and mycotoxin production (Ayerst, 1969; Magan & Lacey, 1984; Sanchis and Magan, 2004; Magan et al., 2010). Overall these have shown that the marginal temperature x a_w conditions for germination are broader than those for growth and mycotoxin production. Such contour maps are now available for a wide range of mycotoxigenic fungi including *Aspergillus flavus*, *A. parasiticus*, *A. carbonarius*, *Fusarium verticillioides*, *F. culmorum*, *F. graminearum*, *F. langsethiae* and *Penicillium verrucosum* and *Stachybotrys chartarum* (Cairns et al., 2000; Sanchis and Magan, 2004; Mitchell et al., 200X; Medina et al., 2011; Fraser et al., 2012). For example, optimum growth for *A. flavus* was between 30-35^{o}C at 0.995-0.98 a_w, while aflatoxin B_1 production was best at 25 and 35^{o}C and 0.995 and 0.95 a_w respectively (Abdel-Hadi et al., 2012). For *F. verticillioides* growth was optimum at 25^{o}C and 0.995 a_w and also at 30^{o}C and 0.98 a_w. Fumonisin B_1 and fumonisin B_2 were optimum at 0.98 a_w and 20^{o}C and 25^{o}C respectively (Medina et al., 2013). Assumptions about mycotoxin production based on the growth responses need to be done with care. The question arises as to whether such data sets could be combined with more detailed information at a molecular level on relative expression of gene clusters involved in mycotoxin biosynthesis.

3. Mycotoxin gene clusters and biosynthetic pathways for mycotoxin production

The biosynthetic gene clusters involved in the production of key mycotoxins such as the type B trichothecene deoxynivalenol (DON), fumonsins and aflatoxins have been largely identified (Desjardins, 2006; Yu et al., 2004, Kimura et al., 2007; Brown et al., 2007; Brown and Proctor, 2013; Dolezal et al., 2014). The function and role of many of the structural and regulatory genes involved in the biosynthesis of these mycotoxins have now been largely unravelled. For example, the aflatoxin gene cluster consists of 29 genes with the key regulatory genes being *aflS* and *aflR*. Other mycotoxigenic *Fusarium* species such as *F. verticillioides* have 17 genes involved in fumonisin production with the *FUM1* and perhaps *FUM21* genes considered important; *F. sporotrichioides* and *F. oxysporum* have 18 (Proctor et al., 2008) and *Gibberella zeae* 25 genes with the *TRI5* being important; ochratoxin A producing *Penicillia* (*P. verrucosum*, *P. nordicum*) have at least 4 genes involved in ochratoxin A production with the *otapks* gene being important (Geisen et al., 2006). Recently for *A. carbonarius* and *A. westerdijkiae* (ochratoxin A producers) the PKS and P450 related genes have been found to be important markers in the biosynthetic pathway (Gil-Serna et al., 2009; Gallo et al., 2014). The quantitative PCR approach for specific genes has been an important tool for a better understanding of the relationship between environmental factors, other stress factors such as preservatives, transcriptional activation

of biosynthesis genes and phenotypic toxin production (Schmidt-Heydt et al., 2007; Marin et al., 2013).

It has been shown that *aflS* and *aflR* are important regulatory genes in the biosynthetic pathway for aflatoxins. However, other global regulatory genes such as *lae*A and *ve*A may also be important in regulating several secondary metabolite pathways (Georgianna and Payne, 2009). The copy number of the *FUM1* gene has been correlated with fumonisin B_1 production; while the *TRI5* gene is considered to be an important key gene in trichothecene production. In all these cases, both q-PCR and RT-PCR approaches have been developed and used as an indicator of mycotoxin production *in situ* in different food commodities using assays for specific genes and making comparisons with a house-keeping control gene (often β-tubulin).

In the last 5 years whole genome microarrays were developed for *A.flavus*, *A.niger, A.nidulans, A.fumigatus F.graminearum, F.vertcillioides* and are commercially available as Affymetrix chips. These were used to examine the relative numbers of up and down regulated genes on a whole genome wide basis (O´Brian et al., 2003; Pirttilä et al., 2004; Price et al., 2005; Price et al., 2006; O´Brian et al., 2007; Georgianna and Payne, 2009; Schmidt-Heydt et al., 2008; 2011; Woloshuk and Shim, 2014). Today, RNA sequencing (RNA-seq) has superseded microarrays to examine the detailed functioning of gene groups involved in pathogenicity of mycotoxigenic fungi. These can be effectively utilized to examine shifts in relative expression of groups of genes in relation to nutritional factors and in a few cases single environmental factors (Wilkinson, 2011; Yu et al., 2011; Brown and Proctor, 2013; Zhang et al., 2014).

4. Effect of interacting environmental factors on mycotoxin gene clusters and integration of data with growth and toxin production data

We have utilized a specific microarray for the biosynthetic genes involved in mycotoxin production with sub-arrays for the gene clusters for aflatoxins, ochratoxins, fumonisins, trichothecenes A and B, and patulin to study the impact of interacting environmental conditions on growth, biosynthetic gene expression and on toxin production (Schmidt-Heydt & Geisen, 2007). This facilitated detailed studies of the relationship between interacting environmental conditions of a_w x temperature on gene clusters, and differential effects on specific genes, to try and relate this information to both growth and phenotypic mycotoxin production (Jurado et al., 2008; Schmidt-Heydt et al., 2008; 2009; 2011; 2011; Marin et al., 2010a, b; Medina et al., 2013). This is important because in nature these fungi need to be able to tolerate such interacting conditions of environmental stress. Thus studies were carried out with *F. culmorum., F. graminearum, F. verticillioides and A.flavus* and *A. parasiticus* to understand the effect that such interacting environmental stress conditions may have on relative expression of the genes involved in the biosynthesis of specific mycotoxin groups. Figure 1a and 1b show the heat maps of the effect of a_w x temperature effects on expression of all the genes in the trichothecene cluster for *F. culmorum* and *F. graminearum* grown on a conducive YES medium for 9-10 days using the trichothecene sub-array. This shows that the relative expression of individual genes varies as a_w x temperature conditions are changed. Similar data has been obtained for *A. flavus*, *A. parasiticus* and *F.*

verticillioides (Schmidt-Heydt et al., 2009; 2010; Abdel-Hadi et al., 2012; Medina et al., 2013).

The data for *F. graminearum* and *F. culmorum* can be examined in conjunction with the effect of these interacting conditions of a_w x temperature on both growth and DON production. This showed that by examining these factors it was possible to determine which statistically significant interactions between factors occurred. This enabled a model to be constructed based on a polynomial equation which fitted the experimental data for DON production in relation to key genes in the *TRI* gene cluster expression. The multiple correlation coefficient (R^2) was 0.999 and the model explained >99.9% of the variance (R^2_{adj}=0.999, residual error 0.187). The regressed model using this approach for *F. culmorum* is presented below (Schmidt-Heydt et al., 2011):

$$\mathrm{DON}(\mu\mathrm{g/g}) = -5.16 + 1.262X_{a_w} + 1.054X_{T(^{o}C)} + 0.283X_{Tri\,6} - 7.8X_{Tri\,10}$$
$$+ 11.28X_{Tri_4} + 11.0X_{Tri\,5} - 2.35X_{Tri\,12} - 8.22X_{Tri\,13}$$

The polynomial model included expression of 6 of the 25 genes in the cluster plus a_w and temperature effects on growth and DON production. Table 1 shows the statistically significant effect of interactions between DON and the dependent variable and the individual genes over all the interacting conditions of a_w x temperature for both *F. culmorum* and *F. graminearum* (Schmidt-Heydt et al., 2011). However, there were difference between the relative expression of some of the specific genes of the 6 chosen in relation to DON production between *F. culmorum* and *F. graminearum* strains. This suggests that while *TRI5* is a key regulatory gene in type B trichothecene biosynthesis, some others may also be important and their importance will differ with species.

It is also possible to use this approach to relate the relative expression of individual genes to specific environmental conditions and DON production which could be useful for predicting conditions which will allow mycotoxin production and those which will not. This would be especially beneficial by using q-PCR or RT-PCR approaches for specific genes (e.g. *TRI5*) combined with moisture and temperature data to determine the relative risk of mycotoxin production.

Figure 2 shows examples of two dimensional contour plots relating specific gene expression of *TRI4, TRI5* and *TRI7* and a_w levels for *F. culmorum* to predicted DON production using the developed models. For this species it suggests that significant DON would be produced under water stress. A significant difference in the expression of *TRI4* resulted in a two orders of magnitude increase in DON production. This direct correlation between *TRI4* gene expression, a_w and DON production shows that maximum toxin production occurred at lowered a_w and causes a high expression of this gene. The *TRI5* expression was species specific. For *F. culmorum* high DON production and *TRI5* expression was observed under water stress (low a_w), in contrast for *F. graminearum* DON production and *TRI5* expression was at high a_w (Schmidt-Heydt et al., 2011). However, overall, the expression of the *TRI5* was generally low in the time frame of this study. Figure 2 shows the predicted contour maps for DON production based on the available data and the model in relation to temperature and a_w. For both species optimum was at approx. 25^{o}C and >0.98 a_w. This showed that the polynomial model approach was useful and could be used effectively for

determining the relationship between key structural and regulatory genes involved in the biosynthetic pathway of DON production (Schmidt-Heydt et al., 2011).

There has also been significant interest in understanding the functional and regulatory role of the gene clusters in biosynthesis of aflatoxin and related secondary metabolites. The comprehensive review by Georgianna and Payne (2009) considered the major impacts that a range of factors may have on functioning of the aflatoxin pathway as known at present. Previous studies have shown the effect of some factors on the up or down regulation of specific aflatoxin genes. Thus conducive conditions include carbon source (simple sugars), nitrogen source (reduced nitrogen), oxidative stress (oxidants), temperature (<35^{o}C) and pH (acidic, pH4.5). Schmidt-Heydt et al. (2009; 2010) showed that for both *A. flavus* and *A. parasiticus* the ratio of the two regulatory genes (*aflR*, *aflS*) may be important and varied with temperature and water stress. Thus a high ratio was related to high phenotypic aflatoxin B_1 production and a low ratio to low aflatoxin production. The impact on the sugar transporter genes may also be important as there expression also changed with a_w x temperature conditions (Medina et al., 2015b).

Studies have been carried out with both *A. flavus* and *F. verticillioides* to relate interacting conditions of a_w x temperature on growth, toxin gene expression and mycotoxin production using the sub-arrays described previously. This was done to try and integrate this data and develop models which could predict the amount of mycotoxin produced. To this end while data was obtained on all the genes involved in aflatoxin (25 genes) and fumonisin (17 genes) production, a subset of the genes were used. For *A. flavus,* 10 (8 structural; 2 regulatory genes) and for *F. verticillioides,* 9 genes (8 structural; 1 possible regulatory gene) were used (Abdel-Hadi et al., 2012; Medina et al., 2013). Overall, a range of a_w x temperature conditions (0.995-0.90 a_w; 20-35^{o}C) were used which included effects on growth, gene expression and mycotoxin production. Figure 3 shows the effect of the interacting a_w x temperature conditions on the gene expression for the genes utilized for *A. flavus* and *F. verticillioides*. For integration of the data sets a mixed growth model was used (Shuler and Kargi, 2007) which takes account of specific growth rates, relative gene expression and metabolite accumulation. This has been previously used predominantly in the pharmaceutical industry for examining the production of useful secondary metabolites. Below, the example of the treatment of the data sets for *A. flavus* is shown. Using this approach the final model considering a_w, temperature, growth rate and gene expression on the regulation of aflatoxin B_1 was given by:

$$P = [g] \times \left(\alpha + \frac{\beta}{\mu} \right) X_0 e^{-\frac{b_1 a_w}{RT}} \left(e^{\mu \cdot t} - 1 \right)$$

Were P is the aflatoxin production (ppm) and b_1, α and β are parameter estimates from the model. Based on this model it was possible to validate prediction of growth and aflatoxin B_1 production in conditions which were not included in the model. Table 2 shows the prediction at elevated temperatures and drought stress conditions.

Subsequently, information on the gene expression of the key biosynthetic genes involved in mycotoxin production was included as a linear combination with the physical model. As an example, for *A. flavus*, the linear component [g] was added so as to include the expression data of 10 aflatoxin cluster genes:

$$[g] = a_1 aflD + a_2 aflR + a_3 aflS + a_4 aflE + a_5 aflM + a_6 aflO + a_7 aflP + a_8 aflQ$$
$$a_9 aflX + a_{10} aflF$$

In order to plot the contour surfaces of the genetic expression a standardized signal from the microarray was calculated as follows:

$$\text{Standardised value} = \frac{\text{actual value-minimum value}}{\text{maximum value-minimum value}}$$

This enabled ternary diagrams to be produced which could be used to examine the effect of relative expression of specific groups of aflatoxin and fumonisin cluster genes in relation to environmental factors (Abdel-Hadi et al., 2012; Medina et al., 2013).

This approach showed that the integrated model gave a good correlation between observed and predicted aflatoxin B_1 and fumonisin B_1 and B_2 production. It was also possible to validate the model outside of the experimental range for the first time. Figure 4 shows an example of the contour maps developed using the model for relating a_w x temperature conditions for growth and aflatoxin B_1 production. The contour maps show the optimum and marginal conditions predicted based on the model (Abdel-Hadi et al., 2012).

By examining the relative expression of different genes to each other, under different a_w x temperatures it was possible to try and identify which genes are more important than others in tolerating temperature or water stress or their relationship to mycotoxin production. Figure 5 shows ternary diagrams of the relationship between the two key regulatory genes and one other gene in relation to water availability and temperature using this approach for *A. flavus*. Figure 6 shows how these ternary diagrams can be used to identify the relative importance of specific genes in the production of fumonisins. The relationship between relative expression of the *FUM1* gene and two other *FUM* genes and their relationship to relative fumonisin (B_1; B_2) production is shown. This approach may be useful in trying to understand the relationship and function of important genes in the biosynthetic pathways of mycotoxin production and also help in a better understanding of how they respond to abiotic and biotic stresses (e.g. interactions with plants during infection). In *F. verticillioides*, temporal changes in expression of other *FUM* genes, such as *FUM2* and *FUM21,* in relation to a_w x temperature has also been demonstrated (Lazzaro et al., 2012). Studies of relative effects of temperature and water stress on growth of strains of related *Fusarium* section *Liseola* species, *F. verticillioides* (maize) and *F. proliferatum* (broad range of host plants), was found to be similar. However, *FUM1* gene expression, an indicator of fumonisin biosynthesis, was differentially expressed in relation to temperature and water stress by strains of these species (Marin et al., 2010). It was suggested that these differences may be related to relative tolerance to such interacting environmental stresses and help explain the difference in host range.

5. **Effect of three way interacting climate change (CC) environmental factors (water stress x elevated temperature and CO_2 on growth, gene expression and mycotoxin production**

There has been significant interest in examining the three key environmental parameters which are implicated in CC scenarios. These include effects of changes in water stress,

increased temperature (+2-4^{o}C) and x2 or x 3 existing CO_2 values (350-400 vs 700-800 and 1000-1200 ppm) on pests and diseases of staple food production systems (Bebber et al., 2013; 2014; Crespo-Perez et al., 2015).

The European Food Safety Authority (EFSA) examined the potential impact of CC in Europe and has suggested that effects will be (a) regional and (b) detrimental or advantageous depending on geographical region (Battilani et al., 2012; Battilani et al., 2016). This suggests that in northern Europe the effects may be positive, while the Mediterranean basin may be a hot spot where many effects will be negative, with extreme changes in rainfall/drought, elevated temperatures and elevated CO_2 impacting on food production. Effects of CC on staple foods, especially cereals and rice, will be significant and detrimental as ripening in southern and central Europe will occur much earlier than at present. This will influence pests and diseases with decreasing yields and potentially increase mycotoxin contamination. This could have also have profound impacts on food security in different continents.

Recent predictions suggest that, on a global scale, pests and diseases are migrating to the poles at the rate of 3-5 km/year and the diversity of pest populations will also significantly change and have profound economic impacts on food production systems (Bebber et al., 2013; 2014). While these predictions did not focus on mycotoxigenic fungal pathogens, they suggest significant potential impacts on mycotoxin contamination of staple foods/crops. A recent study of wheat diseases and CC suggests that the physiology of wheat is modified when comparing exposure to 390 ppm and 780 ppm CO_2, in terms of leaf physiology and stomatal production on adaxial and abaxial surfaces (Vary et al., 2015). Acclimatisation of *Septoria tritici* blotch (STB) disease and *Fusarium* Head Blight (FHB) for 10-20 generations prior to infection resulted in increased disease of wheat grown under CC conditions. The effect of elevated CO_2 was more pronounced for FHB than STB. This study did not examine effects on type B trichothecenes (e.g. DON) which would have been interesting. Increases in pest reproduction rates would increase damage to ripening crops (during anthesis in wheat; silking in maize) and facilitate more infection by mycotoxigenic fungi and potential contamination with mycotoxins. However, few studies have examined the impact of these three-way interactions on growth and mycotoxin production by *Aspergillus*, *Penicillium* and *Fusarium* species.

Some evidence exists that growth of mycotoxigenic species, e.g. *F. verticillioides*, *F. graminearum*, may be modified by interacting CC conditions (Medina et al., 2015b). Recent studies have tried to integrate effects of CC conditions on both plant physiology as well as the associated mycotoxigenic fungi such as *A. flavus* and *F. verticillioides.* For example, Vaughan et al. (2014) investigated the impact of elevated CO_2 on the interactions between maize and *F. verticillioides*. They found that elevated CO_2 of approx. 800 ppm CO_2 (approx. 2 x current CO_2) increased maize susceptibility to *F. verticillioides* colonization, with an increase in biomass of the pathogen. However, fumonisin B_1 contamination of ripening maize cobs was unaffected by these interactions. Previous studies have certainly suggested that a key gene in the fumonisin biosynthetic pathway (*FUM 1*) is significantly affected by changes in environmental factors (Marin et al., 2010b; Medina et al., 2013).

Medina et al. (2014; 2015 a,b) studied the impact of CC scenarios on growth and AFB_1 production by *A. flavus* on maize-based media and in stored maize. The treatments

included: (a) 34 and 37^{o}C; (b) imposed drought stress – 0.97 to 0.95 and 0.91 a_w; and (c) CO_2 was increased from 350 to 650 and 1000 ppm. The effects on growth of *A. flavus* and expression of aflatoxin cluster genes (*aflD*, *aflR*) and phenotypic AFB_1 production were examined. This was the first time a combination of expected CC factors was used to establish the potential effects of CC scenarios on the ecophysiology of mycotoxigenic fungi. Growth of *A. flavus* was relatively unaffected. In contrast, the three-way interacting conditions had a profound, statistically significant stimulatory effect on AFB_1 production (≈80 x the control), especially under drought stress at 37^{o}C and 650 and 1000 ppm CO_2 exposure (Table 3). Studies of the relative expression of biosynthetic genes (*aflD*, *aflR*) in the aflatoxin pathway also corroborated these findings. Research on maize grain have supported these findings although the relative increase in aflatoxin B_1 production on maize grain under CC conditions was about x3-4 greater than the controls. Work is now in progress to examine the impact that these CC conditions have on the whole transcriptome of *A. flavus* to identify key groups of genes which may be affected including the aflatoxin cluster genes, universal regulators, sugar transportation, other stress-related pathways, and secondary metabolite pathways using RNA sequencing. These are important to identify whether any switches in biosynthetic pathways may occur resulting in other secondary metabolites being produced rather than aflatoxins or cyclopiazonic acid. Other studies with *Aspergillus* section *Circumdati* and Section *Nigri* species (*A. wetserdijkiae*, A*. ochraceus*, *A. steynii*; *A. carbonarius*) *in vitro* and on stored coffee beans in relation to ochratroxin A production under CC change conditions have been carried out (Akbar, 2015; Akbar et al., 2016). These suggest that for some species there may be a stimulation in ochratoxin A production which in other species there was a reduction in toxin contamination (Table 4). This suggests that differential effects may occur and that this needs to be taken account of in such experiments and possible predictions of the impact of CC factors on mycotoxigenic fungi.

Because of their ability to adapt to change, fungal species, especially mycotoxigenic ones, may become of primary concern in the coming 20-25 years. The changes in crop production under CC conditions may allow them to evolve rapidly because of their high degree of plasticity to benefit from changes in interacting environmental factors (Battilani et. al., 2012; Battilani et al., 2016; Vary et al., 2015; Vaughan et al., 2014). Thus, precise forecasting with regard to mycotoxigenic fungal populations and mycotoxin contamination and prevalence in the coming years needs to be more accurate and needs better inputs of factual data sets for better accuracy. However, such data is still very limited and is urgently required. The interactions between CO_2 x temperature and a_w may have differential effects which are related to both plant physiology and fungal pathogenic species involved. While for some fungal species growth or mycotoxin production remains similar under the forecasted conditions, for others environmental changes may have significant effects, e.g., increasing toxin production or a switch in the major mycotoxin produced or the ratio of different mycotoxins. Much more data is required to enable a better understanding of the fungal and plant ecophysiology and the pathogen/host interface to improve the potential for making more accurate and relevant global predictions on the impact on staple food crops. The available data supports the idea that a multifactorial modeling approach is necessary to understand the influence of CC scenarios will have on mycotoxigenic fungi and mycotoxin contamination of staple food crops (Medina et al., 2014; 2015a).

6. Conclusions and further research requirements

Toxin production/mycotoxin biosynthetic gene expression are not related to growth *per se*, so more research is needed to establish the potential effect of these factors and understand how gene expression is related to phenotypic toxin production. Considering the available information, several questions remain unanswered and research efforts are needed to improve our current understanding. Are CC factors going to change the toxin production patterns? Will other mycotoxins considered now as secondary become more abundant and thus more important in the future? Are the current control/mitigation strategies going to be effective in the future? Will the agricultural practices used currently (GAP; HACCP) in order to minimize toxin contamination be suitable when marked environmental changes become usual? Are mycotoxigenic fungal populations going to shift their location in the coming years? This may be related to pest populations and their movement globally. More research is urgently required to address these key questions to effectively predict the level of risk of different mycotoxins in economically important staple food crops and to understand whether they are resilient enough to tolerate the expected CC conditions. Existing global players in the agrifood market include countries such as Brazil and Argentina and parts of Asia including China and India. These and parts of Africa are considered hot spots for the impacts of climate change. Thus, from a food security perspective these questions need to be addressed for more accurate prediction of impacts of CC. Without this type of information food sustainability will be compromised in many regions of the world, with developing countries taking the brunt of the impacts possibly resulting in negative social consequences.

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Table 1. Statistical analyses of the significant effect of interactions between gene expression of 6 trichothecene biosynthesis genes, environmental factors with dexynivalenol production as the dependent variable (Schmidt-Heydt et al., 2011). Key: SIG, significant at P=0.05; NS, not significant.

Species:	***F. culmorum***	***F. graminearum***
Factor		
Water activity	NS	SIG
Temperature	SIG	SIG
TRI6	NS	NS
TRI10	NS	SIG
TRI4	NS	SIG
TRI5	SIG	SIG
TRI12	SIG	NS
TRI13	SIG	SIG

Table 2. Model validation outside of the experimental area for growth and aflatoxin B_1 production by *A. flavus* based on the mixed growth model (from Abdel-Hadi et al., 2012).

Factors		**Condition**		
Temperature (^oC)	37	37	37	40
Water activity (a_w)	0.9	0.95	0.99	0.9
Growth rate, □ (mm/day)	0.29	0.59	0.42	0.139
Observed aflatoxin production (ppm)	3.96±0.20	2.68±0.14	2.42±0.16	0.00
Predicted aflatoxin production (ppm)	4.90±0.00	3.75±0.18	3.78±0.14	0.00

Table 3. Summary of the impact that interactions between the three climate change variables have on relative expression of the structural and regulatory genes (*aflD*, *aflR*), and aflatoxin B_1 production by *Aspergillus flavus* when exposed to interacting climate change conditions on Yeast Extract Sucrose medium (from Medina et al., 2015a).
=: variation lower than 2-fold.
Numbers between brackets refer to the fold-variation with respect to the control

Temp (^oC)	a_w	CO_2 (ppm)	*aflD*	*aflR*	AFB_1
34	0.97	650	=	=	=
		1000	=	=	=
	0.95	650	=	=	=
		1000	=	↑(x3.6)	=
	0.92	650	=	↑↑(x24.4)	↑(x2.6)
		1000	=	↑(x2.0)	↑(x2.0)
37	0.97	650	↑(x4.6)	=	↑↑(x30.7)
		1000	↑(x6.5)	=	↑↑(x23.8)
	0.95	650	↑(x6.4)	↑↑(x14.6)	↑↑↑(x79.2)
		1000	↑(x3.2)	↑↑(x43.9)	↑↑↑(x78.5)
	0.92	650	=	↑↑(x40.4)	↑↑(x15.1)
		1000	↑↑(x22.5)	↑↑↑ (x1680)	↑↑(x23.8)

Table 4. Summary statistical table for growth rate and ochratoxin A production of 5 strains from the *Aspergillus* section *Circumdati* and section *Nigri* grown on coffee beans at different water activity x temperature conditions and in normal or elevated CO_2 (400 vs 1000 ppm) using the Kruskal-Wallis Test (non-normality data) and ANOVA (normality data; from Akbar et al., 2016).

	Temperature (30°C)		
	Factors		
Strains	**CO_2**	**Water activity (a_w)**	**a_w x CO_2**
A. westerdijkiae (B 2)	S^b	S^b	S^b
A. westerdijkiae (CBS 121986)	S^{*b}	NS^b	NS^b
A. niger (A 1911)	S^{*b}	S^b	S^b
A. carbonarius (ITAL 204)	NS^b	S^b	S^b
A. ochraceus (ITAL 14)	NS^a	S^a	N/A
	Temperature (35°C)		
A. westerdijkiae (B 2)	S^a	S^a	N/A
A. westerdijkiae (CBS 121986)	S^a	NS^a	N/A
A. niger (A 1911)	NS^b	S^b	NS^b
A. carbonarius (ITAL 204)	NS^a	S^a	N/A
A. ochraceus (ITAL 14)	NS^a	S^a	N/A

S significant (P<0.05); NS not significant (P>0.05); [a] Kruskal-Wallis test; [b] ANOVA
N/A Not Applicable

Figure legends

Figure 1. Heat maps for the relative expression of the trichothecene biosynthetic geen cluster for (a) *F. culmorum* and (b) *F. graminearum* in relation to water activity x temperature conditions on a conducive YES medium. The ligfhter the colour the higher the relative expression.

Figure 2. Two dimensional contour maps of the predicted DON (ppm) production in relation to expression of the (a) *TRI4*, (b) *TRI5* and (c) *TRI7* genes and water activity conditions for *F. culmorum* respectively, based on the polynomial model. The numbers on the isopleths refer to DON levels (μg g^{-1}).

Figure 3. Expression of (a) 10 aflatoxin genes of the aflatoxin biosynthetic gene cluster and (b) 9 fumonisin genes at different water activity x temperature conditions by *A.flavus* and *F.verticillioides* repectively on a conducive YES medium (from Abdel-Hadi et al., 2012; Medina et al., 2013).

Figure 4. Contour maps of the effect of water activity and temperature on growth and aflatoxin B1 production by *A. flavus* based on the mixed growth model developed in Abdel-Hadi et al. (2012).

Figure 5. Ternary diagrams of the relative gene expression of the two regulatory genes *aflS*, *aflR* and the structural gene *aflD* in relation to (a) water activity and (b) temperature (Abdel-Hadi et al., 2012).

Figure 6. Ternary diagram of the relative expression of FUM1, FUM14 and FUM 19 in relation and how they relate to the production of (a) fumonisin B1 and (b) fumonisis B2 based on the mixed growth model and the relative expression of different genes used (Medina et al., 2013).

(a)

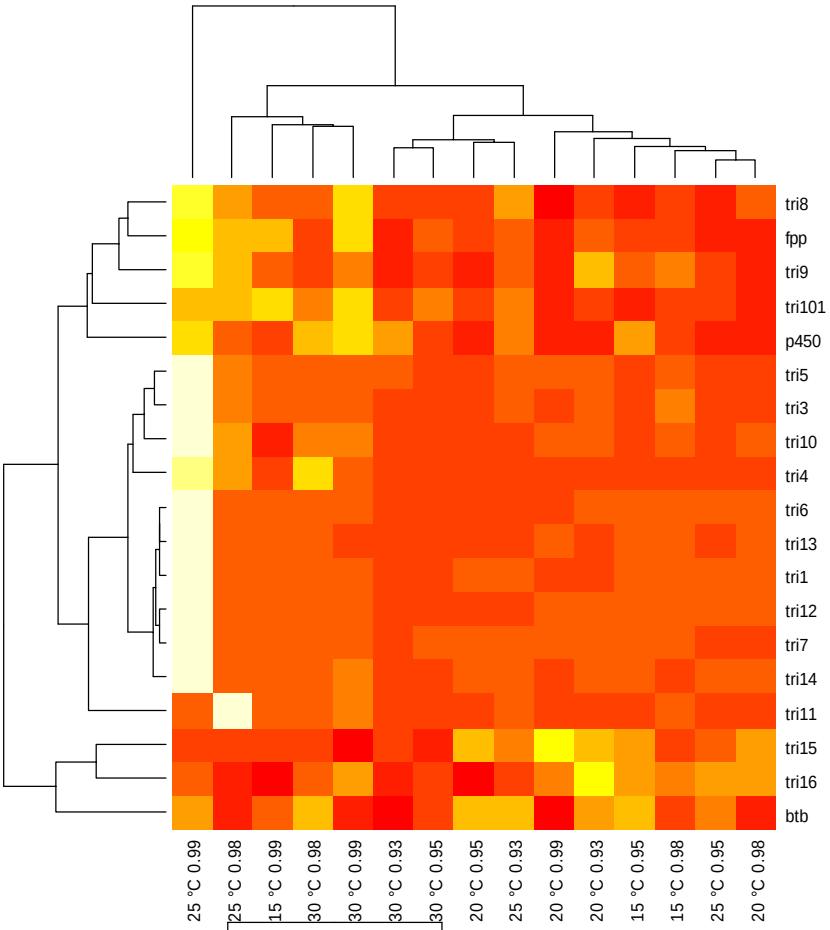
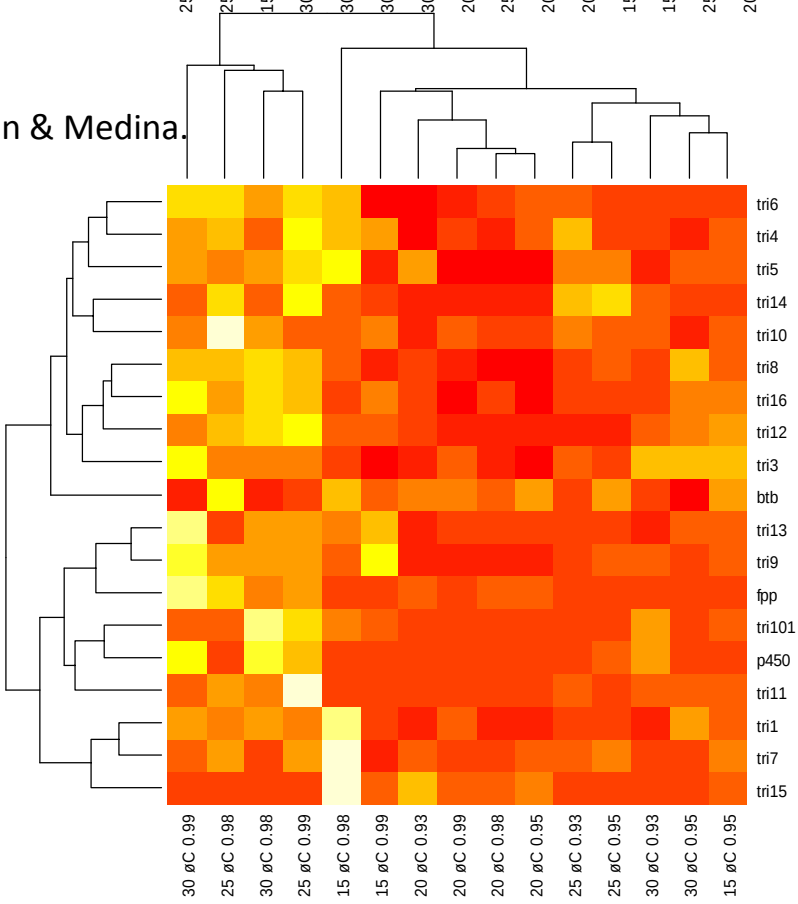


Figure 1. Magan & Medina.

(b)



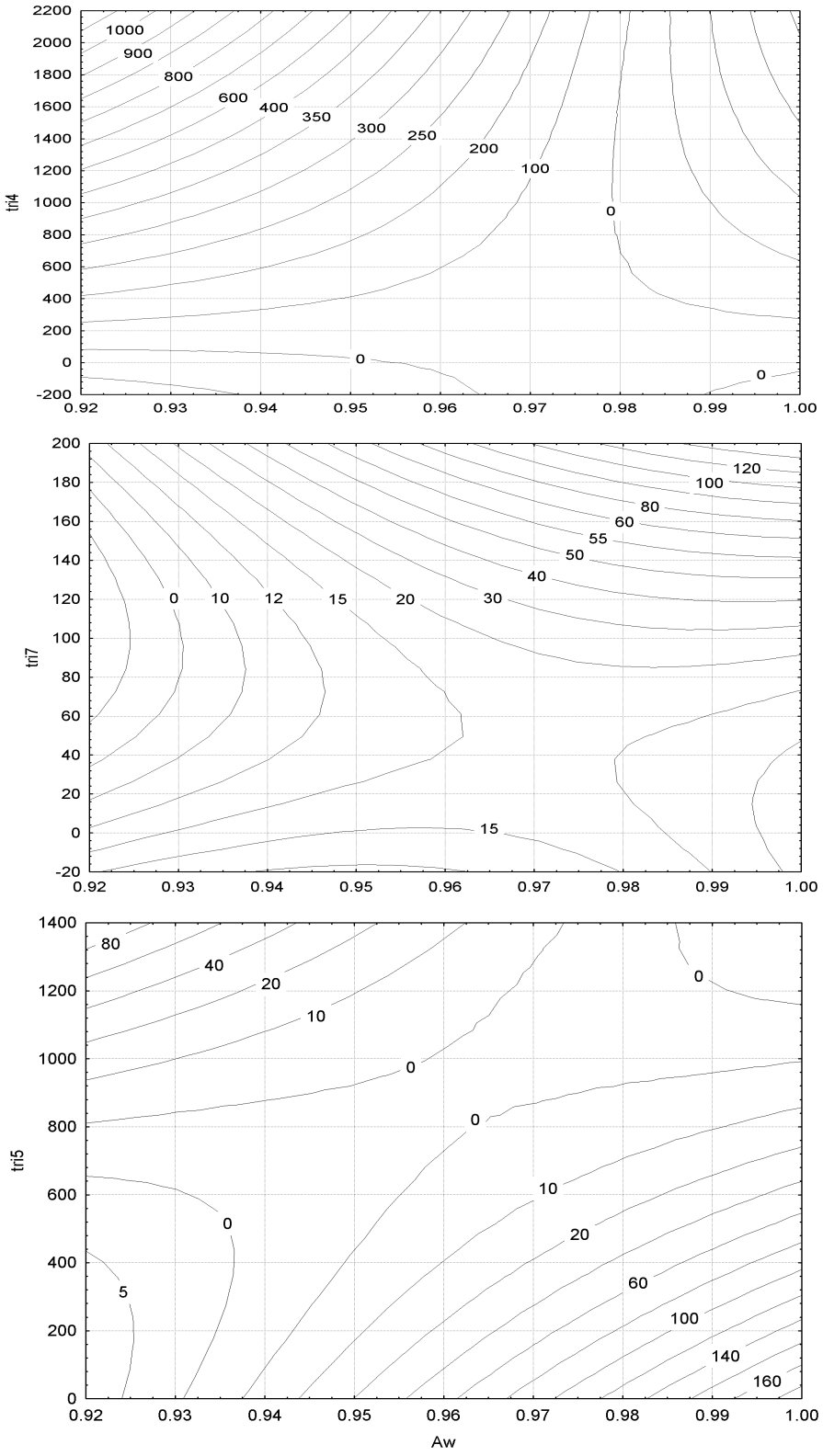


Figure 2. Magan & Medina

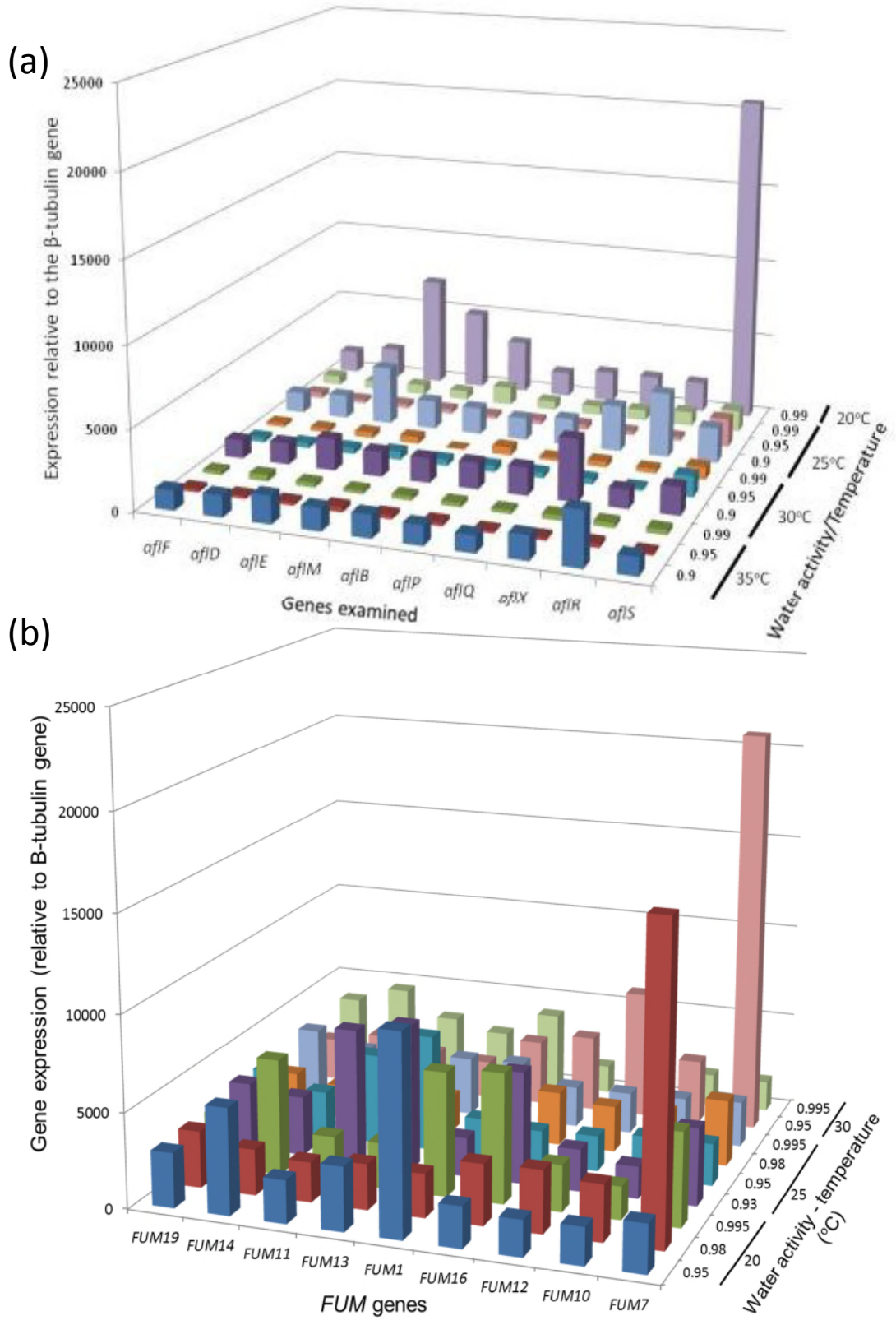
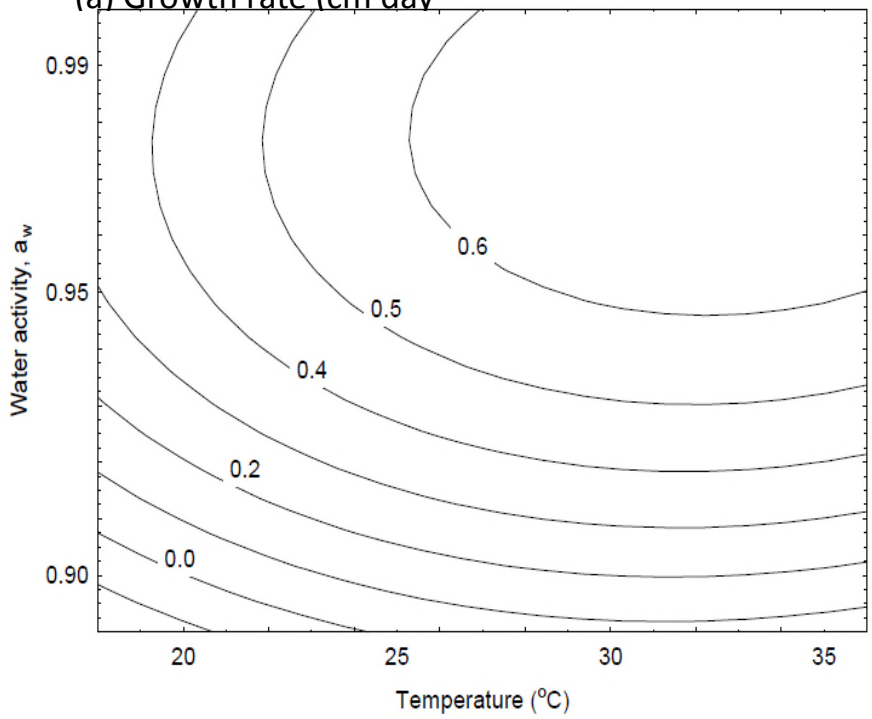


Figure 3. Magan & Medina

(a) Growth rate (cm day^{-1})



(b) Aflatoxin B_1 (µg g^{-1})

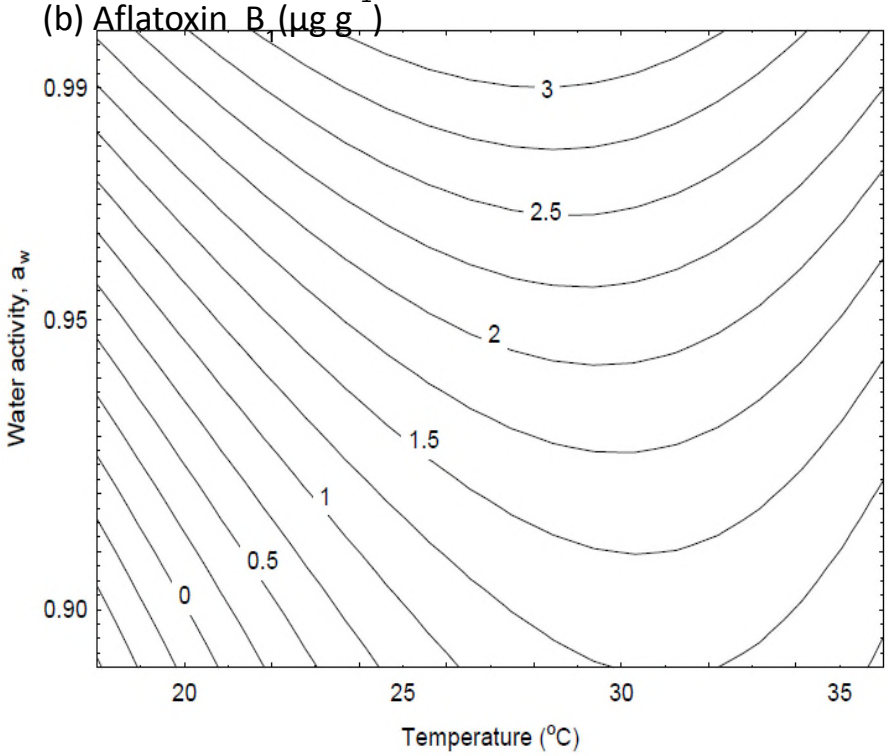


Figure 4. Magan & M<dina.

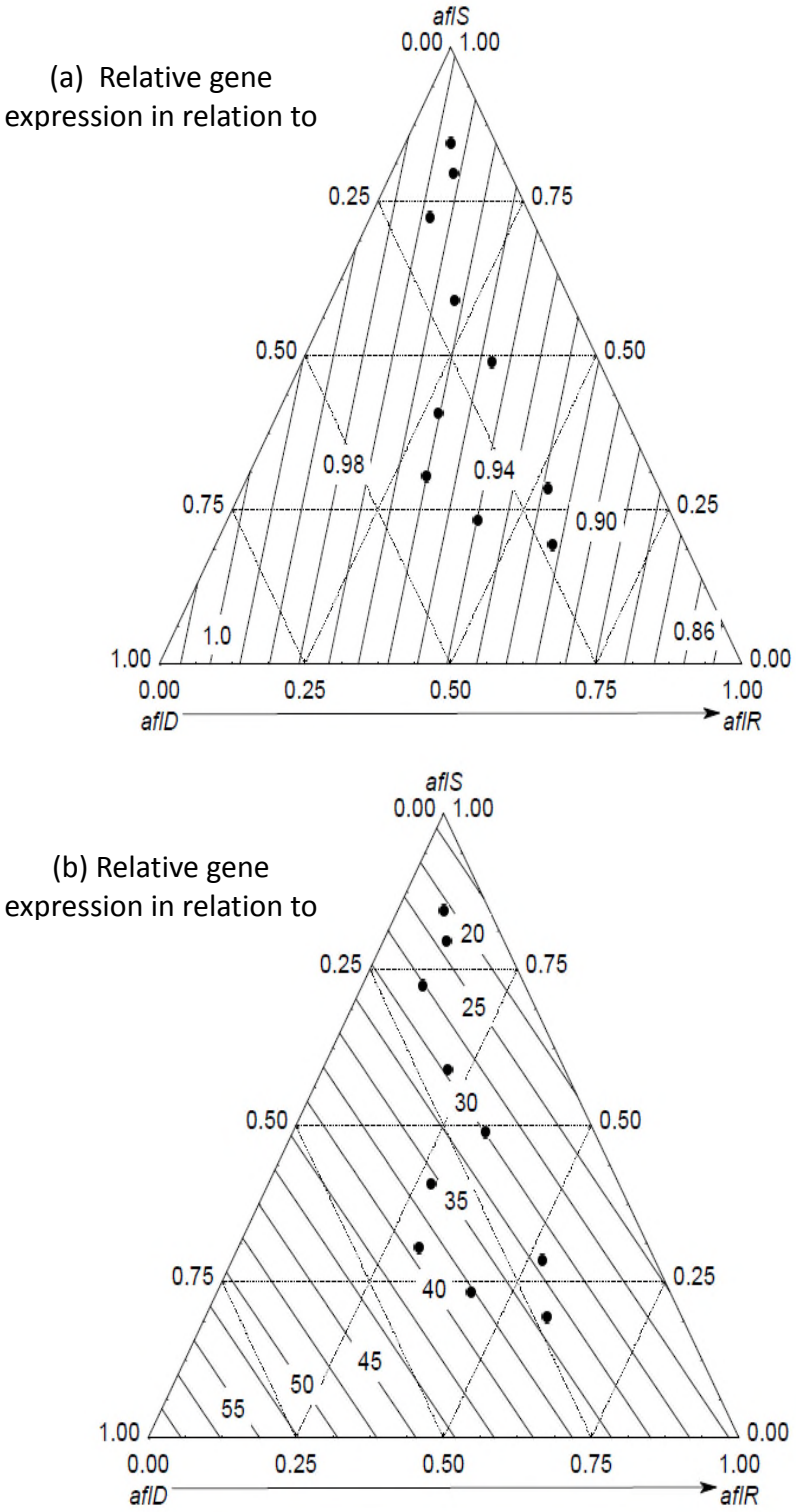
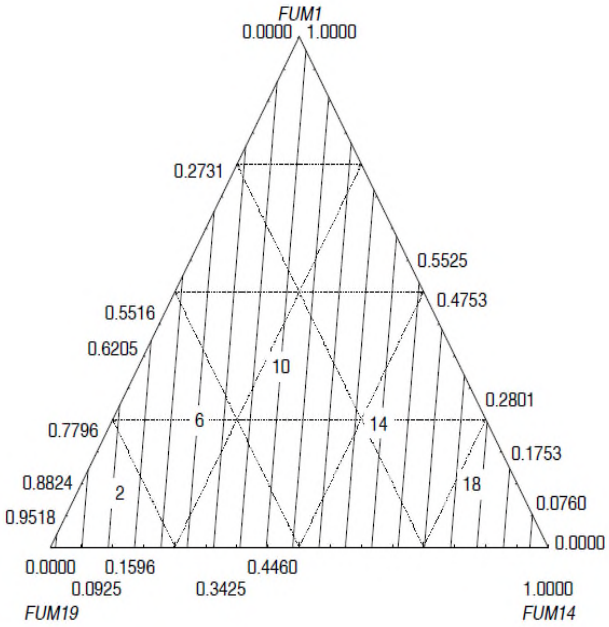


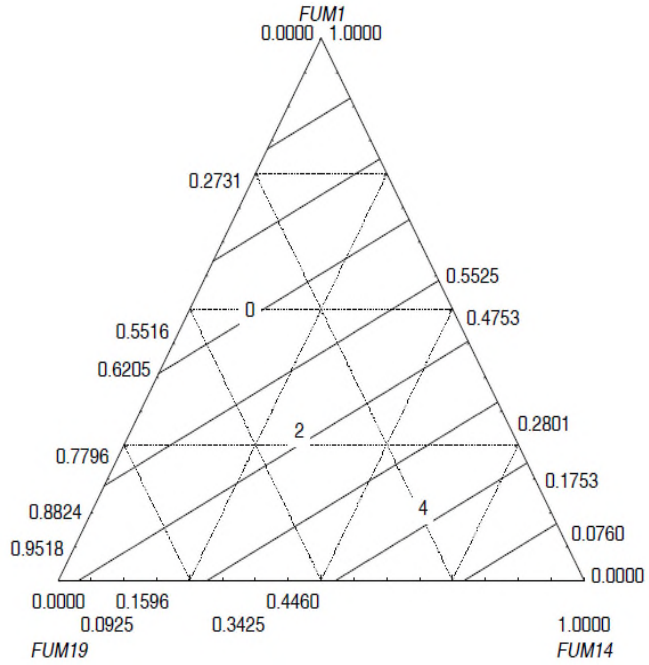
Figure 5. Medina & Magan

(a



FB_1 g/ml = 0.80*FUM19+19.70*FUM14+8.70*FUM1

(b



FB_2 g/ml =1.83*FUM19+5.92*FUM14-2.98*FUM1

Figure 6. Magan & Medina



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Integrating gene expression, ecology and mycotoxin production by Fusarium and Aspergillus species in relation to interacting environmental factors

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