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Modelling the relationship between environmental factors, transcriptional genes and deoxynivalenol mycotoxin production by strains of two *Fusarium* species

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Abstract

The effect of changes in temperature/water activity (a_w) on growth, deoxynivalenol (DON) production and trichothecene gene cluster expression (18 genes) for strains of *Fusarium culmorum* and *Fusarium graminearum* was studied. The expression data for 6 key transcription genes (*TRI4, TRI5, TRI6, TRI10, TRI12 and TRI13*) were analysed using multiple regression analyses to model the relationship between these various factors for the first time. Changes in a_w and temperature significantly (P=0.05) affected growth and DON. Microarray data on expression of these genes were significantly related to DON production for both strains. Multi-regression analysis was done and polynomial models found to best fit the relationship between actual/predicted DON production relative to the expression of these *TRI* genes and environmental factors. This allowed prediction of the amounts of DON produced in two dimensional contour maps to relate expression of these genes to either a_w or temperature. These results suggest complex interactions between gene expression (*TRI* genes), environmental factors and mycotoxin production. This is a powerful tool for understanding the role of these genes in relation to environmental factors and enables more effective targeted control strategies to be developed.

1. INTRODUCTION

Fusarium culmorum (W.G.Sm.) Sacc. and *Fusarium graminearum* Schw. (*Giberella zeae* (Schein.) Petch are the two most important Fusaria responsible for wheat scab in Europe and North America and responsible for the contamination of cereal grain with trichothecenes, especially deoxynivalenol (DON). This has resulted in legislative limits being set in raw and processed cereals for human food and animal feed for DON in many countries. Thus significant effort has gone into development of prevention strategies for minimising contamination of such commodities with DON (Aldred & Magan 2004).

The key environmental factors which influence germination, growth and the biosynthesis of DON and other trichothecenes have been demonstrated to include water availability and temperature (Magan *et al.* 2006). Indeed, detailed profiles have been developed for the effect of a_w, temperature and time on both DON and Nivalenol (NIV) production by strains of these species (Hope & Magan 2003; Hope *et al.* 2005). The

biochemical and genetic control of the biosynthetic pathways for these trichothecenes have been studied (Dejardins 2007; Alexander *et al.* 2009). This suggests that some of the key regulatory and transcriptional genes involved in trichothecene biosynthesis includes *Tri4* (a cytochrome P450 multifunctional monooxygenase which catalyses four steps in the conversion of trichodiene to isotrichodermin and trichothecene), *TRI5* (trichodiene synthase which catalyses isomerisation of farnesyl pyrophosphate to form trichodiene), *TRI6* (a zinc finger protein which is a transcription factor and positive regulator of the trichothecene pathway genes), *TRI10* (regulates transcription of *TRI6*), *TRI12* (trichothecene efflux pump which transports the metabolites out of the cell) and *TRI13* (a cytochrome P450 oxygenase). While a significant amount of work has been done on examining the *TRI5* gene in relation to biotic and abiotic factors (Dejardins 2007) practically no information is available on the effect of environmental stress on these other key genes in *F. culmorum* and *F. graminaerum*. A systems approach to try and relate the different parameters from a molecular to a phenotypic production of the secondary metabolite in relation to environmental factors has not been previously attempted.

A few studies have examined the influence of some abiotic stress factors in relation to the actual biosynthetic genes involved in mycotoxin biosynthesis (Feng & Leonhard 1998; Geisen 2004; Llorens *et al.* 2004; O'Brian *et al.* 2007; Schmidt-Heydt *et al.* 2007; Jurado *et al.* 2008; Schmidt-Heydt *et al.* 2008). These studies have confirmed that environmental factors do have an influence on gene activation and transcription. Complex relationships occur between such abiotic environmental factors and mycotoxin biosynthesis at the transcriptional level and that obtained when phenotypic mycotoxin production is quantified.

The recent development of a microarray which has sub-arrays for the gene clusters of key mycotoxigenic fungi has provided an excellent tool for examining in more detail the impact that changes in interacting environmental factors may have on relative expression of these gene clusters and relating this to phenotypic mycotoxin production (Schmidt-Heydt and Geisen 2007; Gardiner *et al.* 2009). Thus, the objectives of this study were to (a) examine the effect of a_w x temperature interactions on growth, DON production and relative gene expression of six key genes (*TRI4, TRI5, TRI6, TRI10, TRI12, TRI13)* in the *TRI* gene cluster using a mycotoxin gene microarray and (b) attempt to model the relationship between these *TRI* genes, environmental factors and DON production for represenatative strains of two species, *F. culmorum* and *F. graminearum.*

2. MATERIALS AND METHODS

2.1 Fungal strains

The strains used were from the culture collection of the Max Rubner-Institute, Karlsruhe, Germany. *Fusarium culmorum* strain BFE928 produces both DON and NIV and a strain of *F. graminearum* BFE1006, was a DON producer. They were both isolated from infected wheat grain. These were routinely maintained on a conducive yeast extract sucrose medium (YES, 20 g yeast extract l^{-1}, 150 g sucrose l^{-1}, 15 g agar l^{-1} in water) and incubated at 25^{o}C for seven days before use in experiments.

2.2 Modification of water activity of media, incubation and growth assessment.

The YES medium (0.995 a_w) was modified with glycerol/water solutions to different water activity levels (% w/v of YES medium: 0.98/13.1; 0.95/19.9; 0.93/24.5). The media were prepared in 9 cm Petri plates and allowed to cool. All treatments and replicate agar media were overlayed with sterile cellophane sheets (8.5 cm, P400, Cannings Ltd., Bristol, U.K.) before inoculation with a 3 mm agar disc from the growing margin of 7 day old cultures of

each species. This facilitated both growth measurements and removal of the fungal biomass for RNA extractions.

For measurement of the diametric mycelial growth rate, the diameter of the colony was measured in two directions at right angles to each other. The temporal increase in colony radius was plotted and the linear regression lines for the linear phase were used to obtain the relative growth rates (mm day^{-1}).

The plates were incubated at 15, 20, 25 and 30ºC for 9 days, and the experiment consisted of a fully replicated set of treatments with 3-4 replicates per treatment. The experiments were carried out twice to confirm results and also to ensure that enough biomass was available for RNA extraction and microarray analyses.

2.3 Isolation of RNA from samples.

To perform microarray and real time PCR experiments RNA was isolated using the RNAeasy Plant Mini kit (Qiagen, Hilden, Germany). An amount of 1 g of the mycelium was ground with a mortar and pestle in liquid nitrogen. About 250 mg of the resulting powder was used for isolation of total RNA. The powder was resuspended in 750 µl lysis buffer, mixed with 7.5 µl β-mercaptoethanol and 10 glass beads with a diameter of 1 mm (B. Braun Biotech International GmbH, Melsungen, Germany) in a 2 ml RNase free micro reaction tube. The extracts were mixed thoroughly and incubated for 15 min at 55 °C and 42 kHz in a S10H ultrasonic bath (Elma, Singen, Germany). All further procedures were essentially the same as suggested by the manufacturer of the kit.

cDNA synthesis. For cDNA synthesis 12 µl of the DNase I treated total RNA were used along with the Omniscript Reverse Transcription kit (Qiagen). The reaction mixture was essentially as described by the manufacturer (Qiagen, Hilden, Germany) and incubated at 37 °C for 1 h. The cDNA was stored at –20 °C.

*Microarray experiments.*The microarray used in this study has the mycotoxin genes for a number of fungi and includes two sub-arrays with the *TRI* genes for *Gibberella zeae* and *F. sporotrichoides*. This has been recently described in detail by Schmidt-Heydt and Geisen (2007). For labelling of cDNA an amount of 10 - 50 µg of the DNase I treated total RNA was used according to the specifications of the manufacturer of the Micromax cDNA Direct Labeling kit (Perkin Elmer Life and Analytical Sciences, Inc. Boston, USA). After cDNA synthesis and labelling, the cDNA was purified with a QiaQuick MinElute-97 Kit (Qiagen, Hilden, Germany). The labelled and purified cDNA was brought to dryness in a Speed Vac concentrator (Savant Instruments, Farmingdale, USA), suspended in 60 µl hybridization buffer (Scienion, Berlin, Germany), heated for 2 min. at 95 °C and hybridized for 18 h at 42°C to the microarray by using an automatic hybridization station (Perkin Elmer). After hybridization the array was scanned with a confocal laser scanner system (Scanarray lite, Perkin Elmer) at a resolution of 5 µm. The analysis of the results was performed using the Scanarray software (Perkin Elmer, Boston, USA). The results were normalized using the Lowess algorithm (locally weighted scatter plot smoothing) and the subtraction of the background signal intensity. As control, the constitutively expressed β-tubulin gene was used.

2.4 Quantitative determination of deoxynivalenol (DON) by HPLC.

Mycotoxin extraction and analyses was performed using a modified method of Cooney *et al.* (2001). Each sample (10 g) was homogenised and mixed well. The sample was extracted by mixing with acetonitrile/methanol (14:1; 40 ml) shaken for 2 h and then filtered through Whatman No. 1 (Whatman International Ltd, Maidstone, U.K.) filter paper. For analysis a 2-

ml aliquot was passed through a cleanup cartridge consisting of a 2-ml syringe (Fisher Ltd, 3Loughborough) packed with a disc of Whatman filter paper No. 1;, a 5-ml luger of glass wool and 300 mg of alumina/activated carbon (20:1, 500 mg). The column was washed with acetonitrile/methanol/water (80:5:15; 500 ml), and the combined eluate was evaporated (compressed air, 50^{o}C) to dryness and then resuspended in methanol/water (5:95; 500 µl).

Quantification of DON was performed using a Luna C18 reversed phase column (100 x 4.6 mm; 5 µm particle size; Phenomenex, Macclesfield, UK) connected to a guard column SecurityGuard (4 x 3 mm) filled with the same stationary phase. Separation was achieved using an isocratic mobile phase of methanol/water (12:88, v/v) at a flow rate of 1.5 ml min$^{-1)}$. Eluates (injection volume of 50 µl) were detected using a UV detector (Gilson 117, Anachem, Luton, U.K.) set at 220 nm with an attenuation of 0.01 AUFS. The retention time for DON was 13.3 min. Quantification was relative to external standards of 1 to 8 µg ml^{-1}) in methanol/water (5:95). The quantification limit was 5 ng g^{-1}.

2.5 Multiple regression analyses and statistical analyses.

The analysis was performed using a multiple regression analysis for the expression of six key *TRI* genes which are involved in the trichothecene biosynthetic pathway (*TRI6, TRI10, TRI4, TRI5, TRI12 and TRI13),* temperature and a_w conditions with DON concentration considered as the selected dependent variable. The microarray expression data for each gene were used to obtain a normalised relative expression value by dividing the actual copy number by the maximum recorded expression values for a particular gene (e.g. normalised *TRI4* expression = actual *TRI4*/maximum *TRI4* over all conditions).

These were used to examine the relationship between several independent or predictor variables and the dependent variable, in this case, DON concentration. The analysis which was applied to predict DON concentration based on the independent factors was a good tool to obtain vectors showing the significance of each factor on the independent variables. The model was used to predict the dependent Y-variable or response as a function of n > 1 independent of X-variables or predictors. STATISTICA software (version 6.0, StatSoft Inc., Tulsa, USA) was used for response surface regression of the data obtained. The statistical analysis of the model was performed in the form of an analysis of variance (ANOVA). This analysis included the Fisher's test (overall model significance), its associated probability P(F), correlation coefficient R, and determination of the R^2 coefficient measure the goodness of fit of the regression model. It also included the t-value for the estimated coefficients and associated probabilities. Non-linear models were examined but they did not provide a good fit to the data. The use of less parametres, e.g. 2-5 gene expression data were also evaluated and gave a worse fit.

3. RESULTS

3.1 Effect of water activity and temperature on growth

Figure 1 shows the effect of a_w on relative growth rates of *F. culmorum* (BFE928) and *F. graminearum* (BFE1006) strains on a conducive YES medium modified with the non-ionic solute glycerol. Overall, the highest growh was observed at 30^{o}C and 0.98 a_w for *F. culmorum* and 0.995 for *F. graminearum*. Similar growth was observed at 0.995 a_w, but only for *F. culmorum* at 25^{o}C. A significantly slower growth was observed at 15^{o}C for both strain over the a_w spectrum tested. At 30^{o}C the optimum a_w was 0.98, while at all other temperatures for both species this occurred when water was freely available (0.995 a_w). The limits for growth varied with temperature, but were between 0.93-0.95 a_w. For both strains

some growth was observed at 0.93 a_w and temperatures >15°C, but not at 0.90 a_w over the experimental period.

3.2 Phenotypic deoxynivalenol production and environmental factors

The effect of a_w x temperature conditions on DON production showed that there were differences between the two strains over the 9 day time scale (Figure 2). *F. culmorum* produced no DON at 15°C during this period but overall this strain produced significantly more than the *F. graminearum* strain used. For the latter species, although less DON was produced, the toxin was produced at 15°C and 0.995 a_w. Optimum DON production was at 20 and 25°C for *F. culmorum* and *F. graminearum* respectively. This was different from that for growth.

3.3 **TRI** *gene expression in relation to environmental factors*

Figure 3 summarises the relative expression of the six genes (*TRI4, TRI5, TRI6, TRI10, TRI12 and TRI13)* under the different environmental treatments used. This shows that there were different patterns of gene expression depending on abiotic conditions and species. Furthermore, the expression data for the strain of *F. culmorum* were relatively much higher than for the *F. graminearum* one reflecting the lower DON levels produced by the latter strain. The *TRI5* gene, a key one in the biosynthetic pathway of trichothecenes, was expressed over all the conditions examined. The expression data for all six key genes (*TRI4, TRI5, TRI6, TRI10, TRI12, TRI13)* were used for modelling the impact of environmental interactions on phenotypic DON production.

3.4 Modelling of the relationship between gene expression and DON production

(i) F. culmorum strain: In order to find the statistically significant interactions between factors, a model based on a polynomial equation fitting the experimental data for DON production in relation to the expression of the 6 *TRI* genes and the environmental parameters was developed. The determination co-efficient (R^2) was 0.9709. The regressed model is presented below:

$$\begin{aligned}\mathrm{DON}(\mu g\ g^{-1}) = {} & 5.85 + 0.216X_{a_w} - 1.1X_{T(^oC)} - 2.5X_{Tri\,6} + 4.03X_{Tri\,10} \\ & + 3.16X_{Tri\,4} - 2.01X_{Tri\,5} - 10.8X_{Tri\,12}\, 6.42X_{Tri\,13}\end{aligned} \quad \text{Eq. 1}$$

Where X = coded factor and the subscript name the factor in each term of the model. For the gene expression a standardised value was used defined as $X_{gen} = \frac{Actual\_value}{Maximum\_value}$ therefore the gene expression will be in the range of 0<X<1. The coded values for temperature and water activiy are the coded levels tested from the lowest (1) to the higest (4) values.

(ii) F. graminearum strain: The gene expression data for this species was also used for development of a model based on a polynomial equation which fitted the experimental data for DON production by *F. graminearum.* This had a multiple correlation coefficient (R^2) of 0.9542 . The regressed model is presented below:

$$\begin{aligned}\mathrm{DON}(\mu g\ g^{-1}) = {} & -5.16 + 1.262X_{a_w} + 1.054X_{T(^oC)} + 0.283X_{Tri\,6} - 7.8X_{Tri\,10} \\ & + 11.28X_{Tri\,4} + 11.0X_{Tri\,5} - 2.35X_{Tri\,12} - 8.22X_{Tri\,13}\end{aligned} \quad \text{Eq. 2}$$

Using these models the observed versus predicted DON concentration in relation to the expression of the 6 genes and a_w and temperature are shown in Figures 4a and b. This Figure suggests that while there is some under and over prediction the model does generally fit the experimental data, especially with the *F. culmorum* strain.

Table 1 shows the overall statistical fit of the model and the significance of the factors (*TRI* genes, a_w, temperature) in relation to DON production for the *F. culmorum* strain. This Table shows that DON production was statistically related to the experession of a number of *TRI* genes (e.g. *TRI5, TRI12, TRI13*). For the *F. graminearum* strain there appeared to be a much more co-ordinated expression of these *TRI* genes with both a_w and temperature, and expression of *TRI4, TRI5, TRI10* and *TRI13* all being statistically significant factors (Table 2a, b).

Figure 5 shows examples of two dimensional contour plots relating specific gene expression and a_w levels for both species to predicted DON production for *TRI5, TRI6* and *TRI13* as examples using the developed models. This shows clearly that under both a_w optimum and minima there is an increased expression of these genes and this can be related directly to predicted DON production levels. This shows that environmental stress can result in increased gene expression which in turn relates to phenotypic secondary metabolite production. For the *F. culmorum* strain (Figure 5, a, b, c) this is clear while for the *F. graminearum* one (Figure 5 d, e, f) less so, although there is a pattern for conditions conducive and non-conducive to DON production. The lower DON production by the strain of *F. graminearum* used in our experiments is reflected in the relationship between the expression of these three genes used as examples and the predicted DON production. Similar patterns were obtained with regard to the other *TRI* genes and for temperature (data not shown).

Figure 6 shows the predicted contour maps for relative DON production based on the available data and the model in relation to the key environmental factors of temperature and a_w. For both strains optimum was at 20-25^{o}C and >0.98 a_w.

4. DISCUSSION

This study is one of the first to attempt to relate phenotypic mycotoxin production to key *TRI* cluster gene expression in relation to a matrix of interacting environmental factors for strains of mycotoxigenic fungi. The growth of both strains was shown to be optimum at 30^{o}C and between 0.98-0.995 a_w. However, DON production was optimum at 20-25^{o}C over the 9 day experimental period. This is consistent with some of the previous studies relating a_w x temperature effects to growth and DON production for *F. culmorum* amd *F. graminearum* strains from Europe and Argentina (Hope & Magan 2003; Hope *et al.* 2005; Ramirez *et al.* 2006). Marin *et al.* (2004) showed that the temperature x a_w profiles for germination, growth and phenotypic fumonisin production by strains of *F. verticillioides* and *F. proliferatum* were also different. Similarly, for mycotoxigenic fungi such as *Aspergillus carbonarius* and ochratoxin A production differences were observed with optimum growth at 30-35^{o}C and 0.98 a_w while toxin production was optimum at 15-20^{o}C and 0.98-0.95 a_w (Belli *et al.* 2004; Mitchell *et al.* 2004). However, none of these previous studies attempted to relate specific expression profiles of key genes involved in the biosynthesis of the toxins to growth or phenotypic mycotoxin production. The only studies of this type were those recently reported for the effects of osmotic and matric potential on the kinetics of *FUM1* gene expression by *F. verticillioides* (Jurado *et al.* 2008), on *otapks*PV expression by *Penicillium verrucosum* in relation to suboptimal preservatives and environmental factors (Schmidt-Heydt *et al.* 2007) and the recent study which showed the effect of a_w x temperature conditions on mycotoxin

gene cluster activities for *P. nordicum*, *F. culmorum* and *A. parasiticus* using the microarray used in the present study (Schmidt-Heydt *et al.* 2008).

This study has shown that some of the key genes in the biosynthetic pathway for trichothecene production (*TRI4, TRI5, TRI6, TRI10, TRI12 and TRI13*) are markedly increased and influenced by interacting environmental conditions of temperature x a_w. The increased activity of the *TRI5* (sesquiterpene cyclase) and *TRI4* (cytochrome P450) is particularly important as they are key genes involved in the initial biosynthetic pathway for trichodiene synthase and trichodiene oxygenase. Other important genes downstream from these are those involved in regulation and transport (e.g. *TRI6* transcription factor; *TRI12*, superfamily transporter; *TRI10* regulatory gene) in both species (Desjardins 2007). Indeed, studies by Peplow *et al.* (2003) showed that the *TRI10* is a regulatory gene for four trichothecene pathway-specific genes in *F. sporotrichioides*. This may also be relevant to the *F. culmorum* strain used in our study as it also produces nivalenol.

In the present study we have only focussed on these 6 genes although the data for all the *TRI* genes were obtained using the microarray (data not shown). However, there are complex interactions between gene expression and phenotypic mycotoxin production when one considers the whole cluster of genes involved. A lack of correlation between relative gene expression and toxin production for some genes has been observed previously. For example, this has been found with the relationship for some genes involved in aflatoxin production (Scherm *et al.* 2005). They showed that expression of only a small number of aflatoxin biosynthetic pathway genes were directly coupled with aflatoxin biosynthesis. This complexity was recently demonstrated in work with a mycotoxin microarray (Schmidt-Heydt *et al.* 2009) which showed that there are two clusters of genes which were expressed in clusters and this was influenced by water availability. However, in the studies by Scherm *et al.* (2005) the effect of environmental factors was not considered.

The polynomial models were useful tools to try and unravel the complex correlations between these specific genes in the cluster and the range of interacting environmental parameters studied. The analysis showed that models can be developed to relate gene expression to secondary metabolite production and subsequently used to successfully predict DON production. Moreover, the models show that there may well be strain and perhaps species differences in terms of the patterns of expression of these 6 genes in relation to interacting environmental factors and phenotypic mycotoxin production. This would confirm the differences previously found for both temporal growth and DON production by strains of *F. culmorum* and *F. graminearum* (Hope *et al.* 2004). The next step will be to try and validate this model by using RT-PCR data for these specific genes and relate their expression to the predicted DON production we have observed in this study and then use other data sets to test the model.

Because of the nature of this type of study samples were taken after a specific time of 9 days as initial experiments suggested that this was the optimum for *TRI5* gene expression at 0.98 a_w. However, it may well be that as environmental stress is imposed there is a longer lag time before exponential growth occurs. Thus a wider time frame might need to be included for more detailed kinetic studies. Indeed, work with the *FUM1* gene expression by *F. verticillioides* suggests that under ionic water stress there was a decrease in growth but a significant increase in gene expression as a_w was reduced to 0.95 and 0.93 a_w over a 15 day period (Jurado *et al.* 2008).

We believe that a systems approach by integrating related molecular, ecological and secondary metabolite data can be a powerful tool for more targeted functional studies on the relationship between gene expression and phenotypic mycotoxin production in relation to interacting environmental conditions. Furthermore, it may enable more rapid studies to be carried out in identifying anti-fungal compounds which may inhibit specific key biosynthetic

and regulatory genes in these clusters which can be used for the development of improved prevention strategies to minimise mycotoxins in the food chain.

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Table 1. Regression analyses summary of (a) model fit and (b) statistically significant effect of interactions with DON as the dependent variable and the individual six genes and the two environmental factors for *F. culmorum.* The numbers in bold represent significant factors.
(a)

	SS	**Df**	**Mean**	**F**	***P*-level**
Regression	21.02170	8	2.627712	16.47913	0.000326
Residual	1.27566	18	0.159457		
Total	22.29735				

(b)

	B (regressed model coefficient)	**Std Error**	***P*-level**
Intercept	5.8499	1.181035	**0.001116**
Water activity	0.216	0.143956	0.172016
Temperature (^{o}C)	-1.0774	0.301641	**0.007276**
Tri 6	-2.5075	0.787163	0.012891
Tri 10	4.0330	1.263693	0.012775
Tri 4	3.1598	1.066495	0.018068
Tri 5	-2.0096	0.576268	**0.008232**
Tri 12	-10.8186	1.657092	**0.000183**
Tri 13	6.4161	0.914918	**0.000111**

Table 2. The regression analyses summary of (a) model fit and (b) the statistically significant effect of interactions with DON as the dependent variable and the individual genes and the two environmental conditions for *F. graminearum*. Numbers in bold are statistically significant.

(a)

	SS	**Df**	**Mean**	**F**	***P*-level**
Regression	232.8808	8	29.11010	22.89857	0.00033
Residual	22.8827	18	1.27126		
Total	225.7636				

(b)

	B (regressed model coefficient)	**Std Error**	***P*-level**
Intercept	-5.15931	0.990765	**0.000059**
Water activity	1.262	0.313627	**0.000798**
Temperature (°C)	1.05426	0.323034	**0.004314**
Tri 6	0.28255	1.872558	0.881742
Tri 10	-7.79699	1.425399	**0.000034**
Tri 4	11.27672	1.387046	**0.000000**
Tri 5	11.00253	2.094076	**0.000054**
Tri 12	-2.34974	1.179510	0.061748
Tri 13	-8.21629	1.378790	**0.000012**

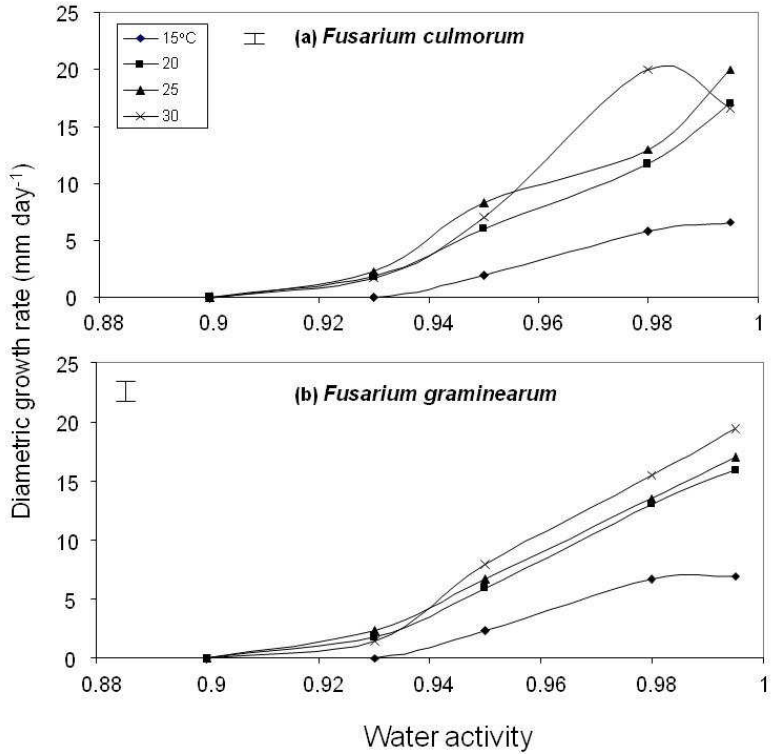

Figure 1. Effect of water activity and temperature on growth rates (mm day^{-1}) of *Fusarium culmorum* and *F. graminearum* strains on YES medium. Bars indicate Least significant differences (*P*=0.05).

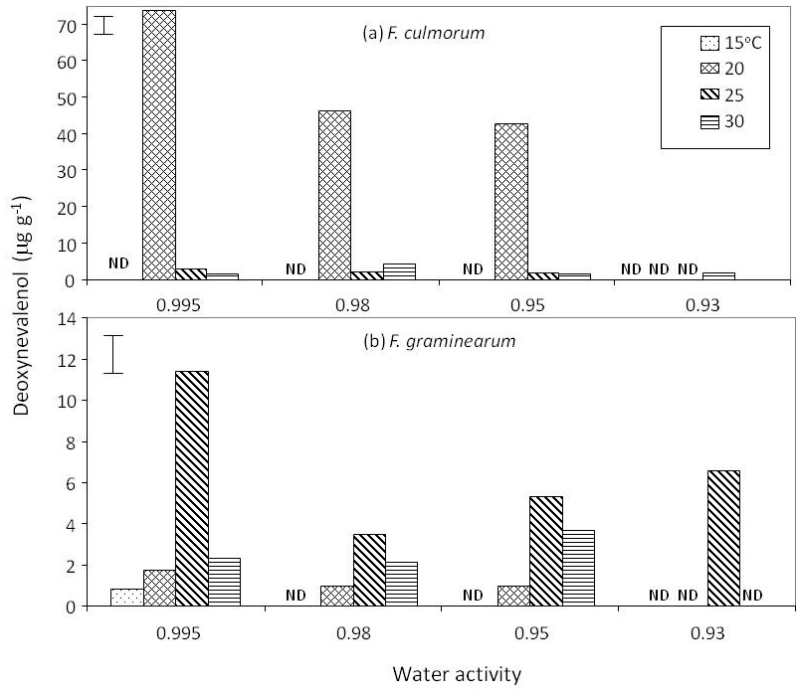

Figure 2. Bar chart of the effect of different water activity and temperature levels on deoxynivalenol production (µg g^{-1}) by (a) *F. culmorum* and (b) *F. graminearum* on a conducive YES medium after 9 days growth. Bars indicate Least significant difference (*P*=0.05). Key: ND, not detected.

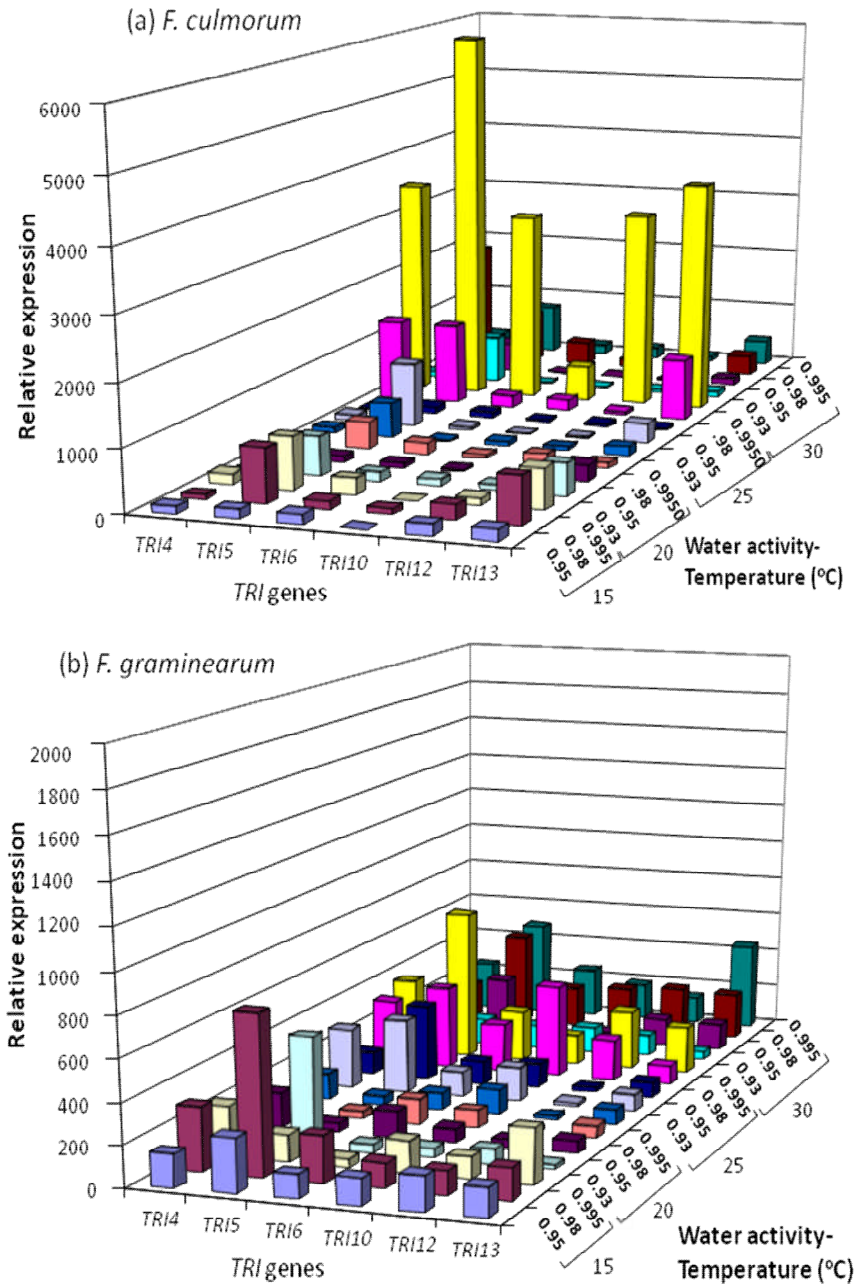

Figure 3. Relative *TRI* gene expression of the six genes analysed in relation to water activity and temperature treatments for (a) *F. culmorum* and (b) *F. graminearum* grown on YES medium for 9 days. The data is relative to the house keeping β-tubulin gene.

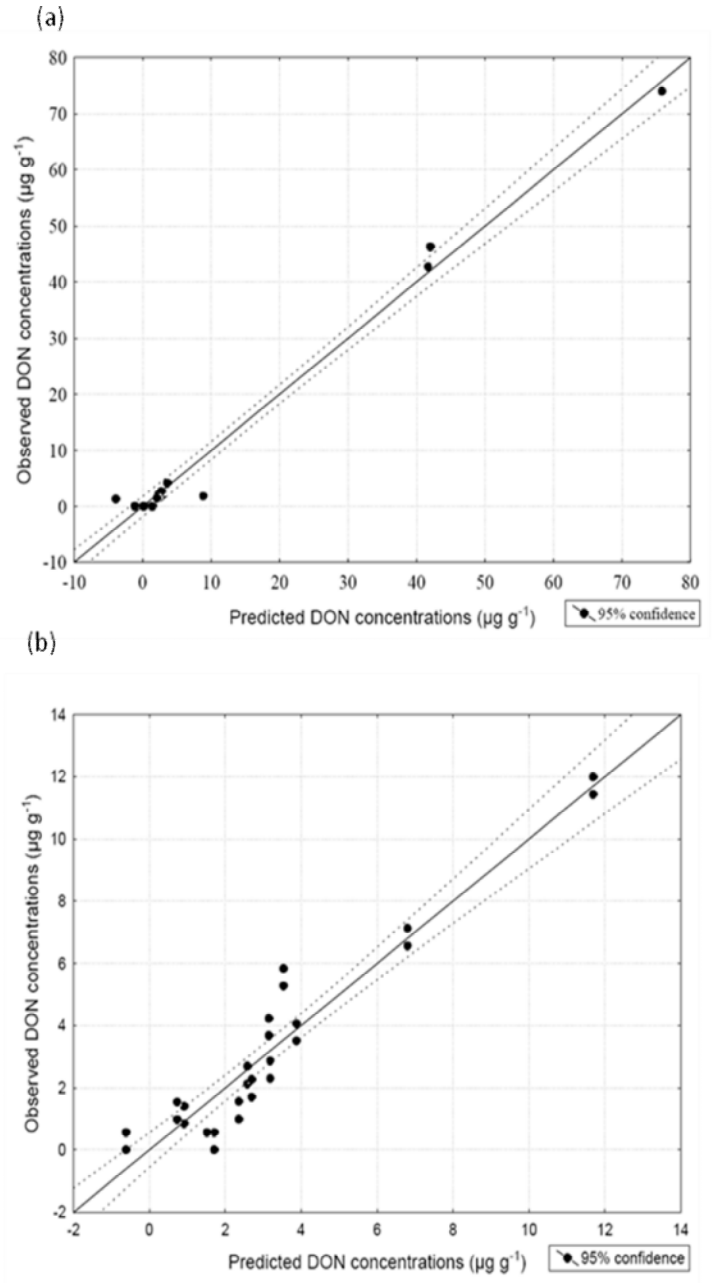

Figure 4. The correlations between the observed and predicted values based on the models developed for deoxynivalenol (µg g^{-1}) (a) *F. culmorum* and (b) *F. graminearum*. The broken lines indicate the 95% confidence limits. Points above or below the diagonal line represent areas of over or under prediction.

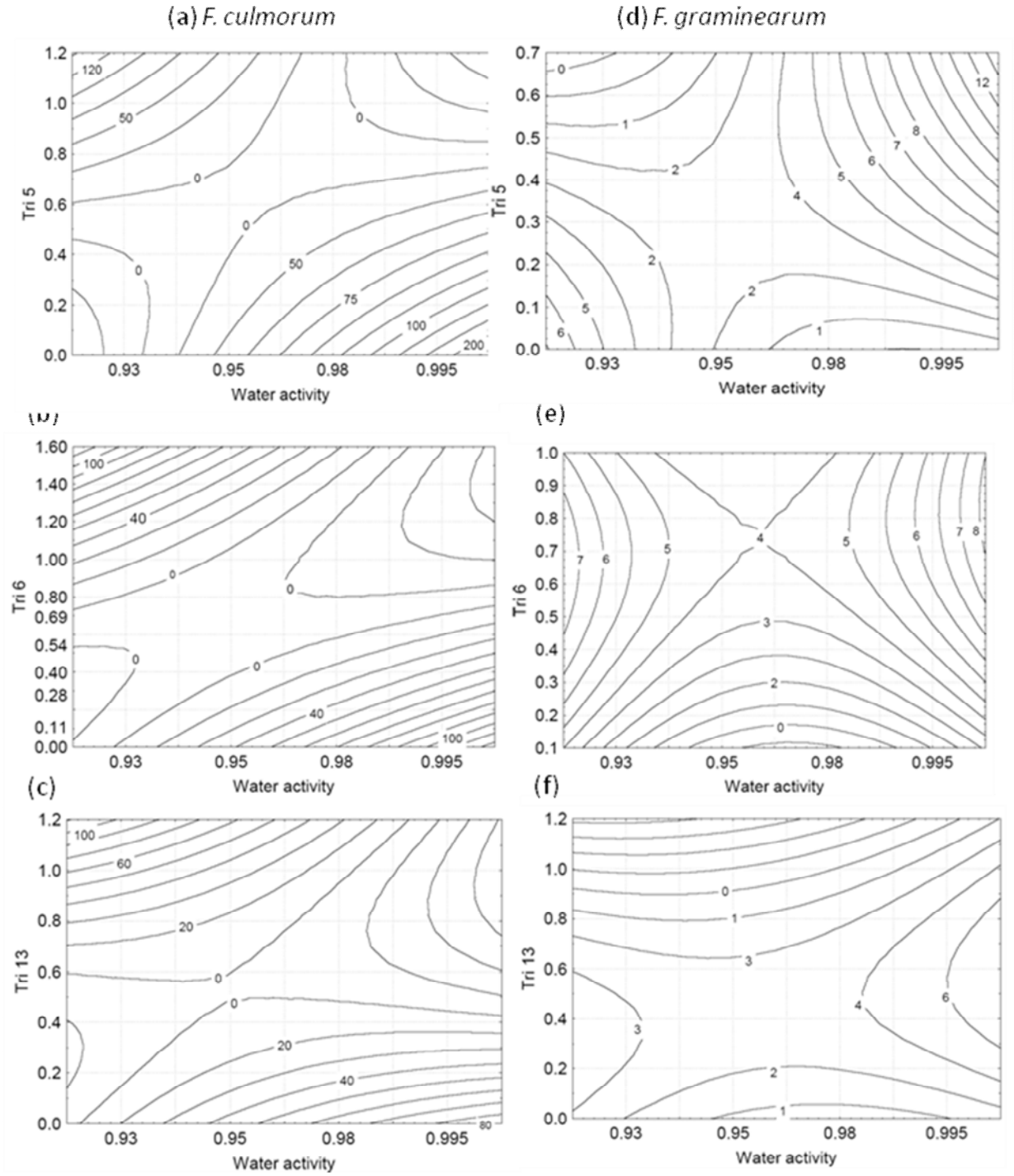

Figure 5. Two dimensional contour maps of the predicted DON (μg g^{-1}) production in relation to expression of (a) and (d) for *TRI5*, (b) and (e) for *TRI6* and (c) and (f) for *Tri13* genes and water activity conditions for *F. culmorum* and *F. graminearum* respectively, based on the polynomial model. The numbers on the contour maps refer to concentrations of DON (μg g^{-1}) based on the relationship between specific gene expression and a range of water activity levels.

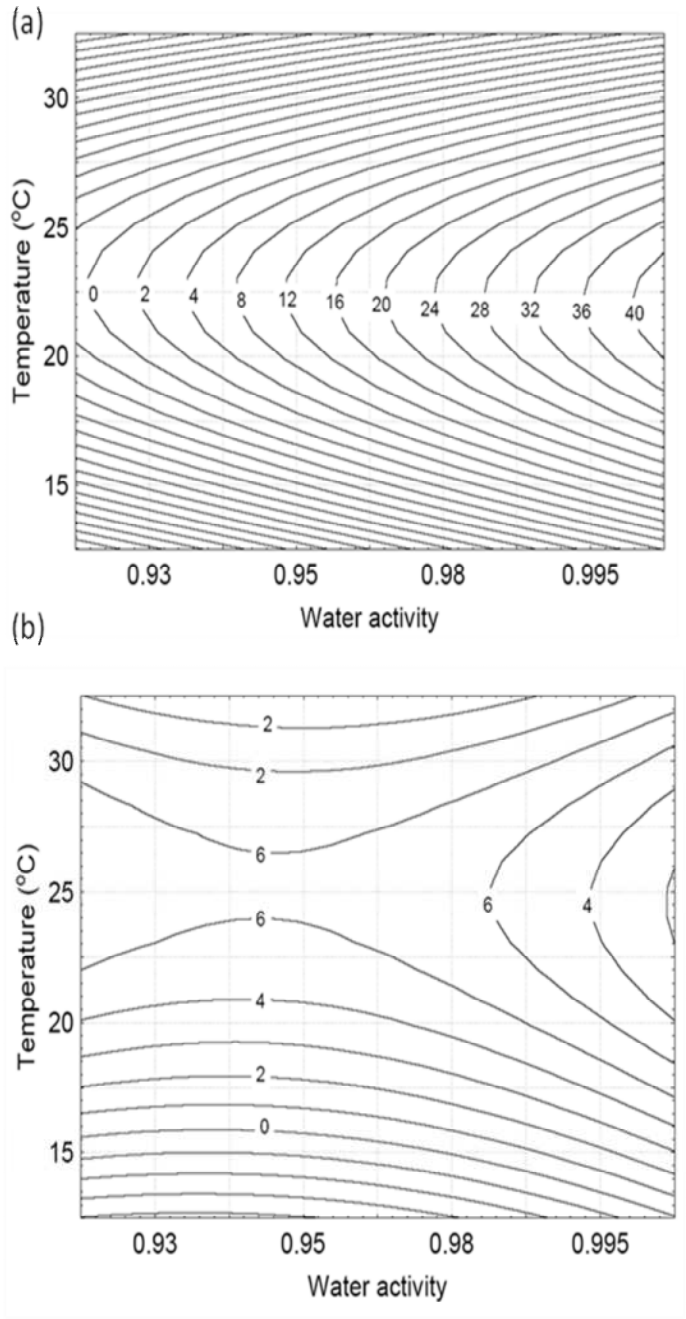

Figure 6. Predicted DON (μg g^{-1}) contour maps of production in relation to temperature and water activity by (a) *F. culmorum* and (b) *F. graminearum* based on the polynomial model developed in this study. Numbers on the contour lines refer to predicted DON concentrations.