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

M. Schmidt-Heydt1,2, R. Parra1,2,†, R. Geisen1,2 and N. Magan1,2,*

1 Applied Mycology Group, Cranfield Health, Cranfield University, Bedford MK43 0AL, UK
2Max-Rubner Institute, Karlsruhe, Germany

The effect of changes in temperature/water activity (aw) 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 six 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 aw 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 aw 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.

Keywords: environmental factors; temperature; trichothecene genes; microarray; deoxynivalenol; multiple regression analyses

1. INTRODUCTION

Fusarium culmorum (W.G.Sm.) Sacc. and Fusarium graminearum Schw. Gibberella zeae (Schein.) Petch are the two most important fusaria responsible for wheat scab in Europe and North America and are 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 the development of prevention strategies for minimizing contamination of such commodities with DON (Aldred & Magan 2004).

The key environmental factors that 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 aw, 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 has been studied (Desjardins 2007; Alexander et al. 2009). This suggests that some of the key regulatory and transcriptional genes involved in trichothecene biosynthesis include TRI4 (a cytochrome P450 multi-functional monoxygenase that catalyses four steps in the conversion of trichodiene to isotrichoderm and trichothecene), TRI5 (trichodiene synthase, which catalyses isomerization 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 (Desjardins 2007), practically no information is available on the effect of environmental stress on one of these key genes in F. culmorum and F. graminearum. 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’Brien et al. 2007; Schmidt-Heydt et al. 2007, 2008; Jurado 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 that has subarrays 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 the relative expression of these gene clusters and relating this to phenotypic mycotoxin production (Schmidt-Heydt & Geisen 2007; Gardiner et al. 2009). Thus, the objectives of this study were to (i) examine the effect of \( a_w \times T \) 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 (ii) attempt to model the relationship between these TRI genes, environmental factors and DON production for representative strains of two species, \( F. culmorum \) and \( F. graminearum \).

2. MATERIAL 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 is a DON producer. They were both isolated from infected wheat grain. These were routinely maintained on a conducive yeast extract sucrose (YES) medium (20 g yeast extract \( \times \) l\(^{-1} \), 150 g sucrose \( \times \) l\(^{-1} \), 15 g agar \( \times \) l\(^{-1} \) in water) and incubated at 25°C for 7 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 glycercol/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 overlaid with sterile cellophane sheets (8.5 cm, P400, Cannings Ltd, Bristol, UK) 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 d\(^{-1} \)).

The plates were incubated at 15, 20, 25 and 30°C for 9 days, and the experiment consisted of a fully replicated set of treatments with three or four 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 RNeasy 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 \( \mu l \) lysis buffer, mixed with 7.5 \( \mu l \) \( \beta \)-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 an S10H ultrasonic bath (Elma, Singen, Germany). All further procedures were essentially the same as suggested by the manufacturer of the kit.

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

2.3.2. Microarray experiments. The microarray used in this study has the mycotoxin genes for a number of fungi and includes two subarrays with the TRI genes for \( Gibberella zeae \) and \( F. sporotrichoides \). This has been recently described in detail by Schmidt-Heydt & Geisen (2007). For labelling of cDNA, an amount of 10–50 \( \mu g \) of the DNase I-treated total RNA was used according to the specifications of the manufacturer of the Micromax cDNA Direct Labelling kit (Perkin Elmer Life and Analytical Sciences, Inc., Boston, MA, USA). After cDNA synthesis and labelling, the cDNA was purified with a QiaQuick MinElute-97 kit (Qiagen). The labelled and purified cDNA was brought to dryness in a Speed Vac concentrator (Savant Instruments, Farmingdale, NY, USA), suspended in 60 \( \mu l \) 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 \( \mu m \). The analysis of the results was performed using the Scanarray software (Perkin Elmer). The results were normalized using the Lowess algorithm.

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(locally weighted scatter-plot smoothing) and the subtraction of the background signal intensity. As control, the constitutively expressed \( \beta \)-tubulin gene was used.

2.4. Quantitative determination of DON by HPLC

Mycotoxin extraction and analyses were performed using a modified method of Cooney et al. (2001). Each sample (10 g) was homogenized 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, UK) filter paper. For analysis, a 2 ml aliquot was passed through a clean-up cartridge consisting of a 2 ml syringe (Fisher Ltd, Loughborough, UK) 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°C) to dryness and then resuspended in methanol/water (5 : 95; 500 \( \mu \)l).

Quantification of DON was performed using a Luna C18 reversed-phase column (100 x 4.6 mm; 5 \( \mu \)m 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 \( \mu \)l) were detected using a UV detector (Gilson 117, Anachem, Luton, UK) 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 1–8 \( \mu \)g 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 that 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 normalized relative expression value by dividing the actual copy number by the maximum recorded expression values for a particular gene (e.g. normalized 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 (v. 6.0, StatSoft Inc., Tulsa, OK, 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 Fisher’s test (overall model significance), its associated probability \( P(F) \), correlation coefficient \( R \) and determination of the \( R^2 \) coefficient measure of the goodness-of-fit of the regression model. It also included the \( t \)-value for the estimated coefficients and associated probabilities. Nonlinear models were examined but they did not provide a good fit to the data. The use of fewer parameters, e.g. two to five sets of gene expression data, was also evaluated, which 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 growth was observed at 30°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°C. A significantly slower growth was observed at 15°C for both strains over the \( a_w \) spectrum tested. At 30°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 and 0.95 \( a_w \). For both strains, some growth was observed at 0.93 \( a_w \) and temperatures greater than 15°C, but not at 0.90 \( a_w \) over the experimental period.

3.2. Phenotypic DON production and environmental factors

The effect of \( a_w \times \) temperature conditions on DON production showed that there were differences between the two strains over the 9 day time scale (figure 2). Fusarium 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 summarizes 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...
Figure 1. Effect of water activity and temperature on growth rates (mm d\(^{-1}\)) of (a) *F. culmorum* and (b) *F. graminearum* strains on yeast extract sucrose (YES) medium. Bars indicate least significant differences (\(p = 0.05\)). Filled diamonds, 15°C; filled squares, 20°C; filled triangles, 25°C; crosses, 30°C.

Figure 2. Bar chart of the effect of different water activity and temperature levels on deoxynivalenol (DON) production (\(\mu g g^{-1}\)) by (a) *F. culmorum* and (b) *F. graminearum* on a conducive YES medium after 9 days’ growth. Bars indicate least significant difference (\(p = 0.05\)). ND, not detected. (a) Bars with dots, 15°C; bars with crosses, 20°C; bars with slant lines, 25°C; bars with horizontal lines, 30°C.
gene, a key one in the biosynthetic pathway of trichotheccenes, was expressed over all the conditions examined. The expression data for all six key genes (TRI4, TRI5, TRI6, TRI10, TRI12 and 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

3.4.1. Fusarium 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 six TRI genes and the environmental parameters was developed. The determination coefficient ($R^2$) was 0.9709. The regressed model is

$$
\text{DON} (\mu g g^{-1}) = 5.85 + 0.216 X_{w} - 1.1X_{\text{TRI}(C)} - 2.5X_{TRI6} + 4.03X_{TRI10} + 3.16X_{TRI4} - 2.01X_{TRI5} - 10.8X_{TRI12} - 6.42X_{TRI13},
$$

where $X$ is the coded factor and the subscript name the factor in each term of the model. For the gene expression, a standardized value was used, defined as $X_{\text{gen}} = \text{actual\_value}/\text{maximum\_value}$; therefore, the gene expression will be in the range of $0 < X < 1$. The

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 are relative to the house-keeping $\beta$-tubulin gene.
coded values for temperature and water activity are the coded levels tested from the lowest (1) to the highest (4) values.

3.4.2. *Fusarium graminearum* strain. The gene expression data for this species were also used for the development of a model based on a polynomial equation that 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

$$
\text{DON} (\mu g g^{-1}) = -5.16 + 1.262 X_{aw} + 1.054 X_{(T/C)} + 0.283 X_{TRI6} - 7.8 X_{TRI10} + 11.28 X_{TRI4} + 11.0 X_{TRI5} - 2.35 X_{TRI12} - 8.22 X_{TRI13}.
$$

(3.2)

Using these models, the observed versus predicted DON concentrations in relation to the expression of the six genes and $a_w$ and temperature are shown in figure 4a,b. This figure indicates 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 expression of a number of $TRI$ genes (e.g. $TRI5$, $TRI12$, $TRI13$). For the *F. graminearum* strain, there appeared to be a much more coordinated 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 2).

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 $a_w$ optima 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 5a–c) this is clear whereas for the *F. graminearum* strain (figure 5d–f) it is 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, the optimum temperature was at 20–25°C and greater than 0.98 $a_w$.

Figure 4. The correlations between the observed and predicted values based on the models developed for DON (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.

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°C and between 0.98 and 0.995 $a_w$. However, DON production was optimum at 20–25°C over the 9 day experimental period. This is consistent with some of the previous studies relating $a_w \times$ temperature effects to growth and DON production for *F. culmorum* and *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 $\times$ $a_w$ 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*, ochratoxin A production differences were observed with optimum growth at 30–35°C and 0.98 $a_w$ while toxin production was optimum at 15–20°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 \textit{FUM1} gene expression by \textit{F. verticillioides} (Jurado et al. 2008), on \textit{otapksPV} expression by \textit{Penicillium verrucosum} in relation to suboptimal preservatives and environmental factors (Schmidt-Heydt et al. 2007) and the recent study that showed the effect of a \textit{w}/C2 temperature conditions on mycotoxin gene cluster activities for \textit{P. nordicum}, \textit{F. culmorum} and \textit{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 (\textit{TRI4}, \textit{TRI5}, \textit{TRI6}, \textit{TRI10}, \textit{TRI12} and \textit{TRI13}) are markedly increased and influenced by interacting environmental conditions of temperature \textit{w}. The increased activity of \textit{TRI5} (sesquiterpene cyclase) and \textit{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 involved in regulation and transport (e.g. \textit{TRI6} transcription factor, \textit{TRI12} superfamily transporter, \textit{TRI10} regulatory gene) in both species (Desjardins 2007). Indeed, studies by Peplow et al. (2003) showed that the \textit{TRI10} is a regulatory gene for four trichothecene pathway-specific genes in \textit{F. sporotrichioides}. This may also be relevant to the \textit{F. culmorum} strain used in our study as it also produces NIV.

In the present study, we have focused only on these six genes, although the data for all the \textit{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 was 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 that 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

Figure 5. Two-dimensional contour maps of the predicted DON (µg g\(^{-1}\)) production in relation to expression of (a,d) TRI5, (b,e) TRI6 and (c,f) 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.
strains and species differences in terms of the patterns of expression of these six 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. 2005). 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 datasets 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 *TR15* gene expression at 0.98 $a_w$ 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 that may inhibit specific key biosynthetic and regulatory genes in these clusters that can be used for the development of improved prevention strategies to minimize mycotoxins in the food chain.

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