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Two-dimensional environmental profiles of growth, deoxynivalenol and nivalenol production by *Fusarium culmorum* on a wheat-based substrate

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ABSTRACT

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Aims: To determine the effect of interacting conditions of water activity (a_w, 0·99–0·85), temperature (15, 25°C) and time (40 days) on growth and production of the mycotoxins deoxynivalenol (DON) and nivalenol (NIV) by *Fusarium culmorum* on a wheat-based agar medium.

Methods and Results: *Fusarium culmorum* grew optimally at 0·995a_w and minimally at 0·90 at both 15 and 25°C. No growth was observed at <0·90a_w. Overall, temperature, a_w and their interaction had a statistically significant effect on the growth rate of *F. culmorum*. Production of both DON and NIV were over a much narrower range (0·995–0·95a_w) than that for growth. The highest concentrations of DON and NIV levels were produced at 0·995a_w and 0·981a_w at 25°C, respectively, after 40 days of incubation. Statistically, a_w, temperature and incubation time, and a_w × temperature and temperature × incubation time had a statistically significant effect on DON/NIV production.

Conclusions: This is the first detailed report on the two-dimensional environmental profiles for DON/NIV production by *F. culmorum* in the UK.

Significance and Impact of the Study: As part of a hazard analysis critical control point (HACCP) approach, this type of information is critical in monitoring critical control points for prevention of DON/NIV entering the wheat production chain.

Keywords: deoxynivalenol, *Fusarium culmorum*, nivalenol, temperature growth, water activity.

INTRODUCTION

Fusarium ear blight (FEB) is a cereal disease responsible for significant reduction in yield and quality of wheat grain throughout the world. FEB is a preharvest disease, but *Fusarium* species can grow postharvest if wet grain is not dried efficiently and quickly. In addition to the degradation in grain quality, *Fusarium* species produce an array of mycotoxins which contaminate the grain (Jennings *et al.* 2000; Magan *et al.* 2002). *Fusarium culmorum* is the most common cause of FEB in the UK and can produce deoxynivalenol (DON) and nivalenol (NIV). DON and NIV are harmful to both animals and humans, causing a wide range of symptoms of varying severity, and are possible immunosuppressants.

Attempts to control *F. culmorum* and other *Fusarium* spp. has relied on the application of fungicides preharvest coupled with effective storage regimes. However, the timing and application of these control measures are critical. For instance, some fungicides are ineffective against FEB, and in some cases result in a stimulation of DON/NIV production, particularly at suboptimal fungal growth conditions and low fungicide doses (D'Mello *et al.* 1999; Jennings *et al.* 2000; Magan *et al.* 2002). It has been shown that moisture conditions at anthesis is crucial in determining infection and mycotoxin production by *F. culmorum* on wheat during grain ripening. Very few studies have been carried out to determine the effect of key environmental factors such as available water and temperature, and time on growth and

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mycotoxin production. Some studies have identified the water activity (a_w) range for germination and growth of *F. culmorum* and other *Fusarium* species (Magan and Lacey 1984a,b). More recently, the effect of $a_w \times$ temperature effects were found to be significant for growth and fumonisin production by *F. verticillioides* and *F. proliferatum* which infect maize (Marín *et al.* 2000).

In the context of a hazard analysis, critical control point (HACCP) approach for controlling the entry of mycotoxins into the food chain, knowledge of the threshold limits for growth, and mycotoxin production are very important. HACCP schemes require detailed profiles of mycotoxigenic fungi in order to quantify hazards and identify critical control points within the production chain. The objective of this study was to examine in detail the impact of water availability, temperature and time on growth, and DON and NIV production by an isolate of *F. culmorum* on a wheat grain-based substrate.

MATERIALS AND METHODS

Fungal isolates and culture

A representative strain of *Fusarium culmorum* (98WW4.5FC; Rothamsted Research Culture Collection, Harpenden, Herts, UK) was chosen from a range examined previously and isolated from UK wheat grain, with a known history of mycotoxin production (Lacey *et al.* 1999; Magan *et al.* 2002). The strain produced mycotoxin levels similar to other strains from the UK and in Europe.

A 2% milled wheat grain agar (Tech. agar no. 3; Oxoid) was used as the basic medium. This was adjusted with glycerol in the range of 0·995–0·850a_w as described by Dallyn (1978). The equivalent moisture contents of the a_w treatments of 0·995, 0·98, 0·95, 0·90 and 0·85 were 30, 26, 22, 19 and 17·5% for wheat grain. Glycerol was used because of its inherent a_w stability over the temperature range of 10–40°C. Media were sterilized by autoclaving for 15 min at 120°C and cooled to 50°C before pouring into 90-mm Petri plates. The a_w of media was confirmed using an Aqualab machine (Decagon, Inc., Pullman, WA, USA). In all cases, the a_w levels were checked at both temperatures and were within 0·003 of the desired treatment level.

Inoculation and growth measurements

Twenty-four plates of each a_w treatment were inoculated centrally with a 5 μl drop of a 10^5-ml^{-1} *F. culmorum* macroconidial suspension obtained from a 7-day-old colony. Macroconidia were obtained by flooding culture with 5 ml of sterile distilled water containing 0·5% Tween-80 and agitating the colony surface with a sterilized glass rod. Plates of the same a_w were enclosed in plastic bags sealed and incubated at 15 or 25°C, for up to 40 days, together with blank agar plates of the same treatment. These were periodically checked and showed that the a_w remained the same over the experimental period. Temporal growth measurements were taken throughout the incubation period, by taking two diametric measurements of the colonies at right angles to each other. Growth rates were determined subsequently by linear regression of the radial extension rates. Three replicates per treatment were removed after 10, 20, 30 or 40 days and analysed for the mycotoxins DON and NIV. The experiment was repeated once.

Mycotoxin extraction and analyses

Mycotoxin extraction was adapted from Cooney *et al.* (2001). The entire agar and mycelial culture from each replicate sample was placed in 40 ml of acetonitrile/methanol (14 : 1) for 12 h and 2 ml was taken for DON and NIV analysis and passed through a cleanup cartridge consisting of a 2-ml syringe (Fisher Ltd, Loughborough, UK) packed with a disc of filterpaper (No. 1; Whatman International Ltd, Maidstone, UK), a 5 ml luger of glass wool and 300 mg of alumina/activated carbon (20 : 1). The sample was allowed to gravity-feed through the cartridge and residues in the cartridge washed out with acetonitrile/methanol/water (80 : 5 : 15; 500 μl). The combined eluate was evaporated (compressed air, 50°C) and then resuspended in methanol/water (5 : 95; 500 μl).

Quantification of DON and NIV was accomplished via HPLC, using a luna C18 reverse-phase column (100 × 4·6 mm i.d.) (Phenomenex, Macclesfield, UK). Separation was achieved using an isocratic mobile phase of methanol/water (12 : 88) at 1·5 ml min^{-1}. Eluates were detected using an u.v. detector set at 220 nm with an attenuation of 0·01 absorption units full scale (AUFS). The retention times for NIV and DON were 3·4 and 7·5 min, respectively. External standards were used to quantify DON and NIV. The level of quantification was 5 ng g^{-1} for DON and 2·5 ng g^{-1} for NIV.

Statistical analysis

The data were analysed using ANOVA (SigmaStat; SPSS, Inc., Chicago, IL, USA), with significance values of $<0{\cdot}05$. Excel 97 (Microsoft) was used for determination of growth rates by linear regression.

RESULTS

Effect of water and temperature effects on growth

Figure 1 shows the radial extension rates (K_r) determined for *F. culmorum* at the $a_w \times$ temperatures studied. The

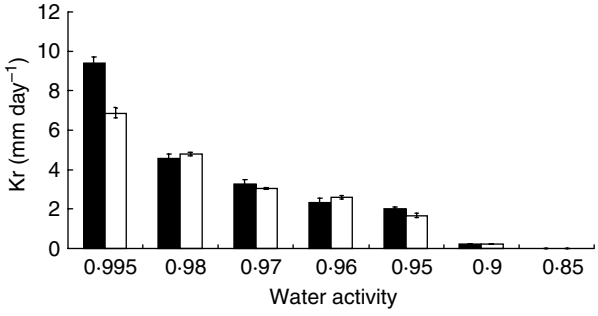

Fig. 1 *Fusarium culmorum* growth rates (K_r) on 2% milled wheat agar adjusted to different a_w levels and at 25°C (black) and 15°C (white)

highest K_r was at 0·995a_w and 25°C. There was no mycelial growth observed at <0·90a_w. Temperature generally affected growth with radial extension rates faster at 25°C than 15°C. This was most noticeable at 0·995a_w, while at lower a_w levels, temperature only affected growth slightly. Statistical analysis showed that a_w, temperature and their interactions significantly affected growth (data not shown).

Effect of water, temperature and time on DON and NIV production

Figure 2 shows the surface response curves for DON production at 15 and 25°C over a 40-day period of growth. The highest DON levels were obtained at 0·995a_w, 25°C after 40 days of incubation on the wheat-based medium. As a_w was reduced DON production rapidly declined. DON production was lower at 15°C than at 25°C and generally increased with incubation time to 40 days. However, the a_w range over which DON was produced by *F. culmorum* was very limited at 25°C (>0·981a_w). At 15°C, the a_w limit for DON production was 0·955a_w. Statistically, the effects of a_w, temperature, incubation time and their interactions were found to be significant (Table 1).

Figure 3 shows the production of NIV under the same treatment conditions. The highest NIV levels were obtained

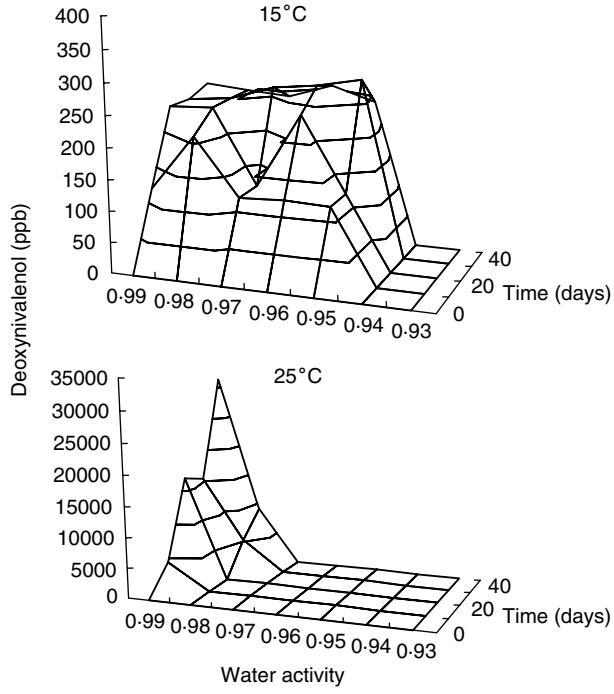

Fig. 2 Deoxynivalenol levels (p.p.b.) produced by *Fusarium culmorum* on 2% wheat agar adjusted to various a_w levels and at 15 and 25°C over a period of 40 days

at 0·981a_w and 40 days of incubation. NIV production was approx. 10 times lower than that of DON at 0·995a_w and 25°C. The a_w range over which NIV was produced was similar at both temperatures, although more NIV was produced at 15°C than 25°C. The a_w, temperature and incubation time had a statistically significant effect on NIV production (Table 2). The interactions between a_w × temperature and temperature × incubation time were also found to be significant. Incubation time × a_w and a_w × temperature × incubation time interactions did not have a statistically significant effect on NIV production.

Source of variation	DF	SS	MS	*F*	*P*
a_w	6	$9{\cdot}14 \times 10^8$	$1{\cdot}52 \times 10^8$	22·547	<0·001*
Incubation temperature	1	$2{\cdot}47 \times 10^8$	$2{\cdot}47 \times 10^8$	36·573	<0·001*
Incubation time	3	64 160 022	21 386 674	3·164	0·027*
a_w × incubation temperature	6	$9{\cdot}07 \times 10^8$	$1{\cdot}51 \times 10^8$	22·358	<0·001*
a_w × incubation time	18	$2{\cdot}4 \times 10^8$	13 318 528	1·971	0·017*
Incubation temp. × incubation time	3	62 325 982	20 775 327	3·074	0·031*
a_w × incubation temp. × incubation time	18	$2{\cdot}44 \times 10^8$	13 561 221	2·007	0·015*
Residual	112	$7{\cdot}57 \times 10^8$	6 758 320		
Total	167	$3{\cdot}44 \times 10^9$	20 570 943		

Table 1 Significance test of experimental factors' effect on production of deoxynivalenol by *Fusarium culmorum*. DF (Degrees of freedom); SS (Sum of squares); MS (Mean squares)

*Indicates that the factor elicited a significant effect ($P < 0{\cdot}05$).

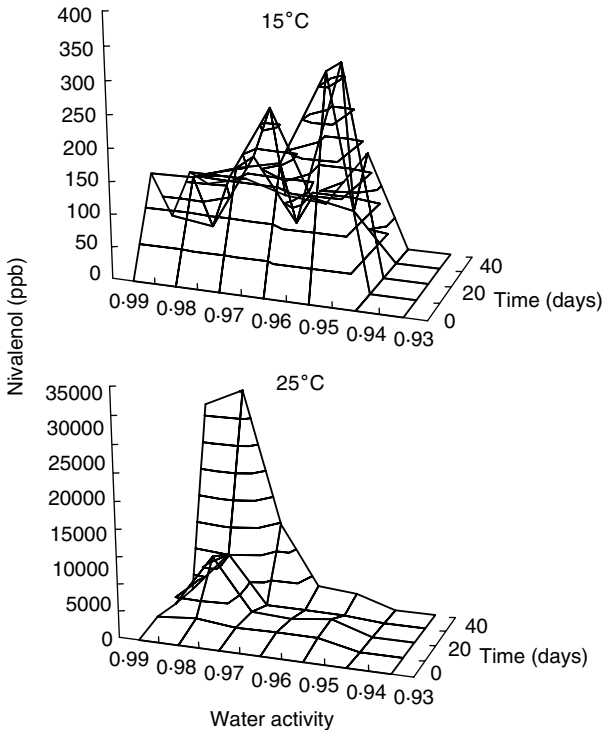

Fig. 3 Nivalenol levels (p.p.b.) produced by *Fusarium culmorum* on 2% wheat agar adjusted to various a_w levels at 15 and 25°C over a period of 40 days

DISCUSSION

This study has shown the dramatic effect that changes in a_w, temperature and time have on growth and DON and NIV production by *F. culmorum*. The a_w range and temperatures used in this study simulate those occurring in ripening grain (Magan and Lacey 1985) and harvested grain in a wet year (19–30%; 0·90–0·995a_w). This study has provided parallel information on growth and DON/NIV production for the first time. Overall, the highest DON and NIV production was at 25°C, 0·995a_w and 0·981a_w, respectively. These conditions were also optimum for growth. In this study, whole colonies were harvested, thus samples contained a gradation of culture age in a single sample. This may explain the early detection of mycotoxins in some treatments. The differential a_w and temperature optimum for NIV compared with DON production was interesting and suggests that *F. culmorum* may respond differently to $a_w \times$ temperature stress. The fungus may produce NIV under sub-optimal growth conditions for improving competitiveness. While it produces less NIV than DON, the former metabolite is more toxic than the latter.

Previous small-plot field-based studies using misting has demonstrated that in the UK *Fusarium* spp. infection and mycotoxin production was highest during wet periods in the summer (Lacey *et al.* 1999). Surprisingly, Birzele *et al.* (2000) suggested that DON was produced by *F. culmorum* at 0·80a_w in wheat grain. These are conditions under which *F. culmorum* and most other *Fusaria* are unable to grow (Magan and Lacey 1984b). This suggests that the accuracy of the a_w measurements may be doubtful in their study.

Previous studies on germination and growth of *F. culmorum* and other temperate *Fusarium* species has shown that a_w thresholds for germination was about 0·88a_w and growth limits were about 0·89–0·90a_w (Magan and Lacey 1984b, 1985). This study shows that the range of a_w conditions for DON/NIV production are much narrower than that for growth on a wheat-based medium. This information is very useful in monitoring critical control points, as it provides the necessary data on the threshold key environmental conditions for growth and mycotoxin production by *F. culmorum*.

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Table 2 Significance test of experimental factors' effect on production of nivalenol by *Fusarium culmorum*

Source of variation	DF	SS	MS	*F*	*P*
a_w	6	4 434 275	739 045·8	2·265	0·042*
Incubation temperature	1	4 039 240	4 039 240	12·381	<0·001*
Incubation time	3	2 951 526	983 842·1	3·016	0·033*
$a_w \times$ incubation temperature	6	5 626 451	937 741·9	2·874	0·012*
$a_w \times$ incubation time	18	5 302 771	294 598·4	0·903	0·576
Incubation temp. × incubation time	3	4 682 219	1 560 740	4·784	0·004*
$a_w \times$ incubation temp. × incubation time	18	5 010 894	278 383	0·853	0·635
Residual	112	36 539 414	326 244·8		
Total	167	68 586 790	410 699·3		

*Indicates that the factor elicited a significant effect ($P < 0·05$).

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