3-Acetyl deoxynivalenol production in a strain of *Fusarium culmorum* insensitive to the fungicide difenoconazole

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Abstract

The production of the mycotoxin, 3-acetyl deoxynivalenol (3-ADON), was investigated in a strain of *Fusarium culmorum* insensitive to the systemic fungicide, difenoconazole. On exposure to graded concentrations of the fungicide, the insensitive strain continued to synthesise 3-ADON when difenoconazole levels of 100 and 200µg/ml media were used. In contrast, a control (sensitive) strain ceased production of 3-ADON at difenoconazole levels of 100 µg/ml. Differences between the two strains were also observed for 3-ADON production with time. Following incubation with fungicide at 0.1µg/ml, 3-ADON production occurred more rapidly in CS than in IS cultures. This is the first report of increased persistence and alteration of the pattern of production of a mycotoxin following the development of fungicide insensitivity in a fungal phytopathogen.

Introduction

Fusarium species of fungi are widely acknowledged as important pathogens of economically important crop plants, particularly cereals. For example, fusarium ear blight of wheat, barley and oats has been linked with over 15 species of this genus (11). Although such diseases generally result in severe losses in yield, quality of harvested grain may also be reduced through contamination with *Fusarium* mycotoxins. These are a diverse group of secondary metabolites of the fungus which are endowed with toxic properties towards humans and animals (5). The most prominent *Fusarium* mycotoxins are the trichothecenes, zearalenone, moniliformin and the fumonisins (3). The trichothecenes are subdivided into four basic groups, with types A and B representing the most important members. The type A trichothecenes include T-2 toxin, HT-2 toxin, neosolaniol and diacetoxyscirpenol, while the type B group includes deoxynivalenol (DON) and its 3-acetyl and 15-acetyl derivatives (3-ADON and 15-ADON, respectively), nivalenol and fusarenon-X. The production of the two types of trichothecenes appears to be species-related. Thus, synthesis of type A trichothecenes predominates in *F. sporotrichioides*

and possibly also in *F. poae*, whereas production of type B trichothecenes is associated primarily with *F. culmorum* and *F. graminearum* (3). *F. culmorum* has been implicated definitively in fusarium ear blight of wheat (13) and partially in at least one recent episode of toxicosis in humans (5). A direct relationship between incidence of ear blight and DON contamination of wheat kernels was established in the study of Snijders and Perkowski (13). Although 3-ADON was not detected in the grain, two of the three strains of *F. culmorum* used in the trial produced this mycotoxin *in vitro*.

Fungicides have been used to control fusarium ear blight of cereals with somewhat indeterminate results (2). For example, in laboratory studies with pure cultures, dicloran, iprodione and vinclozolin were individually effective as inhibitors of diacetoxyscirpenol and zearalenone synthesis in *F. graminearum*. However, tridemorph and carbendazim each enhanced T-2 toxin production in *F. sporotrichioides*. In addition, other studies demonstrated that at levels non-inhibitory to growth, difenoconazole stimulated 3-ADON synthesis in *F. culmorum*, but the effect was temperature-dependent (4). Furthermore, in one field trial with *F. culmorum* a 16-fold increase in nivalenol content of grain was recorded following application of tebuconazole with triadimenol to a wheat crop (6). Whether or not the continued or ineffective use of fungicides has contributed to persistent reports of mycotoxin contamination of cereal grains (2) is a matter of debate. However, a further issue, on which there is currently no published data, relates to the effect of fungicide insensitivity and resistance on mycotoxin production in plant pathogenic fungi. In this paper, we report the results of a study designed to investigate the effects of difenoconazole insensitivity on 3-ADON production by *F. culmorum*.

Materials and Methods

Fungal isolates and difenoconazole treatments

A freeze-dried culture of *F. culmorum* 309344 from IMI was re-suspended in Ringers solution and grown on potato dextrose agar (PDA) in 9 cm Petri dishes which were then incubated at 25^0 C until growth was established. Peripheral plugs from these colonies were used to prepare 5d-old cultures also on PDA. A plug was removed from these 5d-old cultures and placed centrally on to 20 ml PDA containing difenoconazole at 100μg/ml. The fungicide was added as Plover (Ciba Agriculture), containing 250g active ingredient/l. Prior to addition, Plover was diluted in ethanol. Several inoculated plates prepared in this manner were incubated at 25^0 C for 21 days, after which time they were stored at 4^0 C for approximately 11 months. These cultures are designated as 'insensitive' for the purposes of this investigation. Control ('sensitive') cultures were prepared and stored in an identical process, except that equivalent volumes of ethanol without fungicide were added to the PDA prior to inoculation. After storage, control and insensitive cultures were used to prepare 5 d-old colonies on PDA alone for control cultures and on PDA + difenoconazole at 100μg/ml for insensitive strains. Plugs were removed as before and placed centrally on PDA containing difenoconazole at 0, 0.1, 100 and 200μg/ml to obtain a control and insensitive series in factorial combination. Inoculated petri dishes were incubated at 25^0 C for 8, 15 or 22 days.

Growth measurements

On day 8, 15 and 22 of the experiment, radial growth was assessed by measuring colony diameters in cm. Ten replicate cultures were taken for each fungicide treatment.

3-Acetyl deoxynivalenol determinations

On day 8, 15 and 22 of the experiment, entire colonies, including PDA, from two replicates were extracted with chloroform. The extracts were filtered and reduced in volume with a rotary evaporator, dried under N_2 and stored at -20° C prior to TLC analysis. For the determination of 3-ADON, the dried extracts were re-suspended in 100 µl chloroform and 5µl spotted on 20x20 cm TLC plates (silica gel 60, Cat. No. 5721, Merck). The plates were then developed in a toluene, ethyl acetate and formic acid mixture (volumetric proportions: 5, 4, 1 respectively), derivatized by dipping in aluminium chloride solution (1.5g $AlCl_3.6H_2O$ in 15 ml H_2O and 85 ml ethanol). A CAMAG Immersion Device III was used to dip plates which were then heated at 110° C for 10 min prior to examination under UV light. The presence of 3-ADON was evaluated visually by comparing R_f and colour development with pure standard added at 2.5 µg/5 µl spot. Quantification was performed with a CAMAG CD60 densitometer which, in addition, provided the means for positive identification of 3-ADON by spectral analysis. Two-dimensional TLC was also employed to identify and quantify 3-ADON in instances when fungal pigments interfered with chromatography. The lower limit of detection for 3-ADON was 0.02 mg/ml.

Statistical methods

Analysis of variance for a factorial design was performed using Minitab and significant main effects and interactions established by F tests. Assessment of significant differences between treatment means was determined using t tests according to standard procedures (8). Because the levels of 3-ADON were found to be very low for some treatment combinations and below the limits of detection for others, three intersecting analysis of variance were carried out on this variate. More appropriate standard errors for each mean could thus be calculated. Means were compared by t tests.

Results

Growth responses

It is clear from Table 1 that at all three stages of the experiment, growth of the insensitive strain (IS) of *F. culmorum* was superior to that of the control strain (CS) when both strains were subjected to difenoconazole concentrations of 100 and 200 µg/ml. The main effects of strain were highly significant, as were the main effects of fungicide concentration on growth ($P<0.001$). Thus, for all times and for both strains of *F. culmorum*, difenoconazole induced progressive reductions in growth, the effects being significant

(P<0.001) at the two highest levels of fungicide addition. The main effects of time on radial growth were highly significant and, in addition, there were significant interactions between fungicide concentration and strain, and between time and fungicide concentration

Tab 1 — Effects of incubation time and difenoconazole concentrations on radial growth of control (CS) and fungicide-insensitive strains (IS) of *Fusarium culmorum*

	Incubation time (days)						
	8		15		22		
Difenoconazole concentration µg/ml	CS	IS	CS	IS	CS	IS	Mean
		Radial	growth	(cm)			
0.0	6.4	6.8	8.3	8.0	8.3	8.3	7.7
0.1	5.8	6.9	8.2	8.0	8.3	8.3	7.6
100	1.4	4.5	3.0	7.1	3.8	7.8	4.6
200	1.2	2.5	3.1	4.6	4.2	6.7	3.7
SEM (df=207)				0.26			
Mean	3.7	5.2	5.6	6.9	6.1	7.8	

SEM (standard error of the mean) applicable to data in the body of the table.

(P<0.001). A complex interaction involving strain, time and fungicide level also occurred (P<0.01). Of particular interest is the observation that, while there were no significant strain differences (P>0.05) in growth on PDA without fungicide or with fungicide at 0.1 µg/ml, consistent strain differences were recorded at the two higher additions of difenoconazole for all three time periods. Thus, compared to relevant CS cultures, colony diameters were significantly (P<0.001) higher in all groups of the IS series treated with fungicide at 100 and 200µg/ml, irrespective of sampling time.

3-Acetyl deoxynivalenol production

Difenoconazole induced significant effects (P<0.01) on 3-ADON production (Table 2) irrespective of strain of *F. culmorum*. The overall means indicate that at 100 and 200µg/ml, difenoconazole induced a marked reduction (P<0.01) in 3-ADON production when the strain responses are combined. On days 15 and 22, the two highest doses of difenoconazole caused significant decreases (P<0.01) in 3-ADON production in both strains. Furthermore, a highly consistent feature of these reductions was the total elimination of 3-ADON synthesis in the CS series whereas in the IS series, mycotoxin production persisted, albeit at low levels, even when difenoconazole levels of 200µg/ml were used. Indeed, in further *F* and *t* tests 3-ADON values associated with IS treated

with fungicide at 100 μg/ml, were shown to be significantly different ($P<0.05$) from zero on day 8 and 15. The effect of time on 3-ADON production was significant ($P<0.01$) as were the interactions between time and fungicide concentration ($P<0.01$). In both CS and IS cultures without fungicide treatment, the increase in 3-ADON production with time was non-significant ($P>0.05$). However, in CS cultures subjected to difenoconazole at 0.1mg/ml, a significant enhancement ($P<0.01$) in 3-ADON synthesis occurred within 8 days, whereas in IS cultures this increase did not become significant ($P<0.01$) until 22 days had elapsed. When the data for all fungicide treatments are combined, the effects of time on 3-ADON accumulation are still apparent, with significant increases ($P<0.05$) on day 15 in both CS and IS cultures.

Tab 2 — Effects of incubation time and difenoconazole concentrations on 3-acetyl deoxynivalenol (3-ADON) production in control (CS) and fungicide-insensitive strains (IS) of *Fusarium culmorum*

	Incubation time (days)						
	8		15		22		
Difenoconazole concentration μg/ml	CS	IS	CS	IS	CS	IS	Mean
	3-ADON in culture extracts (mg/ml)						
0.0	1.11[1]	0.86[1]	3.93[1]	4.44[1]	3.40[1]	4.51[1]	3.04
0.1	0.69[1]	0.21[1]	6.91[1]	2.86[1]	6.03[1]	6.25[1]	3.82
100	0.00[2]	0.27[3]	0.00[2]	0.39[3]	0.00[2]	0.18[3]	0.14
200	0.00[2]	0.07[3]	0.00[2]	0.15[3]	0.00[2]	0.20[3]	0.07
Mean	0.45	0.35	2.71	1.96	2.36	2.78	

SEM (standard error of means):
[1]SEM=1.337 (df=11)
[2]SEM=0.000
[3]SEM=0.061 (df=5)

Discussion

The results of this study confirm the relative ease with which *Fusarium* species may develop insensitivity to a systemic fungicide such as difenoconazole. Hollomon et al (7) commented in a similar vein regarding the generation of benzimidazole-resistant strains of *F. graminearum* and *F. moniliforme* and further implied that such strains may occur in field populations of these phytopathogens. There is already substantial evidence of the

rapid development of fungicide resistance in *Botrytis cinerea* following the introduction of benzimidazoles, dicarboximides and N-phenylcarbamates (1). It is conceivable that fungicide insensitivity and resistance are factors which may contribute to the perceived ineffectiveness of fungicides in the control of some cereal diseases such as fusarium ear blight (9). However, an additional issue requiring attention relates to the possible effects of fungicide insensitivity and resistance on mycotoxin production in phytopathogenic fungi. The present results (Table 1) show that a difenoconazole-insensitive strain of *F. culmorum* grows more rapidly than a sensitive strain when further exposed to relatively high levels of the same fungicide. Of greater importance is the finding that fungicide insensitivity caused significant ($P<0.05$) changes in 3-ADON production (Table 2). Thus 3-ADON synthesis was consistently more persistent in the insensitive strain than in the control strain after each is subjected to difenoconazole at 100 and 200µg/ml media. Indeed, at these levels of fungicide, 3-ADON production in control cultures was reduced to undetectable values. It may be argued that 3-ADON production is related to growth and that the lack of mycotoxin synthesis in the control cultures, at the three points in time was due to inferior growth relative to that recorded for the insensitive strain. However, by day 22 growth of control cultures subjected to the two highest levels of difenoconazole was similar to or better than that of the insensitive strain on analogous treatments on day 8, but 3-ADON production only occurred in the insensitive strain. In contrast, addition of difenoconazole at 0.1µg/ml induced a more rapid accumulation of 3-ADON in CS cultures, with IS values maximising 22 days after incubaton (Table 2). Thus the pattern of 3-ADON production may be another distinguishing feature of fungicide insensitivity in *F. culmorum.*

At a level non-inhibitory to growth (0.1µg/ml), difenoconazole tended to enhance 3-ADON synthesis, more particularly in CS cultures on days 15 and 22 (Table 2). These effects are consistent with earlier observations (4) demonstrating enhancement of 3-ADON production 14 days after treatment of a sensitive strain of *F. culmorum* with difenoconazole. Taken together with previous data (4, 10, 12) the consensus now emerging is that at sub-lethal applications of fungicides, synthesis of both type A and type B trichothecenes may be enhanced. Although this inference is based on work with pure cultures, there is corroborating evidence from at least one field trial demonstrating increased nivalenol contamination of grain following application of tebuconazole with triadimenol to a wheat crop inoculated with *F. culmorum* (6).

Although the two highest doses of difenoconazole effectively reduced 3-ADON production in the sensitive isolates (Table 2), these observations do not abrogate the disquiet about the general efficacy of fungicides to control *Fusarium* infection and mycotoxin production (2, 6, 9). Consequently, attention has recently focused on the development of disease-resistant cereal genotypes to reduce trichothecene contamination of grain at harvest (13, 14).

It is accepted that difenoconazole is ineffective against fusarium ear blight of cereals. However, its efficacy towards *Septoria tritici* and rust diseases and its relative persistence implies that any residues may affect the secondary metabolism of those *Fusarium* species

which invariably occur on grain even in the absence of overt signs of ear blight (15).

In conclusion, this study has demonstrated the persistence of 3-ADON synthesis in a strain of *F. culmorum* insensitive to difenoconazole. In addition, there is some evidence that the pattern of 3-ADON production in this strain may be different from that in a control strain.

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