Mycotoxin production in a carbendazim-resistant strain of Fusarium sporotrichioides

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Abstract

Mycelial yield and production of three trichothecenes, namely T-2 toxin, diacetoxyscirpenol (DAS) and neosolaniol (NEO) were compared in control (CS) and carbendazim-resistant strains (RS) of *Fusarium sporotrichioides*. Each strain was exposed to graded concentrations of carbendazim (0, 1, 2, and 4 μg/ml media) for 2, 5 and 7 days under shake-culture conditions at an incubation temperature of 25°C. Mycelial yield was significantly ($P<0.001$) affected by strain, carbendazim concentration and incubation time. The strain differences in mycelial mass at 2 days ($P<0.05$) became more pronounced at 5 and 7 days of incubation ($P<0.001$). However, mycelial growth differences between the two strains were greatest following exposure to carbendazim, with the effects becoming more divergent with time. Combined results for the three incubation times showed dose related effects in carbendazim inhibition of T-2 toxin production by CS isolates. In contrast, RS cultures exposed to the 2 μg/ml addition of carbendazim significantly increased T-2 toxin production ($P<0.05$ or better). At 1 and 4 μg/ml additions, T-2 toxin inhibition occurred but the effect was less marked than in the CS series. RS yielded more DAS than CS at 5 days ($P<0.05$) and at 7 days ($P<0.01$) of incubation. The major component of this strain difference arose from the effects of the 2 μg/ml addition of carbendazim ($P<0.01$). NEO production was also higher in RS than in CS, with the difference becoming progressively more pronounced from day 5 ($P<0.05$) to day 7 ($P<0.01$) of

incubation. However, these differences reflected enhanced NEO output with carbendazim addition of 4 μg/ml ($P<0.05$) in day 5 extracts and of both 2 μg/ml ($P<0.01$) and 4 μg/ml additions ($P<0.05$) in day 7 samples. Moreover, the ratio of NEO to T-2 toxin production was affected by an interaction involving incubation time, strain and carbendazim dose ($P<0.05$ or better). On day 5, this ratio was greater in CS exposed to 2 μg/ml, but at 4 μg/ml, the ratio was higher in RS. It is concluded that carbendazim resistance induced genuine differences in the synthesis of T-2 toxin and NEO. It is suggested that the strain difference may reside in the conversion of NEO to T-2 toxin which may be sensitive to fungicide concentration. This would imply that carbendazim resistance induces changes in the terminal rather than initial phases of trichothecene biosynthesis.

Introduction

The genus Fusarium contains many plant pathogenic species that are responsible for disease and yield losses in economically important crops. Thus 15 species have been associated with fusarium head blight (FHB) of cereal plants. A major issue arising from FHB is contamination of harvested grains with Fusarium mycotoxins, including trichothecenes, zearalenone and fumonisins. Both *F sporotrichioides* and *F poae* have been implicated in FHB of wheat (1) and may be responsible for contamination of grain with type A trichothecenes, namely T-2 toxin, diacetoxyscirpenol (DAS) and neosolaniol (NEO) (2). Indeed, T-2 toxin and DAS generally co-occur with type B trichothecenes, particularly deoxynivalenol (DON), produced by other FHB agents including *F graminearum* and *F culmorum* (3), suggesting contamination of cereal crops with more than one Fusarium species.

Recent laboratory and field studies have emphasised the variable efficacy of fungicides in controlling FHB and mycotoxin contamination of cereal grains (4). On the contrary, there is some evidence that certain fungicides may enhance mycotoxin production (4). It has been suggested that development of fungicide resistance by Fusarium phytopathogens might adversely affect fungicide efficacy for mycotoxin control. Such a hypothesis would be difficult to test under field conditions and the absence of relevant data reflects the formidable practical problems associated with such an investigation. However, in laboratory cultures of *F culmorum*, D'Mello et al (5, 6) showed that resistance to difenoconazole induced increased persistence of 3-acetyl DON production compared to a control strain of the pathogen. These strain differences were apparent at each of the three sampling times during the 22-day experimental period. In addition, there was some evidence that the pattern of 3-acetyl DON formation in the resistant culture was different to that in the control strain.

Since the question of fungicide resistance is an important commercial issue, it is relevant to enquire whether the observations of D'Mello et al (5, 6) with type B trichothecene producers are applicable to those Fusarium pathogens with the capacity

to synthesize type A trichothecenes. Past experience in our laboratory (2) indicates that *F sporotrichioides* is an appropriate candidate organism for such an investigation since it readily produces T-2 toxin, DAS and NEO in pure culture. A study with this fungus would also provide an opportunity to examine whether the ratios of the three trichothecenes are altered by the development of fungicide resistance. In addition, T-2 toxin production can be enhanced by fungicide treatment (2) and it is conceivable that the development of resistance might modulate this property. The question also arises as to whether our previous results (5, 6) with difenoconazole would be replicated with other fungicides, such as carbendazim. Accordingly, the experiment described in this paper was conducted to examine mycotoxin production in a carbendazim-resistant isolate of *F sporotrichioides*. An attempt was also made to establish more clearly the relationship between fungal growth and mycotoxin production in control (CS) and resistant (RS) strains. Growth was, therefore measured as mycelial mass instead of colony diameters (5, 6).

Materials and methods

Carbendazim

Carbendazim (98% purity) was supplied by BASF. Due to its particular solubility characteristics, the fungicide was dissolved in ethanol prior to use in the development of carbendazim-resistance and in the main experiment.

Media

A glucose yeast extract peptone (GYP) broth was used in the preparation of control and carbendazim-resistant strains and in the main experiment, as described below. The GYP broth contained 1% glucose, 0.1% yeast extract and 0.1% peptone. The pH was adjusted to 6.5. Potato dextrose agar (PDA), obtained from OXOID (catalogue number CM 139) was used to compare radial growth in the two strains and to provide inoculum for the main experiment.

Fungal isolates

A spore suspension was prepared from a five-day-old culture of Fusarium sporotrichioides. The mycotoxin profile of this organism had been established in a previous experiment in our laboratory (7). The spore suspension was used to prepare control (CS) and carbendazim-resistant (RS) strains for the current study. Two 250 ml Erlenmeyer flasks each containing 100 ml GYP broth were inoculated with the spore suspension containing 5 x 103 spores/ml medium and incubated on a shaker at 25° C for 2 days. One of these flasks served as an untreated control culture (CS) and was maintained by inoculation into fresh GYP medium every 14 days. After the 2-day incubation period, the second flask was spiked with carbendazim solution containing 2 µg a.i./ml, respectively and incubated as before for 7 days, at which time fresh cultures in GYP were set up as before using 0.1 ml inoculum from this flask. Growth was allowed to establish for 2 days after which time carbendazim at 4 µg a.i./ml was added to the culture. After 4 and 8 days of incubation, carbendazim (4 µg a.i./ml) was added to this culture at each of these times. Four days later, a 0.1 ml inoculum was used to set up fresh cultures in GYP. The culture was challenged in this manner for a period of 6 months. At the end of this period, this culture had superior radial growth rates than controls when challenged with carbendazim incorporated into solid media

using PDA. The carbendazim-treated isolate in GYP was therefore designated to be resistant and referred to as RS in the current study.

Experimental design

The GYP cultures of CS and RS were used to inoculate separate Petri dishes of PDA which were then incubated at 25°C until growth was established. The PDA cultures were used to prepare a spore suspension adjusted to 5 x 103 spores/ml GYP medium for each strain (CS and RS). Each suspension was used to inoculate separate experimental flasks containing GYP. After incubation for 1 day at 25°C on a shaker, carbendazim was added to provide concentrations of 0, 1, 2 and 4 µg a.i./ml of GYP in factorial combination. The 0 µg/ml treatment contained an appropriate volume of ethanol consistent with the level used with the carbendazim treatments. Sufficient flasks were incubated at 25°C on a shaker to provide two replicates at each incubation time for each strain and carbendazim level. Thus, a total of 48 flasks were incubated for the entire experiment. After 2, 5 and 7 days of incubation, sampling was initiated for growth and mycotoxin determinations.

Growth measurements

After 2, 5 and 7 days of incubation, entire cultures from two replicates in each treatment were filtered through filter paper (Whatman No 4). The recovered mycelia were freeze-dried and weighed. Weight of mycelia was used as an indication of fungal growth. The filtrates arising from this stage were retained for mycotoxin analyses, as described below.

Determinations of T-2 toxin, diacetoxyscirpenol and neosolaniol

T-2 toxin was quantified in filtrates arising from all experimental treatments and incubation times, but DAS and NEO were determined only in CS and RS treated with 2 and 4 µg a.i./ml for 5 and 7 days. Each filtrate was washed with three 50 ml volumes of chloroform and the resulting extracts combined and filtered through silicone-treated phase separation paper. Each extract was then reduced in volume using a thin-film rotary evaporator, dried under N2 and stored at –20°C prior to analysis by two-dimensional thin layer chromatography (TLC). For the determination of T-2 toxin, DAS and NEO, the dried extracts were re-suspended in 100 µl chloroform and 5µl spotted on 20×20 cm TLC plates (silica gel 60, Cat. No. 5721, Merck). The plates were then developed to a predetermined mark (10 cm) in a mixture of chloroform and methanol (volumetric proportions: 93 and 7, respectively). The plates were air-dried, turned through an angle of 90o and developed in a mixture of toluene, ethyl acetate and formic acid (volumetric proportions: 50, 40, 10 respectively). With both dimensions, plates were developed in separate equilibrated chambers. Derivatization was accomplished by dipping the plates in 8% sulphuric acid. A CAMAG Immersion Device III was used to dip plates which were then heated at 110°C for 20 min prior to examination under UV light. The presence of the three mycotoxins was assessed visually by comparing Rf values and colour development with respective standards added at 2.5 µg/5 µl spot. Quantification was performed with a CAMAG CD60 densitometer. The output from the densitometer also provided the means for positive identification of the three mycotoxins by spectral analysis.

Statistical analysis

Data were subjected to analysis of variance for a factorial design using Minitab. Significant main effects and interactions were identified by F tests. Significant

differences between means were established using t tests according to standard protocols (8).

Results

Mycelial mass

The mycelial mass results are presented in Table 1. Analysis of variance demonstrated that there were highly significant main effects ($P<0.001$) of incubation time, carbendazim concentration and strain on mycelial yield. The interaction between strain and carbendazim concentration was also highly significant ($P<0.001$), while the interaction of strain, carbendazim concentration and time was significant at $P<0.01$. In addition, mycelial yield was affected by interactions between strain and incubation time ($P<0.05$) and between carbendazim level and incubation time ($P<0.01$). Examination of the overall means (Table 1) indicates that differences in mycelial mass between CS and RS at 2 days ($P<0.05$) became more pronounced at 5 and 7 days of incubation ($P<0.001$). However, mycelial growth differences between the two strains were greatest following exposure to carbendazim, as can be seen from the main body of Table 1, with the effects becoming more divergent with time. Thus, at the 4 µg/ml addition of carbendazim, differences between CS and RS were not significant ($P>0.05$) at 2 days but were highly significant ($P<0.001$) at 5 and 7 days. In contrast, no differences between CS and RS were discernible at any incubation time in the absence of carbendazim or at the 1 µg/ml addition of the fungicide.

Table 1: Effects of incubation time and carbendazim concentrations on mycelial mass in control (CS) and carbendazim-resistant (RS) strains of *Fusarium sporotrichioides*.

Carbend-azim level (µg/ml)	Incubation time (days)					
	2		5		7	
	CS	RS	CS	RS	CS	RS
	Mycelial mass (mg)					
0	194	258	415	400	487	497
1	211	245	416	449	462	431
2	123	229	201	455	20	470
4	23	104	44	255	5	299
Overall means	138	209	269	390	244	425
SEM (df= 23)	36.5					

Each value is a mean of duplicate determinations.
SEM (standard error of mean) applicable to data in the main body of the table, excluding overall means.

T-2 toxin production

Analysis of variance of data for T-2 toxin production (Table 2) indicated that there were highly significant main effects of incubation time, carbendazim concentration and strain ($P<0.001$). The interaction between strain and carbendazim level was also highly significant ($P<0.001$). Other significant ($P<0.01$) interactions included those

involving strain and incubation time; strain, carbendazim concentration and incubation time; as well as carbendazim concentration and incubation time. The strain effect is most clearly seen in its interaction with incubation time, as represented by the overall means in Table 2. Thus only minor differences were apparent on day 2, but progressively higher T-2 toxin production occurred in RS compared with CS by the fifth ($P<0.05$) and seventh ($P<0.001$) day of incubation. These strain differences were also affected by the interaction with carbendazim concentration. Thus, for virtually all comparisons there were no strain effects ($P>0.05$) in the absence of carbendazim or at the 1 µg/ml level of the fungicide. However, at the 2 µg/ml addition, T-2 toxin production by RS was significantly increased on day 5 ($P<0.001$) and day 7 ($P<0.05$) in comparison with values for the lower additions of carbendazim. In direct contrast, T-2 toxin output by CS was significantly reduced by the 2 µg/ml addition of carbendazim on day 2 ($P<0.01$) and on days 5 and 7 ($P<0.001$). Consequently, at this level of carbendazim, T-2 toxin production was significantly higher ($P<0.001$) in RS compared to CS cultures.

Table 2: Effects of incubation time and carbendazim concentrations on T-2 toxin production in control (CS) and carbendazim-resistant (RS) strains of *Fusarium sporotrichioides*.

Carbend-azim level (µg/ml)	Incubation time (days)					
	2		5		7	
	CS	RS	CS	RS	CS	RS
	T-2 toxin production (mg/ml extract)					
0	4.82	4.37	10.47	7.01	10.39	10.06
1	3.02	2.62	7.87	6.78	10.08	10.24
2	0.04	1.55	1.14	12.78	0.09	13.80
4	0.00	0.04	0.31	1.08	0.21	2.37
Overall means	1.97	2.14	4.95	6.91	5.19	9.12
SEM (df= 23)	1.057					

Each value is a mean of duplicate determinations.
SEM (standard error of mean) applicable to data in the main body of the table, excluding overall means.

Diacetoxyscirpenol (DAS) and neosolaniol (NEO) production

Data for DAS and NEO production (Table 3) were obtained for a restricted number of treatments since the major effects on T-2 toxin production (Table 2) occurred at 5 and 7 days with 2 and 4 µg/ml additions of carbendazim. Analysis of variance showed that DAS synthesis was significantly ($P<0.01$) affected by strain, carbendazim concentration and by interaction between these two factors. The main effect of strain can be gauged by the overall means shown in Table 3, which indicate that RS yielded more DAS than CS at 5 days ($P<0.05$) and at 7 days ($P<0.01$) of incubation. The major component of this strain difference arose from the effects of the 2 µg/ml addition of carbendazim ($P<0.01$).
With regard to NEO production (Table 3) the significant main effects, identified in the analysis of variance, were strain ($P<0.01$) and carbendazim concentration ($P<0.05$). The strain effect is most clearly indicated by the overall means, which show consistently higher production of NEO in RS compared to CS, with the differences becoming progressively more pronounced from day 5 ($P<0.05$) to day 7 ($P<0.01$) of

incubation. These differences reflected enhanced NEO output following carbendazim application of 4 μg/ml ($P<0.05$) in day 5 extracts and of both 2 μg/ml ($P<0.01$) and 4 μg/ml ($P<0.05$) in day 7 samples.

Table 3: Effects of incubation time and carbendazim concentrations on diacetoxyscirpenol (DAS) and neosolaniol (NEO) production in control (CS) and carbendazim-resistant (RS) strains of *Fusarium sporotrichioides*.

Carbendazim level (μg/ml)	Incubation time (days)			
	5		7	
	CS	RS	CS	RS
	DAS production (mg/ml extract)			
2	0.12	1.58	0.11	1.80
4	0.09	0.17	0.08	0.27
Overall means	0.10	0.87	0.10	1.04
SEM (df=7)	0.266			
	NEO production (mg/ml extract)			
2	4.01	6.97	0.25	7.15
4	0.12	4.09	0.42	4.66
Overall means	2.07	5.53	0.33	5.90
SEM (df=7)	1.170			

Each value is a mean of duplicate determinations.
SEM (standard error of mean) applicable to data in the main body of the table, excluding overall means.

Discussion

Profound differences were observed in the responses of CS and RS cultures of *F sporotrichioides* following treatment with carbendazim; in addition, there were important interactions with time of exposure to the fungicide (Tables 1-3). As in previous studies (5, 6) the differences were most pronounced at the higher levels of application of fungicide. Thus at 4 μg/ml, mycelial yield (Table 1) and mycotoxin production (Tables 2-3) were significantly higher ($P<0.05$ or better) in RS than in CS isolates. It is now clear that the development of fungicide resistance in Fusarium species affects the production of both type A and type B trichothecenes (5, 6). However, it appears that fungicide resistance is expressed more strongly in *F sporotrichioides* than in *F culmorum* (5, 6), although other factors including fungicide type, substrate and culture conditions may contribute to this difference. The current findings have important implications since they help to explain the persistence of trichothecene contamination of cereal grains despite regular applications of fungicides to control mycotoxin-producing diseases such as fusarium head blight (3, 4). Furthermore, mixtures of fungicides are often recommended to offset the effects of resistance to a particular fungicide. However, this and previous studies have shown the relative ease with which toxigenic Fusarium pathogens develop resistance to carbendazim (Tables 1-3) and to difenoconazole (5, 6). In addition, other investigations (9) have shown that *F graminearum* and *F moniliforme* readily develop resistance to benzimidazole and it has been suggested that such strains may already exist in field populations of these fungi. Further work is, therefore, required to elucidate the effects of multiple fungicide resistance on mycotoxin production in Fusarium phytopathogens.

A fundamental question is whether the development of fungicide resistance introduces intrinsic changes in the biosynthesis of mycotoxins or whether all of the observations in this (Tables 2-3) and in earlier papers (5, 6) exemplify an indirect response to the primary effect on fungal growth. In this respect, it is instructive to consider the data presented in Tables 1-2 in terms of % inhibition rather than absolute values. The data in Figs 1 and 2 have been obtained from Tables 1-2 by combining values for the three incubation times and by plotting % inhibition as a function of carbendazim addition. It will be seen that the inhibitory effects of carbendazim on mycelial yields were dose-related within the range 2-4 µg/ml but were greatest in CS than in RS cultures (Fig 1).

Consequently, at the 4 µg/ml level, growth inhibition was almost complete (93%) in CS, whereas in RS cultures inhibition was only 43%. Carbendazim inhibition of T-2 toxin production (Fig 2) in CS isolates largely followed the dose-response pattern for mycelial yield. In contrast, RS cultures exposed to the 2 µg/ml addition of carbendazim significantly increased T-2 toxin production ($P<0.05$ or better). It will be noted that T-2 toxin production in CS was not enhanced at any level of carbendazim even over the range that allowed growth which was comparable with that observed in the RS series (Tables 1-2). It is conceivable that under the conditions employed in the current experiment, carbendazim was an appropriate fungicide to elicit strain differences in T-2 toxin production (Fig 2). It should be noted that under other conditions, tridemorph stimulated T-2 toxin synthesis even in standard cultures of *F sporotrichioides* (10). It is clear that much still needs to be understood about the mode of action of fungicides on T-2 toxin production.

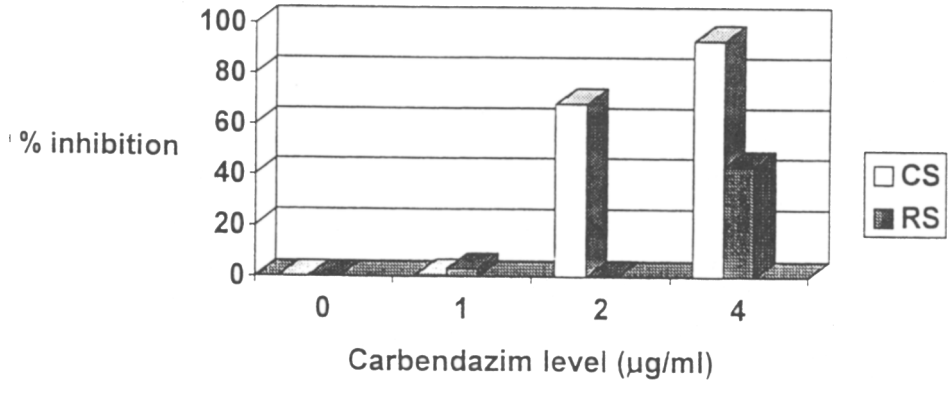

Figure 1 – Growth inhibition by carbendazim in control (CS) and resistant (RS) cultures of *Fusarium sporotrichioides*. Data calculated from values in Table 1.

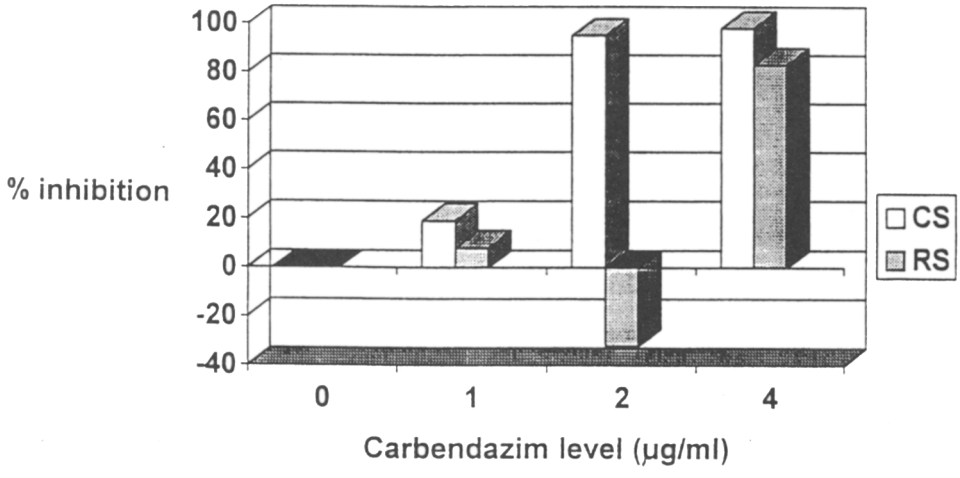

Figure 2 – T-2 toxin inhibition by carbendazim in control (CS) and resistant (RS) cultures of *Fusarium sporotrichioides*. Data calculated from values in Table 2.

Additional evidence of strain differences may be seen in the data for DAS and NEO production (Table 3). With both mycotoxins, higher levels of production were recorded for RS than CS cultures. Furthermore, although NEO output was depressed with incubation time in CS isolates, no such effect occurred in RS cultures. Indeed, it appeared that NEO synthesis in RS was less readily inhibited by carbendazim even at levels that caused 83% inhibition of T-2 toxin production (Fig 2). Ratios of NEO to DAS, T-2 toxin to DAS and NEO to T-2 toxin were calculated from the values in Tables 2-3. It was considered that such ratios might be useful in generating additional evidence for genuine strain differences in trichothecene production. In general, these ratios were not significantly different ($P>0.05$) between CS and RS samples. However, NEO to T-2 toxin ratios were significantly ($P<0.01$) affected by interactions between fungicide concentration and strain. The interaction of fungicide concentration, strain and time was also significant ($P<0.01$). In particular, at the 2 µg/ml addition of carbendazim and after 5 days of incubation, the ratio was 3.52 in CS which was significantly higher ($P<0.05$) than the ratio of 0.55 in RS (Tables 2-3). However, at the 4 µg/ml addition of carbendazim, the ratio was higher in RS (ratio = 3.79) compared to CS (ratio = 0.39) after 5 days of incubation ($P<0.01$). It will be noted that NEO, which is formed from DAS, is an immediate precursor of T-2 toxin in the pathway for type A trichothecenes (11). It is conceivable, therefore, that the strain difference may reside in this particular stage of the pathway, since the other ratios calculated (NEO to DAS; T2 toxin to DAS) were not significantly affected by strain, incubation time or fungicide concentration ($P>0.05$). However, growth patterns and incubation time may also be important for synthesis of T-2 toxin from its precursor.

It is appropriate to compare the efficacy of fungicides for FHB control and the implications for mycotoxin production. In a review, D'Mello et al (4) indicated that

the evidence concerning the efficacy of fungicides was conflicting, with several instances of enhanced production of trichothecenes at certain doses of carbendazim, difenoconazole, tridemorph and tebuconazole applied with triadimenol. In the light of substantial evidence it was concluded that fungicides were not effective for mycotoxin control since they were designed for use against diseases and not necessarily against the accompanying mycotoxins. It was suggested that if chemical control were to become more effective in the future, additional criteria would be required in evaluation protocols for potential fungicides. The present results (Tables 1-3) and those of earlier studies (5, 6) indicate that the development of fungicide resistance and its impact on mycotoxin production should be considered in future evaluation procedures. In respect of overall fungicide use, it is salutary to note that in a new study, Jones (12) concluded that the prospects for the effective chemical control of FHB remained limited. With malting barley, users are adopting a near-zero tolerance for DON contamination, but the results of Jones (12) showed that commonly recommended fungicides including tebuconazole were only partially effective in controlling DON contamination of grain. In most instances, DON residues in grain exceeded the advisory limit of 1 mg/kg proposed by the FDA (4). With iprodione, DON contamination was enhanced over levels found in grain from untreated plots.

The current and previous studies reinforce the potential value of pure culture models in screening studies on fungicide efficacy. Thus, in common with field trials, pure culture systems have shown a variety of common fungicides to be only partially effective in controlling trichothecene production. It might be argued that instances of enhanced mycotoxin production in response to fungicide application (Table 2), (7, 10) are unique to in vitro conditions. To the extent that stimulation of T-2 toxin synthesis (Fig 2) has only been demonstrated with laboratory cultures, such an argument is justified. However, in field studies applications of propiconazole, thiabendazole and tebuconazole induced small but consistent increases in DON content of wheat kernels (13). In another field trial, tebuconazole with triadimenol caused a 16-fold increase in NIV contamination of wheat kernels (14), while in the most recent study (12) iprodione enhanced grain levels of DON.

In conclusion, the current study showed that T-2 toxin production in a carbendazim-resistant strain of *F sporotrichioides* was stimulated on further exposure to the fungicide; such an enhancement did not occur in the control strain. Carbendazim-induced inhibition of NEO production was more pronounced in the control than in the resistant strain. Furthermore, the ratio of NEO to T-2 toxin was affected by interaction between strain and carbendazim concentration. It is suggested that the strain difference may reside in the conversion of NEO to T-2 toxin in the trichothecene biosynthetic pathway, which in turn may be sensitive to fungicide dose. In summary, the current study implies that fungicide-resistance in *F sporotrichioides* may be accompanied by increased competitiveness of the pathogen resulting in more persistent or even enhanced production of associated mycotoxins.

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