Impact of Competitive Fungi on Trichothecene Production by *Fusarium graminearum*

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Bioassays were used to determine the production of the trichothecene mycotoxin, deoxynivalenol (DON), by two isolates of *Fusarium graminearum* when grown in association with potentially competitive fungi and an antifungal chemical, 6-pentyl-α-pyrone (6PAP). The presence of 6PAP in the culture medium reduced DON production by as much as 80%, but this effect was reduced for the *F. graminearum* isolate that most efficiently metabolized the added 6PAP. A 6PAP-producing *Trichoderma* isolate grown in a competition assay system with the *F. graminearum* isolates was also able to substantially reduce DON production. When *Fusarium* isolates (*F. crookwellense*, *F. culmorum*, *F. subglutinans*, *F. poae*, *F. equiseti*, *F. avenaceum*, and *F. sambucinum*), which co-occur with *F. graminearum* in New Zealand maize plants (*Zea mays*), were grown in competition assays, the effect on DON production was variable. However, all isolates of *F. subglutinans* tested were shown to cause reductions in DON production (by 13−76%, mean = 62%). *F. subglutinans* frequently co-occurs with *F. graminearum*, but its presence can vary with location and time of the season. When the competitive fungus tested was also a trichothecene producer (e.g., of nivalenol), both toxins were produced in the assay medium. The results indicate that mycotoxin production by *F. graminearum* can be affected by the presence of particular competitive fungi. These results have implications for an ecological understanding of pathogenicity and of mycotoxin accumulation in plants. Early establishment of *F. subglutinans*, for example, may act as a biological control mechanism providing a temporary protection against invasion by more commonly toxigenic fusaria such as *F. graminearum*.

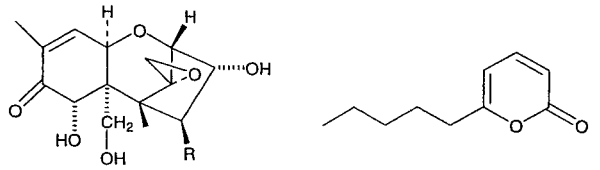
Keywords: *Biocontrol; Fusarium; mycotoxins; trichothecenes; deoxynivalenol; nivalenol; Trichoderma; 6-pentyl-α-pyrone*

INTRODUCTION

Fusarium spp. infection is a problem in grain crops worldwide. As well as causing reduced grain quality, it also leads to grain contamination by toxic metabolites (mycotoxins) such as the tricyclic sesquiterpenoid trichothecenes, deoxynivalenol (DON) and nivalenol (NIV) (Figure 1). DON has been associated with the pathogenic virulence of *F. graminearum* and is suggested to assist in its colonization of host tissue (*1*). Minimizing DON production, therefore, may afford a mechanism for protection against invasion by toxigenic fusaria (*2*).

As part of our studies into mechanisms of biocontrol, we had earlier determined that antibiotic-producing *Trichoderma* gave enhanced production of the antibiotic 6-pentyl-α-pyrone (6PAP) (Figure 1) in response to the presence of pathogenic fungi (*3*, *4*).

The purpose of this study was to measure the impact of competitive fungi or of antibiotic chemicals on the production, by pathogens, of metabolites such as the trichothecenes, which could contribute to pathogenicity or virulence. We initially investigated the effect of 6PAP, applied in pure form, and of a 6PAP-producing *Trichoderma* on the production of DON by some isolates of *F. graminearum*. The investigation was then extended to examine the effect of some potentially competitive *Fusarium* species such as *F. subglutinans* on the production of DON by the same toxigenic isolates. *F. subglutinans* frequently co-occurs with *F. graminearum* in New Zealand grown maize (*Zea mays*) (*5*, *6*).



NIV: R=OH
DON: R=H

6PAP

Figure 1. Chemical structures of the *Fusarium* trichothecene mycotoxins DON and NIV and of 6PAP, a secondary metabolite produced by *Trichoderma* spp.

MATERIALS AND METHODS

Fungal Isolates. Fungi used in this investigation were *F. graminearum* isolates 245AP4 and 34W23.4F9, *F. subglutinans* isolates 15BLK16, 245AK2, 5E27, and 25C/D, *Fusarium culmorum* isolate 10E8, *Fusarium crookwellense* isolate 23280A9, *Fusarium poae* isolate 174BLR6, *Fusarium equiseti* isolate 15BUK33, *Fusarium avenaceum* isolate 62BUA6, and *Fusarium sambucinum* isolate 63BUA/G (all supplied by M. E. di Menna, AgResearch, Hamilton, New Zealand) and *Trichoderma* isolate THF2/3 (supplied by R. A. Hill, HortResearch, Hamilton, New Zealand, identified as *Trichoderma harzianum I* by G. Samuels, USDA, ARS, Beltsville, MD).

Bioassay Technique. *Fortified Agar Slants.* Agar slants fortified with either 6PAP or DON were prepared by dispens-

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ing the appropriate quantity of either 6PAP or DON in 5 or 10 μL of solution (in methanol/water 85:15) into a glass tube containing liquefied MYRO agar [2% agar in MYRO medium as per Lauren et al. (*7*)] (4 mL) maintained at 60 °C in a water bath after autoclaving. The tubes were vortexed and allowed to solidify on an angle to give a uniform slope. These agar slants (three replicates at each rate of 6PAP or DON) were inoculated by point inoculation of mycelium and spores from 6-day-old cultures grown on potato−glucose−agar at 25 °C, and the resulting slants were incubated for 7 days at 25 °C. Control tubes at each rate were uninoculated. Samples (the entire agar/mycelium matrix) were extracted by mixing with acetonitrile/methanol (14:1; 8 mL) and allowing to stand overnight. A 2 mL aliquot was removed for trichothecene analysis, or a 400 μL aliquot was removed for 6PAP analysis, as appropriate.

Tubular Bioassay. The tubular bioassay system used was a modification of that described earlier (*3*). Briefly, minitubes were formed from dialysis tubing filled with MYRO agar and sliced into 3 cm lengths. One end of each tube was point inoculated with the pathogenic isolate (*F. graminearum*) and the other end point inoculated with the competitive test organism, either the *Trichoderma* isolate or a *Fusarium* isolate as appropriate. Control tubes were inoculated with the pathogen only. After 7 days of incubation at 25 °C, each tube was sliced into 3 × 1 cm length sections (A, B, and C, where A is the pathogen inoculation end, B is the midsection, and C is the competitive fungi inoculation end), which were individually extracted with acetonitrile/methanol (14:1; 3 mL). A 2 mL aliquot was removed for trichothecene analysis, and, where appropriate, a 400 μL aliquot was removed for 6PAP analysis.

Workup for Analysis. *Trichothecenes*. The procedure was adapted from the method described earlier for NIV and DON (*8*). A Pasteur pipet was plugged with glass wool and dry-packed with alumina/carbon (20:1; 300 mg) to form a mini-cleanup column. A 2 mL aliquot of extract was applied to the column and allowed to drain under gravity and the eluate collected. The column was washed with acetonitrile/methanol/water (80:5:15; 500 μL), and the combined eluate was evaporated to dryness (N_2, 50 °C). The residue was treated with 0.2 N sodium hydroxide in methanol/water (90:10; 100 μL), vortexed, and then allowed to stand at room temperature (~20 °C) for 60 min to hydrolyze any acetylated trichothecene to the parent alcohol. The solution was neutralized by the addition of 0.25 N hydrochloric acid in methanol/water (95:5; 100 μL), vortexed, and then further diluted by the addition of methanol/water (5:95; 500 μL) prior to analysis by HPLC.

6PAP. A 400 μL aliquot of extract was diluted with water (400 μL) and analyzed by HPLC.

HPLC Analysis. *Trichothecenes*. Analytical HPLC was performed on a Zorbax SB-C8 column (15 cm × 4.6 mm i.d.) (Phenomenex) using a dual UV detector set at 245 and 254 nm. Separation was achieved with an isocratic mobile phase of methanol/water (12:88) at 1 mL/min. The retention times of DON and NIV were 10.5 and 4.9 min, respectively. A rapid solvent flush cycle to methanol/water (65:35) was used to remove any more strongly retained coextractives from the column between injections.

6PAP. Analytical HPLC was performed on a Prodigy 5 ODS-2 (15 cm × 4.6 mm i.d.) (Phenomenex) using a UV detector set at 300 nm. Separation was achieved with an isocratic mobile phase of methanol/water (64:36) at 1 mL/min. The retention time of 6PAP was 6.1 min. After every five samples, a rapid solvent flush cycle to 100% methanol was used to remove any more strongly retained coextractives from the column.

Effect of 6PAP on DON Production by *F. graminearum*. *F. graminearum* isolates 245AP4 and 34W23.4F9 were grown on MYRO agar slants fortified with 6PAP at the following concentrations: 125, 62.5, 25.0, 12.5, 6.25, and 0 μg/mL (three replicates per rate). Samples were treated as described in the bioassay technique for fortified agar slants.

Effect of DON on 6PAP Production by *T. harzianum*. *T. harzianum* isolate THF2/3 was grown on MYRO agar slants fortified with DON at the following concentrations: 125, 62.5, 25.0, 12.5, 6.25, and 0 μg/mL (three replicates per rate). Samples were treated as above.

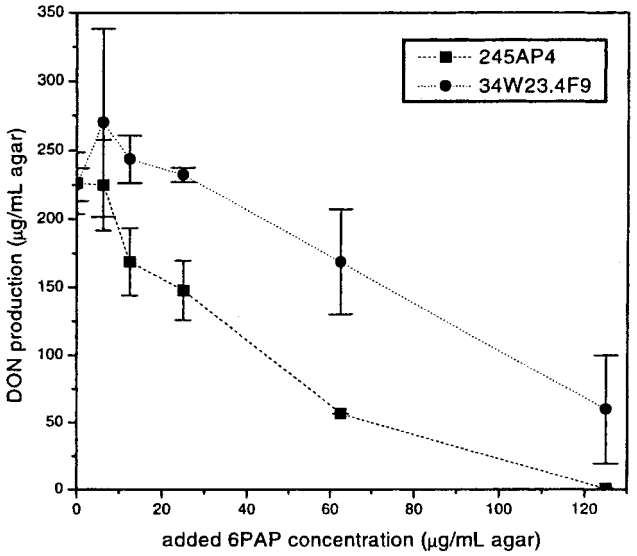

Figure 2. DON production by *F. graminearum* isolates 245AP4 and 34W23.4F9 grown on agar fortified with different concentrations of 6PAP. Points are mean values with SD shown by error bars.

Effect of Competitive Fungi on DON Production by *F. graminearum*. *Experiment One*. Minitubes inoculated at one end with either *F. graminearum* isolate 245AP4 or 34W23.4F9 were inoculated at the other end with either *F. subglutinans* isolates 15BLK16, 245AK2, 5E27, and 25C/D or *T. harzianum* isolate THF2/3 (three replicates of each). Three control tubes were inoculated with the *F. graminearum* isolate at one end only, and another three were inoculated with the *F. graminearum* isolate at both ends. Samples were treated as described in the bioassay technique for the tubular bioassay.

Experiment Two. Minitubes inoculated at one end with either *F. graminearum* isolate 245AP4 or 34W23.4F9 were inoculated at the other end with either *F. poae* isolate 174BLR6, *F. equiseti* isolate 15BUK33, *F. avenaceum* isolate 62BUA6, *F. sambucinum* isolate 63BUA/G, *F. culmorum* isolate 10E8, or *F. crookwellense* isolate 23280A9 (three replicates of each isolate). Three control tubes were inoculated with the *F. graminearum* isolate at one end only. Separate control tubes were also inoculated with the competitive fungi at one end only to test for NIV production (three replicates of each). Samples were treated as described above.

DON Recovery from Fortified Agar Slants Inoculated with *F. subglutinans*. *F. subglutinans* isolates 25C/D, 5E27, 245AK2, and 15BLK16 were grown on MYRO agar slants fortified with DON at 125 μg/mL agar (three replicates of each isolate). Control tubes were fortified but uninoculated. Samples were treated as described in the bioassay technique for fortified agar slants.

RESULTS AND DISCUSSION

6PAP incorporated into the agar culture medium was visually observed to reduce the vigor of *F. graminearum* growth and was also found to reduce DON production in the cultures (Figure 2). Addition of 62.5 μg/mL 6PAP, or above, reduced DON production by as much as 80%. It was also observed that both *F. graminearum* isolates tested were able to metabolize the 6PAP present (Figure 3). HPLC analysis showed that this occurred by hydroxylation of the pentyl side chain to give mixtures of isomers of monohydroxylated 6PAP. The characterization of these biotransformation products was reported in an earlier publication detailing the metabolism of 6PAP by a number of fungal plant pathogens (*9*). It has

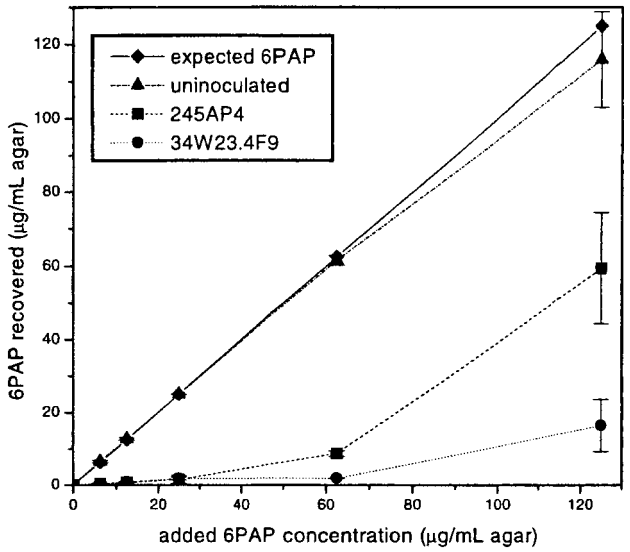

Figure 3. 6PAP recovered from fortified agar after 7 days of incubation with *F. graminearum* isolates 245AP4 and 34W23.4F9. Points are mean values with SD shown by error bars.

been suggested elsewhere that the transformation of hydrocarbon moieties to such hydroxylated metabolites is part of a fungal strategy to eliminate metabolites which would otherwise be toxic (*10*). In the present work it is interesting to note that isolate 34W23.4F9, which most effectively metabolized the 6PAP, also produced the most DON in the presence of 6PAP.

To determine whether the presence of a 6PAP-producing *Trichoderma* isolate would also be effective in inhibiting DON production by *F. graminearum*, the two organisms were grown in competition using the tubular bioassay system in which the competing fungi are inoculated at different ends of a short tube of agar. This bioassay allows analytical measurement of the local production and concentration of antagonistic chemicals at the competitor/pathogen interface. The experiments showed that the presence of the *Trichoderma* isolate, THF2/3, inhibited DON production by *F. graminearum* from 66% (245AP4) to 81% (34W23.4F9) (Figure 4). As noted in earlier studies (*4*) the *Trichoderma* produced enhanced levels of 6PAP in response to the presence of a competitive fungus. This was particularly obvious in the two sections, A and B, closest to the pathogen inoculation end (Figure 4). The levels of 6PAP produced by the *Trichoderma* in the tubular competition system (25−65 µg/mL) were in the range shown to reduce DON production in the dosing experiments (see Figure 2). The increased production of 6PAP was considered not to be due to the production of DON by the *Fusarium*, as similar increases in 6PAP were not found when DON was incorporated in pure chemical form into the agar medium on which the *Trichoderma* was grown. It was also shown by HPLC analysis that the added DON was not metabolized by the *Trichoderma*.

The investigation was extended to determine the effect of some potentially competitive *Fusarium*, such as *F. subglutinans*, on DON production by the same toxigenic *F. graminearum* isolates. Tubular bioassay experiments showed that DON production was inhibited (13−76%; mean = 62%) by the presence of each of the four isolates of *F. subglutinans* tested (Figure 5). The decrease in DON production was not due to fungal metabolism, because none of the *F. subglutinans* isolates tested were able to metabolize DON when inoculated alone into DON-fortified agar slants and incubated for 7 days. Inhibition of DON production is also unlikely to be due to nutrient depletion as minitubes inoculated

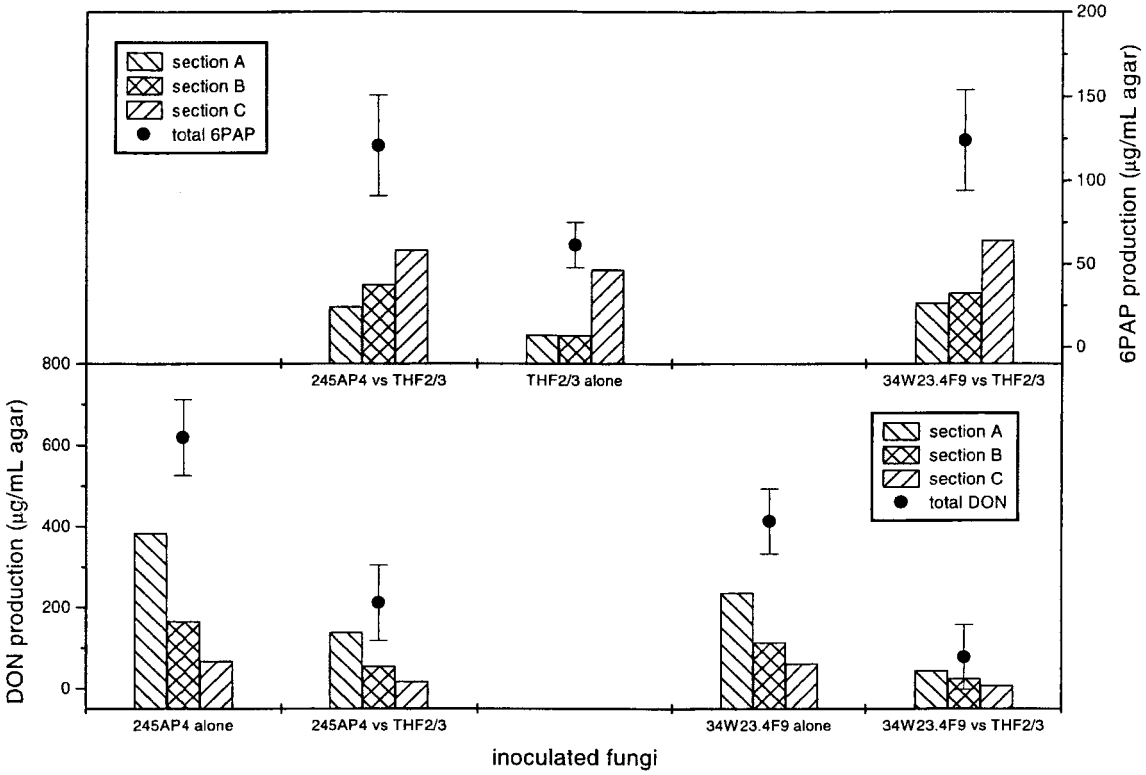

Figure 4. DON and 6PAP concentrations in minitube sections (A, B, and C as defined under Materials and Methods) by *F. graminearum* isolates 245AP4 and 34W23.4F9 when challenged by *T. harzianum* isolate THF2/3. Individual section means are shown without error bars. Points for means of totals are shown with SD error bars.

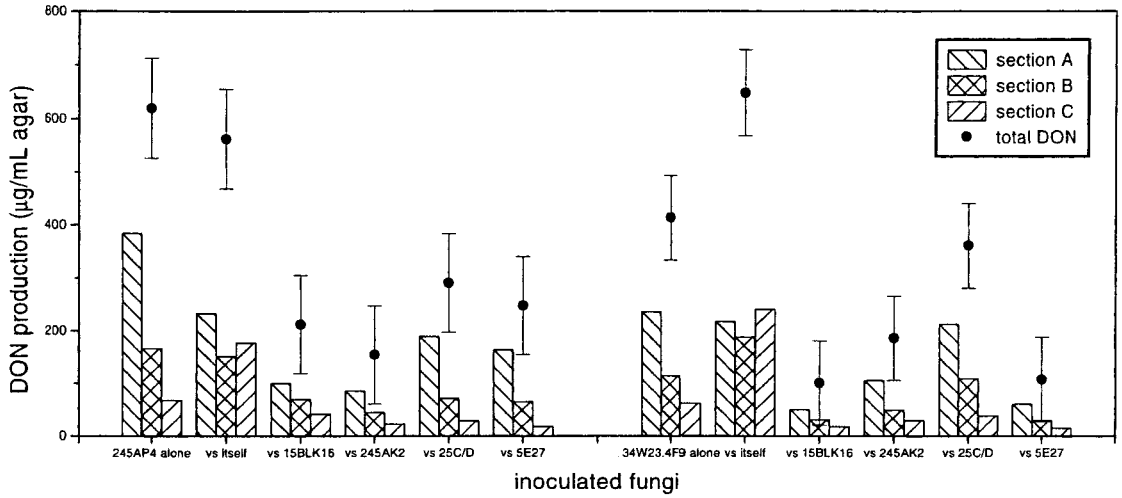

Figure 5. DON production in minitube sections (A, B, and C as defined under Materials and Methods) by *F. graminearum* isolates 245AP4 and 34W23.4F9 when challenged by four individual isolates of *F. subglutinans*. Individual section means are shown without error bars. Points for means of totals are shown with SD error bars.

Table 1. Effect of Competitive Fungi on Total DON Production by *F. graminearum* Isolates 245AP4 and 34W23.4F9

	% DON[a]	
competitive fungus	isolate 245AP4	isolate 34W23.4F9
alone	100	100
vs *F. poae* 174BLR6	68	48
vs *F. equisiti* 15BUK33	55	47
vs *F. avenaceum* 62BUA6	75	133
vs *F. sambucinum* 63BUA/G	61	75
vs *F. culmorum* 10E8	77	34
vs *F. crookwellense* 23290A9	44	87

[a] DON concentration normalized to 100% DON for the *F. graminearum* isolates inoculated at one end of the agar minitube only. Results are the mean of three replicates.

at both ends with *F. graminearum* gave the expected high production of DON throughout the complete minitube (Figure 5).

The effects by other *Fusarium* species sometimes isolated from maize kernels along with *F. graminearum* (*F. poae*, *F. equiseti*, *F. avenaceum*, *F. sambucinum*, *F. culmorum*, and *F. crookwellense*) were variable, with one isolate actually enhancing DON production (Table 1). When both fungi tested in the competitive system were trichothecene-producing isolates (e.g., *F. graminearum* versus NIV-producing isolates of *F. culmorum* and *F. crookwellense*), HPLC analysis showed production of both toxins (NIV and DON). The amount of NIV produced was variable, and no real trends could be discerned.

Our studies reveal that trichothecene mycotoxin production by *Fusarium* species (e.g., *F. graminearum*) can be affected by the presence of particular competitive fungi. In the field situation this would translate into different levels of accumulation of mycotoxins in developing plants depending on the particular co-occurring species present. It is possible, therefore, that early establishment of a species such as *F. subglutinans*, which is able to reduce trichothecene production, may act as a natural control mechanism and temporarily protect the plant from colonization by toxigenic species such as *F. graminearum*. It may therefore create a time window for harvesting a crop at low mycotoxin levels. This could help to explain observations by Lauren and di Menna (*6*) that a maize hybrid first colonized by *F. subglutinans* showed a delay of several weeks before invasion by toxigenic species (*F. graminearum* and *F. crookwellense*) with subsequent toxin production. In contrast, another hybrid growing nearby that was first invaded by toxigenic species (*F. graminearum* and *F. crookwellense*) showed an immediate accummulation of mycotoxins in the kernels. In a study by Chulze et al. (*11*) on Argentinian corn, initial infection by *F. subglutinans* was followed by the dominance of *F. proliferatum* and *F. verticillioides* with the production of fumonisins. However, the information from that study did not allow any conclusion to be made on whether the initial dominance of *F. subglutinans* delayed colonization by the toxin-producing organisms.

The results provided here have indicated that *F. subglutinans* may act as a biological control organism against *F. graminearum*. However, any proposal to use *F. subglutinans* for applied biological control must consider that some isolates of this species are themselves able to produce toxins, for example, beauvericin, moniliformin, and fusaproliferin (*12*, *13*). Nevertheless, these studies have provided new information on the limitation of toxin and virulence factor production by a major crop pathogen. A full understanding of the genetic interactions that allow *F. subglutinans* to reduce DON production by *F. graminearum* may allow development of improved mechanisms for crop protection and for the development of resistant crops.

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