

NEMATODE-ANTAGONISTIC TRICHOHECENES FROM *Fusarium equiseti*¹

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Abstract—A strain of the fungus *Fusarium equiseti* isolated from soybean cyst nematode secretes nematode-antagonistic compounds. Bioassay-guided fractionation of an extract of the culture broth was undertaken to identify the compounds. Fractions were assayed for activity against a root-knot nematode (*Meloidogyne incognita*), a plant pathogen that attacks the roots of numerous plant species. Two trichothecene compounds were isolated that inhibited egg hatch and immobilized second-stage juveniles of this nematode: 4,15-diacetoxy-12,13-epoxy-3,7-dihydroxytrichothec-9-en-8-one (4,15-diacetylinalenol) and 4,15-diacetoxy-12,13-epoxy-trichothec-9-en-3-ol (diacetoxyscirpenol). This is the first published report of these compounds affecting plant-parasitic nematodes.

Key Words—*Fusarium equiseti*, fungus, *Meloidogyne incognita*, root-knot nematode, trichothecene, nematocide, 4,15-diacetoxy-12,13-epoxy-3,7-dihydroxytrichothec-9-en-8-one, 4,15-diacetoxy-12,13-epoxytrichothec-9-en-3-ol, 4,15-diacetylinalenol, diacetoxyscirpenol.

INTRODUCTION

Certain strains of *Fusarium* fungi, when grown in laboratory cultures, secrete compounds that are toxic to plant-feeding nematodes (Mani and Sethi, 1984; Meshram

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¹Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

and Goswami, 1989; Hallman and Sikora, 1996; Nitao et al., 1999). The presence of these compounds is manifested by the inhibition of egg hatch or the immobilization of juvenile nematodes in culture broth filtrates; however, the precise identities of the nematode-antagonistic compounds generally are undetermined. In one of the few examples where structures were partially characterized, Mani et al. (1986) determined that a mixture of long-chain alkanes was responsible for the toxicity of *Fusarium solani* cultures to the root-knot nematode, *Meloidogyne incognita*, a major pathogen that attacks hundreds of crop and ornamental plant species. Although not isolated specifically from a nematode-antagonistic fungus strain, several commercially available mycotoxins commonly produced by *Fusarium* species were tested and found to be nematocidal to *Meloidogyne javanica* at low concentrations (Ciancio, 1995).

Identification of nematode-antagonistic compounds from *Fusarium* species is important for at least three reasons. First, nematode-antagonistic compounds potentially can be used to manage nematode pathogens (Jatala, 1986). Natural products from fungi and bacteria are being sought as alternatives to the use of fumigant and nonfumigant nematicides. Second, in studies searching for biological control agents of pathogenic nematodes, *Fusarium* strains are frequently isolated from nematodes or their egg masses (Qadri and Saleh, 1990; Hay and Skipp, 1993; Oduor-Owino and Waudu, 1996; Chen et al., 1996; Viaene and Abawi, 1998). Identifying the nematode-antagonistic compounds produced by these fungi would help determine if such compounds contribute to the detrimental effects of these antagonists. Third, although it is unknown if compounds produced by *Fusarium* in vitro play a role in natural interactions between fungi and plant-parasitic nematodes, identification of nematode-antagonistic compounds can be the first step toward examining such interactions in future studies.

As part of a study searching for biological control agents, the culture broths of over 250 fungi isolated from soybean cyst nematodes (*Heterodera glycines* Ichinohe) were screened for nematode-antagonistic activity (Meyer et al., 1998). *Fusarium* species were common among those isolates producing broth filtrates that inhibited in vitro egg hatch of soybean cyst nematode and root-knot nematode, *M. incognita*. One of these strains, an isolate of *Fusarium equiseti*, was especially active against *M. incognita* (Nitao et al., 1999), and bioassay-guided isolation of culture broth constituents that caused the nematode-antagonistic activity was undertaken. We report herein the isolation and structure of two *F. equiseti* metabolites responsible in part for inhibiting in vitro egg hatch and juvenile mobility of *M. incognita*.

METHODS AND MATERIALS

Root-knot Nematode Bioassay. Bioassays were conducted in vitro as described previously (Nitao et al., 1999). Eggs were obtained from *Meloidogyne*

incognita (Kofoid & White) Chitwood cultured on tomato plants and were surface-disinfested with 0.5% sodium hypochlorite (Nitao et al., 1999). Eggs were placed in 24-well tissue culture plates with sterile-filtered test solutions. Test compounds were solubilized in DMSO before mixing in water (final concentration: 0.5% DMSO). Control solution was 0.5% DMSO in water. After seven days, the number of hatched second-stage juveniles and the numbers of those juveniles that were mobile and immobile were recorded. The percentage of eggs that hatched and the percentage of juveniles that were mobile were calculated. Each bioassay trial consisted of five replicate wells per treatment (200–280 eggs/well).

Differences in egg hatch and juvenile mobility between test and control solutions were tested with one-way analysis of variance (ANOVA) followed by Dunnett's test to compare each treatment against the control. When two trials were conducted for each set of treatments, results from individual trials were observed to be normally distributed with equal variances. Pooling of data from trials resulted in nonnormality and/or unequal variances that could not be corrected by standard transformations. This indicated that combining data sets would be inappropriate. Therefore, data from trials were analyzed separately, but for brevity, means and standard errors are reported on pooled data since the pattern of means was similar among trials. Kruskal-Wallis test on ranks was used when results from one bioassay trial did not satisfy assumptions of normality and homogeneous variances.

Fungus Culture. *Fusarium equiseti* was isolated from soybean cyst nematode females (*Heterodera glycines*) collected in the People's Republic of China in cooperation with Dr. Xing-Zhong Liu (Chinese Academy of Agricultural Sciences) and was identified by Dr. Jean Juba (Fusarium Research Center, Pennsylvania State University) and Dr. Richard Humber (USDA, ARS, Plant Protection Unit, New York) (Meyer et al., 1998). The fungus was deposited and is maintained in the USDA, ARS, Nematology Laboratory, Beltsville, Maryland, as isolate L128. The fungus was grown on potato-dextrose agar for seven days at 25°C. One-liter Erlenmeyer flasks containing 250 ml potato-dextrose broth were inoculated by blending agar plate cultures into the broth. Broth cultures were incubated for three days on a shaker (240 rpm, 25°C).

Isolation of Nematode-Antagonistic Compounds. After incubation, the cultures were centrifuged, and the broth was filtered through a glass-filter funnel. A total of 18.75 liters of broth was processed in six batches. The broth filtrate was extracted by mixing for 2 hr with Amberlite XAD-16 resin equilibrated in H₂O (1 volume gel–6 volumes broth). The resin was removed from the broth, washed with 2.5 bed volumes H₂O, and eluted with 2.5 bed volumes methanol (MeOH). The MeOH eluate was evaporated under vacuum, and the residue (5.1 g) was partitioned in 7% MeOH in H₂O and ethyl acetate (EtOAc). Earlier studies demonstrated that the nematode-antagonistic activity resided in the EtOAc fraction after partitioning (Nitao et al., 1999).

After evaporating to dryness, the EtOAc fraction (0.68 g) was separated in batches of 70–430 mg by using vacuum liquid chromatography (VLC) (silica gel, Merck grade 9385) and eluted with 100% EtOAc followed by MeOH. Five 25-ml EtOAc fractions and one 50-ml MeOH fraction were collected. VLC fractions from one of these batches were bioassayed at 100 $\mu\text{g}/\text{ml}$ in one trial (5 replicates).

The first two VLC EtOAc fractions and equivalent fractions from other VLC batches were combined after examining with thin-layer chromatography (TLC). Silica gel plates were developed in 100% EtOAc or 20 : 1 chloroform–methanol (CHCl_3 –MeOH); C_{18} plates were developed in 70% acetonitrile in H_2O . Spots were visualized by 50% sulfuric acid spray and charring. The pooled VLC fraction (97 mg) was subjected to medium pressure liquid chromatography (MPLC) (silica gel, Merck, grade 9385). A step gradient (80 : 1, 40 : 1, 20 : 1, and 10 : 1 CHCl_3 –MeOH, and 100% MeOH) was used to elute 21 fractions, and fractions were combined into nine fractions based on TLC. Of these pooled fractions, the five fractions eluted with 40 : 1 and 20 : 1 CHCl_3 –MeOH had masses substantial enough for bioassay and further processing and were assayed at 50 $\mu\text{g}/\text{ml}$ in two trials (five replicates per trial).

Nematode-antagonistic compounds were purified from MPLC fractions with high-performance liquid chromatography (HPLC) (10 \times 250 mm Alltima C_{18} column, Alltech, Deerfield, Illinois; acetonitrile– H_2O gradient, 2 ml/min, 214 nm detection). Purified compounds were bioassayed at 25 $\mu\text{g}/\text{ml}$ (two trials; five replicates per trial).

Identification of Compounds. Compounds were identified by mass spectrometry, NMR spectroscopy, and X-ray crystallography. ^1H NMR and ^{13}C NMR spectra were acquired in CDCl_3 on a Bruker QE 300-MHz spectrometer; chemical shifts are relative to CDCl_3 . Mass spectra (EI, CI using NH_3) were obtained on a Finnegan INCOS 50 mass spectrometer and Finnegan MAT solid probe. X-ray crystallography was performed on a crystal obtained from aqueous CH_3CN . A colorless crystalline plate (0.43 \times 0.06 \times 0.03 mm) was optically centered on a Bruker SMART1000 single crystal CCD diffractometer. The SAINT+ data reduction program (Bruker Analytical X-ray Systems, Madison, Wisconsin) with 2θ max set to 55.0° was implemented to correct for Lorentz and polarization effects. An empirical absorption correction was applied to 11,861 reflections ($\pm h \pm k \pm l$) with 3328 being unique [$R(\text{int}) = 0.0399$] based upon equivalent reflection measurements using Blessing's method (Blessing, 1995) in the program SADABS (Sheldrick, 1996). The XPREP program (Sheldrick, 1994) was implemented to determine the space group and generate the initial files. The structure was determined by direct methods with the program XS (Sheldrick, 1990) and resulted in the successful location of many atoms comprising the molecule. Full-matrix least-squares refinement with the program XL (Sheldrick, 1993) coupled with difference-Fourier maps was used to locate, input, and complete the configuration of the nematode-antagonistic molecule and an accompanying water molecule.

Hydrogen atoms were placed initially in calculated positions and then allowed to refine freely; those hydrogen atoms comprising the water molecule were located directly from a difference-Fourier map and allowed to refine freely.

RESULTS

Isolation of Nematode-Antagonistic Compounds. Fractions from VLC had a significant effect on egg hatch when tested at 100 $\mu\text{g/ml}$ (ANOVA, $P < 0.001$) (Figure 1). The proportion of eggs that hatched was lower in each fraction compared to the proportion in the control solution (Dunnnett's test, $P < 0.05$). Egg hatch in VLC fractions 1 and 5 was 90% lower than that in the control. Of those juveniles that hatched, the percentage that was mobile also was affected by the *Fusarium* fractions (Kruskal-Wallis test, $P < 0.001$). The proportion of juveniles mobile in the fractions eluted with EtOAc (VLC fractions 1–4) was lower than in the control (Dunnnett's test, $P < 0.05$). All or almost all of those juveniles that hatched in these fractions were immobile. However, mobility was not reduced by the MeOH fraction (VLC fraction 5) (Dunnnett's test, $P > 0.05$).

Combined VLC fractions 1 and 2 were chosen for further separation on MPLC due to their relatively higher activity against both hatch and mobility and the larger

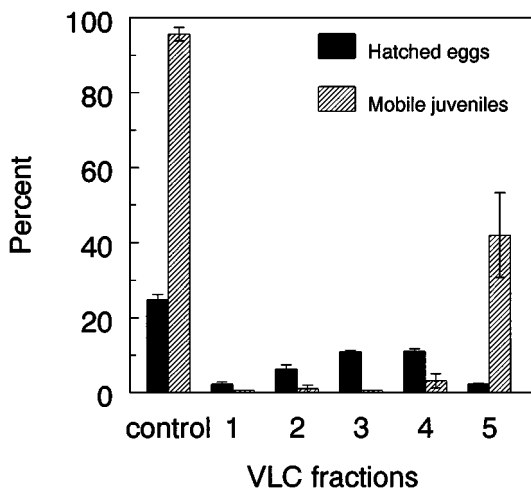


FIG. 1. Percentage of *Meloidogyne incognita* eggs hatched and percentage of hatched juveniles that were mobile after one week in vacuum liquid chromatography (VLC) fractions of *Fusarium equiseti* culture broth. VLC fractions 1–4 were eluted with ethyl acetate; fraction 5 was eluted with methanol. Fractions were tested at a concentration of 100 $\mu\text{g/ml}$. Data are mean \pm SE of five replications in one trial (230 eggs/replicate).

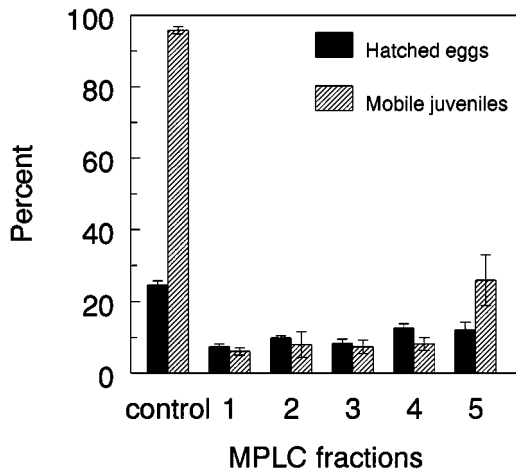


FIG. 2. Percentage of *Meloidogyne incognita* eggs hatched and percentage of hatched juveniles that were mobile after one week in medium-pressure liquid chromatography (MPLC) fractions of *Fusarium equiseti* culture broth. MPLC was performed on combined VLC fractions 1 and 2. Fractions were tested at a concentration of 50 $\mu\text{g/ml}$. Data are mean \pm SE of 10 replications in two trials (250 and 280 eggs/replicate).

available quantity. In both bioassay trials of MPLC fractions, hatch and juvenile mobility were lower in each MPLC fraction than in the control when tested at 50 $\mu\text{g/ml}$ (ANOVA, $P < 0.001$; Dunnett's test, $P < 0.05$) (Figure 2). Hatch was reduced 25–40% by the fungus fractions compared to hatch in the control solution, and the proportion of juveniles that were mobile was as much as 94% lower.

A major component contained in MPLC fractions 1–3 was purified by HPLC and crystallized (6 mg). X-ray crystallography revealed the crystal unit cell to consist of one molecule of 4,15-diacetoxy-3,7-dihydroxy-12,13-epoxytrichothec-9-en-8-one (4,15-diacetylivalenol) and one water molecule in proximity to the epoxide group (Figure 3). Crystal data: monoclinic; $\text{C}_{19}\text{H}_{26}\text{O}_{10}$; $[\text{C}_{19}\text{H}_{24}\text{O}_9][\text{H}_2\text{O}]$; FW = 414.40; space group = $\text{P}2_1$; unit cell dimensions: $a = 10.4225(13)$ Å, $b = 7.7034(10)$ Å, $c = 11.9740(15)$ Å, $\beta = 100.072(2)^\circ$; $V = 946.6(2)$ Å³; $Z = 2$; $D_x = 1.454$ g/cm³; $\lambda(\text{MoK}\alpha) = 0.71073$ Å; $\mu(\text{MoK}\alpha) = 0.118$ mm⁻¹, $F(000) = 40$, $T = 173(2)$ K. The absolute structure parameter (Flack, 1983), $\text{Flack}(x)$, was refined to a value of $-1.0(8)$, indicating that while the structure is both correct and complete, its absolute configuration is inconclusive.

The identification of the molecule as 4,15-diacetylivalenol (Figure 4) was consistent with NMR and MS analyses: ¹H NMR: δ 1.10 (3H, s, H-14), 1.90 (3H, br d, H-16), 1.91 (3H, s, H-20), 2.16 (3H, s, H-18), 3.08 (2H, m, H-13), 3.83 (1H, d, H-2), 4.22 (1H, dd, H-3), 4.27 (2H, dd, H-15), 4.73 (1H, d, H-11), 4.88 (1H, s, H-7), 5.23 (1H, d, H-4), 6.64 (1H, m, H-10). ¹³C NMR APT: δ 7.5 (C-14, CH₃),

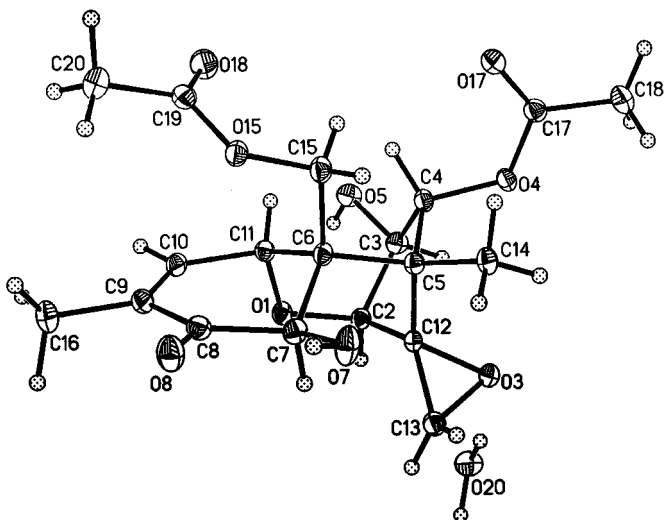


FIG. 3. Oak Ridge Thermal Ellipsoid Plot derived from X-ray crystallography showing the relative conformation of 4,15-diacetylnivalenol. The unit cell of the crystal included one water molecule (O20).

15.2 (C-16, CH₃), 20.6 (C-18, CH₃), 21.0 (C-20, CH₃), 46.1 (C-13, CH₂), 49.4 (C-5, C), 52.2 (C-6, C), 61.8 (C-15, CH₂), 64.3 (C-12, C), 69.3 (C-11, CH), 135.8 (C-9, C), 73.2 (C-7, CH), 78.4 (C-3, CH), 79.7 (C-2, CH), 83.5 (C4, CH), 138.3 (C-10, CH), 170.0 (C-17, C=O), 172.6 (C-19, C=O), 198.9 (C-8, C=O). EI-MS, *m/z* (rel. int.): 396 (1) [M]⁺, 336 (6), 294 (9), 247 (10), 217 (10), 189 (30), 179 (100), 125 (37), 98 (53), 77 (57), 55 (48). CI-MS, *m/z*: 414 [M + NH₄].

A second compound (3 mg) was purified from MPLC fraction 1. Based on NMR analysis, this compound was structurally similar to 4,15-diacetylnivalenol, but the hydroxyl group at C-7 and the carbonyl at C-8 were each replaced by

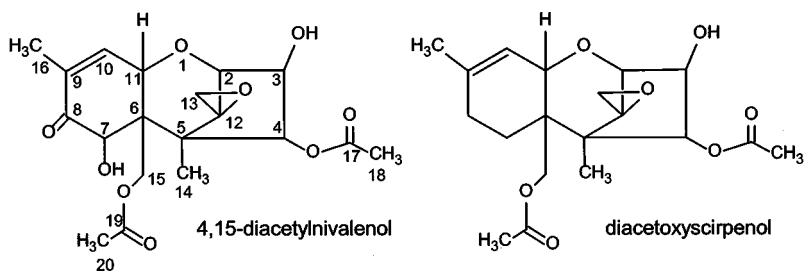


FIG. 4. Nematode-antagonistic trichothecene compounds isolated from *Fusarium equiseti*.

hydrogens. This compound was deduced to be 4,15-diacetoxy-12,13-epoxytrichothec-9-en-3-ol (diacetoxyscirpenol) (Figure 4): $^1\text{H NMR}$: δ 0.82 (3H, s, H-14), 1.71 (3H, s, H-20), 1.72 (3H, br s, H-16), 1.90 (2H, m, H-13), 2.14 (3H, s, H-18), 2.77 (2H, d, H-9), 3.06 (2H, d, H-7), 3.69 (1H, d, H-2), 4.06 (2H, dd, H-15), 4.09 (1H, d, H-11), 4.16 (1H, dd, H-3), 5.12 (1H, d, H-4), 5.53 (1H, m, H-10). $^{13}\text{C NMR APT}$: δ 6.9 (C-14, CH_3), 20.9 (C-18, CH_3), 21.0 (C-20, CH_3), 21.3 (C7, CH_2), 23.2 (C-16, CH_3), 27.9 (C-8, CH_2), 43.9 (C-13, CH_2), 47.8 (C-5, C), 48.7 (C-6, C), 63.6 (C-15, CH_2), 65.1 (C-12, C), 68.0 (C-11, CH), 84.9 (C-4, CH), 78.4 (C-3, CH), 78.9 (C-2, CH), 118.5 (C-10, CH), 140.5 (C-9, C), 171.7 (C-17, C=O), 173.8 (C-19, C=O). EI-MS, m/z (rel. int.): 366 (0.01), 306 (10), 278 (5), 233 (5), 187 (10), 175 (15), 159 (20), 124 (72), 105 (100), 91 (76), 79 (33), 55 (24). CI-MS, m/z : 384 [M + NH_4].

4,15-Diacetylinalenol, diacetoxyscirpenol, and a third unidentified compound (2 mg) isolated from MPLC fraction 3 decreased egg hatch and juvenile mobility in both trials when tested at 25 $\mu\text{g/ml}$ (ANOVA, $P < 0.001$; Dunnett's test, $P < 0.05$) (Figure 5). Egg hatch was reduced by approximately 40% compared to that in the control. The number of juveniles still mobile after hatching in

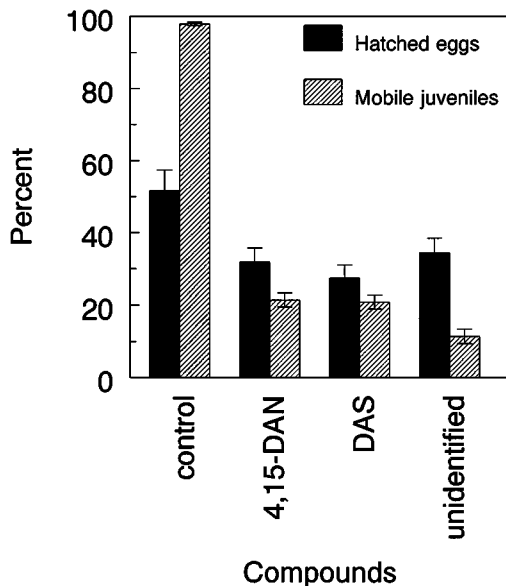


FIG. 5. Percentage of *Meloidogyne incognita* eggs hatched and percentage of hatched juveniles that were mobile after one week in compounds purified from *Fusarium equiseti* culture broth. Fractions were tested at a concentration of 25 $\mu\text{g/ml}$. 4,15-DAN = 4,15-diacetylinalenol; DAN = diacetoxyscirpenol. Data are mean \pm SE of 10 replications in two trials (200 and 250 eggs/replicate).

the test compounds was reduced by approximately 80% relative to the number in the control solution.

DISCUSSION

4,15-Diacetylivalenol and diacetoxyscirpenol are trichothecenes, an important group of tricyclic sesquiterpene mycotoxins that inhibit protein synthesis and are commonly produced by *Fusarium* species (Sweeney and Dobson, 1998). These two compounds were first identified from *F. equiseti* as phytotoxins (Brian et al., 1961; Dawkins et al., 1965) but occur in a number of other *Fusarium* species (De Nijs et al., 1996). The broad-spectrum biological activity of diacetoxyscirpenol (DAS) has been especially well documented (Beasley, 1989); however, this is the first published report of activity against plant-parasitic nematodes for either compound.

The similarity in bioactivity between 4,15-diacetylivalenol and diacetoxyscirpenol suggests that common structural elements are involved in their nematode-antagonistic activity. The C-7 hydroxyl group and the C-8 carbonyl group are not required for this activity, which suggests a hydrophilic binding site including the oxygens on C-3, C-4, and C-13 in a rigid conformation. The importance of these oxygens for biological activity is consistent with trichothecene structure-activity relationships known for protein synthesis inhibition and anticancer activity (Beasley, 1989). The X-ray data, which is the first such published analysis for 4,15-diacetylivalenol, suggest a labile hydrophilic binding site could also occur that involves the two acetate groups. The acetate group attached to C-15 folds over the six-membered ring such that the C-16 and C-20 methyl groups are spatially close to each other, bringing the acetate carbonyl close to the C-9 double bond and the O-1 oxygen. The acetate group on C-15 along with that on C-4 is also involved in anticancer activity (Beasley, 1989). The hydrophobic portion of the molecule C-5, C-6, C-7, C-8 is structurally and/or conformationally different between the two compounds and appears not to be involved in nematode-antagonistic activity.

Nematode-antagonistic activity against *M. incognita* was detected in many different fractions during bioassay-guided isolation, indicating that compounds in addition to 4,15-diacetylivalenol and diacetoxyscirpenol are contributing to the activity of the culture broth. Some of these other compounds, as well as the third nematode-antagonistic compound that was purified but unidentified, could also be trichothecenes, as *F. equiseti* is reported to produce at least four other trichothecenes (De Nijs et al., 1996). One of these, T-2 toxin, has been shown to be nematocidal to *Meloidogyne javanica* (Ciancio, 1995). Despite the predisposition of *Fusarium* species to produce mycotoxins, this should not discourage further investigation of *Fusarium* cultures to find useful natural products with a narrower activity range. Screening procedures to exclude broad-spectrum mycotoxins would increase efficiency of a search for nematode-antagonistic compounds.

It is not known if trichothecenes produced by *Fusarium* strains that parasitize plant nematodes play a role in interactions between fungi and nematodes under natural conditions. Enzymes secreted by parasitic fungi are known to help colonization of nematode eggs by penetrating the egg shell (Segers et al., 1991), but the contribution of nonenzymatic toxins to the success of parasitism has not been studied. If the biology of microbial colonization of insects is an appropriate analogy, then such a contribution would be probable. *Photorhabdus* bacteria that are released into insect hosts by entomophagous nematodes secrete toxins that kill the host and inhibit colonization of the carcass by competing microbes (Bowen et al., 1998). As *Fusarium* species are commonly found in soil environments, there should be ample opportunities for these fungi and their secondary metabolites to interact with root-feeding nematodes.

The broad-spectrum toxicity and nonspecific mode of action of trichothecenes indicate that they would be impractical by themselves as chemical control agents of plant nematode pathogens without significant modification. The production of trichothecenes in a strain isolated from a plant-parasitic nematode suggests that if *Fusarium* species are used for biological control, production of mycotoxins by these strains should be evaluated if incidental exposure to humans is a concern. The occurrence of mycotoxins with broad-spectrum toxicity in a potentially beneficial strain does not automatically preclude use of that fungus for biological control since artificial selection may eliminate or reduce mycotoxin production in that strain. On the other hand, should trichothecenes and other mycotoxins be found to enhance efficacy of parasitism and biological control, the benefits of their production will have to be balanced against their potential negative side effects.

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