# Furanocoumarin Phytoalexins, Trichothecene Toxins, and Infection of *Pastinaca sativa* by *Fusarium sporotrichioides*

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## ABSTRACT

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Many plant pathogenic fusaria produce trichothecenes, which are potent phytotoxins and inhibitors of protein synthesis in eukaryotes. In this study we show that a wild-type, trichothecene-producing strain of *F. sporotrichioides*, NRRL 3299, is pathogenic on *Pastinaca sativa* (parsnip) roots and produces T-2 toxin in planta. Parsnip roots infected with this strain also accumulate high levels of fungitoxic furanocoumarins, mainly xanthotoxin and angelicin. Analysis of serial sections of infected roots showed that furanocoumarin concentrations decrease sharply at the infection boundary to levels apparently insufficient to completely block fungal growth or trichothecene production. The involvement of trichothecenes in pathogenesis was investigated by using three previously isolated, complementary mutants of NRRL 3299, blocked at different steps in T-2 toxin biosynthesis. T-2 toxin production is restored when these mutants are grown together pairwise and each mutant accumulates different end products. In this study the ability of these mutants to infect parsnips correlates with their ability to produce certain trichothecenes. Thus, the mutant that accumulates 4,15-diacetoxyscirpenol, a trichothecene nearly as toxic as T-2 toxin, is as pathogenic as the wild-type parent. In contrast, neither the mutant that accumulates the less toxic calonectrin analogues nor the mutant that accumulates the nontoxic trichothecene precursor, trichodiene, is pathogenic. Furthermore, coinoculation of these latter two mutants on parsnip roots results in infection.

*Additional keywords*: mycotoxins, phytoalexin metabolism.

Various *Fusarium* species produce dry rots of *Pastinaca sativa* L. (parsnip) roots in storage (29), but the factors involved in infection are unknown. Two possible factors are phytoalexins produced by the plant and trichothecene toxins produced by the fungus. Low levels of xanthotoxin, angelicin (Fig. 1), and other fungitoxic furanocoumarins are normal constituents of parsnip roots. Johnson and co-workers (14) have established xanthotoxin as a phytoalexin in parsnip by demonstrating its 20-fold induction in roots inoculated with several fungi nonpathogenic to parsnip. The trichothecenes, which are secondary metabolites, are potent phytotoxins and inhibitors of protein synthesis in eukaryotes (17,27). Although proof is lacking, there is considerable circumstantial evidence for a role for trichothecenes in plant pathogenesis, including correlations of virulence with ability to produce toxins in vitro (15) and isolation of toxins from diseased plant tissues (16,20). Chlorosis, necrosis, and other symptoms are produced in a wide variety of plants treated with very low ($10^{-5}$–$10^{-6}$ M) concentrations of trichothecenes (8,10).

Although originally isolated from corn (16), *Fusarium sporotrichioides* Sherb. NRRL 3299 was found in the present study to be highly pathogenic on parsnip roots, which allowed a simple and reliable bioassay. In liquid culture and on various solid substrates, this strain produces high levels of trichothecenes (6,16). In a previous study (11), trichothecene toxin production by strain NRRL 3299 in liquid cultures was completely inhibited by furanocoumarins at concentrations well below those reported to accumulate in infected parsnips (14). These studies suggested that toxin production might be limited during pathogenesis in plants such as parsnips that accumulate furanocoumarins. A corollary is that trichothecene toxins might not be important for pathogenicity, at least in parsnips. One way to test this hypothesis would be to use mutants blocked or altered in trichothecene production. A number of such mutants have recently been generated from *F. sporotrichioides* NRRL 3299 by UV treatment (6,7).

In this study we have investigated the roles of furanocoumarin phytoalexins and trichothecene toxins in the pathogenicity of *Fusarium* on parsnip. High-performance liquid chromatography (HPLC) and combined gas chromatography-mass spectroscopy (GC-MS) were used to measure concentrations of fungal and plant metabolites in tissue sections collected from parsnip roots infected with *F. sporotrichioides* NRRL 3299. We also used a complementary, genetic approach to further examine this complex plant-pathogen system and compared the pathogenicity of the wild-type trichothecene-producing strain to that of three mutants blocked or altered in trichothecene biosynthesis.

## MATERIALS AND METHODS

**Cultures and growth conditions.** Each fungal strain originated from a single spore. *F. sporotrichioides* strain NRRL 3299 (ATCC 24043) is from the Agricultural Research Service Collection, Peoria, IL. Trichothecene biosynthetic mutants of NRRL 3299 (MB1716, MB2972, and MB5493) were previously obtained following UV mutagenesis (6,7). Wild-type *F. sporotrichioides* NRRL 3299 accumulates predominately T-2 toxin and small amounts of 4, 15-diacetoxyscirpenol and neosolaniol (Fig. 1). Mutant strain MB1716 accumulates predominately 4, 15-diacetoxyscirpenol (6); and strain MB2972 accumulates 15-deacetylcalonectrin and 3, 15-dideacetylcalonectrin (Fig. 1) (7). Strain MB5493 accumulates trichodiene (Fig. 1), the nontoxic precursor of the trichothecenes, and does not produce any 12, 13-epoxytrichothecenes (7).

Cultures were routinely grown on V-8 juice agar medium (26) slants or plates on an alternating 12-hr, 25 C light/20 C dark schedule. For long-term storage, strains were maintained on V-8

juice agar slants at 4 C and were stored as conidial suspensions in 10–15% v/v glycerol at −90 C. For all assays, fresh transfers of the strains were obtained from stock cultures stored at 4 C.

**Pathogenicity assays.** Parsnips obtained from several local suppliers (cultivars unknown) were peeled, washed with tap water, and surface sterilized by dipping for 5–10 sec in 95% ethanol. The roots were cut into approximately 5-mm-thick disks under aseptic conditions, washed in several changes of sterile distilled water, and transferred to sterile plastic petri dishes containing filter paper moistened with sterile distilled water. The upper surface of each disk was inoculated immediately by placing an agar inoculum plug (5 mm diameter), mycelial side down, in the center core of each disk. Inoculum plugs were cut from the growing margins of cultures less than 10 days old. All fungal cultures used in each experiment were of equal age. The petri dishes were then sealed in plastic bags and incubated at 25 ± 1 C for 7–10 days in the dark. Pathogenicity was estimated gravimetrically. Root disks were weighed at the end of each experiment; then rotted tissue was removed with a spatula and the remaining tissue was reweighed.

For uniformity in localization experiments, parsnip root cortex tissue was used instead of root disks and was cut longitudinally into strips approximately 45 mm long, 15 mm wide, and 5 mm thick. The upper surface of each of 15 strips was inoculated immediately with strain NRRL 3299 by placing an agar inoculum (5 mm deep and equal to the strip in width), mycelial side down, across one end of the strip. Each strip was incubated in a moist dish as described above for 6 days until the zone of discoloration, which was homogeneous from the upper to the lower surface, was halfway across the strip. Then the eight most similar strips were each cut by hand into 14 approximately equal sections (3 mm thick). Equivalent slices from three strips were surface sterilized with 0.5% sodium hypochlorite and placed on V-8 juice agar for fungus reisolation. Equivalent slices from five strips were also pooled, weighed, and extracted by shaking overnight at room temperature with 50 ml of ethyl acetate. The localization experiment was repeated twice with virtually identical results; data from one experiment are presented here.

**Toxicity assay for furanocoumarins.** Toxicity of xanthotoxin and angelicin to fungal strains was examined in an agar medium. Xanthotoxin and angelicin were purified as described previously (25). Duplicate 35 × 10 mm plastic petri dishes containing 1 ml of V-8 juice agar and 2% dimethyl sulfoxide v/v with or without xanthotoxin or angelicin alone or together at 25, 50, 100, 150, 200, 300, and 400 μg/ml were inoculated with plugs (4 mm diameter) cut from the growing margin of V-8 cultures less than 10 days old and placed with the mycelial surface appressed to the agar surface at the edge of the plate. Plates were incubated in a humidified box at 25 ± 1 C in the dark. Radial growth (distance from the inoculum plug to the growing margin) was measured daily for up to 10 days. Percent tolerance was determined as ([radial growth rate on amended medium/radial growth rate on control medium] × 100). Plots of dosage versus tolerance were used to determine $ED_{50}$ and $ED_{90}$ values.

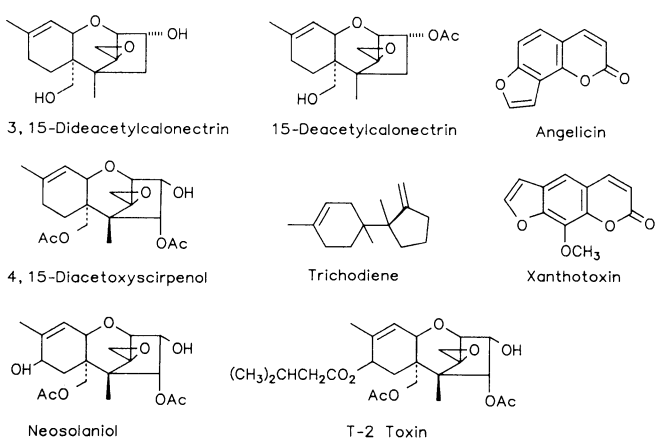


**Fig. 1.** Structures of xanthotoxin, angelicin, trichothecenes, and trichodiene.

**Chemical analyses.** Furanocoumarins in parsnip root tissues were quantitated by HPLC as described previously (25). In one experiment, the effluent represented by the xanthotoxin and angelicin peaks was collected and analyzed by nuclear magnetic resonance spectroscopy (Bruker WM-300) to ensure that these furanocoumarins were indeed responsible for the peaks.

Trichothecenes were analyzed as their trimethylsilyl (TMS) derivatives by GC-MS. An aliquot of the ethyl acetate extract of the tissue equivalent to 500 mg of tissue was evaporated to dryness under nitrogen and 50 μl of Tri-Sil/TBT (Pierce Chemical Co., Rockford, IL) was added. The samples were held at 50 C for 1 hr before analysis of 1 μl by GC-MS. The samples were injected in the splitless mode onto a 15 M by 0.25-mm fused silica DB-1 column (J & W Scientific, Rancho Cordova, CA). The linear flow rate of helium in the column was 50 cm/s. At injection, the GC temperature was 120 C. Two minutes after injection, the column was rapidly heated to 200 C (at 40 C/min) and the temperature programmed to 270 C at 10 C/min. For quantitative analyses, the MS was operated in the chemical ionization mode with isobutane as the reagent gas (0.3 torr). The protonated molecular ion and most intense ion for the trichothecenes of interest were monitored in the selected ion mode. Trichothecenes were identified by their retention time on the column and by the presence of the correct ratio of the protonated molecule and the most intense ion. Quantitation was based on the most intense ion. Response factors were determined from the response of known amounts of standards and were used to quantitate the amount of individual trichothecenes in the samples. The detection limit for T-2 toxin was 0.5 ng, which was equivalent to approximately 0.1 nmoles/g of parsnip fresh weight.

The parsnip extracts were analyzed for trichodiene without derivatization. The samples were injected into a capillary GC column as above, and after 5 min the column oven was temperature programmed to 250 C at 10 C/min. For quantitation, the MS was operated either in the EI mode, with selected ion monitoring of masses 67, 108, and 109, or in the CI mode, with selected ion monitoring of the protonated molecule, 205, and the fragments 109 and 95. In either case, trichodiene was identified by its retention time and the presence of the correct ratio for the measured signals. Quantitation was based on the fragment ion 109 by using response factors determined for standards. The detection limit for trichodiene was 0.1 ng, equivalent to approximately 0.05 nmoles/g of parsnip fresh weight.

## RESULTS

**Pathogenicity of wild-type strain.** Within 48 hr after inoculation with *F. sporotrichioides* NRRL 3299, a dark brown lesion developed under the inoculum plug in the central xylem core of the parsnip root disk. Over 4–8 days of further incubation, the infected area continued to enlarge, extending throughout the softer cortex tissue, and often reached the edge of the root disk. The necrotic areas were usually dry and punky in texture, and the advancing margins were quite distinct. Mycelia and spores were usually sparse on the disk surface. All parsnip roots tested were susceptible to strain NRRL 3299.

**Localization of *Fusarium*, furanocoumarins, and trichothecenes during infection by wild-type strain.** Colonization of parsnip root strips by *F. sporotrichioides* NRRL 3299 was measured by isolation of viable fungus directly from surface-disinfected pieces of plant tissue placed on an agar culture medium. Attempts to measure fungal growth by measuring ergosterol content were unsuccessful due to lack of assay sensitivity under these experimental conditions. *F. sporotrichioides* was reisolated from most of the visibly brown tissue sections, but not from any surface-disinfected tissues in advance of browning.

In uninoculated, peeled parsnip roots, furanocoumarin levels were very low; <0.1 μmoles of total furanocoumarins was found per gram of freshly cut roots, and <0.5 μmoles/g was found after up to 6 days incubation under sterile conditions. Furanocoumarin levels are substantially higher in the peelings than in peeled roots (13); for uniformity, peeled roots were used for all experiments in

this study. The two major furanocoumarins detected by HPLC in parsnips in all experiments in the present study were xanthotoxin and angelicin, which together consistently accounted for more than 95% of furanocoumarins detected. Other furanocoumarins such as psoralen and bergapten, which previously have been found in parsnip (13), were either not detected or present in much lower levels than xanthotoxin and angelicin under all experimental conditions.

The quantitative distribution of xanthotoxin and angelicin was measured in serial cross sections of parsnip root cortex strips infected with *F. sporotrichioides* NRRL 3299. Maximum furanocoumarin concentrations were 5.1 μmoles and 8.4 μmoles/g of fresh weight for xanthotoxin and angelicin, respectively (Fig. 2). The combined furanocoumarin concentration was more than 13 μmoles/g of fresh weight, which greatly exceeded the in vitro $ED_{50}$ and $ED_{90}$ for radial growth of *F. sporotrichioides* NRRL 3299 on V-8 juice agar medium amended with xanthotoxin, angelicin (Table 1), or an equal weight mixture of the two (data not shown). This sensitivity of *F. sporotrichioides*' radial growth to xanthotoxin was similar to that previously reported for *Ceratocystis fimbriata* (14).

The furanocoumarin concentration changed sharply at the leading edge of infection, as measured by visible browning of tissue and by fungal reisolation. The concentration of total furanocoumarins in advance of infection was from 0.4–1.0 μmoles/g of fresh weight, which was enough to slow, but less than that necessary to completely inhibit fungal growth in vitro (Table 1). Furanocoumarin concentrations were highest in tissue sections collected several millimeters behind the leading edge of infection. Lower furanocoumarin levels in tissue sections closer to the original inoculum could result from furanocoumarin lability or decreased synthesis, or from metabolism of furanocoumarins by the plant or by the pathogen. *F. sporotrichioides* NRRL 3299 was able to completely metabolize a variety of furanocoumarins, including xanthotoxin, added to 24-hr liquid cultures to a final concentration of up to 5 mM (angelicin was not tested) (11) and was also able to completely metabolize xanthotoxin and angelicin added at 0.5 mM to agar cultures, a concentration at which radial growth was inhibited by 50% (data not shown). Both xanthotoxin and angelicin were fungistatic, but not fungicidal, to *F. sporotrichioides*. Cultures of strain NRRL 3299 kept for more than 2 wk at furanocoumarin concentrations completely inhibitory to growth had the same growth rate as control cultures when transferred to agar media without furanocoumarins.

The quantitative distributions of the fungal metabolites T-2 toxin and trichodiene were measured by combined GC-MS of ethyl acetate extracts of serial cross sections of parsnip root cortex strips infected with *F. sporotrichioides* NRRL 3299 (Fig. 2). Maximum concentrations observed in one localization experiment were approximately 180 nmoles and 10 nmoles/g of fresh weight for trichodiene and T-2 toxin, respectively. Concentrations of both fungal metabolites were highest near the initial inoculation site and decreased gradually toward the leading edge of the infection. T-2 toxin, if present, was below the GC-MS detection limit (0.1 nmoles/g of fresh weight) in the two infected tissue sections nearest the infection boundary where trichodiene was detected. Neither T-2 toxin nor trichodiene was detected in root strips in advance of visible browning or fungal infection as measured by reisolation.

**Pathogenicity of mutant strains.** Detection of T-2 toxin in infected tissues is only circumstantial evidence that trichothecenes have a role in pathogenicity of *Fusarium* on parsnip root. *F. sporotrichioides* is amenable to genetic manipulation by mutational analysis, which allows a more rigorous test of the role of trichothecenes in this disease. Therefore, the pathogenicity of three mutants of *F. sporotrichioides* NRRL 3299 altered in trichothecene biosynthesis was investigated. Mutant strain MB1716, which produces 4,15-diacetoxyscirpenol, was consistently as pathogenic as the wild-type strain on parsnip root disks (Table 2 and Fig. 3). In contrast, mutant strain MB2972, which produces 15-deacetylcalonectrin and 3, 15-dideacetyl-

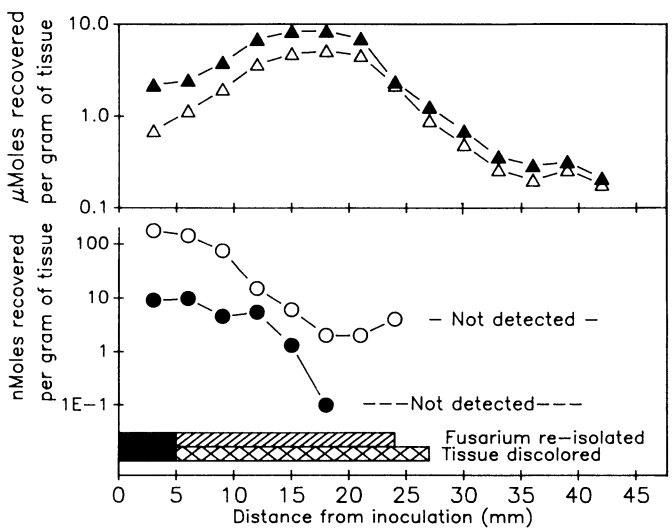


**Fig. 2.** Concentrations of xanthotoxin (Δ), angelicin (▲), T-2 toxin (•), and trichodiene (o) in parsnip root cortex strips 6 days after inoculation with *Fusarium sporotrichioides* NRRL 3299 and incubation at 25 C in the dark. Root strips were cross sectioned, extracted, and analyzed by HPLC and GC-MS, all as described in the text. The extent of fungal penetration was determined by subculture of excised tissue cross sections. For clarity of presentation, concentrations are shown on logarithmic plots. The dark rectangle indicates the area of the initial mycelial inoculum.

TABLE 1. Toxicity of xanthotoxin and angelicin to *Fusarium sporotrichioides* NRRL 3299 and mutant strains in agar culture[a]

| Strain | Xanthotoxin | | Angelicin | |
|---|---|---|---|---|
| | $ED_{50}$ (mM) | $ED_{90}$ (mM) | $ED_{50}$ (mM) | $ED_{90}$ (mM) |
| NRRL 3299 | 0.5 | 1.2 | 0.5 | 2.0 |
| MB1716 | 0.8 | 1.9 | 0.4 | 1.4 |
| MB5493 | 0.8 | 1.8 | 0.5 | 1.2 |

[a] Toxicity assays were conducted as described in the text by measuring radial growth (duplicate plates) of strains on agar media with and without furanocoumarins.

TABLE 2. Pathogenicity of *Fusarium sporotrichioides* NRRL 3299 and mutant strains on parsnip root disks[a]

| Exp. no. | Percentage of tissue rotted (mean ± s.d.)[b] | | | | |
|---|---|---|---|---|---|
| | NRRL 3299 | MB1716 | MB2972 | MB5493 | MB5493 and MB2972 |
| 1 | 53 ± 29 (4) | 49 ± 31 (4) | 1 ± 1 (3) | 3 ± 3 (3) | nd |
| 2 | 50 ± 27 (10) | 62 ± 38 (10) | 0 (8) | 0 (7) | nd |
| 3 | 87 ± 19 (10) | 83 ± 17 (9) | 4 ± 5 (8) | 0 (8) | nd |
| 4 | 53 ± 15 (5) | 44 ± 15 (6) | 2 ± 2 (8) | 1 ± 3 (7) | nd |
| 5 | 62 ± 17 (5) | 85 ± 21 (5) | 0 (10) | 0 (10) | 20 ± 12 (7) |
| 6 | 57 ± 13 (5) | nd | 1 ± 1 (5) | 0 (5) | 25 ± 11 (20) |
| 7 | 41 ± 15 (5) | nd | 1 ± 1 (5) | 0 (5) | 24 ± 15 (20) |

[a] Pathogenicity assays conducted as described in the text. Pathogenicity was assessed at 6 days after infection, except for exp. 7, which was assessed at 10 days.

[b] Mean weight of all disks used in each experiment was 8.90 ± 1.52 (exp. 1), 4.38 ± 0.96 (exp. 2), 2.72 ± 0.84 (exp. 3), 5.51 ± 1.28 (exp. 4), 5.80 ± 1.28 (exp. 5), 8.62 ± 2.1 (exp. 6), and 8.86 ± 2.70 (exp. 7). Number in parentheses indicates number of disks measured for each treatment; nd, not done.

calonectrin, was weakly pathogenic under the same conditions (Table 2 and Fig. 3). Furthermore, mutant strain MB5493, which produces only the nontoxic trichothecene precursor, trichodiene, was not pathogenic on parsnip root disks (Table 2 and Fig. 3).

A mixed culture of strains MB2972 and MB5493 is able to produce high levels of T-2 toxin in liquid media (7). This result demonstrates the ability of these two mutants to complement one another. When these two strains, which individually were nonpathogenic, were co-inoculated on parsnip root disks, an intermediate level of pathogenicity was recovered (Table 2 and Fig. 3). The three *F. sporotrichioides* mutants were very similar to the wild-type strain in growth rate in liquid culture (7) and in radial growth on V-8 agar medium (data not shown). In addition, strains MB1716 and MB5493 were similar to the wild type in sensitivity to xanthotoxin and angelicin in a V-8 agar medium (Table 1) and were able to metabolize all xanthotoxin and angelicin added at 0.2 mM to agar cultures (data not shown). Strain MB2972 was not tested for furanocoumarin sensitivity or metabolism.

Infected portions of parsnip root disks either inoculated with the wild-type strain NRRL 3299 or co-inoculated with mutant strains MB5493 and MB2972 were extracted with ethyl acetate and analyzed by GC-MS for T-2 toxin and trichodiene. This experiment was repeated twice, and in both experiments trichodiene was detected in root disks either inoculated with the wild-type strain or co-inoculated with the two mutants. T-2 toxin, however, was above the detection limit (0.1 nmole/g of fresh weight) in only one of the four extracts, which was obtained from parsnips inoculated with strain NRRL 3299. Trichodiene was detected, but trichothecene toxins were not, in an extract of parsnip root disks infected with strain MB1716 (one test).

## DISCUSSION

Infection of parsnip roots with *F. sporotrichioides* NRRL 3299 caused tissue browning with a concomitant, marked increase in the levels of xanthotoxin and angelicin. Both of these furanocoumarins fulfill several requirements necessary to be considered phytoalexins in parsnip. Both xanthotoxin and angelicin exhibit antifungal activity in vitro, which is circumstantial evidence that they contribute to limiting fungal growth in plant tissues. Both xanthotoxin and angelicin occur as natural constituents of numerous plant species, but neither is known to be a product of fungal metabolism (22). Levels of xanthotoxin and angelicin are very low in healthy parsnip tissues, but their concentrations in lesion areas greatly exceed those shown to inhibit fungal growth in vitro. There is also considerable evidence that xanthotoxin and other linear furanocoumarins are phytoalexins in celery (5,9).

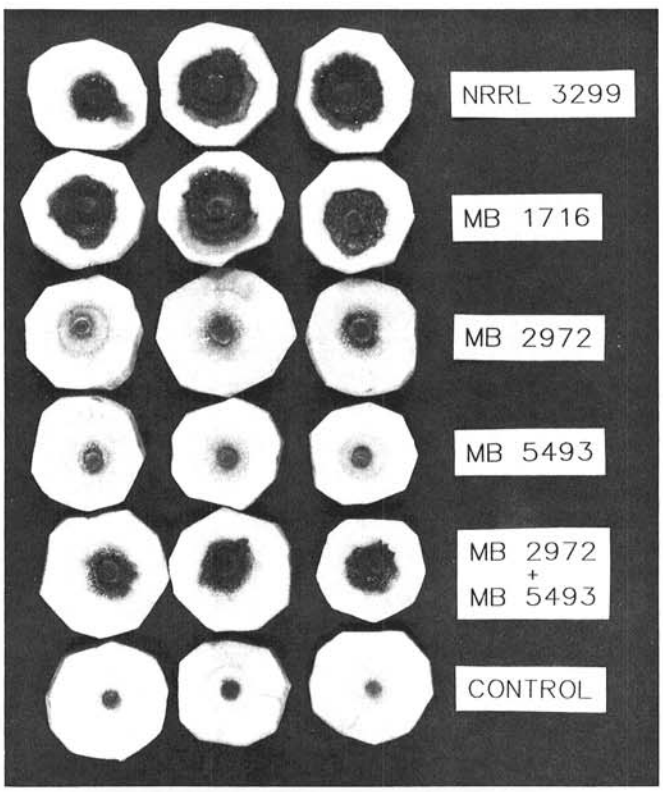


**Fig. 3.** Pathogenicity of *Fusarium sporotrichioides* NRRL 3299 and mutant strains on parsnip root disks. For each treatment, six root disks were inoculated with 5 mm agar inoculum plugs and incubated for 7 days at 25 C in the dark. The three disks shown include the least pathogenic and most pathogenic tests in each treatment group. The control disks were inoculated with a plug of agar medium. Inoculum plugs were not removed before photography.

The mechanism by which furanocoumarins accumulate in diseased plant tissues remains highly speculative. It is uncertain whether furanocoumarins are, like isoflavonoids (24), synthesized de novo from remote phenylpropanoid precursors or from preformed intermediates or glycosides. Known intermediates in the biosynthesis of xanthotoxin and angelicin, including coumarin, umbelliferone, marmesin, psoralen, and columbianetin, were, if detected, present at very low levels in uninfected parsnip roots analyzed by HPLC (data not shown), which suggests that these precursors are not readily available as aglycones. Coumarin can exist in sweet clover as a glucoside (22), and similar bound forms of furanocoumarins have recently been found in celery seeds (1). Whether furanocoumarin glycosides are present in parsnips was not investigated in the present study.

In the susceptible interaction between parsnip and *F. sporotrichioides* NRRL 3299, xanthotoxin and angelicin accumulate in root tissue to levels that are greatly in excess of those inhibitory to the fungus in vitro. But the furanocoumarins apparently do not accumulate rapidly enough to levels that can completely block fungal growth and, consequently, the pathogen can continue to spread through tissue where the furanocoumarin concentration remains relatively low. Similar results have been obtained in more detailed studies of other plant-fungal systems, including the interactions between *Phaseolus* and *Colletotrichum* (2) and *Glycine* and *Phytophthora* (12). Our data are consistent with the hypothesis that furanocoumarins are a resistance mechanism in parsnip, but some anomalies remain. In parsnip tissues, furanocoumarins reach levels sufficient to almost completely block fungal growth in vitro, yet the fungus can still infect the plant. This anomaly may occur, in part, because the in vitro bioassay does not duplicate the complex environment of the infected parsnip root. Further study will be necessary to clarify the exact spatial and temporal relationships of parsnip cell death, furanocoumarin accumulation, and the inhibition of fungal growth, and to determine the importance of furanocoumarins as a resistance mechanism.

In agar and in liquid media, *F. sporotrichioides* can metabolize xanthotoxin and angelicin to apparently less toxic and as yet unidentified products. Detoxification of xanthotoxin and other furanocoumarins by *F. sporotrichioides* in vivo may contribute to reducing the amounts of furanocoumarins that accumulate, particularly at the infection boundary. The ability of plant pathogenic fusaria and other fungi to detoxify their host's phytoalexins and its importance in pathogenesis has been amply demonstrated in other systems (28). We have screened more than 60 strains of the parsnip pathogen *Gibberella pulicaris* (*F. sambucinum*) and found a strong correlation between virulence and the ability to metabolize xanthotoxin and angelicin. All strains sensitive to either furanocoumarin are unable to infect parsnip root (A. E. Desjardins, G. F. Spencer, and R. D. Plattner, *unpublished*).

Xanthotoxin and other furanocoumarins completely block trichothecene biosynthesis in liquid cultures of *F. sporotrichioides* NRRL 3299, with the concomitant accumulation of the hydrocarbon precursor trichodiene (11). There is some indirect evidence that furanocoumarins may partially limit trichothecene biosynthesis in parsnip roots infected with strain NRRL 3299. In infected, viable root tissues, xanthotoxin and angelicin accumulated to high levels, whereas T-2 toxin, which is produced

at high levels in liquid culture, was present at very low levels, and trichodiene, which is not detectable in liquid culture, accumulated. In contrast, in microwave-cooked parsnip root infected with strain NRRL 3299, very low levels of furanocoumarins and trichodiene were detected, and the level of T-2 toxin was comparable to that observed in liquid cultures.

The studies reported in this paper provide the following preliminary evidence for a causal relationship between certain trichothecene toxins and plant pathogenesis. T-2 toxin was found in infected plant tissue, although not in all experiments and not at the infection boundary as determined by GC-MS analysis of extracts of parsnip root cross sections. Although toxicity of T-2 and other trichothecenes to parsnip root cells is not known, levels of T-2 toxin in infected parsnip root strips were within the range (1–10 $\mu$M) known to be toxic to other plants (8,10). The reasons for the variability in recovery of T-2 toxin from infected parsnip root are not known. Attempts of others to detect trichothecenes in infected plant tissues also have not always been successful; for example, Bean and co-workers (4) found no trichothecenes in muskmelon seedlings infected with toxin-producing strains of *Myrothecium roridum*. As discussed by Yoder (31), Mitchell (21), and others, the inability to detect toxins in infected plant tissues does not necessarily indicate that toxins are not involved in pathogenesis. Toxins may be unstable or inactivated by plant enzymes, or may not be detectable due to interference by plant constituents. Some evidence to support these hypotheses has been obtained in *Fusarium* infections of grains. Levels of the trichothecene deoxynivalenol declined in wheat heads naturally and experimentally infected with *F. graminearum* (19,23), and suspension cultures of wheat have been found to transform added deoxynivalenol to a variety of products (18).

The pathogenicity of *F. sporotrichioides* NRRL 3299 on parsnip root and the availability of mutants blocked in T-2 toxin biosynthesis have allowed a mutational analysis of the role of trichothecene toxins in this chemically complex plant-fungal system. The results from this study revealed that the ability of three stable, previously isolated and characterized T-2 toxin-minus mutants to infect parsnip roots parallels their ability to produce toxic trichothecenes. These mutants had been derived from NRRL 3299 and were shown by complementation tests and chemical analysis each to be blocked in a different step in the T-2 toxin biosynthetic pathway and to accumulate different end products (6,7; M. N. Beremand and R. D. Plattner, *unpublished*). Mutant MB5493 is blocked early in the pathway and accumulates the nontoxic trichothecene precursor, trichodiene, and no detectable trichothecenes (7). Mutants MB2972 and MB1716 are blocked later in the pathway; MB2972 adds four of the six oxygens found in T-2 toxin to produce the calonectrin analogues, 15-deacetylcalonectrin, and 3, 15-dideacetylcalonectrin (7), and MB1716 adds five of the six oxygens to produce 4, 15-diacetoxyscirpenol (6). It is most interesting that only the mutant that accumulates diacetoxyscirpenol is as pathogenic as the T-2 toxin producing parent; the mutant that does not produce trichothecenes is nonpathogenic, and the mutant that produces the calonectrin analogues is only slightly pathogenic. One possible interpretation of these results is that not only are trichothecenes important for pathogenicity, but also that particular, highly oxygenated trichothecenes may be required.

Use of blocked mutants allows a critical analysis of the role of fungal toxins in pathogenicity as long as the mutants are blocked specifically in the process being studied. As with any induced mutants, it is possible that the T-2 toxin-minus mutants used in this study carry multiple mutations or are the result of a genetic lesion that has a pleiotrophic effect that also alters trichothecene production. Some precautions have been taken to reduce these possibilities. These mutants were carefully screened to eliminate any T-2 minus mutants that might be caused by common pleiotropic mutations such as auxotrophy (all are prototrophs) or reduced growth (all retain wild-type growth rates) (6,7). UV irradiation was used to generate these mutants because UV-induced mutants are less likely than chemically induced mutants to carry multiple defects. Finally, and perhaps most significantly, we used three independently isolated mutants blocked at different steps within the trichothecene biosynthetic pathway. Two of these mutants proved to also be nonpathogenic. Furthermore, these two mutants complement one another to restore T-2 toxin production in vitro (7) and also complement one another to restore pathogenicity. It seems unlikely that two independently isolated, complementary mutants, selected solely for their inability to produce T-2 toxin and blocked at different steps within the T-2 toxin biosynthetic pathway, would also both carry second site mutations that not only render them nonpathogenic, but that are also complementary. The results therefore strongly suggest that the two processes, T-2 toxin (or 4, 15-diacetoxyscirpenol) production and pathogenicity, are interrelated.

The trichothecenes are known to vary in toxicity and in effectiveness as protein synthesis inhibitors. The most toxic trichothecenes are those, like T-2 toxin and 4, 15-diacetoxy-cirpenol, that inhibit the initiation and not the elongation or termination of protein synthesis (3). Studies by Wei and McLaughlin (30) showed that 15-deacetylcalonectrin was not an initiation inhibitor, but rather an elongation and termination inhibitor. The phytotoxicities of T-2 toxin and 4, 15-diacetoxyscirpenol have been well documented, but the phytotoxicities of the calonectrin analogues are not known. There is considerable indirect evidence, however, that the calonectrin analogues, which are not oxygenated at C 4, are less toxic than the C 4 oxygenated compounds T-2 and 4, 15-diacetoxyscirpenol. Another possible explanation for the low pathogenicity of mutant strain MB2972 is that it produces less toxin than the wild-type strain in parsnip root tissues. In any case, the results from the present study demonstrate that a correlation exists between the ability of a strain or mutant to produce trichothecene toxins in culture and its ability to cause disease symptoms on parsnips. These results support the hypothesis that trichothecenes play an important role in this plant-fungal disease interaction.

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