Toxin Production by *Fusarium solani* From Fibrous Roots of Blight-Diseased Citrus

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ABSTRACT

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Single-spore isolates of *Fusarium solani,* obtained from fibrous roots of field-grown citrus trees exhibiting blight symptoms and from a diseased greenhouse-grown tree, were evaluated for toxin production. Several isolates from roots of blight-diseased trees produced culture extracts that severely inhibited root growth of radish seedlings. Inhibitory effect of extracts appeared to be due primarily to the presence of naphthoquinone derivatives. Toxins purified and identified from culture extracts included fusarubin, javanicin, and anhydrofusarubin; phytotoxicity was largely due to fusarubin.

Blight, a complex of symptoms also called Young Tree Decline, Sand Hill Decline, and Rough Lemon Decline, is a disease of unknown etiology that threatens most of the citrus plantings of Florida. Originally thought to affect only citrus budded on rough lemon (*Citrus limon* (L.) Burm. f.), it has now been observed to some degree on most scion/rootstock combinations (4). In severely affected groves, tree losses may reach 22% annually, resulting in the almost complete replacement of the grove after several years. It has been estimated that 10–15 yr after the first occurrence of blight in a grove all trees will be affected (5). No vector has been implicated in the apparently random spread of the disease, nor has transmission by grafting been observed.

One of the earliest symptoms of the disease is increased susceptibility to wilting during periods of moisture stress (1). As the disease progresses, partial defoliation and dieback become severe. Other symptoms include xylem dysfunction resulting in reduced water uptake (4), zinc and manganese deficiency patterns in leaves, increased zinc levels in xylem (16), and increased xylem vessel plugging (10). Infected trees seldom die, but persist for years in an unproductive state (1).

It has been suggested that the soil fungus *Fusarium solani* (Mart.) Appel et Wr. emend. Snyd. et Hans. may be responsible for blight symptoms (10). This weak parasite is commonly found in the soils of citrus groves, particularly the acid, sandy soils of Florida. A ubiquitous inhabitant of both healthy and diseased root surfaces, it may invade fibrous roots following injury or stress conditions. The root pruning resulting from this limited invasion would probably not significantly affect tree health unless it became severe (10). However, *F. solani* causes leaf necrosis and root rot in some herbaceous crops by producing phytotoxic compounds. Phytotoxins elaborated by certain isolates of this species include naphthazarins (7), trichothecenes (17), and fusaric acid (3). Absorption and translocation of fungal toxins by fibrous roots may cause the characteristic symptoms of blight. Toxin production by isolates of *F. solani* from citrus roots has not been reported. We undertook the present work to determine the toxicogenic potential of *F. solani* from citrus roots and to identify any toxins produced.

MATERIALS AND METHODS

Citrus seedling inoculation. The Florida isolate of *F. solani* used to inoculate rough lemon seedlings was cultured from wood of fibrous roots from blight-affected trees with root rot symptoms. The California isolate was obtained from a citrus tree with dry root rot symptoms. Both were cultured 5 days on liquid Fries' medium, with shaking. After 5 days, seedling roots were dipped for 5 min in a portion of the medium containing the fungus, and other seedlings were dipped in a fraction filtered (0.2-μm, Millipore) to remove the fungus. Both isolates produced red pigments on potato-dextrose agar (PDA) slants.

Inoculated seedlings were rated for wilt, root rot and vessel plugging. Wilt and root rot symptoms were rated on a scale of 0–5, in which 0 represented no evident aboveground or belowground symptoms, and 5 indicated a wilted plant near death or a root system extensively rotted because of infection. Vessel plugging was rated on a scale of 0–3 in which 0 = no vessel plugs, 1 = 1–50, 2 = 51–100, and 3 = >100 plugs in 30, 1-cm-long pieces of stele per plant.

Toxin production. Single-spore isolates of *F. solani* derived from cultures obtained from diseased roots of blighted citrus trees and from a diseased greenhouse-grown tree infected with *F. solani* were grown on PDA slants at 4 C. Spore suspensions obtained by shaking sterile distilled water with PDA slants were used to inoculate liquid media. The liquid medium used to evaluate isolates for toxin production had the following composition (in mg/L of medium): NH_4NO_3, 400; NaH_2PO_4, 100; KCl, 300; $MgSO_4 \cdot 7H_2O$, 40; $CaCl_2 \cdot 2H_2O$, 40; H_3BO_3, 1.0; $CuSO_4$, 0.1; $FeSO_4$, 1.0; $MnSO_4$, 1.0; Na_2MoO_4, 1.0; $ZnSO_4$, 1.0; and glucose, 2×10^4. Mineral and glucose solutions were autoclaved separately and mixed aseptically after cooling. Cultures (200 ml of medium in each 500-ml Erlenmeyer flask) were grown in the dark for 15 days at 27 C without shaking. After incubation, cultures were filtered through cheesecloth, and filtrates with pH >3 were adjusted to that value with HCl (8). Filtrates were extracted three times with 200-ml volumes of ethyl acetate, the combined extracts dried with anhydrous sodium sulfate, and reduced in volume to 10 ml on a vacuum rotary evaporator.

Toxin purification. One isolate of *F. solani* was inoculated into 16 L of medium and grown as described above, then filtered and extracted twice with ethyl acetate. The extract was evaporated to dryness, dissolved in acetone, and fractionated by preparative thin-layer chromatography (TLC) on 20 cm silica gel HF 254 (type 60) plates (EM Laboratories, Elmsford, NY). Plates were developed to the top twice with benzene-acetone (85:15, v/v). Seven bands were collected and eluted from the gel with acetone; any remaining colored material was eluted with ethanol. Both acetone and ethanol eluates were diluted to 100-ml volumes and assayed for toxicity in a root growth test. Compounds possessing toxic activity were further

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purified from these eluates by TLC with hexane-acetone (75:25) or hexane-chloroform-ether (1:1:1). Toxins were crystallized from benzene, benzene-methylene chloride, or methanol.

Identification of compounds. *Fusarubin.* Crystallized from benzene, mp 196–198 C uncorrected (195–197 C [6]; charring about 200 C, but m. 218 C[15]; mp 210 C dec. [2]). MS (m/e): 306, 288, 273, 259, 246, NMR, $CdCl_3$: 1.68 (3H, s, CH_3), 2.27 (d, J=2, OH, disappears on addition of D_2O), 2.72 (1H, dd, J=18, J=2, CH_2), 3.05 (1H, d, J = 18, CH_2), 3.94 (3H, s, CH_3-O), 4.90 (2H, s, -CH_2-O-), 6.18 (1H, s, H-6), 12.69 (s, O-H), 12.95 (s, OH).

Javanicin. Crystallized from benzene, mp. 207–208 C uncorr. (207–208 C [19]). MS (m/e): 290, 248, 230, 219, 205, 43. NMR, $CdCl_3$: 2.22 (3H, s, CH_3), 2.28 (2H, s, CH_2), 3.88 (2H, s, CH_2), 3.91 (3H, s, CH_3), 6.18 (1H, s, H-6), 12.82 (s, O-H), 13.21 (s, O-H).

Anhydrofusarubin. Crystallized from benzene-methylene chloride, mp. 197–198 C uncorr. (204 C [15]). MS (m/e): 288, 272, 244, 217. NMR, $CdCl_3$: 2.04 (3H, s, CH_3), 3.94 (3H, s, CH_3-O), 5.24 (2H, s, CH_2), 6.02 (1H, s, CH), 6.15 (1H, s, H-6), 12.70 (s, O-H), 13.05 (s, OH)

Succinic acid. Melting point and infrared spectrum were identical to those of an authentic sample.

Toxicity assay. The 10-ml extracts and 100-ml eluted fractions were prepared for testing by diluting 0.2 ml with 2-ml of ethanol, applying to sterile 9-cm-diameter Whatman No. 1 filter paper disks in petri dishes, and drying in a sterile laminar-flow hood. After all solvent was removed, 2.5 ml of sterile water was added, and 20 surface-disinfected White Icicle radish seeds were distributed evenly on the paper disk. Seeds were germinated and grown for 3 days at 27 C in the dark, then examined for root growth. Elongation of primary roots was expressed as a percent of control root growth.

Purified toxins were tested for inhibition of citrus root growth by using germinating rough lemon seeds. Toxins were dissolved in acetone, applied to sterile filter paper as described above, and 10 surface-disinfected rough lemon seeds were placed on the wetted paper. After 13 days at 27 C, total root growth (rough lemon is polyembryonic) was measured and compared to control growth.

TABLE 1. Reaction of rough lemon seedlings inoculated with *Fusarium solani* liquid cultures and with culture filtrates

F. solani treatments[a]	Host response: Wilt[b]	Root rot[c]	Vessel plugging[d]
FL-M-1	3.2	2.6	0.070
FL-M-1 filtrate	0.0	0.0	0.000
CA-118	2.2	1.8	0.034
CA-118 filtrate	0.0	0.0	0.000
Control	0.0	0.0	0.000

[a] FL (Florida) and CA (California) isolates of *F. solani* grown 5 days on liquid Fries' medium. Culture filtrates were obtained by Millipore filtration (0.2 μm).

[b] Rating scale, 0–5: 0 = no wilt, 1 = mild leaf roll to 5 = severe wilt and leaf desiccation.

[c] Rating scale, 0–3: 0 = no root rot to 3 = severe epidermal and cortical sloughing.

[d] Rating scale, 0–3: 0 = no vessel plugs, 1 = 1–50, 2 = 51–100, and 3 = 100 + in 30 1-cm-long pieces per plant. Five plant replicates per treatment.

TABLE 2. Color and pH of aqueous filtrates of *Fusarium solani* cultures, and radish root growth inhibition by ethyl acetate extracts of filtrates

Isolate	Color	pH	Root growth inhibition (%)
1	Orange	2.65	81
2	Orange	2.65	83
3	Orange	2.70	89
4	Light yellow	2.55	33
5	Orange	2.65	86
6	Orange	2.70	53
7	Red-orange	2.65	97
8	Orange	2.55	25
9	Clear	3.20	0
10	Clear	3.10	11
11	Clear	2.55	0
12	Clear	2.65	0
13	Clear	2.70	0
14	Light orange	2.55	29
15	Light orange	2.90	33
16	Clear	2.70	11
17	Light yellow	2.65	6
18	Light yellow	2.60	8
19	Clear	2.70	0
20	Light orange	2.60	25
21	Light orange	2.55	46

RESULTS AND DISCUSSION

Both Florida and California isolates of *F. solani* caused wilt, root rot, and some plugging in rough lemon seedlings when roots were dipped in unfiltered cultures (Table 1). Culture filtrates did not evoke any response. Wilt symptoms in seedlings inoculated with both isolates of *F. solani* were irreversible at the termination of the test, 12 days after inoculation. Root rot symptoms were identical to those appearing on blighted trees in the field (10), and the vessel plugging that developed in inoculated roots was the resin type associated with infected roots on blighted trees in the field (12). These results compliment the observations made in another study on infection and vessel plugging as a result of inoculation (11).

Of the 15 *F. solani* isolates from blighted grove trees (Nos. 1–15), 5 yielded culture extracts that inhibited radish root growth by 80% or more (Table 2). The most toxic extract, that from isolate No. 7, reduced root growth by 97%. Extracts of cultures with clear, light yellow, or light orange aqueous filtrates were relatively nontoxic; none inhibited root elongation by more than 46%. Aqueous filtrates of all highly toxic isolates were orange or reddish orange, suggesting the presence of naphthazarin toxins. None of the isolates (Nos. 16–21) obtained from a greenhouse-grown tree infected with *F. solani* yielded extracts of high toxicity or strong color. With the exception of isolates 9, 10, and 15, all cultures produced acidic filtrates with pH in the range of 2.55 to 2.70. Acidity of filtrates did not correlate with toxicity or color formation.

TABLE 3. Radish root growth inhibition by fractions from thin-layer chromatography separation of *Fusarium solani* culture filtrate extracts. See Materials and Methods for details of separation

R_f	Color	Eluant	Root growth inhibition (%)	Toxin
0.74	Purple	Acetone	14	Anhydrofusarubin
0.68	Yellow	Acetone	17	...
0.63	Red	Acetone	78	Javanicin
		Ethanol	42	...
0.46	Red	Acetone	99	Fusarubin
		Ethanol	23	...
0.10	Red-purple	Acetone	29	...
		Ethanol	94	Naphthazarin derivative
0.04	Yellow	Acetone	32	Succinic acid
0	Purple (origin)	Acetone	29	...
		Ethanol	11	...

TABLE 4. Root growth inhibition in rough lemon by naphthazarin toxins from *Fusarium solani*

Toxin	Concn (ppm)	Root growth (% of control)
Fusarubin	100	38
Fusarubin	50	60
Javanicin	100	62
Javanicin	50	85
Anhydrofusarubin	100	59
Anhydrofusarubin	50	82

These data show that *F. solani* from roots of a blighted citrus tree has the potential to produce phytotoxins. Only inorganic salts and glucose (which would be available in the root cortex) are required for the elaboration of these toxins. As expected, some isolates were not highly toxic; strains of *F. solani* are known to vary widely in toxin production (8). If infection by *F. solani* is a factor in the generation of blight symptoms, this variability in toxin production could help explain the random appearance of the disease. Most strains occurring in the rhizosphere probably produce little or no toxin and cause only fibrous root pruning. Appearance and spread of toxin-generating strains may be random, or may be favored by changes in nutritional status or stress of the tree.

The most toxic isolate, No. 7, was cultured in quantity for isolation and identification of the toxic principles. Preparative TLC of the ethyl acetate extract separated a number of highly colored compounds (Table 3). When these fractions were eluted and tested for toxicity to radish roots, most of the activity was found in three fractions: the acetone eluates of bands at R_f 0.46 and 0.63, and the ethanol eluate of the band at R_f 0.10. All of these fractions were red or reddish purple. Toxins eluted with acetone from the major red bands (R_f 0.63 + 0.46) were identified as javanicin and fusarubin, respectively (Fig. 1). These two naphthazarin toxins have been isolated from cultures of *F. solani* obtained from diseased herbaceous plants, such as peas infected with root rot (8). Both are known phytotoxins that disrupt plant metabolism by inhibiting anaerobic and oxidative decarboxylation reactions (9).

Ethanol extraction (following acetone extraction) of the region at R_f 0.10 yielded a dark red solution that was quite toxic to roots. The coloration of this extract suggested that a naphthazarin derivative was present, but quantities adequate for complete characterization were not obtained. When concentrated, the acetone extract of the band at R_f 0.04 formed colorless crystals that were insoluble in benzene and soluble in water. These crystals were found to be succinic acid, which has been reported to be a metabolite of several *Fusarium* species (14).

The top band (R_f 0.74) contained a purple compound identified as anhydrofusarubin. For comparison, an authentic sample of this compound was formed by dehydration of fusarubin according to the procedure of Ruelius and Gauhe (15). This compound was not as toxic to radish roots as fusarubin and was not found in quantities sufficient to cause appreciable root inhibition.

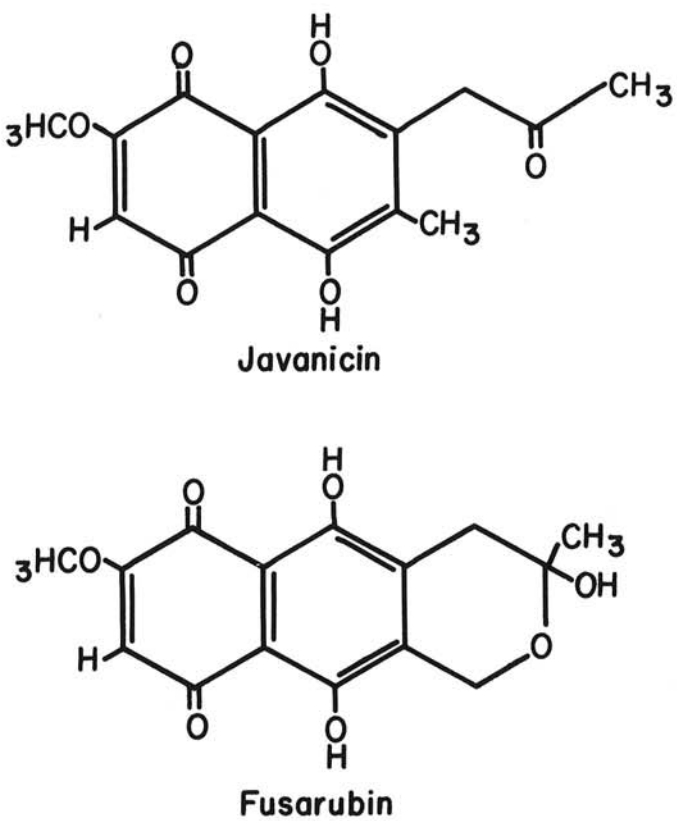

Fig. 1. Structures of javanicin and fusarubin.

When purified toxins were tested on germinating rough lemon seeds, fusarubin was most effective in reducing root growth (Table 4). At 100 ppm, this naphthazarin reduced growth to 38% of the control value. Since this toxin was also produced in the greatest amount, it appears that in still cultures, the primary toxin produced by *F. solani* isolates from rough lemon roots is probably fusarubin. Toxicity of cultures is augmented to a lesser extent by javanicin and an unknown toxin that is probably another naphthazarin derivative. These toxins could be absorbed by the root after formation in the rhizosphrere, or could be produced within the root cortex by invading *F. solani*. If toxins are instrumental in causing wilt and vessel plugging, our data on inoculated rough lemon seedlings indicate that infection and colonization of root tissues must precede these events. After invasion, this pathogen readily spreads through the cortex and stele of rough lemon fibrous roots (10). In pot tests, infection of citrus seedlings with *F. solani* caused growth reductions (12,13,18) and chlorosis (10). Pathogenicity was increased in the presence of any factor facilitating penetration, such as nematodes or physical damage (13). It has been suggested that the variations in pathogenicity seen in greenhouse seedling tests may be due to differing toxigenic capacities of the isolates studied (12). Whether the toxins described here cause blight remains to be determined. However, even if not solely responsible for blight symptoms, absorption of these phytotoxic compounds would further increase the stress to which trees are subjected. The complex of symptoms described as blight may be the result of an accumulation of physical and chemical stresses affecting the root system.

LITERATURE CITED

1. Anderson, C. A., and Calvert, D. V. 1970. Mineral composition of leaves from citrus trees affected with declines of unknown etiology. Proc. Fla. State Hortic. Soc. 83:41-45.
2. Chilton, W. S. 1968. Isolation and structure of norjavanicin. J. Org. Chem. 33:4299-4300.
3. Claydon, N., Grove, J. F. and Pople, M. 1977. Fusaric acid from *Fusarium solani*. Phytochemistry 16:603.
4. Cohen, M. 1974. Diagnosis of young tree decline, blight, and sand hill decline of citrus by measurement of water uptake using gravity injection. Plant Dis. Rep. 58:801-805.
5. DuCharme, E. P. 1971. Tree loss in relation to young tree decline and sand hill decline of citrus in Florida. Proc. Fla. State Hortic. Soc. 84:48-52.
6. Hardegger, E., Steiner, D., Widmer, E., and Schmidt, T. H. 1964. Wilting agents and antibiotics. XXXIII. Selective cleavage of the ethers of chelated phenols and synthesis of javanicin. Helv. Chem. Acta 47:2031-2037.
7. Kern, H. 1972. Phytotoxins produced by fusaria. Pages 35–48 in: R. K. S. Wood, A. Ballio, A. Graniti, eds. Phytotoxins in Plant Diseases. Academic Press, New York.
8. Kern, H., and Naef-Roth, S. 1965. Zwei neue, durch *Martiella*-Fusarien gebildete Naphthazarin-derivate. Phytopathol. Z. 60:316-324.
9. Kern, H., Naef-Roth, S., and Item, H. 1970. Parasitogene Naphthazarin derivate als Hemmstoffe der Decarboxylierung von α-Ketocarbonsauren. Phytopathol. Z. 67:1-14.
10. Nemec, S. 1978., Symptomatology and histopathology of fibrous roots of rough lemon (*Citrus limon*) infected with *Fusarium solani*. Mycopathologia 63:35-40.
11. Nemec, S., Baker, R., and Burnett, H. 1980. Pathogenicity of *Fusarium solani* to citrus roots and its possible role in blight etiology. Proc. Fla. State Hortic. Soc. 93:36-41.
12. Nemec, S., Burnett, H. C., and Patterson, M. 1977. Observations on a citrus fibrous root rot involving *Fusarium solani* in blight-diseased groves. Proc. Fla. Soil Crop Sci. Soc. 37:43-47.
13. O'Bannon, J. H., Leathers, C. R., and Reynolds, H. W. 1967. Interactions of *Tylenchulus semipenetrans* and *Fusarium* species on rough lemon (*C. limon*). Phytopathology 57:414-417.
14. Ranson, S. L., and Yeoman, M. M. 1961. Nonvolatile carboxylic acids in plants. Pages 958–975 in: C. Long, ed. Biochemists' Handbook, E. & F. N. Spon Ltd., London.
15. Ruelius, H. W., and Gauhe, A. 1950. Fusarubin, a naphthoquinone

coloring matter from Fusaria. Liebig's Ann. Chem. 569:38-59.
16. Smith, P. F. 1974. Zinc accumulation in the wood of citrus trees affected with blight. Proc. Fla. State Hortic. Soc. 87:91-95.
17. Ueno, Y., Sawano, and Ishii, K. 1975. Production of trichothecene mycotoxins by *Fusarium* species in shake culture. Appl. Microbiol. 30:4-9.
18. Van Gundy, S. D., and Tsao, P. H. 1963. Growth reduction of citrus seedlings by *Fusarium solani* as influenced by the citrus nematode and other soil factors. Phytoapthology 53:488-489.
19. Widmer, E., Meyer, J. W., Walser, A., and Hardegger, E. 1965. Wilting agents and antibiotics. XXXIV. Synthesis of isojavanicin, and a simplified synthesis of javanicin. Helv. Chem. Acta 48:538-555.