ELISA and Immunocytochemical Detection of *Fusarium solani*-Produced Naphthazarin Toxins in Citrus Trees in Florida

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ABSTRACT

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Naphthazarin toxins of *Fusarium solani* were detected by competitive ELISA analysis in xylem fluid of roots rotted by *F. solani* and symptomless scaffold roots and branches of healthy-appearing and diseased citrus trees in ridge and flatwoods Florida groves. Studies concentrated on blight, a wilt disease with an undefined cause and etiology, to determine if *F. solani* is a causal factor of the disease. Healthy-appearing roots of trees with blight symptoms in six groves contained up to 11.4 times more toxin than roots of healthy trees in the same groves. In blight-diseased trees from these groves, median toxin values per root and the percentage of roots positive for toxin were higher than for healthy trees. Root-rotted roots from blight-diseased trees in two groves contained 112 and 3.4 times more toxin than healthy-appearing roots from the diseased trees. In two groves, one containing tristeza-diseased trees and the other foot rot-diseased trees, toxin concentrations were greater in diseased compared with healthy trees only in the foot rot site. Toxin concentrations were not different in healthy-appearing roots of healthy tangerine and sweet orange trees on *Citrus limon* 'Milam' in adjacent groves in a burrowing nematode site. Significantly more toxin was present in branches of blight than in healthy trees in two of three groves. In fibrous roots infected by *F. solani,* immunocytochemical localization of naphthazarins was present in fungal cell walls and associated electron-dense substances on the outer surface of the hyphae. In the fungal cytoplasm, the toxin was localized in nonmembrane-bound electron-lucent areas. The presence of naphthazarin toxins in blight-diseased trees as well as those with other diseases suggests the nonspecificity of *F. solani* pathogenic activity on various rootstocks. Therefore, in situ toxin concentrations high enough to trigger pathogenic effects in susceptible rootstocks may be required to cause blight.

Additional keywords: isomarticin, phytotoxins.

Fusarium solani (Mart.) Sacc. is a common soil fungus that causes root rot symptoms in peas, beans, lentils, and cucurbits (2,3,9,26). On citrus in Florida, it is a primary colonizer of healthy-appearing fibrous roots (24) causing fibrous and scaffold root rots (7,14). Fusaria are producers of a wide range of toxins that may be synthesized in diseased roots. Florida citrus isolates of *F. solani* produce at least 11 structurally related naphthazarin toxins (27) but do not produce detectable levels of fusaric acid (S. Nemec, *unpublished data*) or trichothecenes (R. Baker and O. Kawamura, *personal communication*) in culture. Naphthazarins of *F. solani* are not host-specific, and their toxicity has been demonstrated to tomato, rice, radish, bean, pea, and citrus (1,4,10,11). Pea seeds exposed to isomarticin in solution lost electrolytes at 5 $\mu g \cdot l^{-1}$ and had reduced plant growth at 10 $\mu g \cdot l^{-1}$ (6). In another study, 25 $\mu g \cdot l^{-1}$ of isomarticin and 10 $\mu g \cdot l^{-1}$ of dihydrofusarubin had growth-repressing effects on citrus seedlings (17).

Until recently, no satisfactory method was available to detect naphthazarins in *F. solani*-infected plants because of potentially low in situ quantities that standard extraction methods were not sensitive enough to detect. An enzyme-linked immunosorbent assay (ELISA) now has been developed for isomarticin (22), one of the naphthazarins. This ELISA procedure was used in our studies to measure naphthazarins in xylem fluid of citrus with various diseases. These studies principally were done in citrus groves where blight disease occurred because the etiology and causal agent for this disease has not been defined and described (15), and because the data generated may provide clues to the cause of the disease.

Blight is an irreversible wilt disease of citrus with vascular symptoms of vessel plugging and impaired hydraulic conductivity (15). Diseased trees exhibit external fibrous and scaffold root rot symptoms caused by *F. solani* (18,19). *F. solani* infects the wood of these roots; however, no other potential biological causal agents have been detected microscopically in wood (although *Xylella fastidiosa* Wells can be detected by ELISA in, and cultured from, a very low percentage of trees with blight [8]). No causally related, transmissible biological agent has been isolated nor detected in graft-transmission studies (15).

In addition to ELISA, immunocytochemical techniques were used to detect naphthazarins in citrus fibrous roots infected with *F. solani* from blight-diseased trees.

MATERIALS AND METHODS

Citrus groves—roots. Healthy-appearing, pencil-sized to 2.5-cm-diameter scaffold roots were cut from blight-diseased and healthy trees in six central and southeastern Florida groves either by removing trees with a tractor or by digging them out of the soil with a shovel. Scaffold roots used had either *F. solani*-infected fibrous roots or scars of where rotted roots had been attached. Trees in these groves either were previously diagnosed as having blight using the water-uptake (5) and wood Zn (25) diagnostic methods or were tested to confirm the presence of blight by the syringe water-uptake method (12). Healthy-appearing trees were selected as controls in most groves; in others where all trees had some symptoms, the healthiest were selected as controls. Roots from two groves were collected before the December 1989 freeze; all other groves were sampled in areas of the state that escaped with only minor freeze injury. These groves were sampled after recovery from the freeze was evident. Most groves contained sweet

orange cultivar Valencia (*Citrus sinensis* (L.) Osbeck) scion on rough lemon (*C. limon* (L.) N. L. Burm.) rootstock. The exception was a grove at Indiantown, FL, where healthy trees of sweet orange cultivar Pineapple were resets on sour orange (*C. aurantium* L.) rootstock; no healthy-appearing trees on rough lemon remained in that grove. The soil types in which they were grown ranged from Astatula fine sand (hyperthermic-uncoated typic quartzipsamments) to Myakka and Immokalee fine sands (siliceous, hyperthermic aeric haplaquods) in the flatwoods area of the state. Trees were rated for disease on a scale of 0–5. Zero was assigned to visibly healthy trees; 1 was assigned to trees with the earliest blight symptoms, leaf curl, or Zn deficiency; 5 was assigned to trees with extensive dieback.

Scaffold roots were sampled from tristeza-diseased Valencia on sour orange rootstock in a grove near Vero Beach, FL; from 7-yr-old cultivar Hamlin on Rangpur lime (*C. reticulata* Blanco var. *austera* hybrid) with symptoms of *Phytophthora parasitica* Dastur foot and root rots in a grove near Indiantown; and from healthy 7-yr-old tangerine cultivar Sunburst (*C. reticulata* Blanco × [*C. paradisi* Macfady × *C. reticulata*]) and 20-yr-old Valencia on rough lemon cultivar Milam in adjacent groves in a site near Bartow, FL, where the burrowing nematode *Radopholus citrophilus* Huettel, Dickson, Kaplan caused spreading decline symptoms on the previous grove planted on this site. Trees in these four groves did not have blight.

Scaffold roots were collected from healthy Valencia on rough lemon in a 4-yr-old, healthy-appearing grove and from 27-yr-old, blight-diseased Valencia on rough lemon in a adjacent grove near Fort Pierce, FL. Similarly, roots were collected from healthy Valencia on rough lemon in a 5-yr-old grove and from 20-yr-old, blight-diseased Valencia on rough lemon in a adjacent grove near Holopaw, FL.

Healthy-appearing and root-rotted scaffold roots were sampled from blight-diseased Valencia on rough lemon trees in two groves, one near Bartow and the other near Holopaw.

Citrus groves—branches. Healthy-appearing 1- to 2-cm-diameter branch pieces about 25–30 cm long were cut from blight-diseased and healthy trees in three groves. One grove, a 15-yr-old Palestine Sweet Lime (*C. aurantifolia* (L.) Swingle) on rough lemon, was located near Holopaw. The other two were 12-yr-old Valencia on Carrizo citrange (*Poncirus trifoliata* (L.) Raf. × *C. sinensis*) and 12-yr-old Hamlin sweet orange on Carrizo citrange rootstock blocks near Fort Pierce.

Extraction of xylem fluid. About six to 12 root pieces, 0.3–0.6 m long, and about eight to 10 branch pieces were removed from each tree, and at least three trees of each health category were sampled from each grove. Roots and branches were refrigerated in transit between the field and laboratory, and roots were washed free of soil in the laboratory. Roots and branches were prepared for pressure removal of xylem fluid by cutting 2–3 cm from each end of each piece and inserting one end into thick-walled Tygon (Norton Plastics and Synthetics Div., Akron, OH) tubing. The other end of the tubing was attached to a high-pressure hose fitted to an air tank pressurized at 2.76×10^5 Pa. Xylem fluid was collected in sterile plastic petri dishes or lids by releasing pressure to the tubing at the tank. Accumulated fluid from each root was collected by syringe and placed in 1-ml glass vials and stored at −20 C.

ELISA procedures. ELISA was conducted on thawed xylem fluid samples by pipetting 200 μl of each sample into separate test tubes and adding to each tube 200 μl of tracer prepared as described by Phelps et al (22). The contents of each tube was mixed by vortexing. The same volume of isomarticin standards was prepared in additional test tubes, to which 200 μl of tracer was added to each standard.

ELISAs were performed in 96-well, flat-bottomed polystyrene plates (Dynatech Immulon 2, Dynatech Laboratories, Inc., Chantilly, VA) and were coated with antiisomarticin IgG as previously described (22). Each plate was amended with 11 unknown xylem fluid-tracer samples and 10 isomarticin-tracer standards, four replicate wells per sample or standard. After removing these solutions from the wells and washing the wells with TBS buffer (0.15 M NaCl, 25 mM Tris-HCL, pH 8.5, 0.02 M NaN_3), a development solution of 0.1 M diethanolamine, pH 9.8, 1 mM $MgCl_2$, and 15 mM *p*-nitrophenol phosphate was added to the wells. Plates were read with an Automated Microplate Reader, model EL 309 (Bio-Tek Instruments, Inc., Winooski, VT). Standard values were corrected for unspecific tracer binding, and a standard sigmoidal curve was generated. Sample concentrations of naphthazarins were determined directly from the standard curve.

Tissue preparation for transmission electron microscopy. Fibrous roots infected with *F. solani* were collected from Valencia on rough lemon trees with blight symptoms. Infection by this fungus was verified by microscopic examination of roots for presence of conidia and chlamydospores and by culturing the fungus from roots on potato-dextrose agar. Root pieces were fixed in 3% glutaraldehyde in 100 mM potassium phosphate buffer, pH 7, during 5 h at room temperature followed by postfixation in 1% osmium tetroxide in the same buffer for 1 h. After fixation, the root segments were dehydrated in acetone and embedded in Epon Araldite as previously described (16).

For controls, rough lemon seedlings were grown in vitro in MS medium according to Murashige and Skoog (13) and in steam-pasteurized Astatula fine sand in pots in a greenhouse. Coatless seeds of in vitro-grown seedlings were sterilized first in 95% (v/v) ethanol for 2 min, then in 50% bleach (2.5% sodium hypochlorite) for 30 min. After this treatment, seeds were rinsed once in 0.1 N HCL in sterile distilled water to remove any traces of bleach, then in two consecutive baths of sterile distilled water. The sterilized seeds were soaked overnight in sterile distilled water and then transferred to culture tubes containing the MS medium. The seeds were germinated for 4 wk at 24 C under a light intensity of 15 $\mu m \cdot m^{-2} \cdot s^{-1}$ for 16 h per day. Root segments from in vitro and greenhouse-grown citrus seedlings were fixed in 3% glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.4, for 3 h followed by postfixation in 1% osmium tetroxide in the same buffer for 2 h. Roots were rinsed in buffer, dehydrated in a series of ethanol, and embedded in Epon 812. Ultrathin sections of gold interference color from infected fibrous roots and noninfected seedling roots (controls) were picked up on 200-mesh nickel grids coated with Formvar and processed for immunocytochemical labeling.

Immunocytochemistry. Ultrathin sections of roots first were treated with a blocking agent consisting of 5% powdered skim dry milk in 500 mM Tris-buffered saline (TBS), pH 7.4, for 5 min. Sections then were transferred to the antiisomarticin (100 μg ml^{-1} in TBS) diluted to 1/40 with TBS for 1 h. After two rinses in TBS, sections were incubated with the secondary goat antirabbit IgG-gold (10 nm diameter) antibody (Sigma Chemical Co., St. Louis, MO) diluted 1/50 in TBS during 30 min at room temperature. After two rinses in TBS, sections were stained with 2% aqueous uranyl acetate followed by lead citrate (23). The sections were examined with a JEOL 1200 EX electron microscope (JEOL Limited, Nakagami Akishima, Tokyo, Japan).

The specificity of the immunolabeling was assessed using the following controls: 1) adsorption of the antibody with its specific antigen (100 mM isomarticin), 2) use of nonimmune serum instead of the specific antiserum, 3) use of nonlabeled secondary antibody before the goat antirabbit IgG-gold complex, and 4) use of the secondary antibody alone.

RESULTS

Citrus groves—roots. Naphthazarins were detected in roots of diseased as well as healthy trees in all six groves containing trees of similar age (Table 1). The highest quantity detected was 100,000 ng ml^{-1}, the upper limit of the tests conducted. Significant differences were calculated only for mean values, and data between diseased and healthy trees were significant for two of the groves. Median toxin values were consistently higher for roots of diseased than of healthy trees, and a higher percentage of roots containing toxin was present on diseased trees in all groves.

In two sites where xylem fluid was extracted from roots of healthy trees in young groves and from older blight-diseased trees

in adjacent groves, toxin levels were higher in blight-diseased trees. Median toxin values were higher in diseased trees and percentage of roots positive for toxin was higher in diseased trees (Table 1).

In locations where symptoms of tristeza and foot rot were present and plantings were reset in a burrowing nematode-infected area, toxin was present in roots of trees in all locations (Table 2). In the grove with tristeza symptoms, toxin concentrations were about the same between diseased and healthy trees. The foot rot-diseased trees had extensive fibrous root rot symptoms, and if infected by *F. solani,* that could account for the significantly higher toxin concentration in scaffold roots of the diseased trees. In the burrowing nematode-infested area, toxin concentrations were almost threefold greater in the trees of the younger grove than in the older grove. The older grove tree condition was given a score of 1.0 because of residual damage from the December 1989 freeze.

The highest mean toxin concentrations were detected in *F. solani* root-rotted scaffold roots of two groves (Table 3) and were significantly higher than toxin concentration from healthy-appearing scaffold roots of the same trees. Xylem fluid extracted from visually healthy scaffold roots was clear and colorless, while fluid from root-rotted roots was generally light brown due to discoloration of diseased roots.

TABLE 1. *Fusarium solani* naphthazarin toxins in xylem fluid of healthy-appearing scaffold roots of blight-diseased and healthy trees in Florida citrus groves

Grove location	Scion/rootstock	Tree age	Tree condition (0–5)[a]	Trees sampled (number)	Roots sampled (number)	Range of naphthazarins in roots (ng/ml)	Mean (ng/ml)[b]	Median (ng/ml)	Roots positive for toxin (%)
Trees of similar age in the same grove									
Holopaw	Valencia/Rough lemon	6	2.0	5	23	0–1,500	271 NS	90	78
		6	0.0	4	24	0–1,300	185	53	58
Holopaw	Valencia/Rough lemon	20	2.3	16	56	0–100,000	4,173 NS	550	80
		20	0.3	8	35	0–100,000	4,292	1	51
Indiantown	Pineapple/Rough lemon	15	3.0	4	28	35–100,000	15,686*	3,000	100
	Pineapple/Sour	8	0.0	3	28	0–18,000	1,666	340	68
Clermont	Valencia/Rough lemon	29	3.0	4	12	0–35,000	4,075 NS	408	83
		29	0.0	6	18	0–4,000	1,381	265	78
Fort Pierce	Valencia/Carrizo	12	3.0	3	10	75–11,000	4,480***	2,250	100
		12	0.0	3	23	0–3,300	394	130	70
Indiantown	Valencia/Rough lemon	27	3.2	6	21	0–100,000	9,737 NS	4,500	90
		27	0.2	4	16	0–100,000	9,762	215	69
Trees of different ages in adjacent groves									
Fort Pierce	Valencia/Rough lemon	4	0.0	3	16	0–4,300	664 NS	0	44
		27	4.0	3	18	0–100,000	12,877	1,377	89
Holopaw	Valencia/Rough lemon	5	0.0	6	21	0–38,000	3,780 NS	11	43
		20	2.3	16	56	0–100,000	4,173	550	80

[a] 0 = No symptoms, 1 = Zn deficiency and leaf curl, and 5 = a tree with extensive dieback.
[b] Mean values of toxin concentration for healthy compared with blight-diseased trees by Student's *t* test. * = $P \leq 0.05$; *** = $P \leq 0.001$; NS = not significant.

TABLE 2. *Fusarium solani* naphthazarin toxins in xylem fluid of healthy-appearing scaffold roots of citrus with foot rot and tristeza symptoms and trees grown in a burrowing nematode site

Grove location and disease	Scion/rootstock	Tree age	Tree condition (0–5)[a]	Trees sampled (number)	Roots sampled (number)	Range of naphthazarins in roots (ng/ml)	Mean (ng/ml)[b]	Median (ng/ml)	Roots positive for toxin (%)
Vero Beach (tristeza)	Valencia/Sour	25	3.2	4	24	0–100,000	19,405 NS	2,000	92
		25	0.2	4	29	0–100,000	16,866	2,800	97
Indiantown (foot rot)	Hamlin/Rangpur lime	7	3.7	3	18	700–100,000	22,467*	6,500	100
		7	0.0	3	27	0– 28,000	6,083	3,200	81
Bartow (burrowing nematode)	Valencia/Milam rough lemon	20	1.0	3	15	0– 73,000	11,940 NS	2,800	67
	Sunburst tangerine/ Milam rough lemon	7	0.0	3	9	7,000– 90,000	27,967	10,000	100

[a] 0 = No symptoms, 1 = early leaf symptoms, and 5 = a tree with extensive dieback.
[b] Mean values of toxin concentration for healthy compared with blight-diseased trees by Student's *t* test. * = $P \leq 0.05$; NS = not significant.

TABLE 3. *Fusarium solani* naphthazarin toxins in xylem fluid of root-rotted and healthy-appearing scaffold roots of blight-diseased citrus trees in Florida

Grove disease	Scion/rootstock	Tree age	Tree condition (0–5)[a]	Root health	Roots sampled (number)	Range of naphthazarins in roots (ng/ml)	Location (ng/ml)[b]	Median (ng/ml)	Roots positive for toxin (%)
Holopaw	Valencia/Rough lemon	20	3.0	root-rotted	8	1,300–100,000	75,975***	100,000	100
				healthy	23	0–4,500	679	140	61
Bartow	Valencia/Rough lemon	25	4.0	root-rotted	7	2,000–100,000	83,143**	100,000	100
				healthy	10	1,000–100,000	24,160	4,750	100

[a] 0 = No symptoms, 1 = Zn deficiency and leaf curl, and 5 = a tree with extensive dieback.
[b] Mean values of toxin concentration for healthy roots compared with rooted roots by Student's *t* test. ** = $P \leq 0.01$; *** = $P \leq 0.001$.

Citrus groves—branches. Xylem fluid from branches of trees in three groves was positive for naphthazarins (Table 4). Mean toxin concentrations were significantly higher in diseased trees than healthy trees in only one of the three groves.

Localization of isomarticin. Infected fibrous roots contained *F. solani* hyphae in the lumen and cell walls of vessel members and fibers (Fig. 1A and B). In vessel members, hyphae eroded secondary walls (Fig. 1B). The immunocytochemical localization of toxin in infected roots revealed intense labeling over both fungal and host cells. In the fungus, toxin was localized over the cell wall of active and degenerated hyphae (Fig. 1A and C). Labeling also was present outside the fungal cells over an electron-dense and fibrillarlike substance that appeared closely associated with the fungal cell wall (Fig. 1C, arrow). Moreover, toxin also was localized in nonmembrane-bound electron-lucent areas of the fungal cytoplasm (Fig. 1D, asterisk). All other fungal organelles, such as lipid bodies, mitochondria, and nuclei, were not labeled (Fig. 1A and C). In the host cells, different degrees of labeling were observed in the secondary walls of vessel members. In infected tissue, gold particles were predominantly localized over the innermost (S_2) layer of vessel member secondary walls (Fig. 1A). However, the density of labeling appeared higher when hyphae were present in close proximity to the wall (Fig. 2A). In tissues of noninfected in vitro- and greenhouse-grown plants, the labeling showed a uniform density of distribution over the cell wall layers of vessel members (Fig. 2B). No labeling was detected in any other host cellular compartments.

Specificity of labeling for naphthazarins was demonstrated by the control tests that showed complete abolition of gold labeling (Fig. 2C).

DISCUSSION

This study reports the first in situ detection of *F. solani* naphthazarin toxins in citrus by ELISA. This assay is specific for the core structure of 2-methoxynaphthazarin that isomarticin and other naphthazarin toxins contain (22). The use of xylem fluid offers a more suitable alternative to host tissue for the extraction and detection of these fungal metabolites. Host leaves, especially those of woody species, contain substances that can actively interfere with ELISA detection procedures. Citrus leaves and roots are known to contain large quantities of coumarins (21) that have been suspected of forming complexes with other metabolites when extracted from citrus tissue. Such complexes may have inhibited the activity of alkaline phosphatase in preliminary leaf extraction-ELISA studies by Phelps et al (22).

The presence of toxin, in trees with blight, tristeza, or foot rot symptoms, and in older healthy trees was not unexpected. *F. solani* is an early colonizer of citrus roots in many citrus-producing countries (20,24) and, compared with soil, there is a high enrichment of *Fusarium* in the rhizosphere of citrus roots (20). There is a naturally high level of fibrous root rot on trees with tristeza symptoms because of the failure of the phloem to translocate food to the roots. These roots could readily be saprophytically colonized by *F. solani.* Foot rot-diseased trees with root rot as extensive as that which occurred on trees in this test may have been invaded by *F. solani* as well as *P. parasitica.* Healthy trees in blight sites have fibrous root rot (18), but not as much as blight-diseased trees. Even roots on young trees in new groves experience some infection by *F. solani,* but if the fungus produces naphthazarins in the citrus rhizosphere, as it does around pea roots (28), they may be absorbed by intact roots.

Trees in groves with blight were included in this study because the disease still has an undefined etiology. It is evident from the six blight groves examined (Table 1) that a considerable variation in toxin is found from grove to grove and that a wide range occurs in individual roots. This variation in toxin content within and among trees in groves makes it difficult to quantify differences between healthy and diseased trees and requires that large numbers of root samples be tested to obtain an estimate of these differences. Data from the mean, median, and percentage of roots positive for toxin indicate that most roots from a tree have a relatively low toxin content, generally markedly less in healthy-appearing trees, and that fewer roots contain toxin in healthy trees. Roots rotted by the fungus (14) had the highest mean content of toxin. The lower toxin levels in roots of healthy trees may be due to the lower incidence of fibrous root rot on these trees, a phenomenon revealed in a physical examination of roots in an earlier study (18). However, mean concentrations of toxin in roots examined in this study were higher than the minimum quantities of the naphthazarins tested, dihydrofusarubin (10 ng/ml) and isomarticin (25 ng/ml), that had toxic effects on citrus seedlings in a solution culture (17).

Unusually large quantities of toxin were detected in xylem fluid of branches. In the limited number of groves where branches were sampled, no trends in toxin concentration between healthy-appearing and blight-diseased trees could be determined. That the toxin could be detected in all branches sampled from healthy-appearing and diseased trees implies that during its translocation from the roots to the top, it becomes more evenly distributed. No comparison of toxin concentration between aerial parts and roots could be made because of the different time periods during which roots and branches were sampled.

The immunocytochemical tests provide strong evidence of the localization of naphthazarins in *F. solani.* The absence of significant labeling with the use of different control tests indicated that the resultant staining depended only on antibody-antigen binding sites. An important limitation of using chemically fixed tissue for immunogold labeling is that only cross-linked or sequestered forms of antigen are detected, whereas soluble molecules (e.g., free form) are lost during fixation and embedding procedures and thus are not accessible to the antibody. The use of cryosectioning techniques might well prevent the leaking of the toxin.

Cytochemistry demonstrated that the toxin is present in fungal cytoplasm, namely, in the electron-lucent areas and over cell walls. This may indicate that the toxins accumulate in cellular compartments, then are translocated to the fungal cell walls and secreted to the outside. In addition to fungal cells, labeling also was present in host cell walls, although the intensity of labeling and its distribution were not similar between infected and noninfected hosts. In the infected host, the abundance of labeling in the innermost cell wall layers of vessels that are in close

TABLE 4. Naphthazarin toxins of *Fusarium solani* in xylem fluid of branches from healthy and blighted citrus trees in Florida

Grove location	Scion/rootstock	Tree age	Tree condition (0–5)[a]	Trees sampled (number)	Branches sampled (number)	Range of naphthazarins in roots (ng/ml)	Mean (ng/ml)[b]	Median (ng/ml)	Branches positive for toxin (%)
Holopaw	Palestine sweet lime/Rough lemon	15	3.0	3	7	3,800–100,000	76,257*	100,000	100
		15	0.3	3	13	700–100,000	36,654	8,000	100
Fort Pierce	Hamlin/Carrizo	13	2.7	3	8	5,500–100,000	46,313 NS	22,500	100
		13	0.0	3	15	6,900–100,000	34,227	20,000	100
Fort Pierce	Valencia/Carrizo	12	2.3	3	3	600–100,000	35,200 NS	5,000	100
		12	0.7	3	4	1,200–100,000	51,050	51,500	100

[a] 0 = No symptoms, 1 = Zn deficiency and leaf curl, and 5 = a tree with extensive dieback.
[b] Mean values of toxin concentration for healthy compared with blight-diseased trees by Student's t test. * = $P \leq 0.05$; NS = not significant.

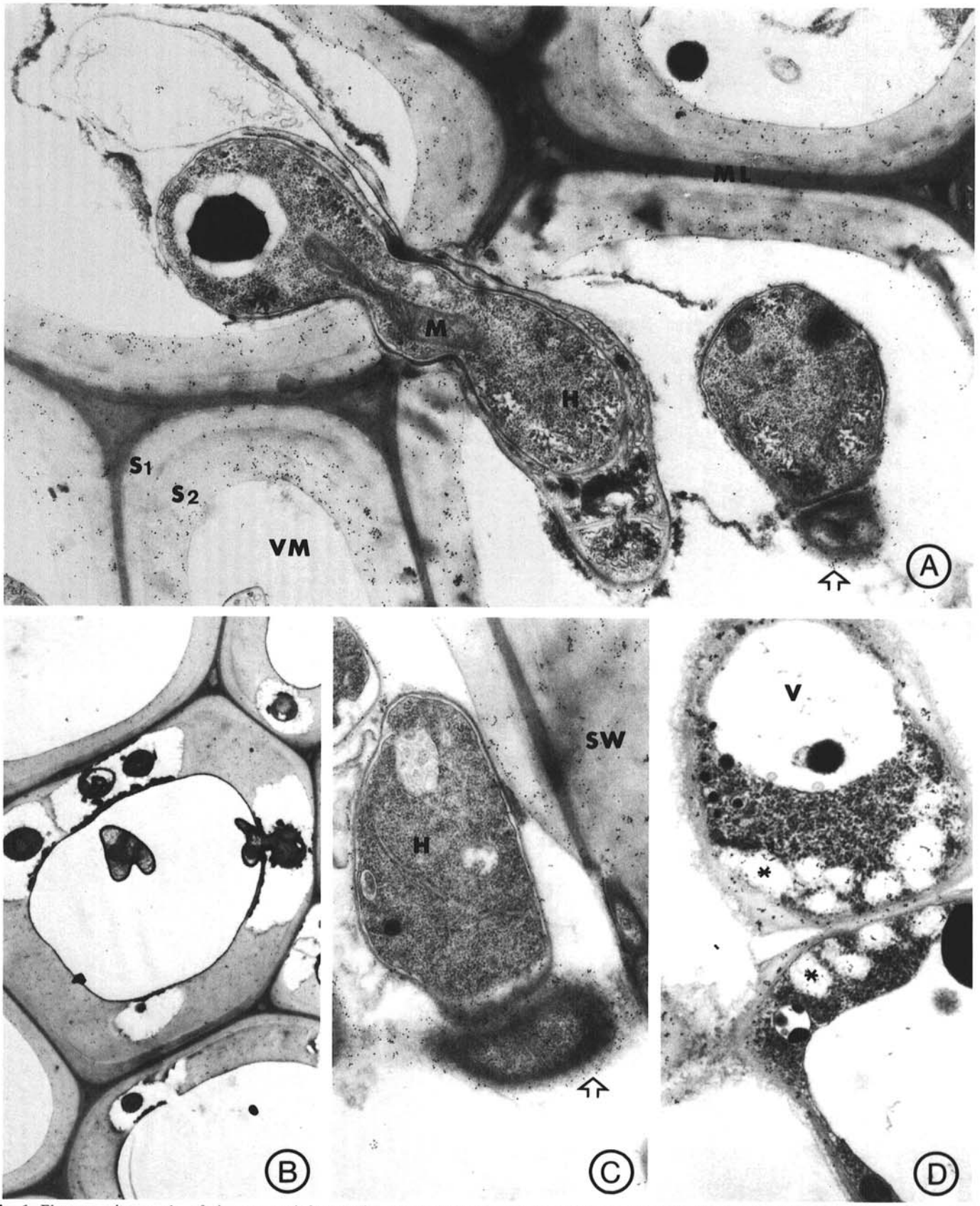

Fig. 1. Electron micrographs of citrus roots infected with *Fusarium solani* and labeled immunocytochemically. **A,** Penetration of vessel member by a hypha of *F. solani* through a bordered pit-pair. The cell wall of the young hypha is strongly labeled (arrow) as well as the S_2 layer. Note that fungal organelles such as mitochondria are not labeled (×21,000). **B,** Extensive degradation of secondary cell wall of vessel member after *F. solani* colonization. Note that the host secondary cell wall is significantly labeled (×6,500). **C,** A young hypha located very close to the host cell wall with intense labeling (arrow) over the fibrillar material that is associated with the fungal cell wall. The secondary wall of the vessel member also is labeled (×21,500). **D,** Section of a hypha of *F. solani* located in the lumen of a vessel member of citrus root secondary xylem. Labeling for naphthazarins is localized in nonmembrane-bound electron-lucent areas (asterisk) in the fungal cytoplasm. Large vacuoles are devoid of labeling (×24,000). H, hypha; M, mitochondrion; ML, middle lamella; S_1, S_2, secondary wall layers; SW, secondary wall; V, vacuole; VM, vessel member.

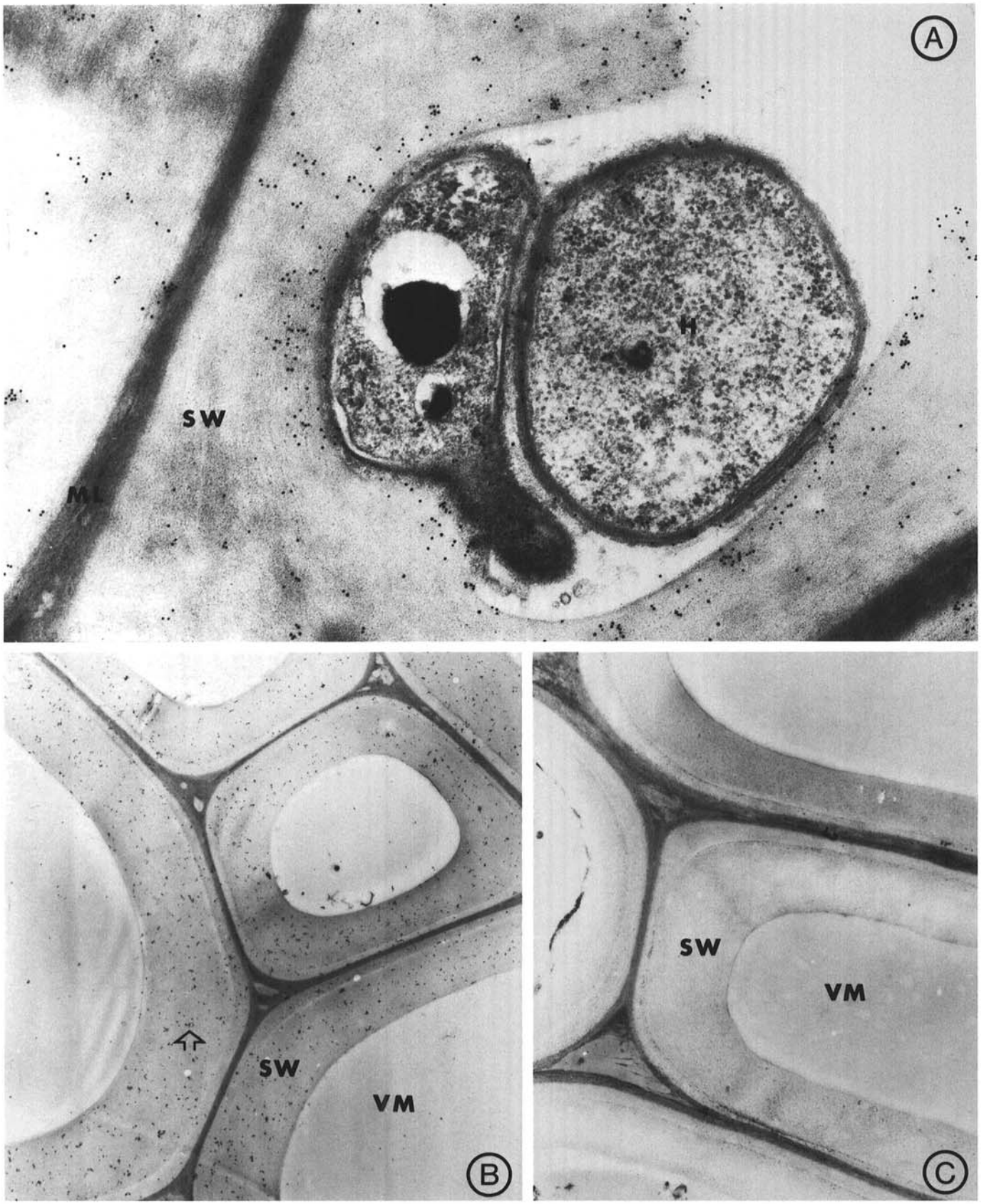

Fig. 2. Immunocytochemical labeling of sections of control and infected citrus roots. **A,** Two hyphae of *Fusarium solani* localized in the lumen of a vessel member. The density of the naphthazarin labeling in the secondary wall is intense in the close proximity of hyphae (×42,500). **B,** Secondary xylem of noninfected citrus root. The isomarticin labeling (arrow) is uniformly distributed over the different layers of the secondary wall of vessel members (×10,500). **C,** Secondary xylem of noninfected citrus root. Control experiment where the antiisomarticin was omitted before the incubation with the goat antirabbit IgG secondary antibody. Labeling was not evident (×16,000). H, hypha; ML, middle lamella; SW, secondary wall; VM, vessel member.

proximity to fungal cells is suggestive of leakage of toxin into the walls from the fluid in the lumen. The uniform distribution of labeling over the cell wall layers of vessel members of control roots is not surprising because they should contain phenolic and terpene compounds that can react with the tracer (22). Cytochemistry of phenolic compounds in cell walls only indicates their presence, not their concentration, in the wall. It is unlikely they are leached into the vessel in quantities large enough to effect tracer displacement because a high frequency of xylem fluid samples were negative for toxin. Conversely, isomarticin and dihydrofusarubin, when tested for their cross-reactivity to limonin (one of the terpenes displacing 50% of the tracer at 0.1 mg/ml [23]) in a radioimmunoassay, had cross-reactivities of 0.0015 and 0.0011%, respectively—too low to make a significant contribution to limonin testing (R. Mansell, *personal communication*).

The results of this study indicate that if *F. solani* toxins are involved in blight etiology their effect would not be apparent immediately in young trees, but that a high proportion of roots from older trees would have to contain toxin before the onset of symptoms occur. Toxin action likely would be by stimulation of vessel plugging (14) causing constraints in hydraulic conductivity under conditions of stress. Even though toxins were present in rootstocks other than rough lemon, preliminary data indicate (17) that one of the toxins, dihydrofusarubin, causes more vessel plugging in rough lemon, the rootstock most susceptible to blight, than other rootstocks. This variation of rootstocks to produce vessel plugging to toxins or other factors could be the most important factor causing some rootstocks to retain a tolerance of blight.

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