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Host-Parasite Relationships of Susceptible and Resistant Elm Callus Cultures Challenged with *Ophiostoma ulmi* (Buisman)Nannf.[1]

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Abstract

Ultrastructural effects of *Ophiostoma ulmi* (Buisman) Nannf..(= *Ceratocystis ulmi* Buisman C. Moreau) on susceptible American elm (*Ulmus americana* L.) callus tissue consisted of extensive vacuolation, accumulation of cytolytic substances, and plasmolysis when viewed with electron and light microscopy. Infected resistant American elm callus tissue revealed slight cell wall damage with some phenolic-like deposits but adjacent cells, membranes, mitochondria and nuclei were intact. Fungal hyphae that adhered to callus grown from susceptible American elm plants were regularly observed. Sparse hyphal growth was not suspended above resistant American elm callus cells. Callus from nonhost and resistant elms showed reduced hyphal growth between host cells compared to susceptible elm with increased accumulation of extracellular, phenolic-like deposits than in callus of American elm. The fungal structures on resistant tissue showed evidence of irregular growth and morphology. The latter suggest a phytoalexin-like response by the resistant American elm callus tissue.

Index words: transmission and scanning electron microscopy, light microscopy, vascular wilt disease.

Species used in this study: American elm (*Ulmus americana* L.); Siberian elm (*Ulmus pumila* L.); Petunia (*Petunia* x *hybrida*).

Significance to the Nursery Industry

Dutch elm disease (DED) caused by the fungus, *Ophiostoma ulmi*, was studied using callus cultured from susceptible and resistant American elm , siberian elm and petunia sources. Light and electron microscopy were used to see any differences in interactions with the DED fungus. Ultra-structural differences in the host cells of susceptible elm calli corresponded to those known in the intact tree. Reaction of the callus tissue to fungal culture filtrate varied depending on the tolerance or susceptibility of the host. Close contact of the fungal hyphae were routinely observed on susceptible calli. Resistant elm and nonhost callus tissue showed little indication of hyphal penetration or visible resistant tissue response in the form of phenolic-like deposits. Fungal structures on resistant tissue showed evidence of irregular growth and shape. Conventional elm breeding and screening for DED resistance requires several years to determine relative success. This study provides possible visual clues to physical and chemical resistance mechanisms using cell culture. Reduced time needed for screening of candidate DED resistant germplasm could result with additional research.

Introduction

Dutch elm disease (DED), caused by *Ophiostoma ulmi* (Buisman) Nannf.(= *Ceratocystis ulmi* Buisman C. Moreau), has been a highly destructive shade tree disease in many parts of the world for over 60 years (15). Numerous investigations of the host-parasite interaction have determined the nature of infections and mechanisms of resistance in elm *Ulmus americana* (7, 8, 10). One day after infection with *O. ulmi*, tyloses and other internal cytological changes occur in both susceptible and resistant elms (8). Pit membrane alterations (11) and cell wall breakdown (12) occur with other cytological changes. With transmission electron microscopy (TEM), Krause and Wilson (7) observed direct penetration of vessel cell walls and invasion of parenchymal cells of *Ulmus americana L* by *O. ulmi*.

O. ulmi has been isolated from inoculated, symptomless elm, indicating host tolerance to the pathogen (8). Ultrastructural studies have established that host-parasite interactions in tissue cultures and intact plants can be similar (9). Callus has been used successfully to reveal reactions of plants to pathogens (4, 6). The nature of the plant-microbe interaction may be studied by observing the DED fungus on elm calli (3). *O. ulmi* is able to grow differentially on callus cultures from susceptible and resistant elm cultivars (3).

In vitro studies of *U. americana* callus exposed to *O. ulmi* culture filtrate showed appreciable differences between the fine structural changes in callus tissue from resistant and susceptible elm (13). The present study, using light and electron microscopy, examined the cytological nature of host-parasite interactions of the fungal pathogen on callus tissues of various susceptible and resistant elm species and of nonhost species.

Materials and Methods

Callus tissue was developed from stock plants propagated from 1) American elm seedling susceptible to DED (susceptible American elm); 2) American elm 8630, a resistant selection (resistant American elm); 3) Siberian elm, *Ulmus pumila* , a resistant species (resistant Siberian elm); and 4) Petunia (*Petunia* x *hybrida*), a nonhost. Callus cultures were initiated from young leaves on Murashige and Skoog (MS)

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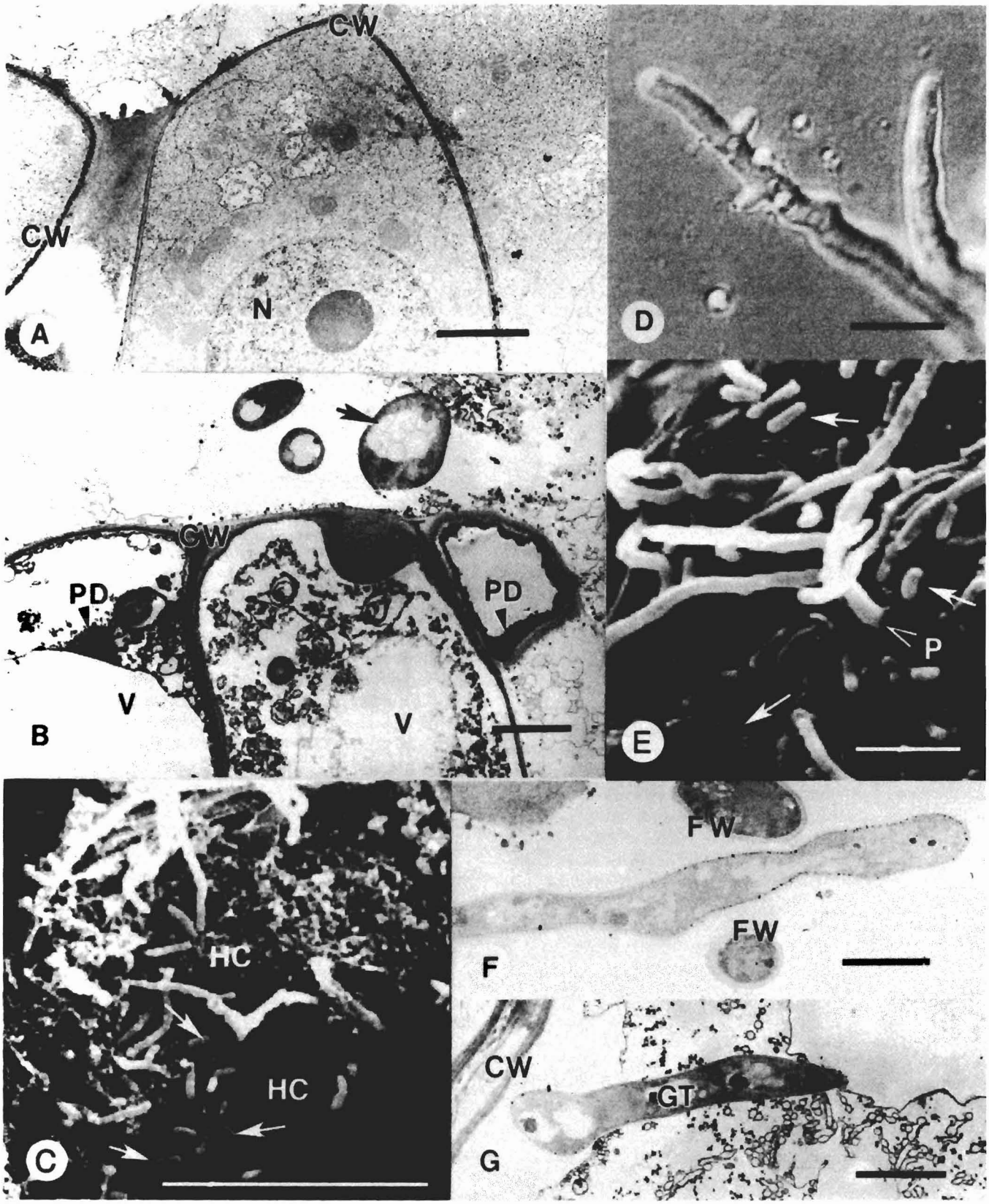

Fig. 1. Transmission electron micrographs A, B, F, and G; scanning electron micrographs C and E; light micrograph D of susceptible American elm callus inoculated with *Ophiostoma ulmi* showing a susceptible reaction. (A) Uninoculated American elm callus tissue with intact membranes, nucleus (N), nucleolus, mitochondria and cell wall (CW). Bar = 1 µm. (B) Inoculated elm callus showing plasmolyzed cells with vacuoles (V) and electron dense phenolic-like deposits (PD). Note fungal hyphae (arrow) in the intercellular space near host cell wall (CW). Bar = 1 µm. (C) Dense hyphal growth in intimate contact with host callus cell (HC). Note scattered conidia (arrows). Bar = 20 µm. (D) Hypha of uniform diameter with normal branching. Bar = 5 µm. (E) Penetration (P) of hyphae into the host callus cell. Note abundant conidia (arrows). Bar = 5 µm. (F) Conidia with electron transparent fungal wall (FW) and fungal hypha showing normal ultrastructural morphology. Bar = 1 µm. (G) Conidium with a normal germ tube (GT) next to a disrupted host cell wall (CW). Bar = 1 µm.

culture medium, supplemented with 200 mg/liter casein hydrolysate, 8 µM 6-benzylaminopurine, 0.5 µM 2,4-dichlorophenoxyacetic acid, 3% (w/v) sucrose, and 0.7% (w/v) Difco Bacto Agar (pH 5.7). Routine subculturing was carried out every 6 to 8 wk on half-strength MS medium fortified with the same supplements. Cultures were grown in the dark at 22 ± 1C (72 ± 1F). A final transfer of 10–15 mm (0.39–0.56 in) diameter callus pieces on half-strength fortified MS medium was made 11 days prior to inoculation.

Aggressive *O. ulmi* isolate PMP1 (3, 13) was recovered from a diseased American elm at Delaware, Ohio. PMP1 was grown on cellophane-covered potato dextrose agar (PDA: Difco, Detroit, MI) for 5 days at 24C (75 ± 1F). A suspension of 2×10^6 conidia/ml was made by diluting the washings from the surface of the plates with sterile distilled water. Filter paper discs (3mm or 0.12 in) (Difco, Detroit, MI) were cut into quarters producing 1.5 mm (0.06 in) triangles, sterilized, and inoculated by pipeting 20 µl of the spore suspension onto each triangle. After air-drying, the inoculated filter paper triangles were placed on the top center of the calli. The plates were incubated in the dark at 22 ± 1C (72 ± 1F).

Callus samples (1–1.5 mm thick (0.04–0.06 in)) were prepared for microscopy studies at 4, 8, 18, 24, 36, 48, 60 and 72 hr after inoculation. Samples (1 mm^2) were fixed for 18 hours in 3% glutaraldehyde in 0.05M cacodylate buffer (pH 7.2) at 4C (39F). After three rinses in the same buffer, subsequent fixation was in 2% osmium tetroxide in cacodylate buffer, pH 7.2, for 4 hr at 4C (39F). The samples were rinsed three times in buffer and dehydrated in a graded ethanol series and critical-point dried for scanning electron microscopy (SEM). Samples were mounted, sputter-coated with gold (200–300 Å thickness) and 32 random observations made for each time period on a Hitachi Model S-500 SEM at 20 kV using a Noran digital analysis system, Model TN 5402. Sister preparations were embedded in a firm mixture of Spurr's plastic for transmission electron microscopy (TEM). Ultrathin sections were cut using a diamond knife on a Sorval Porter-Blum MT2 microtome and post-stained with 5% uranyl acetate and lead citrate for 7 min (14). TEM photographs were taken on a Hitachi Model HU 11E at 75 kV accelerating voltage. Observations with the light microscope were made using the same material prepared from both fresh wet mounts and 1–2 µm-thick sections cut from the same block used for TEM.

Results and Discussion

Reactions in susceptible tissue. Uninoculated, susceptible American elm callus tissue (Fig. 1A) exhibited a normal complement of organelles under TEM. Callus cell walls were regular with intact plasma membranes, cytoplasm, nuclei, and mitochondria (Fig. 1A). Inoculated, susceptible American elm callus displayed extensive vacuolation, accumulation of electron-dense, phenolic-like deposits and various

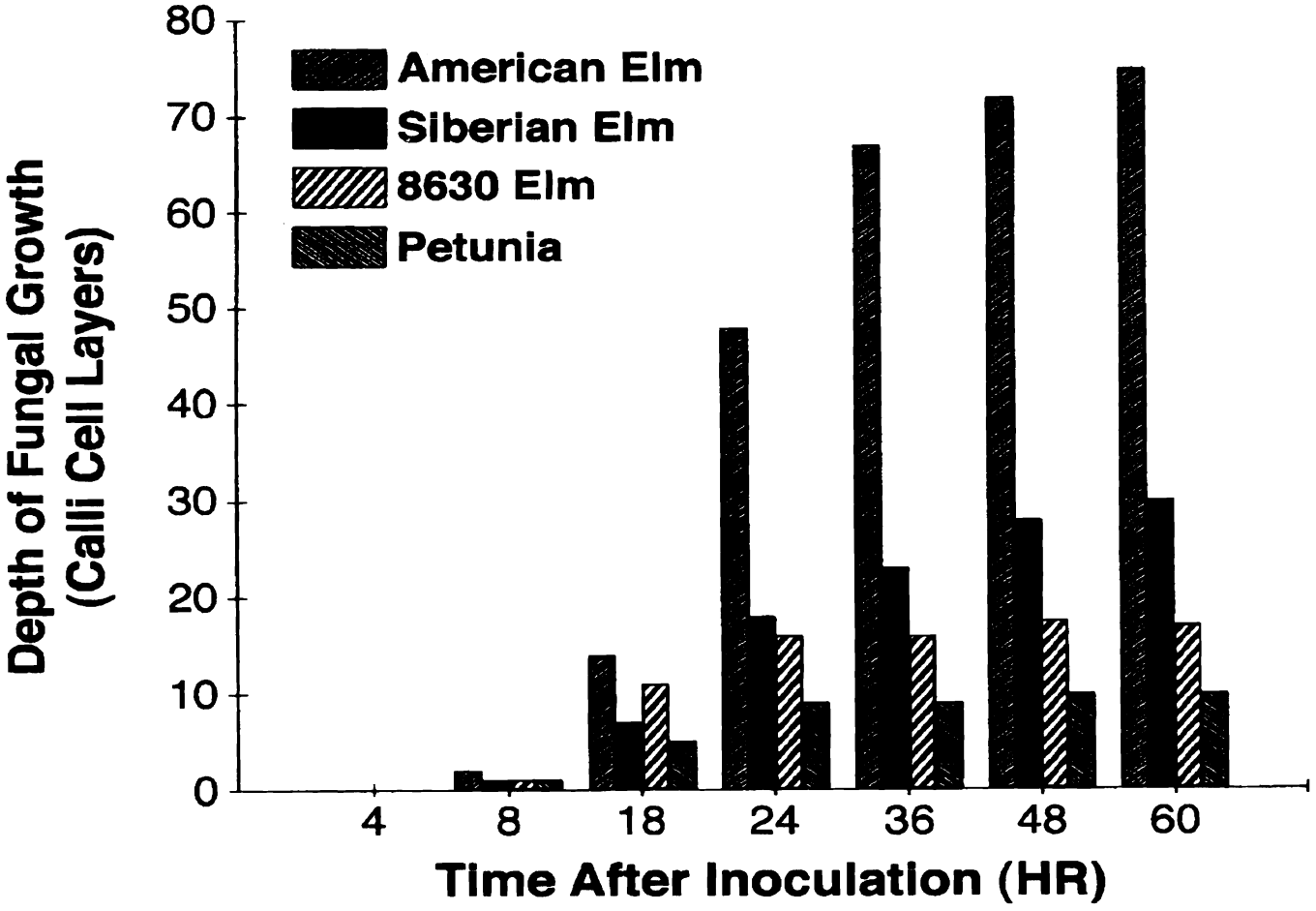

Fig. 2. Depth of *Ophiostoma ulmi* colonization into callus tissue of host and nonhost species.

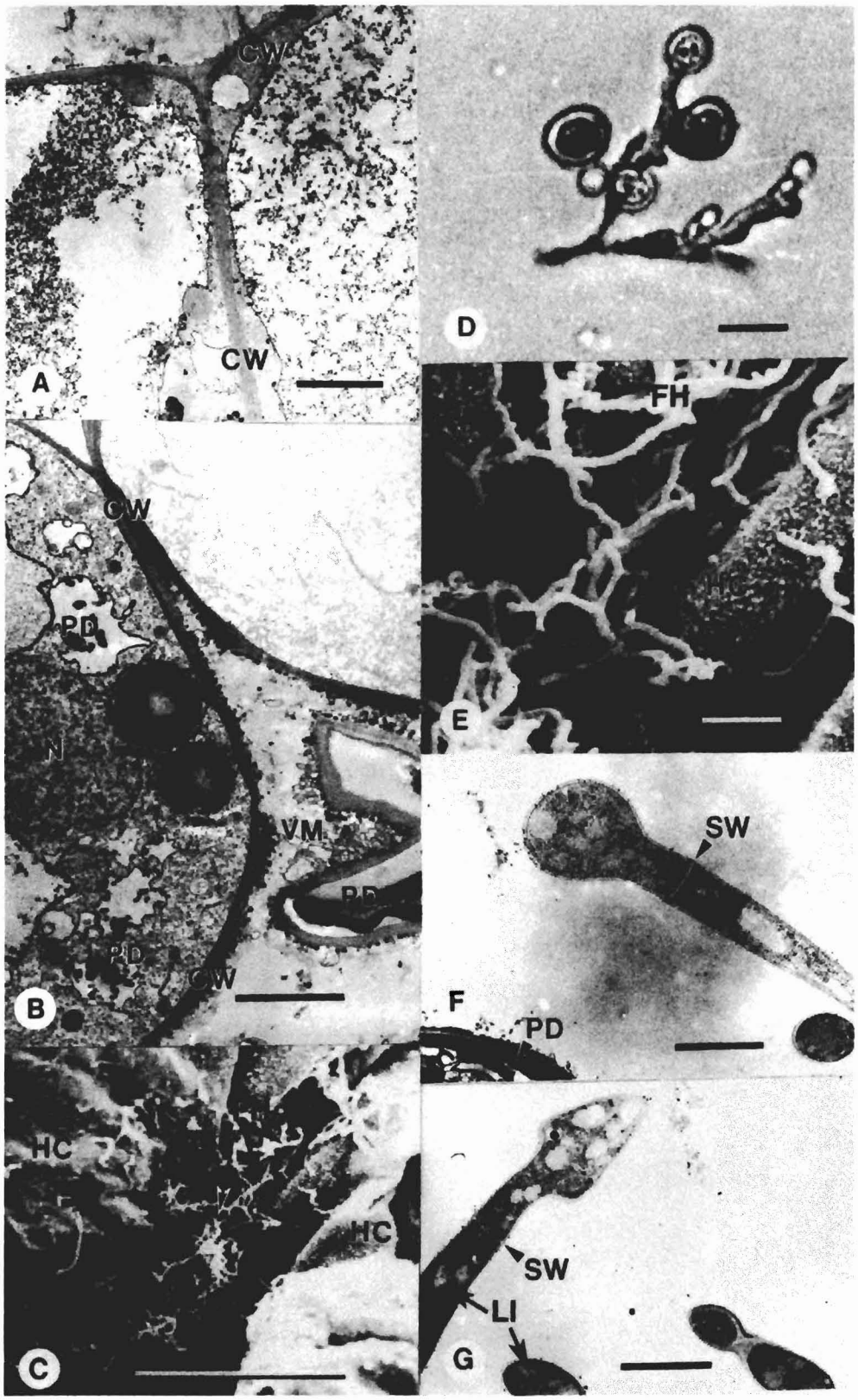

Fig. 3. Transmission electron micrographs A, B, F, and G; scanning electron micrographs C and E; light micrograph D of resistant American elm callus inoculated with *Ophiostoma ulmi* showing a resistant reaction. (A) Uninoculated callus tissue from resistant American elm showing normal ultrastructure and cell wall (CW). Bar = 1 μm. (B) Infected callus cells showing electron-dense, phenolic-like deposits (PD) in vacuoles. Nucleus (N) bounded by host cell walls(W) with fibrillar deposits in extracellular matrix (VM). Bar = 1 μm. (C) Sparse growth of fungal hyphae(FH) are not in intimate contact or covering host callus cells (HC). Conidia are lacking. Bar = 25 μm. (D) Abnormal hyphal morphology. Note contorted growth with bulges and swollen tips. Bar = 5 μm. (E) Fungal hyphae(FH) are twisted with short branches. Hyphal penetration of host callus(HC) and mature spores are lacking. Bar = 5 μm. (F) Young conidium is separated from conidiophore by septal walls (SW) developing in close proximity to callus cell wall. Note heavy accumulation of phenolic-like electron dense material (PD) 60 hr. after inoculation. Bar = 1 μm (G) Fungal hyphae showing extensive vacuolation and lipid-like inclusion(LI). Note septal walls (SW) of *O. ulmi*. Bar = 1 μm.

stages of plasmolysis (Fig. 1B). Regularly-shaped fungal hyphae were present in the intercellular spaces. Extensive, sporulating, dense hyphal growth was observed with SEM on host callus cells within 24 hr after inoculation (Fig. 1C). Fungal growth was in intimate contact with the calli (arrows). Light microscopy revealed hyphae of *O. ulmi* with a uniform and regular shape growing on susceptible American elm callus cells (Fig. 1D). Direct penetration by the hyphae into a susceptible American elm callus cell was observed with SEM (Fig. 1E). Numerous conidia (arrows) were observed on calli surfaces. TEM of conidia revealed a regular fungal wall pattern, lipid inclusions and a healthy spore coat (Fig. 1F). A germinated conidium with a germ tube of uniform diameter was observed with TEM next to a disrupted American elm callus cell (Fig. 1G). Hyphal colonization and fungal penetration were greatest on calli surfaces near inoculation sites. SEM observations revealed that the pathogen penetrated into susceptible American elm callus tissue to a depth of more than 75 cell layers within 60 hr after inoculation (Fig. 2).

Reactions in resistant tissue. Uninoculated, resistant American elm callus tissue (Fig. 3A) displayed a normal array of cellular organelles (Fig. 3A). Infected, resistant American elm callus tissue (Fig. 3B) revealed slight cell wall damage with some electron-dense, phenolic-like deposits. Within adjacent cells, however, cell membranes, mitochondria, and nuclei were intact. Vesicular materials accumulated in extracellular matrices (Fig. 3B) but were not observed in susceptible elm calli. SEM observations revealed cells that had not collapsed and appeared to be unaffected by the disease even at the point of inoculation. Exfoliate growth of fungal hyphae on resistant 8630 host callus was less dense (Fig. 3C) than on susceptible American elm callus (Fig. 1C). The hyphal growth was contorted and hyphal tips were swollen (Fig. 3D). Irregular bulges in the hyphae were common in contrast to the uniform hyphal morphology observed in the susceptible reaction (Fig. 1D). *O. ulmi* was not observed to penetrate the resistant American callus (Fig. 3E). Fungal hyphae were sparse and were found only in intercellular spaces and on the upper surfaces of the callus tissue. Macroscopically, hyphae did not extend beyond the callus edges by 60 hr. after inoculation. Penetration into callus of resistant American elm, Siberian elm and Petunia was limited to 30, 18 and 8 cell layers, respectively, 60 hr after inoculation (Fig. 2).

Little sporulation was observed on resistant American elm callus tissue(3E). Conidial formation often appeared to be arrested when the fungus was in close proximity to the resistant American elm callus in which heavy accumulation of phenolic-like, electron dense material were present (Fig. 3F). Septal wall formation appeared normal, but free conidia were rarely observed. In addition to bulging hyphal tips (Fig. 3G) and irregular twisted growth, fungal hyphae showed increased vacuolation and lipid-like inclusions.

Dramatic differences in the response of the pathogen, *O. ulmi*, to various callus sources were observed. Typical DED host-pathogen interactions occurred when the fungus colonized, sporulated, and invaded susceptible American elm callus cultures. Eventually, the susceptible calli cells lost their integrity. Tyloses, pathogenic vascular structures that form in response to *O. ulmi* in intact trees, were not present in any callus cultures since cellular differentiation had not occurred. Reactions of *O. ulmi* on resistant American elm cultures were expressed as bulging hyphal tips, and twisted growth suggesting a phytoalexin-like response (1). The latter could partially explain the cellular basis of resistance by resistant American elm to DED. The absence of cellular membrane disruption and apparent phenolic deposits in resistant host cytoplasm also indicates a resistance response. The latter response is similar to that described by Pijut et al (13) when American elm calli was exposed to medium containing culture filtrate of *O. ulmi*. While resistant responses of the intact resistant elm tree selection to *O. ulmi* may be quite different than responses observed on undifferentiated tissue culture, certain cytological responses of the host-pathogen interactions remain similar to phenolic deposits and extracellular matrix formations observed by Krause and Wilson (7).

In TEM preparations, electron-dense materials were clearly evident along with vacuolation and cytoplasm disruption of susceptible American elm callus cells. The electron-dense substances appeared phenolic-like by TEM. However, this could not be confirmed by histological methods. Fine, electron-dense deposits were found in the extracellular space of the host-pathogen complex. The similarity between the micrographs of susceptible callus exposed to *O. ulmi* culture filtrate observed by Pijut et al (13) and susceptible cells interacting with the fungus suggests that extracellular enzymes or toxins of the fungus may degrade the host cell walls.

The extracellular matrix of vesicular material found in resistant elm callus may represent sheath-like substances of callus origin that may play a role in resistance. Jacobi et al (5) observed a similar network on cultured loblolly pine callus inoculated with *Cronartium fusiforme* and suggested the senescing susceptible cells produced the material in response to stress. Beech and Gessler (2) also observed electron-dense fibrillar deposits in the host-pathogen complex of apple callus tissue infected with *Venturia inaequalis*.

SEM observations were quite useful in detecting hyphal penetration and the extent of pathogen colonization. The rapid and complete colonization by *O. ulmi* of the susceptible callus tissue was in contrast to the limited and disassociated pattern seen on resistant American elm and Siberian elm callus tissue and on petunia.

There were histopathological differences between resistant and susceptible callus cells as observed by light and electron microscopy. This may be an indication of DED-resistant elm cells responding to infection by producing an indeterminate factor or factors that inhibit normal growth of the DED pathogen. Identification of these factors and further clarification of the plant-microbe interactions could be used for relatively rapid screening of candidate DED-resistant callus rather than time-consuming conventional screening methods using entire trees.

Literature Cited

1. Barz, W., W. Bless, G. Borger-Papendorf, W. Gunia, U. Mackenbrock, D. Meier, Ch. Otto, and E. Super. 1990. Phytoalexins as part of induced defense reactions in plants: Their elicitation, function, and metabolism. *In*: Symposium on Bioactive Compounds in Plants. D.J. Chadwick and J. Marsh (Eds.). John Wiley and Sons, New York, NY. pp 140–141.

2. Beech, J. and C. Gessler. 1986. Interaction between *Venturia inaequalis* and apple callus tissue cultures; an electron microscopic study. J. Phytopathology 116:315–322.

3. Domir, S., L.R. Schreiber, and J.M. Ichida. Factors affecting growth of *Ophiostoma ulmi* on elm callus tissue. J. Environ. Hort. 9:211–215.

4. Helgeson, J.P. 1983. Studies of host-pathogen interactions in vitro. *In*: Use of Tissue Culture and Protoplasts in Plant Pathology. J.P. Hegeson and B.J. Deverall (Eds.). Academic Press, New York, NY pp. 9–38.

5. Jacobi, W.R., H.V. Amerson, and R.L. Mott. 1982. Microscopy of cultured loblolly pine seedlings and callus inoculated with *Cronartium fusiform.* Phytopathology 72:138–143.

6. Jang, J.C. and F.H. Tainter. 1990. Cellular responses of pine callus to infection by *Phytophthora cinnamomi*. Phytopathology 80:1347–1352.

7. Krause, C.R. and C.L. Wilson. 1972. Fine structure of *Ceratocystis ulmi* in elm wood. Phytopathology 62:1253–1256.

8. MacDonald, W.L. and H.S. McNabb. 1974. Electron microscope observations of *Ceratocystis ulmi*-induced tylosis development in *Ulmus*. Eur. J. For. Pathol. 4:2–10.

9. Miller, S.A. and D.P. Maxwell. 1983. Evaluation of Disease Resistance. *In*: Handbook of Plant Cell Culture. Vol. 1. Techniques for Propagation and Breeding. D.A. Evans, W.R. Sharp, P.V. Ammirato, and Y.Y. Yamada (Eds.). Macmillan, New York. pp. 853–879.

10. Ouellette, G.B. 1978. Fine structural observations on substances attributable to *Ceratocystis ulmi* in American elm and aspects of host cell disturbances. Can. J. Bot. 56:2550–2566.

11. Ouellette, G.B. 1978. Ultrastructural observations on pit membrane alterations and associated effects in elm xylem tissues infected by *Ceratocystis ulmi*. Can. J. Bot. 56:2567–2588.

12. Ouellette, G.B. 1978. Light and electron microscope studies on cell wall breakdown in American elm xylem tissues infected with Dutch elm disease. Can. J. Bot. 56:2666–2693.

13. Pijut, P.M., R.D. Lineberger, S.C. Domir, J.M. Ichida, and C.R. Krause. 1990. Ultrastructure of cell of *Ulmus americana* cultured in vitro and exposed to culture filtrate of *Ceratocystis ulmi*. Phytopathology 80:764–767.

14. Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell. Biol. 17:208–212.

15. Went, J.C. 1954. The Dutch elm disease-summary of fifteen years hybridization and selection work (1937–1952). Tijdschr. PlZiekt 60:109–127.

Identification of Genetic Diversity among *Loropetalum chinense* var. *rubrum* Introductions[1]

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Abstract

Loropetalum chinense (R.Br.) Oliv. is an Asiatic evergreen shrub with many desirable landscape characteristics. It is fast-growing, tolerant to disease and insects, and produces an abundance of attractive flowers. Recently, the horticultural community has shown an increased interest in the species since several pink-flowering selections *(L. chinense* var. *rubrum)* have become available. Due to the relatively rapid introduction and commercialization of *Loropetalum chinense* var. *rubrum*, there has been some confusion concerning the identity and distinctness of cultivars. This research used Randomly Amplified Polymorphic DNA (RAPDs) to examine the genetic diversity of 14 *L. chinense* var. *rubrum* accessions, and 2 accessions of *L. chinense*. Results indicate the presence of 4 groupings among the introductions, with many of the introductions having at least 1 or 2 other closely related selections. Most named introductions were closely related to other unnamed introductions.

Index words: RAPD, DNA fingerprinting, breeding.

Species used in this study: *Loropetalum chinense* (R.Br.) Oliv., *Loropetalum chinense* var. *rubrum* Yieh.

Significance to the Nursery Industry

Many *Loropetalum chinense* var. *rubrum* introductions have been established into North America since the 1980s. Many of these selections have been introduced by different individuals; however, plant material has been obtained from the same sources. This circumstance may lead to different cultivars being named from the same material. This research uses DNA markers to examine *L. chinense* var. *rubrum* introductions to identify diversity among introductions. The results indicate the presence of 4 groupings among the introductions. Many of the introductions, however, have at least 1 or 2 other closely related selections. This information will provide growers with a basis for decision-making to avoid redundancy in cultivar propagation. Also, the data presented are useful when making breeding decisions concerning *L. chinense* var. *rubrum.*

Introduction

Loropetalum chinense (R.Br.) Oliv. is an Asiatic evergreen shrub that belongs to the witchhazel family. Its natural range is in the Chinese provinces from Shantung to Yunnan, and in a small area of Japan (1). Traditionally, *Loropetalum* has been used for medicinal purposes by the Chinese (2, 3), and for charcoal by the Japanese (1).

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