Ultrastructure of Cells of *Ulmus americana* Cultured in Vitro and Exposed to the Culture Filtrate of *Ceratocystis ulmi*

Paula M. Pijut, R. Daniel Lineberger, Subhash C. Domir, Jann M. Ichida, and Charles R. Krause

First and second authors, Department of Horticulture, The Ohio State University, Columbus 43210; third, fourth, and fifth authors, USDA-ARS, Nursery Crops Research Laboratory, Delaware, OH 43015.
Present address of first author: USDA-Forest Service, North Central Forest Experiment Station, 1992 Folwell Ave., St. Paul, MN 55108.
Present address of second author: Department of Horticulture, Clemson University, Clemson, SC 29634.
Accepted for publication 12 February 1990.

ABSTRACT

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

Calli of American elm susceptible and resistant to Dutch elm disease were exposed to a culture filtrate of a pathogenic isolate of *Ceratocystis ulmi*. Cells from untreated tissue exhibited typical internal composition associated with healthy, actively growing cells. All cells exposed to culture filtrate showed appreciable ultrastructural changes. Cells from susceptible American elm displayed mitochondrial and cytoplasmic disruption. Damage to membranes was evident by the appearance of incomplete cell walls and distinctive plasmolysis. Cells of the resistant American elm differed dramatically with the presence and accumulation of phenoliclike deposits. The response seen with the resistant elm could be associated with a defensive mechanism against toxic metabolites of *C. ulmi*.

Various internal symptoms of Dutch elm disease occur in xylem vessels of elm trees infected with *Ceratocystis ulmi*. Tyloses formation was noted to occlude conducting vessels of elm twigs as early as 1 day after infection with *C. ulmi* (4). Certain vessels are occluded following infection with a fibrillar material of varying density (7–9). The occluding components observed in vessels were reported to be of fungal origin, possibly degradation products of host secondary walls (7). Alterations in vasicentric parenchyma cells included retraction or distortion of the plasmalemma, vacuolated cytoplasm with altered organelles, and dilated endoplasmic reticulum.

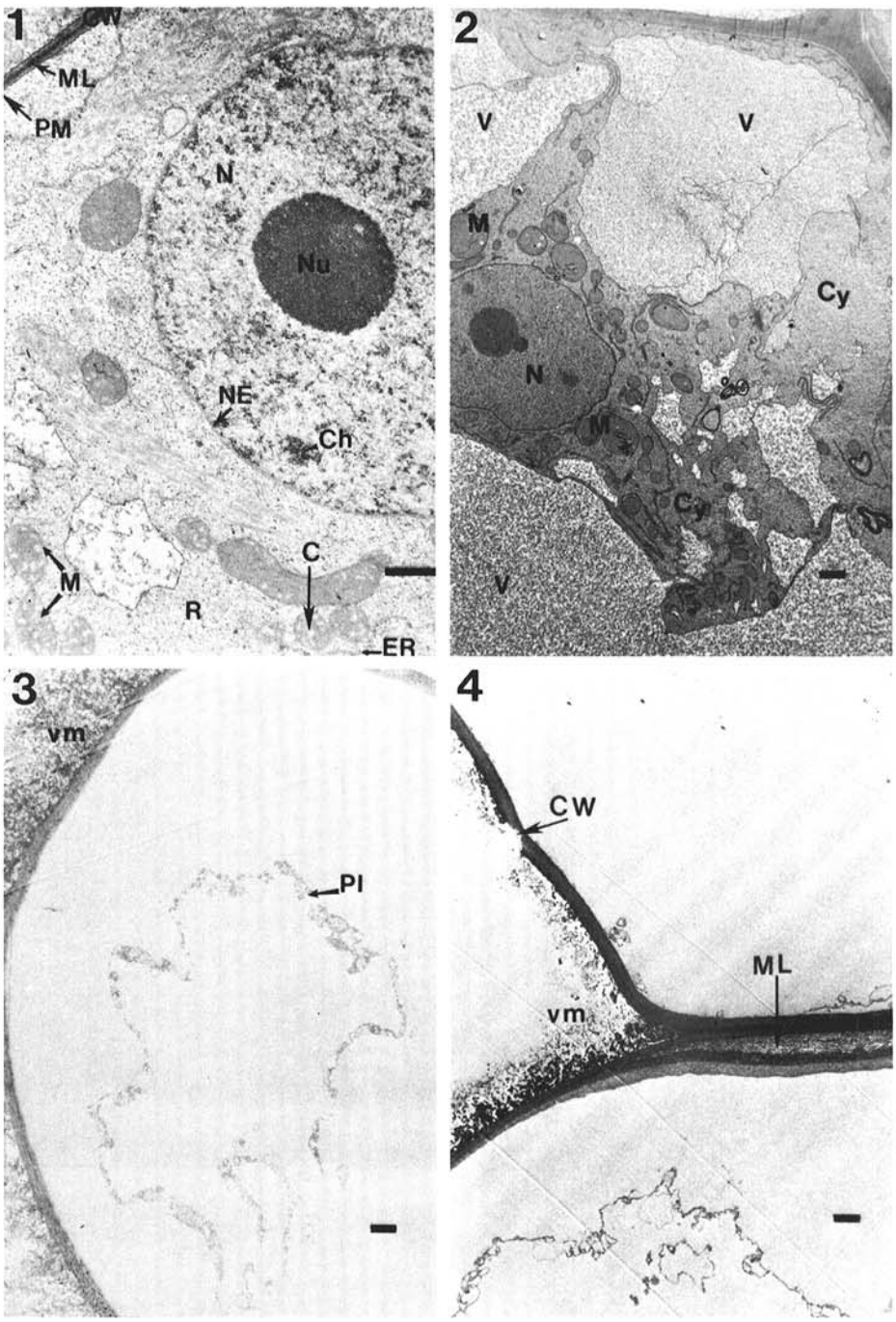
Extensive cell wall disruption and breakdown occur consistently in elm xylem tissues infected by *C. ulmi* (9). Ouellette (10,11) investigated the ultrastructural cell wall modifications that result from or are enhanced by disease development in American elm.

Aggressive isolates affect xylem vessel walls more severely than do nonaggressive isolates (12). The toxin, cerato-ulmin, which is produced by *C. ulmi*, also has been reported to induce symptoms indistinguishable from those of Dutch elm disease (16). The following study was undertaken to describe the ultrastructural alterations in American elm cells cultured in vitro following exposure to the culture filtrate of a pathogenic isolate of *C. ulmi*.

MATERIALS AND METHODS

Callus cultures from a Dutch elm disease-susceptible American elm (*Ulmus americana* L.) and a resistant American elm selection #8630 were initiated from young leaves of mature trees (growing at the Nursery Crops Research Lab, Delaware, OH). Leaf segments including a portion of the midvein were plated adaxial side up on a Murashige and Skoog (MS) medium (6) supplemented with 200 mg of casein hydrolysate per liter, 10% (v/v) coconut milk, 3% (w/v) sucrose, 8 μM 6-benzylaminopurine, and 0.5 μM 2,4-dichlorophenoxyacetic acid. The pH of the media was adjusted to 5.7 and Difco Bacto agar (0.7%, w/v) added before autoclaving. Cultures were incubated in the dark at 23 $\pm$ 2 C for 1–2 mo. Proliferating callus cultures then were transferred to fresh half-strength MS media containing the same supplements.

Culture filtrates were prepared from liquid shake cultures grown at 23 C for 8 days in the dark. The culture medium was a modified Salemink's medium (15) seeded with an isolate of *C. ulmi* initially



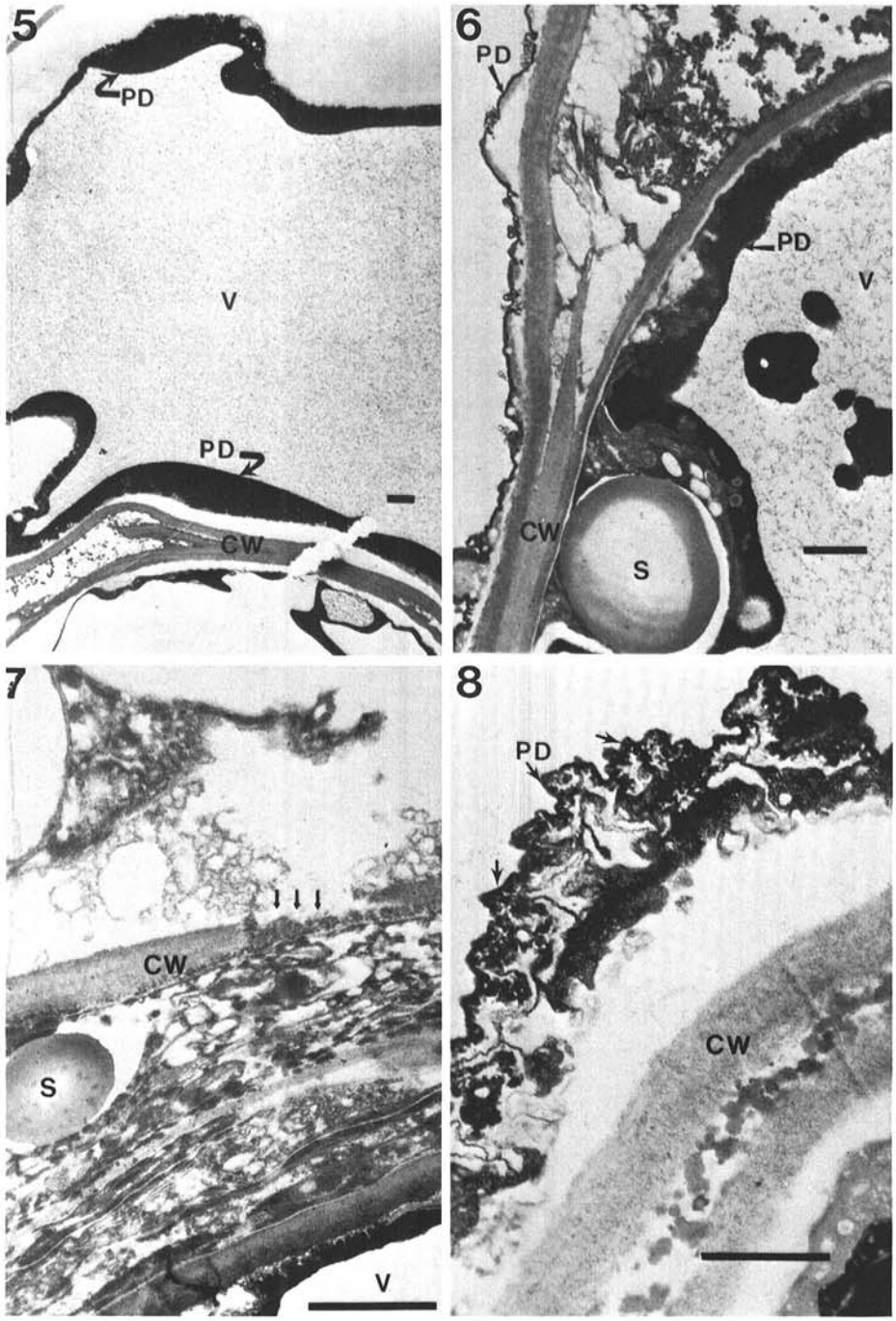
Figs. 1–4. Transmission electron micrographs of cells of *Ulmus americana* cultured in vitro, and treated with culture filtrates of *Ceratocystis ulmi*. C = cristae; Ch = chromatin; CW = cell wall; Cy = cytoplasm; ER = endoplasmic reticulum; M = mitochondrion; ML = middle lamella; N = nucleus; NE = nuclear envelope; Nu = nucleolus; Pl = plasmolyzed cell membrane; PM = plasmalemma; R = ribosomes; V = vacuole; vm = vesicular material. All bars = 1 μm. **1,** Ultrastructural overview of control susceptible elm. **2,** Susceptible elm cell exposed to culture filtrates of *C. ulmi*. **3,** Culture filtrate-treated susceptible elm cell, which appears to be plasmolyzed. **4,** Incomplete cell wall and vesicular material outside the cell of a susceptible elm cell that had been treated with culture filtrates.

derived from a diseased American elm tree in Delaware, OH. After 8 days, the liquid cultures were centrifuged for 30 min at 1,200 *g* to remove spores and mycelia. The pH of the filtrate was adjusted to 5.7, and the filtrate was sterilized with a 0.45-μm Nalgene filter unit.

Sterilized half-strength MS medium was amended with culture filtrate to give a 50% (v/v) final concentration and solidified with 0.7% agar. Calli (0.5 g) of each elm line were placed on the surfaces of the test media and incubated in the dark at 23 C for 4 wk. Controls were obtained from callus cultures 4 wk after subculturing to fresh callus culture medium. Preliminary tests for cell toxicity of fungal growth medium revealed no detrimental effect.

Samples for transmission electron microscopy (TEM) were obtained from 10 replicates per elm. Calli were fixed in 3% (w/v) glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.0, for 19 hr (2). Samples then were rinsed in 0.05 M sodium cacodylate buffer three times at 15-min intervals. After thorough washing the cell material was postfixed in 0.1 M sodium cacodylate buffer containing 1% osmium tetroxide for 2–3 hr at room temperature. The material was again thoroughly rinsed in 0.1 M sodium cacodylate buffer three times at 15-min intervals.

Specimens were dehydrated in a graded ethanol series and infiltrated with Spurr's epoxy resin (13). Ultrathin sections (50–70 nm) were cut and mounted on Cu/Pd grids. Grids with sections were poststained with 2% uranyl acetate (18) for 30 min, followed by lead citrate for 5–10 min (17). Serial sections of controls and treatments were observed (12–15 grids each), and electron micrographs were taken with Kodak electron image plates on a Hitachi HU-11E (NSA, Mountain View, CA) electron microscope operated at 75 kV.



Figs. 5–8. Transmission electron micrographs of cells of *Ulmus americana* exposed to the culture filtrates of *Ceratocystis ulmi*. CW = cell wall; PD = phenoliclike deposits; S = starch grain; V = vacuole. All bars = 1 μm. **5 and 6,** Treated, resistant elm cells containing electron dense phenoliclike deposits. **7,** Disrupted cell wall (arrows) of resistant elm cell after exposure to culture filtrates. **8,** Phenoliclike deposition outside the cell wall of resistant elm cell that had been treated with culture filtrate.

RESULTS

Cultured cells from susceptible and resistant American elms were similar in composition to meristematic parenchyma cells, with a complement of organelles (Fig. 1). Cells were contained within well-demarcated cell walls and membranes. A denser wall layer presumed to be the middle lamella was evident, especially where two cells were adjacent to one another. Untreated cells displayed a large central vacuole usually filled with electron-dense particles encased within the tonoplast membrane. Ribosomes were either free or were attached to the membranes of the rough endoplasmic reticulum cisternae. The nucleus of these cells was of the reticulate type, large, centrally located, with a compact, prominent nucleolus, and delimited by a typical nuclear envelope. Mitochondria were abundant and varied in shape from cylindrical to spherical according to the angle of sectioning. Starch grains were observed in only a few cells.

TEM analysis of cells exposed to the culture filtrates of *C. ulmi* revealed some interesting differences. In the case of the susceptible American elm, very few cells had any normal internal organization. Cytoplasm of these cells was disrupted, and there was a general increase in vacuolation (Fig. 2). The nucleus appeared to be intact, but the mitochondria were swollen and lacked internal structure. In some areas, the outer membrane was disrupted. Most of the treated cells were devoid of organelles and internal structure (Figs. 3 and 4). Plasmolysis seemed to have occurred, and the middle lamella between contiguous cells appeared to have pulled away from the cell wall, which in several instances was incomplete. An increase in vesicular material outside the empty cells also was evident.

As with the susceptible elm, most treated cells of the resistant elm line were highly vacuolated and devoid of organization (Fig. 5). A noticeable electron-dense precipitate was found within the vacuoles of about 40% of the cells. Starch grains were abundant (Fig. 6) and a striking accumulation of an electron-dense precipitate was present along the plasmalemma. Evidence of cell wall damage was also found (Fig. 7). This unusually heavy precipitate also was quite visible and frequently found along the exterior of the cell wall (Fig. 8).

DISCUSSION

Untreated control cells of American elm were of the parenchyma type and exhibited the classical complement of organelles and cell organization associated with healthy, actively growing cells. Cells exposed to culture filtrates of *C. ulmi* showed appreciable ultrastructural changes. Susceptible exhibited overall mitochondrial and cytoplasmic disruption, possibly as a result of damage to cellular membranes. Strobel et al (14) found this to be the case in sugarcane leaves treated with a toxin from *Helminthosporium sacchari*. The swelling of mitochondria and loss of internal structure in treated cells of the susceptible elm suggested that these may be sites of toxin action, as has been suggested in other systems (1,5).

The possibility of damage to the cellular membranes was evident by the appearance of incomplete cell walls and distinctive plasmolysis. The middle lamella appeared to have pulled away from the cell wall, and an increase in vesicular material outside the damaged cells was evident. Ouellette (9) showed that extensive cell wall disruption and breakdown occur consistently in elm xylem tissue infected by *C. ulmi*. Disruption and breakdown of the middle lamella and secondary wall layers frequently were observed. Degrading host cytoplasm retained many vacuolated areas and contained plastids and mitochondrial remnants. Scheffer and Elgersma (12) demonstrated that degradation of cell walls was more severe after infection with an aggressive isolate of *C. ulmi* than with a nonaggressive isolate.

Treated cells of the resistant elm line were characterized by the presence and accumulation of phenoliclike deposits that appeared electron dense. Krause and Wilson (3) and Ouellette (7–9) suggested that the host cytoplasm responds to fungal invasion by depositing phenoliclike material and that this may be a resistance response. The response seen here could similarly be associated with a defensive mechanism against toxic metabolites of *C. ulmi*. This phenoliclike deposition could possibly account for the ability of resistant callus to grow normally and increase in fresh weight in medium containing culture filtrates of *C. ulmi* (unpublished observations). The present work largely compares in vitro results with the in vivo observations of others who examined infected host tissues. While this study has demonstrated a differential response between callus cells initiated from susceptible and resistant selections of American elm, it is important to consider that the ultrastructural alterations found in vitro may or may not be causally related to the mechanism of resistance at the whole tree level in vivo. Further research concerning the nature of the ultrastructural alterations in vitro must be conducted before a causal relationship to disease resistance can be shown.

LITERATURE CITED

1. Aldrich, H. C., Gracen, V. E., York, D., Earle, E. D., and Yoder, O. C. 1977. Ultrastructural effects of *Helminthosporium maydis* race T-toxin on mitochondria of corn roots and protoplasts. Tissue Cell 9:167-177.
2. Feder, N., and O'Brien, T. P. 1968. Plant microtechnique: Some principles and new methods. Am. J. Bot. 55:123-142.
3. Krause, C. R., and Wilson, C. L. 1972. Fine structure of *Ceratocystis ulmi* in elm wood. Phytopathology 62:1253-1256.
4. MacDonald, W. L., and McNabb, H. S. 1974. Electron microscope observations of *Ceratocystis ulmi*-induced tylosis development in *Ulmus*. Eur. J. For. Pathol. 4:2-10.
5. Miller, R. J., and Koeppe, D. E. 1971. Southern corn leaf blight: Susceptible and resistant mitochondria. Science 173:67-69.
6. Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.
7. 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.
8. 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.
9. 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.
10. Ouellette, G. B. 1981. Ultrastructural cell wall modifications in secondary xylem of American elm surviving the acute stage of Dutch elm disease: Vessel members. Can. J. Bot. 59:2411-2424.
11. Ouellette, G. B. 1981. Ultrastructural cell wall modifications in secondary xylem of American elm surviving the acute stage of Dutch elm disease: Fibres. Can. J. Bot. 59:2425-2438.
12. Scheffer, R. J., and Elgersma, D. M. 1982. A scanning electron microscope study of cell wall degradation in elm wood by aggressive and non-aggressive isolates of *Ophiostoma ulmi*. Eur. J. For. Pathol. 12:25-28.
13. Spurr, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26:31-43.
14. Strobel, G. A., Hess, W. M., and Steiner, G. W. 1972. Ultrastructure of cells in toxin-treated and *Helminthosporium sacchari*-infected sugarcane leaves. Phytopathology 62:339-345.
15. Takai, S. 1978. Cerato-ulmin, a wilting toxin of *Ceratocystis ulmi*: Cultural factors affecting cerato-ulmin production by the fungus. Phytopathol. Z. 91:147-158.
16. Takai, S., and Hiratsuka, Y. 1984. Scanning electron microscope observations of internal symptoms of white elm following *Ceratocystis ulmi* infection and cerato-ulmin treatment. Can. J. Bot. 62:1365-1371.
17. Venable, J. H., and Coggeshall, R. 1965. A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. 25:407-408.
18. Watson, M. L. 1958. Staining of tissue sections for electron microscopy with heavy metals. J. Biophys. Biochem. Cytol. 4:475-478.

SUSTAINING ASSOCIATES

ABBOTT AGRIC. RES. CTR., Long Grove, IL

AGRI-DIAGNOSTICS ASSOCIATES, Cinnaminson, NJ

AGRICULTURE CANADA, Vineland Station, Ontario

ALF. CHRISTIANSON SEED CO., Mt. Vernon, WA

AMERICAN CYANAMID CO., Agriculture Center, Princeton, NJ

ATOCHEM NORTH AMERICA, Geneva, NY

BASF CORPORATION, Research Triangle Park, NC

BUCKMAN LABORATORIES, Memphis, TN

CALGENE, INC., Davis, CA

CHEVRON CHEMICAL CO., San Ramon, CA

CIBA-GEIGY CORPORATION, Agric. Div., Greensboro, NC

DEKALB-PFIZER GENETICS, DeKalb, IL

DEL MONTE FOODS USA, Walnut Creek, CA

DNA PLANT TECHNOLOGIES, INC., Oakland, CA

E. I. DUPONT DE NEMOURS & CO., INC., Agric. Chem. Dept., Newark, DE

ELI LILLY & CO., Lilly Res. Labs, Greenfield, IN

FERMENTA ASC CORPORATION, Mentor, OH

FERRY MORSE SEED CO., San Juan Bautista, CA

FUNK SEEDS INTERNATIONAL, INC., Bloomington, IL

GREAT LAKES CHEMICAL CORPORATION, West Lafayette, IN

GRIFFIN CORPORATION, Fresno, CA

GUSTAFSON, INC., Des Moines, IA

HARRIS MORAN SEED CO., Hayward, CA

H. J. HEINZ CO., Bowling Green, OH

HOECHST ROUSSEL AGRI. VET. CO., Somerville, NJ

ICI AMERICAS, INC., Mountain View, CA

ICI AMERICAS, INC., Richmond, CA

ILLINOIS CROP IMPROVEMENT ASSOCIATION, Urbana, IL

ILLINOIS FOUNDATION SEEDS, INC., Champaign, IL

ISTITUTO DI FITOVIROLOGIA, Torino, Italy

JANSSEN PHARMACEUTICA, Piscataway, NJ

LANDIS INTERNATIONAL, Valdosta, GA

MERCK & CO., INC., Rahway, NJ

MOBAY CORPORATION, Kansas City, MO

MONSANTO CO., St. Louis, MO

NOR-AM CHEMICAL CO., Wilmington, DE

NORTHRUP KING CO., Woodland, CA

PEST PROS, INC., Plainfield, WI

PETOSEED CO., INC., Woodland, CA

PFIZER, INC.-TEKCHEM, Chem. Div., New York, NY

RHONE-POULENC AG COMPANY, Research Triangle Park, NC

RICERCA, INC.; Painesville, OH

RJR NABISCO INC., Winston-Salem, NC

ROHM & HAAS CO., Philadelphia, PA

SAKATA SEED AMERICA, INC., Salinas, CA

SANDOZ CROP PROTECTION CORP., Des Plaines, IL

O. M. SCOTT & SONS, Marysville, OH

TWYFORD INTERNATIONAL, INC., Sebring, FL

UNIROYAL CHEMICAL CROP PROT. R&D, Bethany, CT

UNOCAL CHEMICALS, West Sacramento, CA

W-L RESEARCH, INC., Evansville, WI