Histopathology and Ultrastructure of Vascular Responses in Peas Resistant or Susceptible to *Fusarium oxysporum* f. sp. *pisi*

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ABSTRACT

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A histopathological and ultrastructural examination of resistant and susceptible host-pathogen interactions was conducted in the garden pea cultivar Thomas Laxton following inoculation with race 1 and race 2 of *Fusarium oxysporum* f. sp. *pisi*. Responses were characterized and compared with healthy controls at 0.5, 1, 2, 3, 4, 6, 8, 10, and 12 days after inoculation. Vascular plugs, vessel coatings, callose deposits and phenolic compounds that accumulated as host responses were histochemically characterized. No qualitative differences were found to explain resistance or susceptibility. No anatomical or ultrastructural differences in response were observed between resistant or susceptible suscept-pathogen interactions up to 4 days after infection. After 4 days in susceptible interactions the pathogen grew laterally from initially infected vessels into adjacent vessels and parenchyma cells until the vascular bundle was completely colonized, while in resistant interactions the pathogen was confined to vessels initially infected. An increase in cytoplasmic activity of vascular parenchyma cells was detected in both resistant and susceptible interactions.

Although responses to vascular wilts have been intensively studied and characterized in several suscept-pathogen interactions, little histopathological and histochemical research has been conducted on pea infected with *Fusarium oxysporum* Schlechtend. f. sp. *pisi* (Van Hall) Snyder & Hansen. Early work with paraffin-embedded pea stems was performed by Schroder and Walker (21), who noted increased cambial activity in infected plants. Extensive colonization of vessels by the fungus was observed in susceptible plants, while in resistant plants the fungus was found only sparingly.

More recently Tessier (23) characterized the responses of peas to stem infection by race 1 and race 2 of *F. o. pisi* by light microscopy of paraffin-embedded tissues. In susceptible interactions wilting began 4 days after inoculation and progressed vertically through all the leaflets until death of the plant 12–14 days after inoculation. In resistant interactions the first leaflet above the site of inoculation exhibited yellowing between 4 and 6 days after inoculation, but no further symptom development was observed after that time. Vascular responses of the host to infection included vessel occlusion by gels, deposition of callose in some xylem parenchyma cells, and extensive vascular browning. The gels were composed of carbohydrates, protein, and pectin, but tests for phenolic compounds were negative. Tyloses were not found. Most recently Bishop and Cooper (5,6) described the ultrastructure of vessel invasion and vessel occlusion by gels and death of xylem parenchyma cells in susceptible and resistant cultivars.

Information reported here characterizes the processes of pathogen containment in resistant interactions and compares these with the processes occurring in susceptible interactions. Histological and histochemical observations were made from 0.5 to 12 days after inoculation on stem-inoculated pea plants that were embedded in plastic and sectioned at 1.0–1.5 μm. Thin sections of similarly treated material were prepared for examination by electron microscopy.

MATERIALS AND METHODS

Cultures of *F. o. pisi* races 1 and 2 (ATTCC 26043 and 26044, respectively) were grown for 3 or 4 days in 50 ml of Czapek-Dox medium (modified by adding 1.0 g of yeast extract per liter of medium) at 25 C in 125-ml Erlenmeyer flasks. The cultures were continuously agitated on an orbital shaker at 150 RPM. Inoculum was prepared by filtering cultures through a double layer of facial tissue to remove mycelial fragments. The spore suspension then was centrifuged at 1,500 RPM for 5 min. The supernatant was decanted, the remaining spore pellet was resuspended in 5 ml of distilled water, and then adjusted to a final

concentration of 5×10 microconidia/ml. Red vinyl tracer particles were added to the spore suspensions (4). These particles become trapped in the vascular elements and provide a marker for locating the initial infection sites for microscopic examinations.

Seeds of *Pisum sativum* L. 'Thomas Laxton' were grown in 1:1:1, sand/loam/peat mix in plastic flats. They were then kept in a 25 ± 5 C greenhouse and drenched weekly with Peter's 20:20:20 nutrient solution. Plants were inoculated 5 mm above the soil line 12–14 days after seeding by injection of inoculum from a 1-cm^3 tuberculin syringe with a 23 G needle. A 0.1-ml aliquot of inoculum was placed on the stem of each plant, and the syringe needle was passed through the drop into the stem. The needle was passed through the drop twice more to ensure that vascular elements were severed and that the drop of inoculum was drawn up into the vascular system. Inoculated plants were transferred to environmental chambers to provide ambient conditions of 125 $\mu E/m^2/sec$ of light during a 13-hr photoperiod/day at a temperature of 25 ± 1 C.

At intervals of 0.5, 1, 2, 3, 4, 6, 8, 10, and 12 days after inoculation, stem segment samples were removed from each of five uninoculated plants and five plants inoculated with race 1 and race 2. Stem segments 2.5–5.0 mm long were excised 5–10 mm above the site of inoculation and fixed for 90 min in 2.5% glutaraldehyde in 0.05 M phosphate buffer at pH 8.0, washed in 0.05 M phosphate buffer at pH 8.0 for 1 hr, and postfixed for 1 hr in 1% OsO_4 in 0.05 M phosphate buffer at pH 6.8. After fixation, all material was dehydrated in acetone, infiltrated for 4 days with Spurr's low viscosity medium (22), and then flat embedded in Spurr's medium in aluminum weighing dishes. Thick sections were cut at 1.0–1.5 μm with glass knives, mounted on glass slides, stained with 0.1% thionin (18) and examined under an Olympus Vanox light microscope. Thin sections were cut with a diamond knife, mounted on Formvar-coated 100-mesh grids, and examined with a Hitachi HS-9 microscope at 75 kV. For routine observation glutaraldehyde-osmium fixed sections were stained with uranyl acetate and Reynold's lead citrate. Thick sections of glutaraldehyde or glutaraldehyde-osmium fixed tissue were stained for carbohydrates, lipids, protein, callose, and phenolics by the reagents listed in Table 1. Tissue also was treated by the Albersheim procedure (1) for the detection of pectin,

TABLE 1. Histochemical stains used and the compounds detected

Stain	Type of compound	Reference
Coomassie blue	Protein (basic)	8,10
Ponceau 2R	Protein (basic)	11
PAS	Carbohydrates	8,19
Sudan black B	Lipids	7,9
Sudan IV	Lipids	7,9
Aniline blue-UV	Callose	3
O-Dianisidine	Phenolics	8
Diazo red RC	Phenolics	15
Ferric chloride (1%)	Phenolics	19
Ferric chloride (10%)	Phenolics	8
Ferric sulfate (1%)	Phenolics	14
P-Nitroaniline	Phenolics	14
DMB (Gibb's reagent)	Phenolics	14,15
Nitroso reaction	Phenolics	14,15
DCQ reagent	Phenolics	14,15
Swain-Hillis	Phenolics	20
Folin-Ciocalteau	Phenolics	13

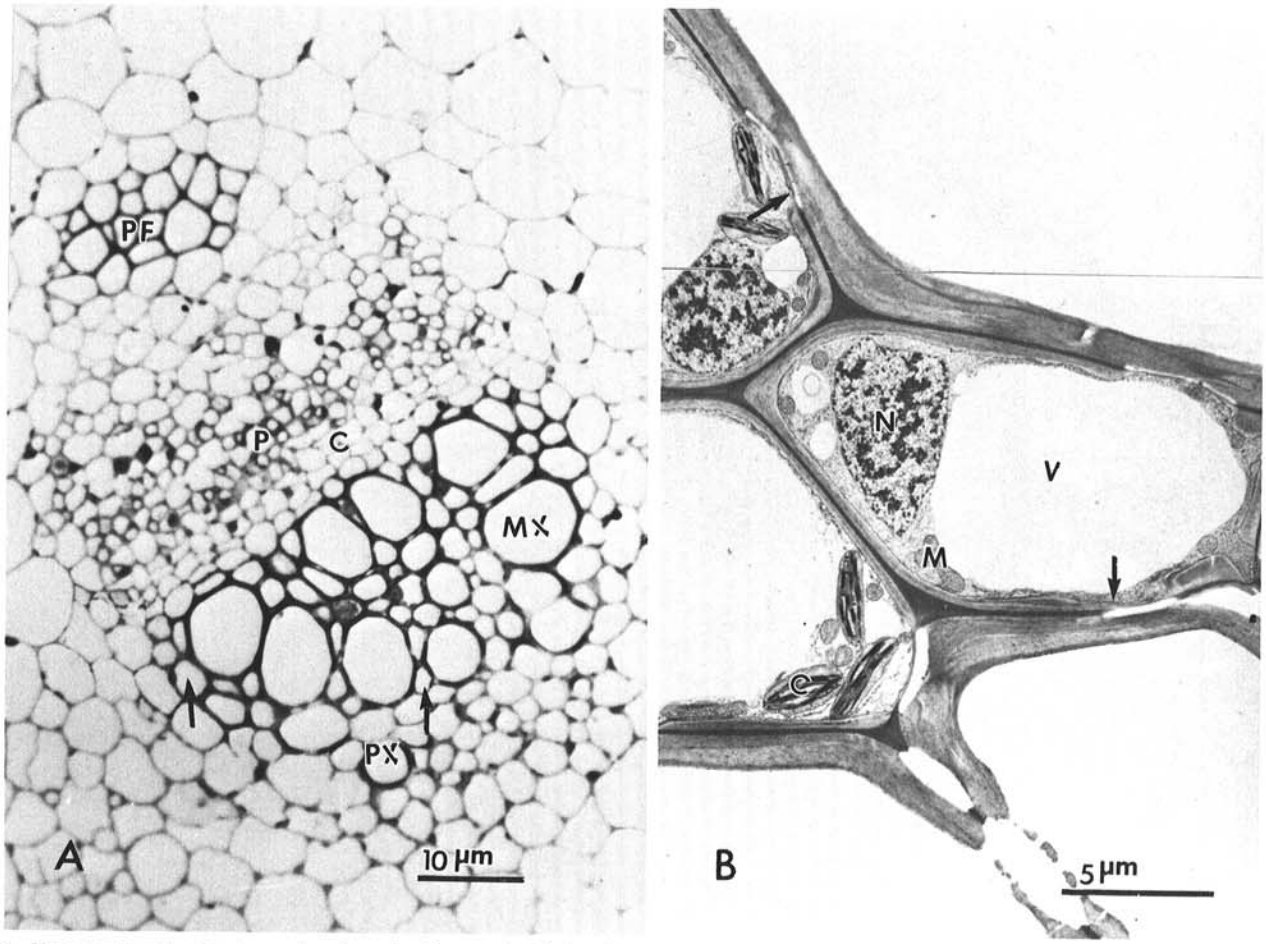

Fig. 1. Vascular bundle structure of uninoculated peas. **A,** Light micrograph of whole bundle. Typical contact cells with only peripheral layers of cytoplasm are indicated by arrows, phloem fibers (PF); phloem (P); cambial region (C); metaxylem (MX); protoxylem (PX). **B,** Electron micrograph of metaxylem vessels and associated contact cells. A large central vacuole (V) in a contact cell is surrounded by a peripheral layer of cytoplasm containing a nucleus (N), mitochondria (M), and chloroplasts (C). Protective layer (arrows) in pit areas is barely visible at this magnification.

dehydrated in an ethanol series, embedded in Spurr's resin, sectioned, and then examined for pectin without further treatment.

RESULTS

Anatomy and ultrastructure of uninfected vascular bundles. Healthy peas corresponded in general anatomy to that described previously (12). The vascular region of stem cross sections consisted of four to six individual vascular bundles interconnected by the vascular cambium. Each vascular bundle was composed of a region of phloem fiber cells, phloem, cambium, and xylem elements. The xylem region of each vascular bundle was composed of meta- and protoxylem elements and xylem parenchyma (Fig. 1A).

Contact cells (those xylem parenchyma in direct physical contact with vessels) adjacent to protoxylem elements had only

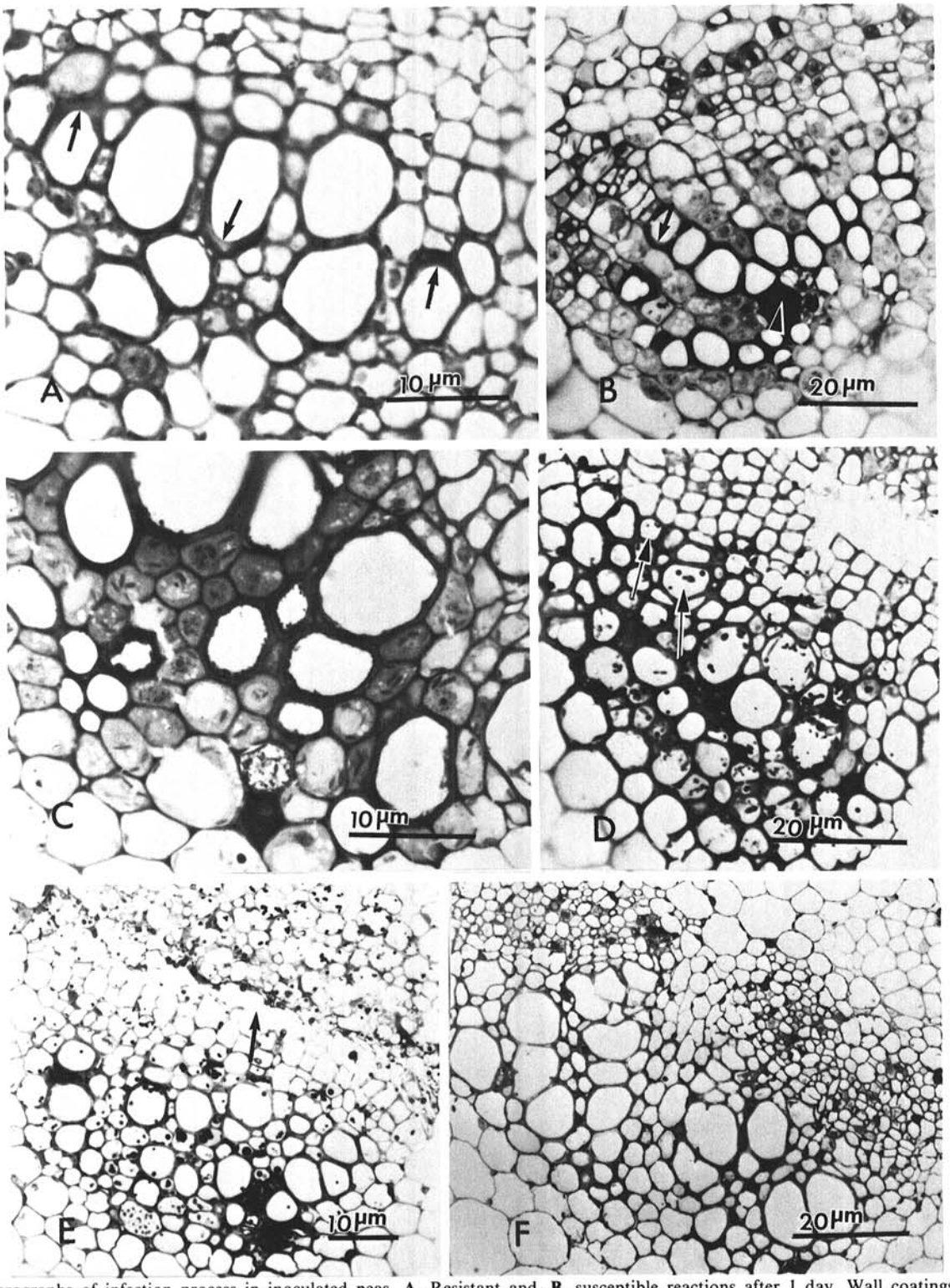

Fig. 2. Light micrographs of infection process in inoculated peas. **A,** Resistant and, **B,** susceptible reactions after 1 day. Wall coatings (arrows) and plugs (arrowheads) are visible. Note the increase in density of cytoplasm in contact cells. **C,** Susceptible after 3 days. Vessel wall reaction, vessel coatings, and active cytoplasm in contact cells are evident. **D,** Susceptible after 6 days. The fungus has grown laterally from initially infected vessels to surrounding vessels (arrows). **E,** Susceptible after 10 days. Fungus has invaded parenchyma throughout bundle, and the cambial layer (arrow) is disintegrating. **F,** Resistant after 10 days. Infection is no longer evident.

a primary wall, whereas contact cells adjacent to metaxylem had secondary walls and large simple pits abutting the vessels (Fig. 1B). A deposit similar to the protective layer as described by Mueller and Beckman (16) was present at pit membranes of mature contact cells. Contact parenchyma cells generally had a large central vacuole and a thin peripheral layer of cytoplasm containing mitochondria, chloroplasts, and the nucleus (Fig. 1B). The chloroplasts were elliptical, with well-formed grana and thylakoids. The mitochondria appeared circular to slightly ellipsodial in transverse sections and were scattered throughout the cytoplasm.

Anatomy and ultrastructure of infected vascular bundles. Vascular elements that contained primary inoculum could be distinguished from uninfected vascular elements and from secondarily invaded elements by the presence of red vinyl tracer particles in vessels. Between 0.5 and 3 days after inoculation the gross anatomy of infected vascular bundles in both resistant and susceptible suscept-pathogen interactions remained the same as described for uninfected plants. Marked changes could be observed, however, at the cellular level within infected vessels and within surrounding vascular parenchyma cells.

One day after inoculation, vascular wall coatings and plugs, not observed in uninfected tissues, could be observed in meta- and protoxylem elements in infected vascular bundles (Fig. 2A and B). Vessel coatings appeared as layers on secondary walls and in pit regions of vessels. Vessel plugs appeared as amorphous or fibrillar material that filled the lumen of the vessels. Vessels also contained fungal hyphae. In contact parenchyma cells, the amount of detectable cytoplasm increased when compared with control plants. This increase was accompanied by an increase in the number and a decrease in the size of vacuoles.

The intensity of the reactions in the vessels and contact cells in both the resistant and susceptible reactions increased to 3 days after inoculation (Fig. 2C). By this time, the secondary walls of both proto- and metaxylem elements had become more intensely stained with thionin, giving the infected vessels a dark blue appearance, whereas the walls of vessels of control plants and those vessels in infected plants that did not contain fungus, plugs, or coatings stained a light blue color. Pit membranes of uninfected vessels were thin and indistinct and stained a light blue with thionin. In contrast, the middle lamella and pit membranes of infected vessels stained dark blue, were distinctly thickened, and coalesced with vessel coatings and plugs. The increased quantity and activity of the cytoplasm in the contact cells adjoining infected vessels was very evident at this time.

At the ultrastructural level, the contact parenchyma cells adjacent to infected vessels had dense granular cytoplasm, containing an extensive endoplasmic reticulum system of both rough and smooth types and increased numbers of dictyosomes and mitochondria (Figs. 3 and 4A). The rough endoplasmic reticulum often contained electron-dense deposits. The nuclei were large, lobed, and centrally located in the cell. The vacuoles were

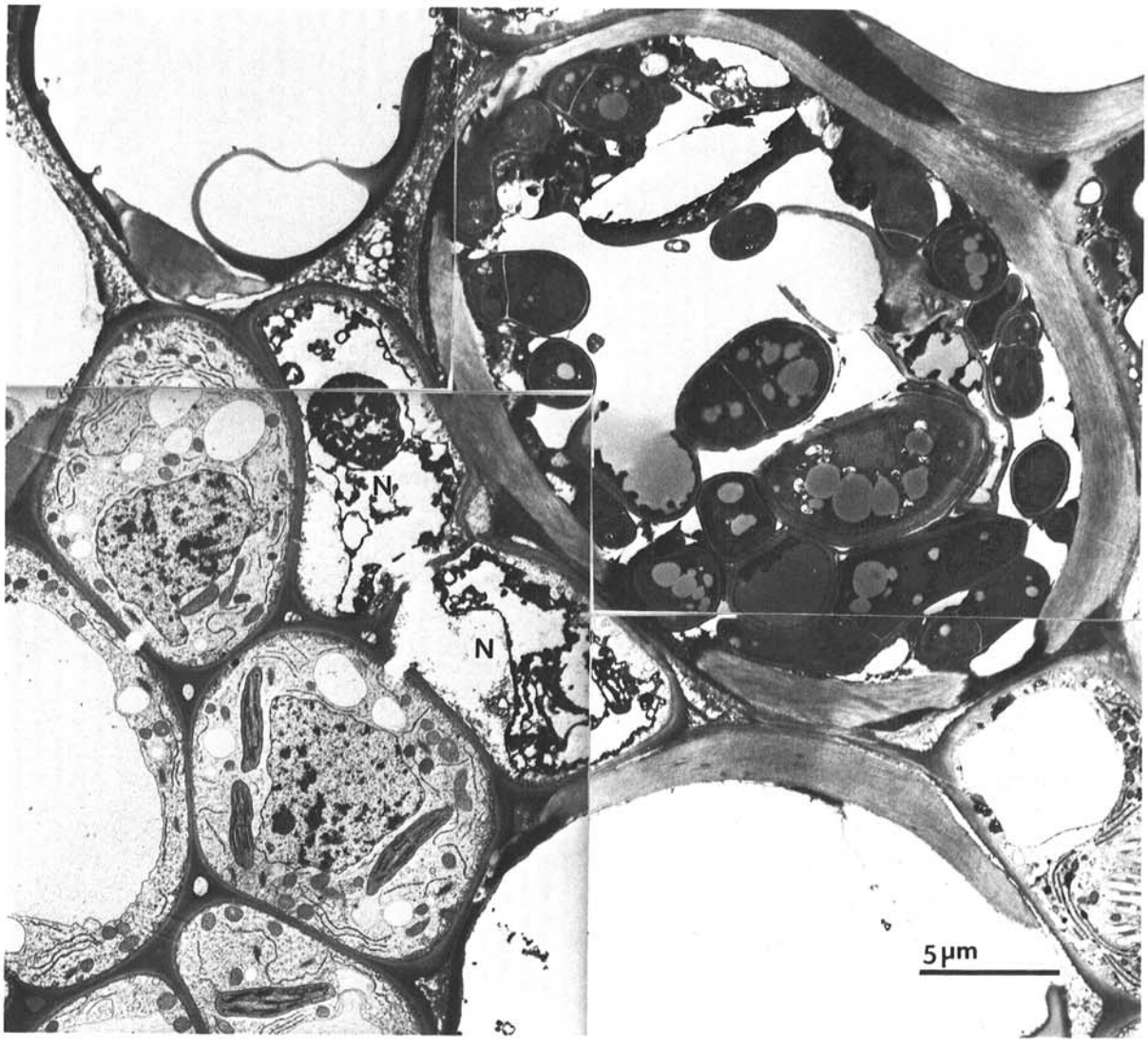

Fig. 3. Electron micrograph of susceptible reaction 3 days after inoculation. The fungus is present in one vessel, and wall coatings are present in other vessels. There is intense cytoplasmic activity in contact cells. Note the small vacuoles, central, lobed nuclei, and extensive endoplasmic reticulum. Two cells that show early necrosis (N) are visible.

numerous and much smaller than the single central vacuole found in the control cells. Granular, electron-dense deposits began to form in these vacuoles (Fig. 5A). An electron-dense deposit between the plasmalemma and the cell wall was evident in most cells. This deposit was thickest in the pit areas and wall adjacent to the infected vessel.

Randomly scattered contact cells in both resistant and susceptible reactions appeared necrotic with extremely electron-dense and completely disorganized cytoplasm (Fig. 3). Fungal hyphae were not found in these cells.

The secondary walls of infected vessels were more electron dense than the walls of uninfected vessels. The membranes in pits between the vessels and contact cells were swollen and thickened and formed a continuum with the vessel coating material. This latter was very electron dense and most frequently appeared as a smooth deposit. Occasionally layered and bubbly coatings were observed. Similar coating frequently surrounded the fungal hyphae present in the vessels.

Two types of plugs were found in infected vessels: a homogeneous electron-dense mass that appeared similar in composition to the smooth wall coatings and an electron-dense fibrillar, netlike plug (Fig. 4B and C). Both types of plugs were found in protoxylem and metaxylem elements.

Morphological and anatomical differences could be detected between the resistant and susceptible reactions 4 days after inoculation. In the resistant reaction the fungus remained confined to the initially infected vessels and no gross symptoms associated with wilting appeared. In the susceptible reaction the fungus grew laterally from the initially infected vessels into adjacent vessels and eventually into the vascular parenchyma cells. Concomitantly, yellowing and wilting became progressively more pronounced on susceptible plants.

No further developments were observed in the resistant reaction after 4 days. The fungus remained confined to the initially infected vessels, and 8 days after inoculation the fungus was difficult to detect in the vessels and the intensity of the staining reaction in the vessel walls declined. The contact cells lost their dense cytoplasmic character and appeared similar to control cells (Fig.

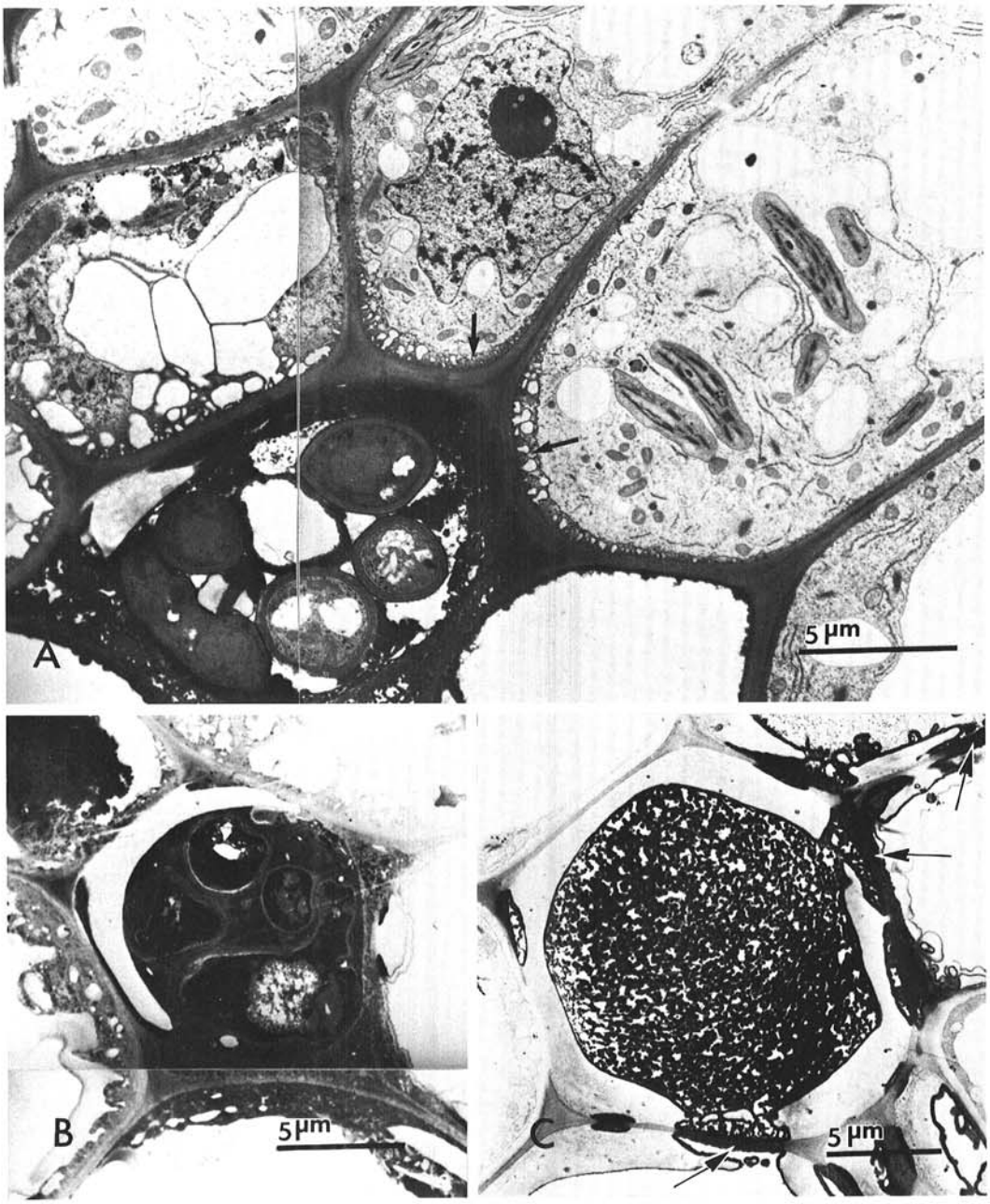

Fig. 4. Electron micrographs of resistant reaction 3 days after inoculation. **A,** Features present are similar to susceptible reaction in Figure 3. The endoplasmic reticulum contains an osmiophilic deposit, and another is present between the plasmalemma and cell wall (arrows). **B,** Homogeneous and, **C,** fibrillar vessel plugs. Note swelling and osmiophilic coating in pit areas (arrows).

5B). After 8 days the vascular bundles in tissue examined with the light microscope could not be distinguished from those in the uninoculated controls (Fig. 2F). No tyloses were found and there was no evidence of external symptoms.

In contrast, 4 days after inoculation the fungus in the susceptible intereaction had moved laterally from the initially infected vessels to the adjacent surrounding vessels (Fig. 2D). This could be determined because fungus hyphae were abundant in vessels that showed no or very little wall coating and did not contain red vinyl particles. Contact cells around initially infected vessels remained highly cytoplasmic; the fungus was not found in these cells (Fig. 5A). In contrast, contact parenchyma around the secondarily infected vessels showed signs of disorganization with membranes and organelles losing their integrity (Fig. 6A). Six days after infection, these cells contained fungal hyphae (Fig. 6B). After 10 days the fungus had colonized the entire bundle including the cambium (Fig. 2E).

External symptoms developed in parallel with the movement of the fungus. Thus, by the sixth day after inoculation, the first and second leaflets above the site of inoculation showed yellowing and wilting. These began to necrose by the eighth day, at which time the third, fourth, and fifth leaves above the inoculation site showed yellowing and wilting. At 10 days these had necrosed and all the remaining leaves showed yellowing. By the 12th day, the whole plant showed wilting and extensive necrosis.

Histochemical analysis of infected vascular bundles. The histochemical analyses of control and infected plants are given in Tables 2 and 3. The 3-day sample was chosen for this purpose because

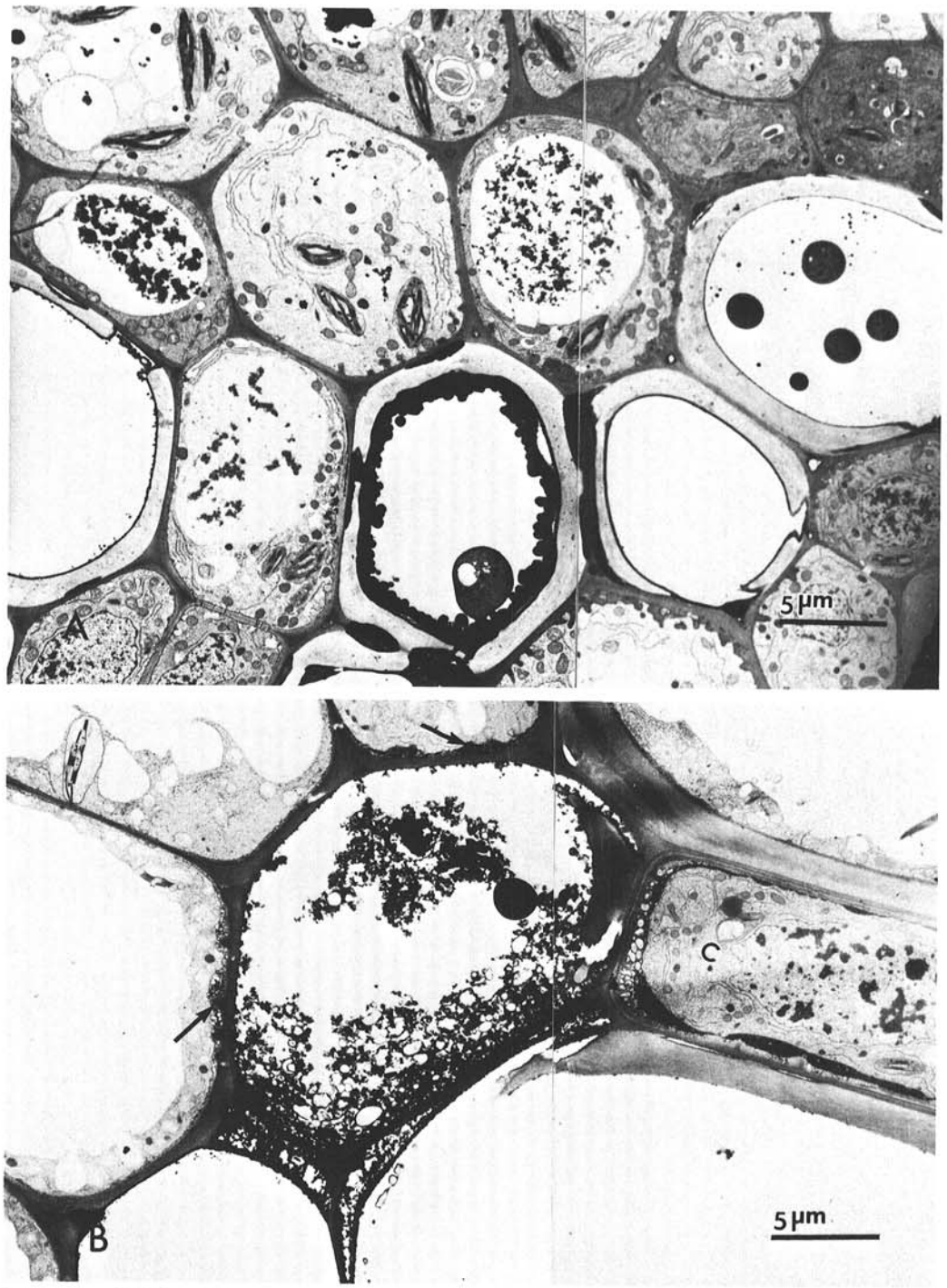

Fig. 5. Electron micrographs of initially infected vessels 8 days after inoculation. **A,** Susceptible reaction. Contact cells adjacent to these vessels continue to react strongly with no sign of fungal invasion. Osmiophilic deposits are visible in the vacuoles. **B,** Resistant reaction. Some contact cells (C) continue to show strong cytoplasmic activity, while others retain osmiophilic deposit between plasmalemma and cell wall (arrows) but have a peripheral layer of cytoplasm similar to contact cells in the uninoculated plants.

extensive host responses could be detected at this time and differences in containment of the pathogen became evident thereafter. No differences were detected in the responses to the histochemical tests in the resistant and susceptible interactions. Vessel plugs and coatings stained for protein, carbohydrates, and pectin; coatings, but not plugs, stained for lipid; neither coatings nor plugs stained for free phenolics. Cell walls in control and infected plants stained for carbohydrates and the middle lamella stained for pectin. Cell walls that stained intensely with thionin also gave a positive phenolic reaction with diazo red and O-dianisidine but not with the other phenolic reagents. Callose deposits were detected in walls in pit areas in some contact parenchyma cells associated with infected vessels in both resistant and susceptible interactions, but it was not determined whether these deposits were correlated with the electron-dense deposits evident in the electron micrographs between the plasmalemma and the cell wall. Fungal hyphae stained for protein, carbohydrate, and lipid but not for pectin and phenolics.

DISCUSSION

Peas responded to infection with *F. o. pisi* as early as one-half day after inoculation by an increase in cytoplasmic activity and quantity in contact parenchyma cells and by the production of vascular plugs and coatings in infected vessels. The amount of coating and plugging material produced increased to a maximum at 3 days after inoculation. By 4 days after inoculation the pathogen was no longer restricted to vessels initially infected in susceptible interactions but was seen in adjacent vessels. Contact cells adjacent to vessels initially infected continued to show a

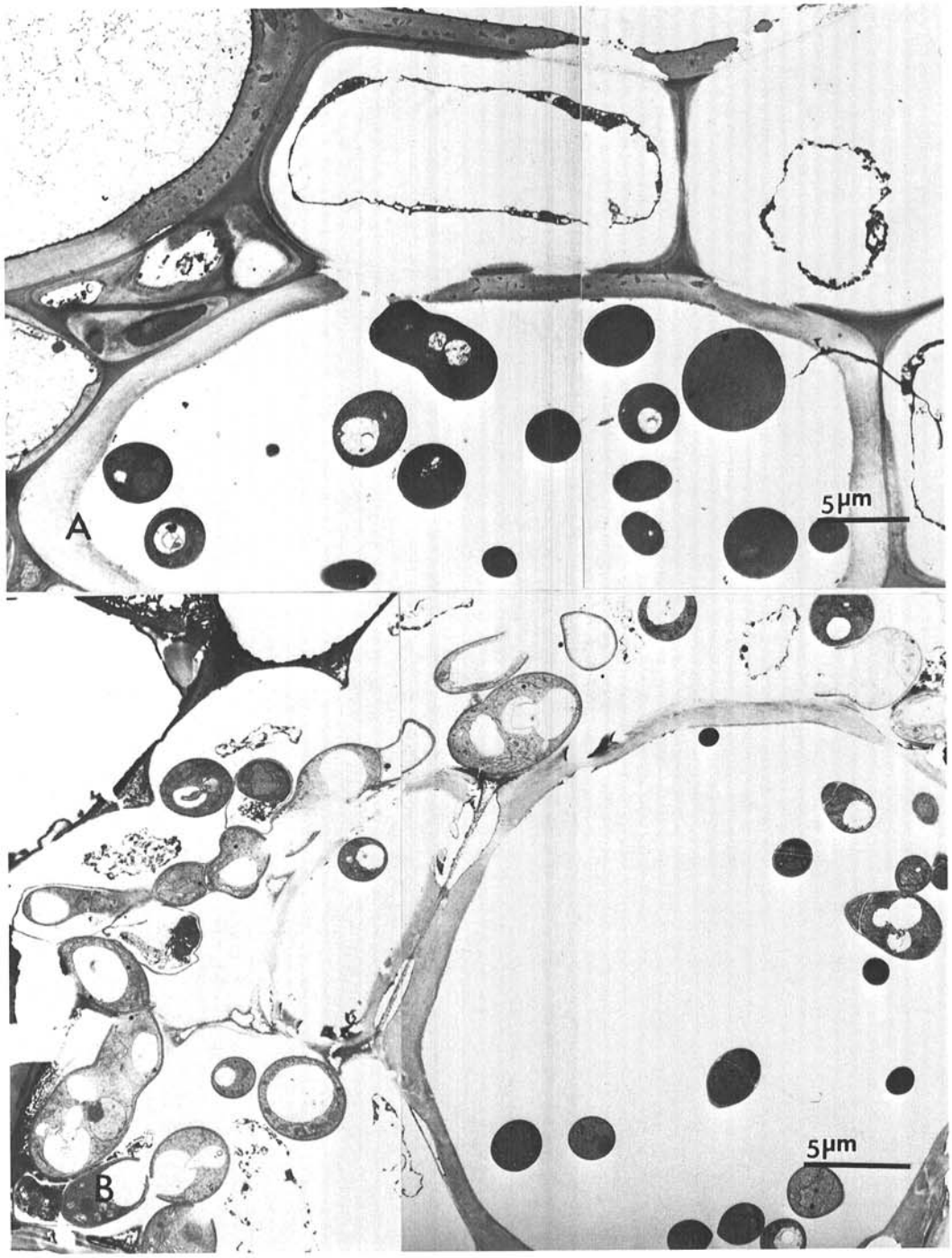

Fig. 6. Electron micrographs of susceptible reaction. **A,** After 6 days the fungus is present in surrounding vessels that do not show wall coatings. Contact cells adjacent to these vessels have become necrotic. **B,** After 8 days the fungus is present extensively throughout the parenchyma.

strong cytoplasmic reaction and apparently resisted penetration by the fungus, whereas those contact cells adjacent to the laterally infected vessels began to show signs of disorganization. The fungus moved from the laterally infected vessels into these adjacent contact cells and then continued to grow laterally through the parenchyma into the cambial/phloem region of the vascular bundle. The separation of the stele from the cortical tissue and death of the plant was associated with extensive colonization of the cambial/phloem and vascular regions of infected bundles. The colonization of the cambium occurred at the time of extensive wilt symptom expression, i e., 8 days after inoculation.

In contrast, the pathogen in resistant interactions was restricted to initially infected vessels. Four to 12 days after inoculation a decrease in cytoplasmic content of contact parenchyma cells and a reduction in other responses was observed, but with no concurrent movement of the pathogen out of initially infected vessels or into the adjacent contact cells. These differences in colonization detected between susceptible and resistant plants confirm the earlier light microscopic observations of Schroder and Walker (21).

TABLE 2. Histochemical analysis of vascular bundles in uninfected control plants of the pea cultivar Thomas Laxton[a]

Cell type stain	Walls	Middle lamella
Vessels		
Protein	−	−
Carbohydrate	+	+
Lipid	−	−
Callose	−	−
Phenolics	−	−
Pectin	−	+
Xylem parenchyma		
Protein	−	−
Carbohydrate	+	+
Lipid	−	−
Callose	−	−
Phenolics	−	−
Pectin	−	+

[a]Positive reactions are indicated by (+); negative reactions by (−).

TABLE 3. Histochemical analysis of vascular bundles in the pea cultivar Thomas Laxton, infected with either race 1 or race 2 of *Fusarium oxysporum* f. sp. *pisi*[a]

Cell type	Cell wall	Middle lamella	Vessel coatings	Vessel plugs
Race 1 Infected				
Vessels				
Protein	−	−	+	+
Carbohydrate	+	+	+	+
Lipid	−	−	+	−
Callose	−	−	−	−
Phenolics	+	+	−	−
Pectin	−	+	+	+
Xylem parenchyma				
Protein	−	−	N/A	N/A
Carbohydrate	+	+	N/A	N/A
Lipid	−	−	N/A	N/A
Callose	+	−	N/A	N/A
Phenolics	+	+	N/A	N/A
Pectin	−	+	N/A	N/A
Race 2 Infected				
Vessels				
Protein	−	−	+	+
Carbohydrate	+	+	+	+
Lipid	−	−	+	−
Callose	−	−	−	−
Phenolics	+	+	−	−
Pectin	−	+	+	+
Xylem parenchyma				
Protein	−	−	N/A	N/A
Carbohydrate	+	+	N/A	N/A
Lipid	−	−	N/A	N/A
Callose	+	−	N/A	N/A
Phenolics	+	+	N/A	N/A
Pectin	−	+	N/A	N/A

[a]Positive reactions are indicated by (+); negative reactions by (−). Reactions not recorded because test was not applicable are indicated by N/A.

No qualitative differences in the host responses examined, as determined by histochemical and ultrastructural tests, were detected. These tests were not performed, however, with the intensity required to detect the subtle differences detected by Newcombe and Robb (17). Vessel coating material stained positively for protein, carbohydrate, pectin, and lipid. Vessel plugging material stained for protein, carbohydrate, and pectin. Vessel coatings and the expanded primary wall/middle lamella seemed to be histochemically and visually similar, while most vessel plugging material differed from the expanded primary wall/middle lamella substance of the pit membranes. Phenolics were detected in walls of vessels and contact cells treated with diazo red and O-dianisidine; tests with nine other phenolic reagents were negative. Aniline blue-fluorescent deposits (callose) were found in contact parenchyma cells in plants infected with either race 1 or race 2. Insufficient numbers of plants were sampled, however, to yield information on the quantitative or qualitative aspects of this callose response.

The response of peas to infection by *F. o. pisi* appears to coincide in many respects with the responses found in other fungus/host combinations (2). Resistance is associated with the localization of the pathogen. Although tyloses are not found in pea, the fungus is confined in initially infected vessels by a rapid and intense accumulation of plugs and vessel coatings, confirming the work of Bishop and Cooper (6) that restriction of the fungus was due to occlusion of the vessels. Concomitantly, the contact cells adjacent to these vessels become metabolically active with the deposition between the plasmalemma and cell wall of a calloselike material that becomes infused with osmiophilic deposits. These cells are not invaded by the fungus. The fungus thus confined may now be susceptible to the action of phytoalexins.

A similar series of reactions occurs in the susceptible reaction. In this case, however, the fungus is capable of moving laterally from the initially infected vessels into the surrounding vessels within 4 days after inoculation. Although no qualitative or quantitative differences in wall coatings were detected here, it is possible that the ability of the fungus to move from vessel to vessel is associated with the subtle differences in wall coatings described by Newcombe and Robb (17). Contact cells adjacent to these laterally infected vessels do not become metabolically active, but the cytoplasm rapidly becomes disorganized. The fungus invades these cells and moves through the parenchyma cells of the bundle, particularly the cambium. Wilting and necrosis of the plant is associated with extensive fungal colonization of the vascular bundles.

Some contact cells adjacent to initially infected vessels in both resistant and susceptible plants rapidly become necrotic. The fungus does not penetrate into these cells, which may correspond to a presumed hypersensitive response attributed to similar appearing cells in the tomato-*Fusarium* interaction (4).

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