Induction of Systemic Resistance to Fusarium Crown and Root Rot in Tomato Plants by Seed Treatment with Chitosan

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ABSTRACT

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Chitosan, derived from crab-shell chitin, was applied as seed coating and substrate amendment prior to infection with the fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Experiments were performed either on a mixture of peat/perlite/vermiculite or on bacto-agar in petri dishes. In both cases, a combination of seed coating and substrate amendment was found to significantly reduce disease incidence as judged by the decreased number of root lesions and the healthier appearance of the root system. Although seed treatment alone could induce a delay in symptom development, it was not efficient enough to confer full seedling protection. Chitosan at concentrations ranging from 0.5 to 1 mg/ml was used for the ultrastructural and cytochemical investigations. Examination of the root tissues at sites of fungal penetration revealed that a pretreatment with chitosan was always associated with the expression of plant defense reactions. In the epidermis, cells showed typical signs of necrosis character-ized by marked disorganization of the cytoplasm. Fungal growth was reduced in but was not restricted to this reacting area. Indeed, the pathogen was detected in the outer cortex where its development was halted. Fungal cells suffered from serious damage and were frequently encircled by an electron-dense material. In the noncolonized inner cortex, strong host reactions were detected that were mainly associated with the deposition of two types of material that differed in their electron density. Gold cytochemistry with a β-1,3-glucanase and a laccase showed that the more electron-dense material was phenolic in nature whereas the other material, occurring either as deposits inserted between the phenolic aggregates or as globular structures lining the host cell walls, was made of β-1,3-glucans. These observations bring further evidence that chitosan is an active inducer of plant defense reactions and, thus, has the potential of becoming a powerful alternative means of disease control.

Additional keywords: ultrastructure, phenolic compounds, ligninlike compounds.

Fusarium oxysporum Schlechtend.:Fr. f. sp. *radicis-lycopersici* W. R. Jarvis & Shoemaker (FORL) is one of the most destructive pathogens of tomato (8). Various strategies for controlling FORL have been introduced over the years (e.g., soil disinfestation, cultural practices, fungicide treatments, and allelopathy) but serious losses still occur, largely because the effectiveness of these approaches is variable and often shortlived (23,24). In addition, tomato cultivars with strong resistance to FORL are not commercially available, although some breeding lines derived from cvs. Vendor and Larma have proven to be good sources of resistance (41). As a result, research efforts have been directed toward developing effective and environmentally safe means of combating Fusarium crown and root rot of tomato (1). Several microorganisms, such as *Trichoderma* spp., *Gliocladium* spp., and *Pseudomonas* spp., have been identified as potential biocontrol agents for use against a number of soil-borne pathogens (28,39). However, commercial applications of these antagonists have been difficult so far, mainly because their efficiency not only requires an excessively large amount of inoculum but also varies with environmental conditions.

Recent advances in our understanding of the mechanisms underlying the expression of plant defense genes upon microbial infection (16) have led to the conclusion that artificial manipulation of the natural plant defense system could provide a biologically, environmentally, and commercially valuable alternative to existing pathogen control methods (26,38). Several lines of evidence have shown that all plants, resistant or susceptible, respond to pathogen attack by the induction of an array of defense reactions designed to affect pathogen growth and viability (27). However, in cases in which a plant-pathogen interaction results in disease establishment, successful host colonization by the parasite is likely due to delayed plant defense expression, rather than to absence or inactivation of defense mechanisms (16). Thus, the speed and extent of the plant response to microbial attack appear to be key determinants in the outcome of a given interaction, and it is reasonable to assume that a faster response to a pathogen may enhance the resistance of a previously susceptible plant.

Research progress over the past decade has strengthened the idea that sensitizing a plant to respond more rapidly to infection could confer increased protection against virulent pathogens (26). Interest in immunization or acquired resistance in plants has increased steadily since the first demonstration by Ross (34) that tobacco plants reacting hypersensitively to TMV developed increased resistance to a wide range of potential pathogens. Enhanced resistance was, later on, found to also occur in response to natural products such as salicylic acid (18) as well as to oligosaccharides and glycoproteins originating from either fungal cell walls (2) or host cell walls (35), the so-called elicitors. The role of fungal oligosaccharides as active inducers of plant defense genes has been the focus of particular interest in recent years. It has been convincingly shown that treatment of plant cells with fungal elicitors resulted in the activation of numerous genes leading to the synthesis of structural compounds (33), secondary metabolites (2), and new proteins (13). However, in spite of the large research effort expended, little has been reported about the cellular biochemistry of host-pathogen interactions following elicitor treat-

ment. Recent advances in this area have led to the exploration of useful tools that could be directly applied on intact tissue sections and provide a detailed picture of the cellular and molecular events in planta (5).

We recently reported that treatment of tomato plants with chitosan fragments resulted in increased resistance to infection by FORL (10). The elicitor, applied by root coating or leaf spraying, was found to sensitize the plants to respond faster to root colonization and, thus, to halt pathogen invasion in the tissues. To further define the signaling role of chitosan as an active inducer of systemic resistance, it was of interest to investigate whether application of this elicitor to tomato seeds could confer resistance to susceptible plants. Over the past 10 years, biological seed treatment has attracted considerable attention, largely because this approach offers the advantage over other control methods of easy application under commercial agricultural conditions (30). While seed coating with antagonistic microorganisms has been abundantly documented, only a few attempts have been reported with biological products such as chitin and chitosan (20,22). We report here that seed coating with chitosan induces systemic resistance to Fusarium infection in tomato seedlings by triggering a hypersensitive-like response at sites of fungal entrance. Evidence is presented that pathogen growth and development are halted by the rapid accumulation of newly formed macromolecules such as β-1,3 glucans, phenols, and ligninlike compounds.

MATERIAL AND METHODS

Fungus culture and growth conditions. The isolate of FORL known to be highly virulent on tomato (*Lycopersicon esculentum* (Mill.) 'Bonny Best') (23) was kindly supplied by P. O. Thibodeau, Service de recherche en défense des cultures, MAPAQ, Québec, Canada. It was recovered from an infected tomato plant and grown on potato-dextrose agar (PDA) at 23–25 C. It was periodically inoculated into and reisolated from ripe tomato fruits.

Preparation of chitosan. Crab-shell chitosan purchased from Sigma Chemical Co. (St. Louis, MO) was ground to a fine powder by extensive grinding in a mortar, washed repeatedly in distilled water, pelleted by low-speed centrifugation, and air-dried. Sheets of chitosan were solubilized by stirring in 0.25 N HCl, centrifuged (10,000 × g, 10 min) to remove insoluble material, and precipitated by neutralization with 2.5 N NaOH. The chitosan pellets, recovered by centrifugation at 25,000 × g for 15 min at 25 C, were extensively washed with deionized water to remove salts and freeze-dried. For experimental use, purified chitosan was dissolved in 0.25 N HCl under continuous stirring and the pH was adjusted to 5.6 using 1 N NaOH. The solution was dialyzed against four, 20 × volumes of deionized water 4 times a day for 4 days at 4 C for salt removal and diluted in sterile distilled water containing 0.01% (v/v) Tween 80 to obtain final chitosan concentrations of 0.1, 0.5, and 1 mg/ml.

Seed treatment with chitosan and fungal inoculation. Seeds of tomato (cv. Bonny Best, highly susceptible to FORL) were surface sterilized by immersion in 2% sodium hypochlorite, thoroughly rinsed in sterile distilled water, and immersed into each of the chitosan solutions (pH 5.5–6.0) at concentrations ranging from 0.1 to 1 mg/ml. After gentle stirring for 15 min, the wetted seeds were air-dried in a sterile cabinet and kept in a dessicator until use. Seeds treated with sterile water containing 0.05% (w/v) NaCl (estimated salt concentration remaining in the chitosan standard solutions) and 0.01% (v/v) Tween 80 and adjusted to pH 6.0 were used as controls.

Control and chitosan-treated seeds were separated into two batches and allowed to germinate under different conditions. Seeds of the first batch (4 seeds per 6-cm pot) were sown in a mixture of peat/perlite/vermiculite (2:1:1) amended or not with chitosan (0.1–1 mg/ml). Each amended substrate was thoroughly mixed in a concrete mixer for 6 h prior to placing 200 cm³ of each treated soil into 6-cm-diameter plastic pots. Although the possibility that some chitosan molecules bind to humic substances in the peat could not be avoided, the presence of perlite and vermiculite (two-thirds of the mixture) allowed chitosan to be

distributed more or less uniformly. Plants were maintained in a glasshouse at about 22 C and a RH of 75%. Tomato seedlings at the three-leaf stage were inoculated by introducing disks of actively growing FORL mycelium close to the root system. Control plants were treated similarly but with fungus-free PDA disks. Eight plants were used for each treatment, and the experiment was repeated twice. Root samples were collected 4 days after FORL inoculation and processed for electron microscopy.

Seeds of the second batch were placed at 0.5 cm around a disk of FORL mycelium deposited on 1% (w/v) bacto-agar in sterile petri dishes. The agar medium was amended with chitosan (0–1 mg/ml) and adjusted to pH 6.0. Controls included seeds placed at 0.5 cm from a PDA disk. After 6 days, root samples were collected from 10 seedlings. The experiment was repeated twice.

Tissue processing for electron microscope studies. Samples (2 mm³), collected from the crown and the main root at sites of fungal entry (root lesions), were fixed by immersion in 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 h at room temperature and post-fixed or not with 1% (w/v) osmium tetroxide in the same buffer for 1 h at 4 C. Root samples were dehydrated in a graded ethanol series and embedded in Epon 812. Ultrathin sections were either contrasted with uranyl acetate and lead citrate or further processed for cytochemical labeling.

Preparation of the gold-complexed probes. Colloidal gold with particles averaging 12 nm in diameter was prepared according to Frens (17) using sodium citrate as a reducing agent.

The β-1,4-exoglucanase-gold complex used for localization of cellulosic β-1,4-glucans was prepared according to Benhamou et al (7). For detection of pectic compounds, the *Aplysia* gonad lectin (AGL), a lectin isolated from the gonads of the sea mollusc *Aplysia depilans*, was complexed to colloidal gold according to a previously described procedure (9). N-acetylglucosamine residues (chitin) were localized by a two-step procedure (6,10) using wheat germ agglutinin (WGA) as a first-step reagent and gold-complexed ovomucoid as a second-step reagent. A purified tobacco β-1,3-glucanase complexed to colloidal gold according to a recently described method was used for localizing β-1,3-glucans (3).

Localization of lignin-associated phenolic compounds was, for the first time, performed by using a laccase (EC 1.10.3.2) purified from the white rot fungus *Rigidoporus lignosus* (19). The enzyme was complexed to colloidal gold at pH 4.0, a pH value close to its reported 3.83 isoelectric point (19). The minimal amount of enzyme necessary for full stabilization of the gold solution was determined by adding 1 ml of colloidal gold (pH 4.0) to 100 μl of serial dilutions of the enzyme. After a few minutes, 100 μl of 10% (w/v) NaCl was added and flocculation estimated visually. The minimum concentration of laccase necessary to stabilize the gold solution at pH 4.0 was estimated to be 10 μg/ml. For preparation of the complex, 100 μg of the purified laccase was mixed with 10 ml of colloidal gold at pH 4.0. The solution was further stabilized by adding 1 ml of 1% (v/v) polyethylene glycol 20,000 (PEG 20,000) and centrifuged at 13,000 rpm for 60 min. The resulting pellet was carefully recovered and resuspended in 0.5 ml of phosphate-buffered saline (PBS), pH 6.0, containing 0.2 mg/ml of PEG 20,000. The gold-complexed laccase was stored at 4 C until use.

Cytochemical labeling. Labeling with the gold-complexed exoglucanase, AGL, β-1,3-glucanase, or laccase was performed by first incubating the ultrathin root sections for 5–10 min on a drop of PBS-PEG at the pH corresponding to the pH of optimal protein activity (6.0 for the exoglucanase, the β-1,3-glucanase, and the laccase; 8.0 for the AGL). Sections were then transferred to a drop of the protein-gold complex for 30 min at room temperature in a moist chamber. After careful washing with PBS, pH 7.2, and rinsing with distilled water, sections were contrasted with uranyl acetate and lead citrate and observed with a JEOL 1200 EX transmission electron microscope operating at 80 kV.

For the indirect labeling of chitin, sections were first incubated on a drop of PBS, pH 7.2, transferred to a drop of WGA diluted 1:30 in PBS, pH 7.2, and finally incubated on a drop of ovomucoid-gold complex (10). Sections were contrasted as described

above.

Specificity of the different labelings was assessed by control tests: 1) incubation on the probe to which was previously added the corresponding substrate (β-1,4-glucans from barley for the exoglucanase, polygalacturonic acids from citrus for the AGL, laminarin or laminaribiose for the β-1,3-glucanase, p-coumaric acid, ferulic acid, or sinapinic acid for the laccase, and N-N'-N''-triacetylchitotriose for the WGA, 1 mg/ml); 2) incubation with the uncomplexed protein followed by incubation with the gold complex; 3) incubation with colloidal gold alone.

RESULTS

Macroscopic observations. Two experiments were undertaken to determine the effect of chitosan-seed coating on the susceptibility of tomato seedlings to FORL attack (Tables 1 and 2).

In experiment 1, tomato seeds, coated or not with chitosan, were allowed to germinate in a mixture of peat/perlite/vermiculite that was previously amended or not with chitosan. Fusarium disease incidence in tomato seedlings at the three-leaf stage was recorded from 1 to 15 days after inoculation with FORL. Typical root symptoms, characterized by the formation of brownish lesions, were visible by 3 days after inoculation in control plants grown in chitosan-free soil. Between 4 and 5 days postinoculation, these plants showed severe symptoms of root rot and wilting. Most of them were dead by 7 to 8 days after inoculation.

Chitosan treatment applied as seed coating resulted in less seedling disease development than occurred with nontreated seeds (Table 1). However, a combination of seed treatment plus soil amendment with chitosan was found to be more effective in protecting tomato seedlings against FORL attack (Table 1). By 5 days after inoculation, seedlings derived from chitosan-treated seeds and grown in chitosan-amended soil were free of apparent symptoms such as wilting or senescence and exhibited a significantly reduced number of root lesions (Table 1). Comparative tests with chitosan concentrations ranging from 0.1 to 1 mg/ml indicated that higher protection occurred when seed coating and soil amendment were performed with concentrations of 0.5 and 1 mg/ml. Although chitosan at a concentration of 0.1 mg/ml induced a delay in disease development (root lesions visible by 4 days after inoculation), emergence of wilting symptoms occurred

between 7 and 10 days postinoculation while death of about 80% of the plants was recorded 1 wk later. At the same time, 95% of the plants treated with chitosan at 0.5 or 1 mg/ml exhibited a healthy appearance as judged by the absence of visible leaf symptoms and by the minimal root damage. By 2 wk after inoculation, more than 90% of the chitosan-treated plants were free of symptoms.

No difference in overall plant growth, leaf size, shape, or color was observed among noninoculated plants whether they were produced from chitosan-treated or chitosan-free seeds.

In experiment 2, tomato seeds, treated or not with chitosan at 1 mg/ml, were deposited on bacto-agar at 0.5 cm from a disk of actively growing mycelium (Fig. 1). Disease incidence was recorded in the emerging seedlings from 1 to 5 days after seed and fungal deposition in the petri dishes (Table 2). In control plates containing nontreated seeds and chitosan-free agar medium, the fungus ramified extensively and, as early as 1 day after plate inoculation, grew on the surface of several seeds (Fig. 1A, large arrows). In spite of this marked colonization, about 70% of the seeds germinated. However, the emerging seedlings appeared thinner and shorter than normal in addition to exhibiting pronounced root damage as visualized by the occurrence of numerous root lesions (Fig. 1B, small arrows).

In plates containing chitosan-coated seeds and chitosan-free agar medium, the fungus was also found to develop rapidly on the agar, but the seed colonization was delayed (not shown). About 90% of the seeds germinated. However, by 5 days after seed germination, severe symptoms of root rot were visible on the young, emerging seedlings (Table 2).

Effective protection against FORL attack was observed with a combination of seed treatment and agar amendment. In these conditions, fungal growth was significantly reduced and seeds germinated in the absence of physical contact with the fungus (Fig. 1C). Interaction with the pathogen occurred upon seedling emergence but did not apparently affect plant development. Between 4 and 5 days after seed germination, symptoms of root rot were seldom seen (less than 1% of the seedlings). For most seedlings, the root system appeared vigorous and was always characterized by the typical formation of tiny root hairs (Fig. 1D). Observations of these seedlings over a 2-wk period after seed germination showed that growth and development were

TABLE 1. Effect of chitosan seed coating and soil amendment on the number of root lesions per plant induced by *Fusarium oxysporum* f. sp. *radicis-lycopersici*

Treatment (chitosan in mg/ml)	Days after inoculation					
	1	2	3	4	5	7
Nontreated seed + nontreated soil (control)	0	1[a] (±0.5)[b]	4 (±1.0)	7 (±1.0)	10 (±0.5)	(plant dead)
Chitosan-treated seed (0.1) + nontreated soil	0	0	2 (±1.0)	3 (±0.7)	5 (±0.5)	8 (±0.5)
Chitosan-treated seed (0.5) + nontreated soil	0	0	1 (±0.8)	2 (±0.5)	5 (±1.0)	8 (±1.6)
Chitosan-treated seed (1.0) + nontreated soil	0	0	1 (±0.2)	2 (±0.4)	3 (±0.3)	6 (±2.3)
Chitosan-treated seed (0.1) + chitosan-treated soil (0.1)	0	0	0	3 (±1.0)	8 (±0.7)	8 (±0.2)
Chitosan-treated seed (0.5) + chitosan-treated soil (0.5)	0	0	0	0	2 (±0.2)	3 (±0.3)
Chitosan-treated seed (1.0) + chitosan-treated soil (1.0)	0	0	0	0	1 (±0.3)	2 (±0.2)

[a] Number of root lesions determined from observations of eight main roots per chitosan treatment per day after fungal inoculation.
[b] Values in parentheses represent standard errors of the mean.

TABLE 2. Effect of chitosan seed coating and agar medium amendment on the number of root lesions per plant induced by *Fusarium oxysporum* f. sp. *radicis-lycopersici*

Treatment (chitosan in mg/ml)	Days after inoculation				
	1	2	3	4	5
Nontreated seed + nontreated agar	...	...	2[a] (±1.0)[b]	4 (±1.0)	4 (±0.8)
Chitosan-treated seed (0.1) + nontreated agar	...	...	0	2 (±0.5)	5 (±1.0)
Chitosan-treated seed (0.5) + nontreated agar	...	...	0	2 (±0.7)	4 (±0.6)
Chitosan-treated seed (1.0) + nontreated agar	...	...	0	1 (±0.9)	4 (±0.6)
Chitosan-treated seed (0.1) + chitosan-treated agar (0.1)	...	...	0	1 (±0.2)	3 (±0.3)
Chitosan-treated seed (0.5) + chitosan-treated agar (0.5)	...	...	0	0	1 (±0.2)
Chitosan-treated seed (1.0) + chitosan-treated agar (1.0)	...	...	0	0	0

[a] Number of root lesions determined from observations of ten main roots per chitosan treatment per day after fungal inoculation.
[b] Values in parentheses represent standard errors of the mean.

similar to those occurring in noninoculated plates.

As mentioned above for experiment 1, treatments with chitosan at concentrations of 0.5 and 1 mg/ml were more effective in reducing disease incidence caused by FORL (Table 2).

Transmission electron microscope observations. In order to determine whether reduction of disease incidence due to chitosan treatment was associated with an increased expression of plant defense reactions, comparative cytological studies were undertaken on root samples harvested at or near (0.5 mm) the infection sites localized by the occurrence of local lesions. Because combinations of seed coating and substrate amendment were found to be more effective in protecting tomato seedlings against FORL infection, our attention was mainly focused on the effect of a dual chitosan treatment on the cytology of infection of tomato root tissues by FORL. Based also on the macroscopic observations, we selected the two concentrations that gave the optimal results in terms of plant protection (standard solutions, 0.5 and 1 mg/ml).

Examination of about 100 ultrathin sections collected from 10 different primary roots revealed that the extent and spatio-temporal distribution of the plant defense reactions were not affected by the type of substrate in which the tomato seedlings were grown. Defense responses, similar in terms of magnitude, speed of expression, and chemical composition, were detected in root tissues from seedlings grown either in soil (experiment 1) or on bacto-agar (experiment 2).

Ultrastructural features of FORL-infected root tissues in control plants. Tomato seedlings derived from nontreated seeds grown in nonamended substrate were highly susceptible to FORL attack. Examination of root samples, collected at 1-day intervals after inoculation, revealed a pattern of fungal colonization similar to that previously described (8,12). The fungus multiplied abundantly on the root surface and penetrated the host epidermis by 24 h after inoculation. Between 1 and 2 days after host penetration, hyphal cells ramified extensively through much of the cortex and rapidly reached the endodermis. Colonization of the vascular stele occurred by 4 days after inoculation and proceeded via the infection of the paratracheal parenchyma cells (Fig. 2B). Pathogen growth in the vicinity of xylem vessels was inter- and intracellular. This massive fungal colonization was always associated with
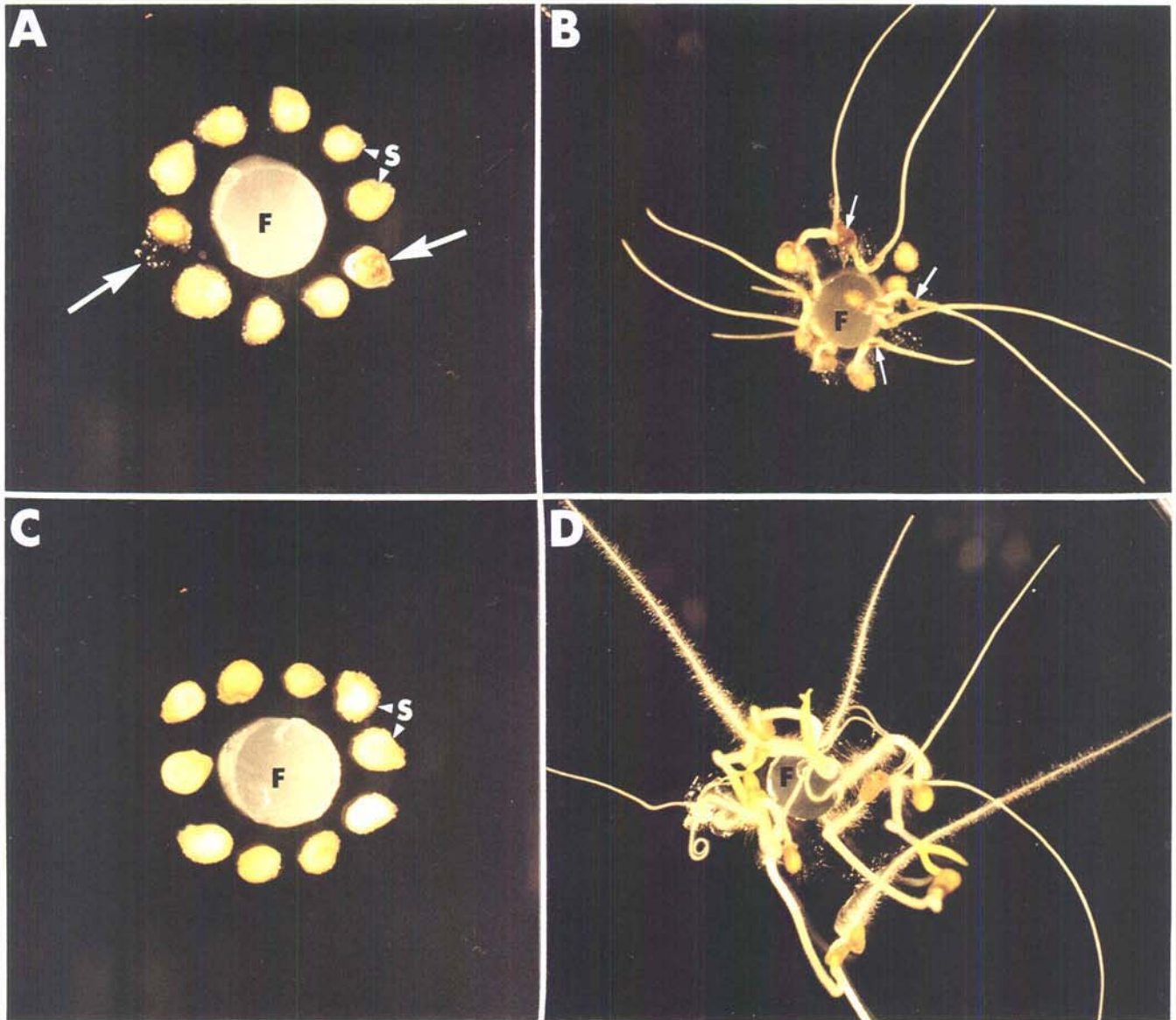

Fig. 1. Tomato seeds, treated (**C, D**) or not (**A, B**) with chitosan (1 mg/ml), are deposited on bacto-agar, amended (**C, D**) or not (**A, B**) with chitosan (1 mg/ml) at 0.5 cm from a disk of *Fusarium oxysporum* f. sp. *radicis-lycopersici* mycelium. In **A** and **B**, the fungus (F) develops rapidly on the agar medium and colonizes the seed (S) surface as soon as 1 day after inoculation (**A**, arrows). By 5 days after inoculation, emerging seedlings appear thinner and shorter than normal and exhibit pronounced root damage characterized by numerous root lesions (**B**, small arrows). In plates containing chitosan-coated seeds and chitosan-amended agar (**C and D**) fungal growth is significantly reduced. The seeds are free of fungus by 1 day after inoculation (**C**). Between 4 and 5 days after seed germination, the emerging seedlings are vigorous and root lesions are not seen (**D**).
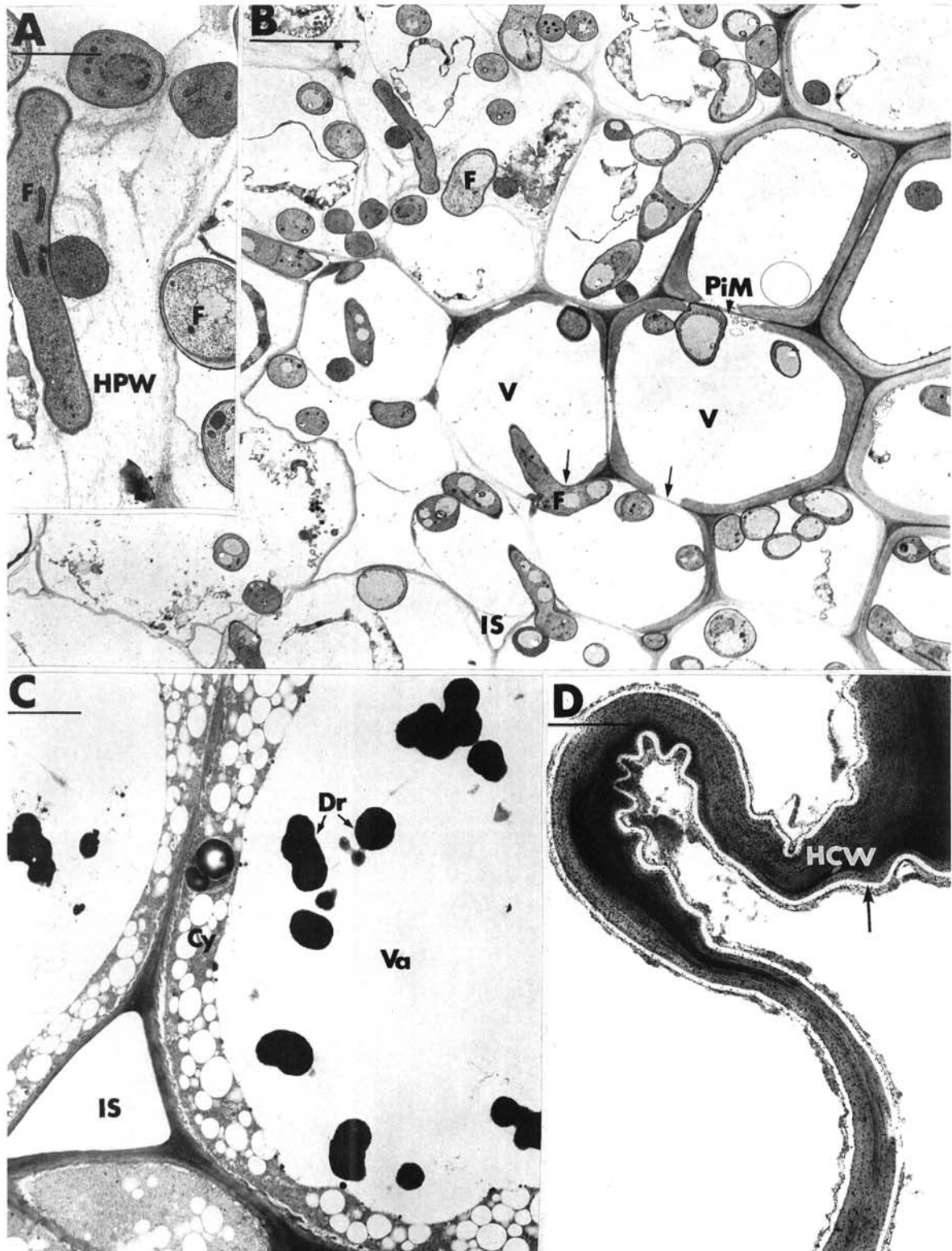
Fig. 2. Transmission electron micrographs of tomato root tissues. **A** and **B**, Tomato root tissues from chitosan-free seeds grown in nonamended substrate (control) and infected with *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL). By 4 to 5 days after inoculation, the fungus (F) multiplies abundantly and colonizes the vascular bundle. Pathogen ingress in the xylem vessel (V) proceeds usually through the pit membrane (PiM, arrows). This massive colonization is accompanied by a marked alteration of host primary walls (HPW), which appear reduced to fine strands of fibrillar material (**B**). **A**, ×2,800, bar = 3 μm; **B**, ×7,500, bar = 2 μm. **C** and **D**, Tomato root tissues from chitosan-coated seeds (1 mg/ml) grown in chitosan-amended substrate (1 mg/ml) and noninfected with FORL. The only visible changes concern the deposition of electron-dense droplets (Dr) in the vacuoles (Va) of some vascular parenchyma cells (**C**) and the formation of an electron-lucent layer (arrow) along the contorted host epidermal cell wall (HCW) (**D**). Abnormal deposits are not seen in the intercellular spaces (IS) and in the cytoplasm (Cy). **C**, ×5,200, bar = 2 μm; **D**, ×14,500, bar = 1 μm.

marked host cell wall alterations (Fig. 2B). Primary walls and middle lamellae were, most often, swollen and reduced to fine strands of fibrillar material (Fig. 2A). Pathogen ingress in the xylem vessels usually proceeded through the fragile pit membranes (Fig. 2B, arrow). By 4 to 5 days after inoculation, about 80% of the xylem vessels were colonized. By this time, early signs of wilting and root rot were visible on the plants.

Effect of chitosan on the ultrastructure of noninfected tomato root tissues. The root system of plants produced by chitosan-coated seeds and grown in chitosan-amended substrate was visually similar to that of nontreated healthy plants (not shown). At the cellular level, observation of at least 50 ultrathin sections, collected from 10 different primary roots, did not reveal any major changes compared with nontreated tissues except for the accumulation of polymorphic, electron-dense droplets in the centrally located vacuole of several vascular parenchyma cells (about 50% of the cells) (Fig. 2C). Such droplets were seldom seen in the cortical area. A close examination of the sections showed that the contorted epidermal cell walls were surrounded by an unusual, electron-lucent layer (Fig. 2D). Typical host defense reactions such as wall appositions were never observed.

Effect of chitosan on the cytology of infection of tomato root tissues by FORL. Time-course studies of fungal colonization in root tissues from tomato seedlings produced by chitosan-treated seeds and grown in chitosan-amended substrate revealed that pathogen growth was restricted to the epidermis and the outermost cortical cell layers. In all examined sections, fungal cells were never detected in inner tissues, including the endodermis and the vascular parenchyma. This localized colonization at infection sites was consistently associated with strong host reactions resembling hypersensitive responses.

Epidermis. Occurrence of fungal cells in the epidermis was recorded between 2 and 3 days after challenge inoculation. The frequency of penetration of epidermal cells from roots originating from seeds treated with chitosan at 1 mg/ml and germinated on media containing 1 mg/ml chitosan was about 10% of that of the untreated control. Epidermal cells at penetration sites exhibited signs of severe disorganization including cytoplasm aggregation and cell wall distortion (Fig. 3A). In such cells, pre-existing organelles such as nuclei and mitochondria were no longer discernible. Interestingly, the contorted epidermal cell walls were not apparently damaged. Instead, they appeared, in some areas, to be impregnated by osmiophilic substances as judged by the marked increase of their electron density (Fig. 3A, arrows). Fungal cells growing in these necrotic epidermal cells also displayed marked changes in their ultrastructure, such as retraction of the plasmalemma from the cell wall and alteration of the cytoplasm (Fig. 3B).

Outer cortex. Pathogen penetration of the outer cortex was detected by 3 days after inoculation and was accompanied by drastic host cell reactions (Fig. 3C). In the following days, most fungal cells (about 80%) suffered severe damage and were often reduced to empty shells (Fig. 3C, arrows). Host reactions in the outer cortex were mainly characterized by the accumulation of electron-dense deposits along the cell walls (Fig. 3C). These polymorphic deposits, made of an amorphous material (Fig. 3D), often extended toward the inside of the cell. Hyphae were frequently found to be trapped by this material through an apparent physical interaction (Fig. 3D). Trapped hyphae were distorted and exhibited marked changes including increased vacuolation (Fig. 3C, arrowhead), retraction of the plasmalemma, and, most often, loss of the protoplast (Fig. 3D). Most intercellular spaces were also coated by a band of electron-opaque material with a texture similar to that of the intracellular deposits (Fig. 3C, double arrows).

Inner cortex. Fungal cells did not penetrate the innermost cortical cells, which were filled with deposits of varying size, shape, and texture (Fig. 4A). In some cells, the deposits accumulated randomly while in others they were regularly distributed along the cell walls as globular structures embedded in a granular matrix (Fig. 4A). Two types of intracellular material could be easily distinguished according to electron density. The first type dis-

played a very high electron density and was always encountered as large aggregates in the cell lumen (Fig. 4A). The second was less electron-dense, amorphous, and of smaller size. It occurred as opaque deposits inserted between the electron-dense aggregates (Fig. 4A, arrows). The globular structures lining the host wall in some cells appeared to be made of the same opaque material. A large number of intercellular spaces were also found to be partially or completely plugged by opaque substances of different textures (Figs. 4B–D).

Endodermis and vascular stele. The intensity and magnitude of host reactions decreased in the noncolonized endodermal and vascular parenchyma cells (Fig. 5). In the endodermis, the deposition of amorphous globules in the band of cytoplasm appressed against the cell walls was the most typical host response (Fig. 5A). These globular structures appeared similar to those found in the innermost cortex but they were less numerous. Impregnation of the host cell walls with osmiophilic substances was another typical feature of reaction (Fig. 5A, arrows). Major structural changes were not detected in the vascular parenchyma cells except for the accumulation of electron-dense droplets in the large vacuoles (Fig. 5B). Xylem vessel coating, known to be induced upon attack by vascular wilt pathogens (17), was never detected in all the examined sections.

Cytochemical observations. Various gold-complexed probes were applied to root tissue sections in order to further elucidate the nature of the induced host defense reactions.

Cytochemical localization of chitin. WGA, a lectin with N-acetylglucosamine-binding affinity, was used in association with gold-complexed ovomucoid for localizing chitin (a polymer of β-1,4-N-acetylglucosamine units) in FORL cell walls. In infected root tissues derived from control plants, labeling with the WGA-ovomucoid-gold complex resulted, as previously described (10), in a regular and intense deposition of gold particles over the fungal cell walls (not shown). By contrast, examination of infected root tissues from seedlings produced by chitosan-treated seeds and grown in chitosan-amended substrate revealed an uneven distribution of gold particles over the disorganized fungal cells found in the epidermis and the outer cortex (Fig. 6A). Labeling was not exclusively associated with the fungal walls since gold particles were frequently found to occur in the cell lumen (Fig. 6A, arrowheads) and also at a short distance from the pathogen (Fig. 6A, arrows). This labeling pattern indicated that molecules were likely released from the fungal walls as a result of the metabolic changes induced in the plants. No labeling was observed upon incubation with the WGA to which was added N-N'-N'' triacetyl-chitotriose prior to section labeling (not shown).

Cytochemical localization of cellulose and pectin. Section labeling with the gold-complexed exoglucanase for localizing cellulosic β-1,4-glucans resulted in a specific deposition of gold particles over the host cell walls (Fig. 6B). Examination of a large number of sections revealed that the aggregates accumulating in the reacting cortical cells as well as the amorphous globules lining the host cell walls in both cortical and endodermal cells were free of labeling (Fig. 6B). A similar labeling distribution was observed upon incubation with the AGL-gold complex used for localizing pectic subunits (Fig. 6C). Gold particles were predominantly associated with the host cell walls and seldom occurred over the electron-dense and opaque deposits induced in response to infection in root tissues of seedlings derived from chitosan-treated seeds (Fig. 6C). With the two probes, cytoplasm, organelles, and vacuoles were usually free of any significant labeling. Incubation of sections with the probes to which were previously added the corresponding substrate molecules resulted in an absence of wall labeling (not shown).

Cytochemical localization of β-1,3-glucans. A β-1,3-glucanase, purified from tobacco plants reacting hypersensitively to TMV infection (3), was used for localizing callose, a polymer of β-1,3-glucans, in infected root tissues from tomato seedlings obtained by chitosan-coated seeds. Incubation with the gold-complexed enzyme resulted in an intense and specific accumulation of gold particles over the opaque deposits plugging the intercellular spaces and occurring in most reacting cortical cells

(Fig. 7A). The amorphous globules lining the host cell walls were also heavily labeled while scattered gold particles occurred over the host cell walls (Fig. 7A). By contrast, the electron-dense material neighboring the opaque deposits in cortical cells was never labeled. Preincubation of the gold-complexed enzyme with laminarin resulted in an absence of labeling (not shown).

Cytochemical localization of phenols and ligninlike compounds. A purified laccase, produced by the white rot fungus *Rigidoporus*
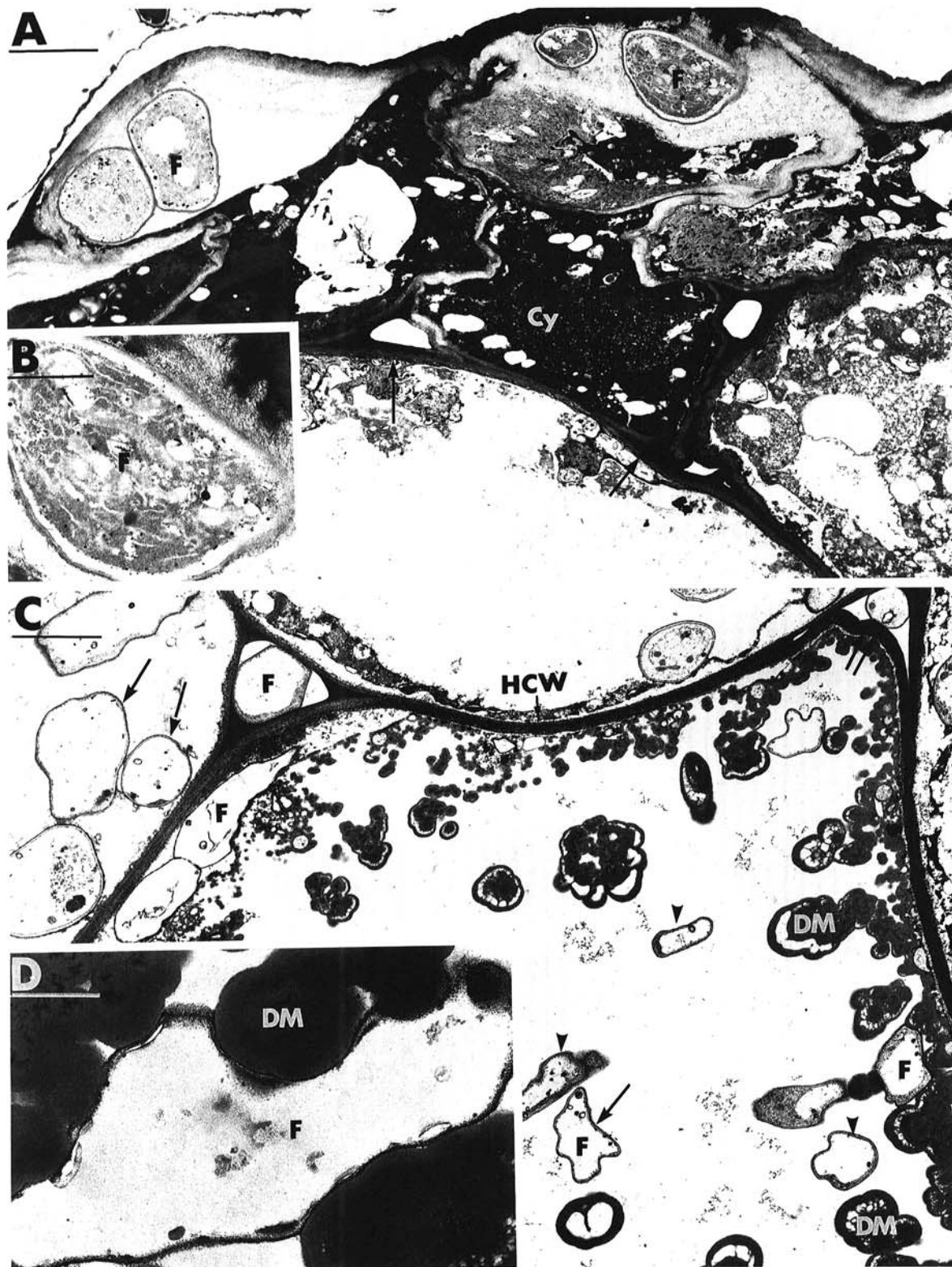

Fig. 3. Transmision electron micrographs of tomato root tissues from chitosan-treated seeds (1 mg/ml) grown in chitosan-amended substrate (1 mg/ml) and inoculated with *Fusarium oxysporum* f. sp. *radicis-lycopersici*. **A and B,** Epidermis. By 3 days after inoculation, some fungal cells (F) are visible in the epidermis, which shows a pronounced disorganization characterized by cytoplasm (Cy) aggregation and cell wall distortion. The host cell walls (arrows) are not apparently damaged. Fungal cells are disorganized (**B**). **A,** ×4,600, bar = 3 μm; **B,** ×12,500, bar = 1 μm. **C and D,** Outer cortex. Between 3 and 4 days after inoculation, pathogen penetration in the outer cortex is accompanied by marked host cell changes such as accumulation of a dense material (DM) that often encases fungal cells (F). Invading hyphae suffer from some damage ranging from increased vacuolation (arrowhead) to complete loss of protoplast (arrows). Intercellular spaces are frequently coated by a layer of electron-opaque material (double arrows). **C,** ×7,000, bar = 2 μm; **D,** ×28,000, bar = 0.5 μm.

lignosus, was used, for the first time, to localize ligninlike compounds and polymerized phenols in the plant material under study. Upon incubation with the gold-complexed enzyme, labeling was found to be predominantly associated with the electron-dense aggregates occurring in the lumen of reacting cortical cells and to a lesser extent with the host cell walls (Fig. 7B). The amorphous globules lining the host cell walls as well as the material plugging the intercellular spaces were unlabeled (Fig. 7B). Similarly, cytoplasm, organelles, and vacuoles were free of labeling. In all examined sections, gold particles appeared randomly distributed over the host cell walls. Such a labeling pattern was never observed in root tissues from infected control plants (Fig. 7C). Incubation
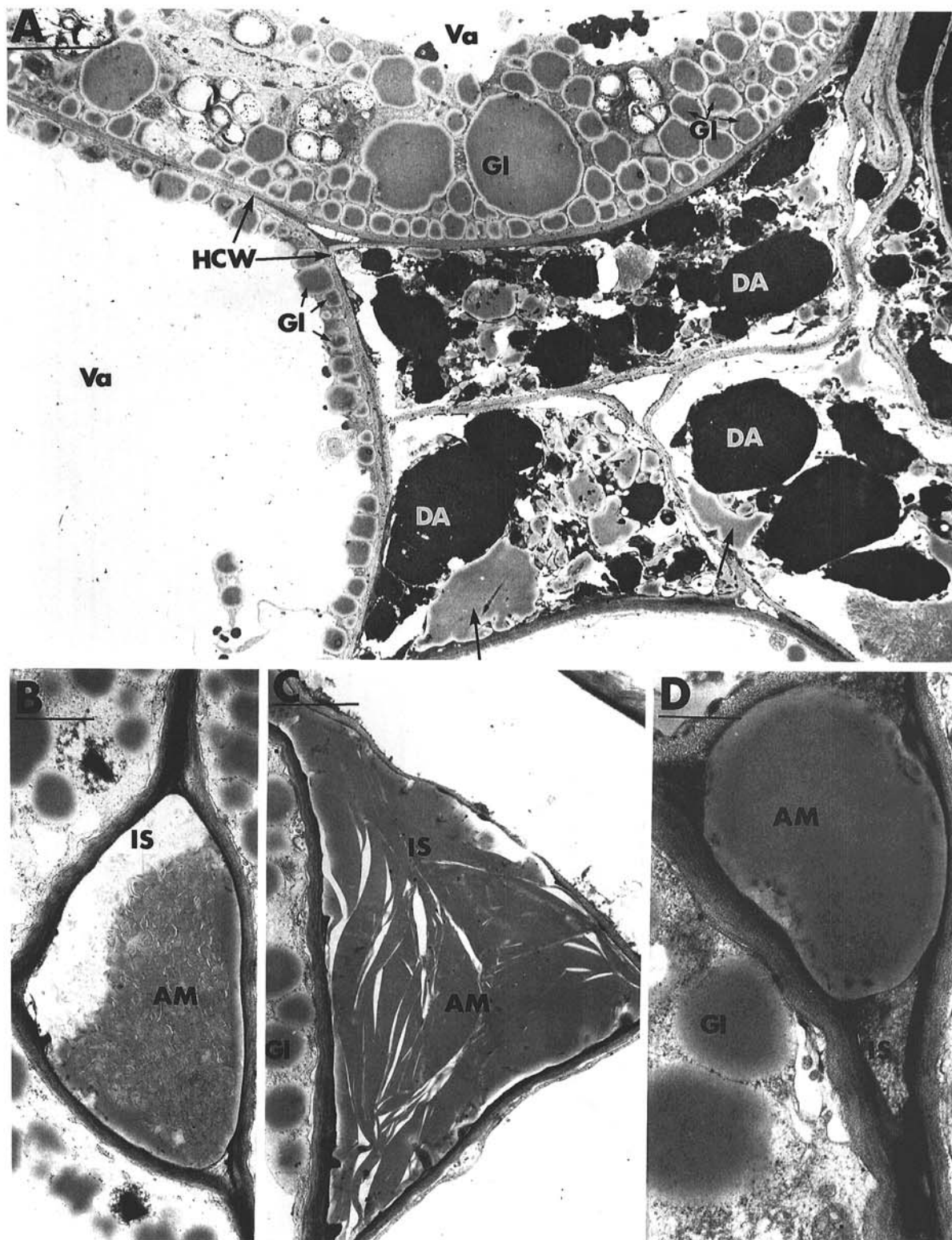

Fig. 4. Transmission electron micrographs of tomato root tissues from chitosan-treated seeds (1 mg/ml) grown in chitosan-amended substrate (1 mg/ml) and inoculated with *Fusarium oxysporum* f. sp. *radicis-lycopersici.* **A–D,** Inner cortex. Fungal cells are not detected in the inner cortical cells, which show considerable changes characterized by the accumulation of two main types of material: large aggregates of very high electron density (DA); and, ovoid globules (Gl) along the host cell walls (HCW) that also occur as polymorphic intracellular deposits (arrows). Intercellular spaces (IS) are also filled with a newly formed material (AM) that can appear **B,** fibrillar, **C,** lamellar, or **D,** amorphous. **A,** ×4,600, bar = 3 μm; **B,** ×12,000, bar = 1 μm; **C,** ×12,000, bar = 1 μm; **D,** ×25,000, bar = 0.5 μm.

of the enzyme-gold complex with either ferulic acid or *p*-coumaric acid prior to section treatment abolished the labeling over the host walls and the osmiophilic deposits (not shown).

DISCUSSION

In the past decade, elicitor-mediated induced resistance has become one of the most challenging research areas in plant pathology (35). Extensive studies, initially using model systems of reduced complexity such as elicitor-treated cell-suspension cultures (37), have provided a conceptual basis for designing new strategies to enhance plant resistance to microbial attack (20,42). Among the known elicitors, chitosan is probably one compound that offers the best prospects as a biocontrol agent due to its commercial availability from the chitin of crustacean shell wastes (21). However, before chitosan-induced resistance can be implemented in plant disease management, efforts need to be directed toward understanding the mechanisms by which this component may affect cell regulation and gene expression in plants challenged by virulent pathogens.

Results of the present study demonstrate that susceptible tomato plants develop a systemically induced resistance to FORL infec-tion in response to chitosan application. Although earlier observa-tions have suggested the potential of chitosan seed treatment (22), the data reported here provide, for the first time, evidence that seed coating in combination with substrate amendment increases resistance of tomato seedlings to FORL attack in correlation with a restricted fungal growth in the root tissues, a decreased pathogen viability, and a marked accumulation of new products in the host cells.

Several lines of experimental evidence have shown that seed treatments with bacterial or fungal antagonists were effective in protecting germinating embryos and seedlings from the damaging action of root pathogens (30). Although the basic mechanisms behind pathogen inhibition are not clearly defined, the possibility that antibiosis, mycoparasitism, and competition may operate synergistically has been suggested (30). Understandably, such mechanisms do not likely account for the restriction of pathogen growth following chitosan seed treatment, although the known antimicrobial properties of chitosan (4) may well be responsible, at least partly, for a reduction of the pathogen population at the seed surface. The present cytological observations indicate that induction of systemic resistance against FORL is one of the main mechanisms by which chitosan contributes to tomato
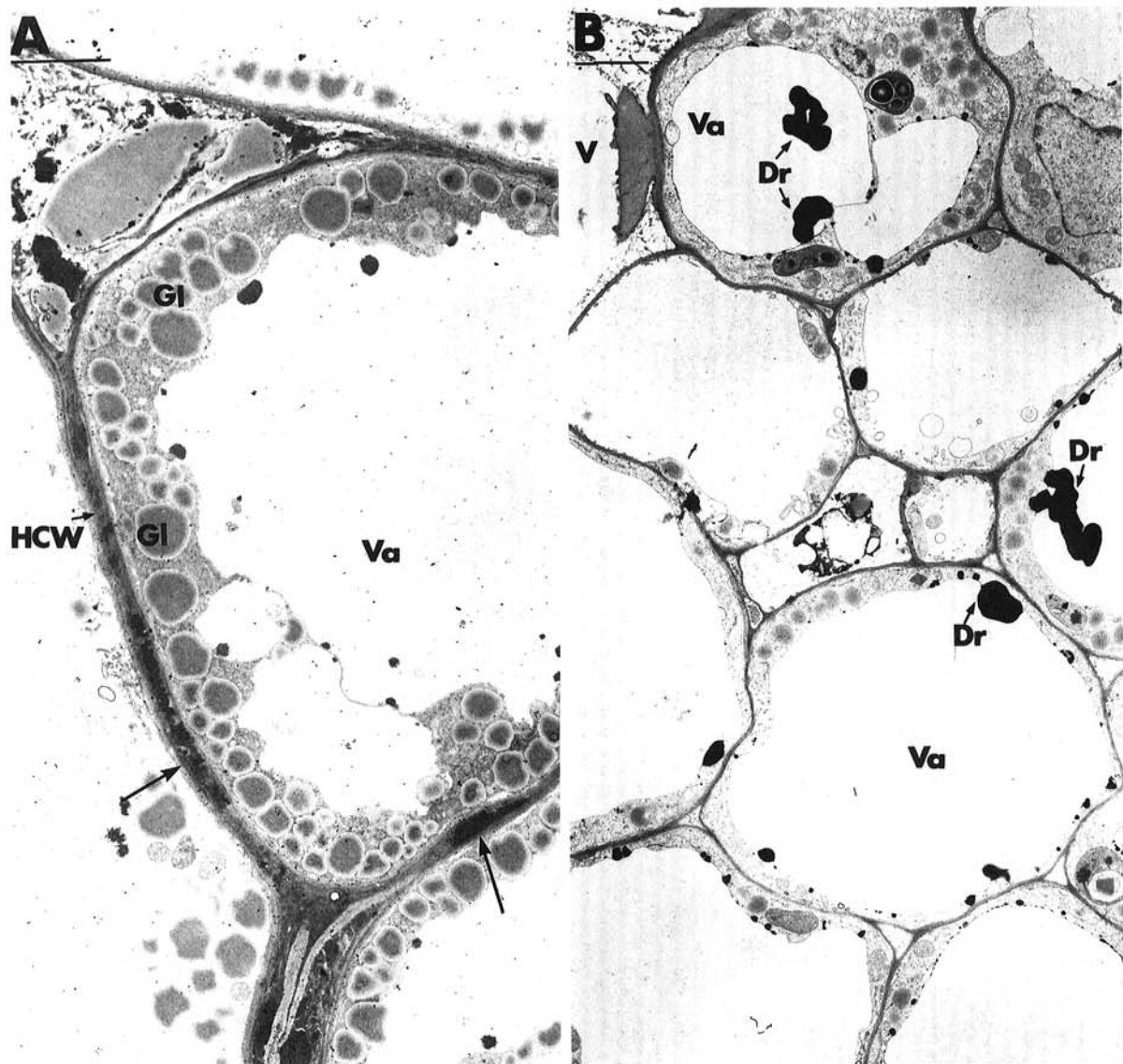

Fig. 5. Transmission electron micrographs of tomato root tissues from chitosan-treated seeds (1 mg/ml) grown in chitosan-amended substrate (1 mg/ml) and inoculated with *Fusarium oxysporum* f. sp. *radicis-lycopersici*. **A,** endodermis. By 4 days after inoculation, the deposition of globules (Gl) in the band of cytoplasm appressed against the host cell wall (HCW) is the main host cell reaction observed. The host cell wall appears impregnated by osmiophilic substances in places (arrows). **B,** In the vascular parenchyma cells, some electron-opaque droplets (Dr) accumulate in the centrally located vacuole (Va). **A,** ×6,500, bar = 2 μm; **B,** ×5,000, bar = 2 μm.

seedling protection. However, if one considers that the seed coat initially is a dry, senescent tissue, the question can be raised as to the extent to which chitosan penetrates the thick seed envelope and interacts with cells of the embryo. At least two possibilities may explain this phenomenon. First, imbibition of the seeds following planting in a wet substrate likely affects seed envelope permeability, causing microscopic ruptures by which chitosan oligosaccharides may diffuse. Second, interaction with chitosan molecules may be initiated upon seed germination only. This would obviously imply that surface interactions between the emerging seedling and the seed coat are established for allowing the spread of chitosan fragments in both the coleoptile and the
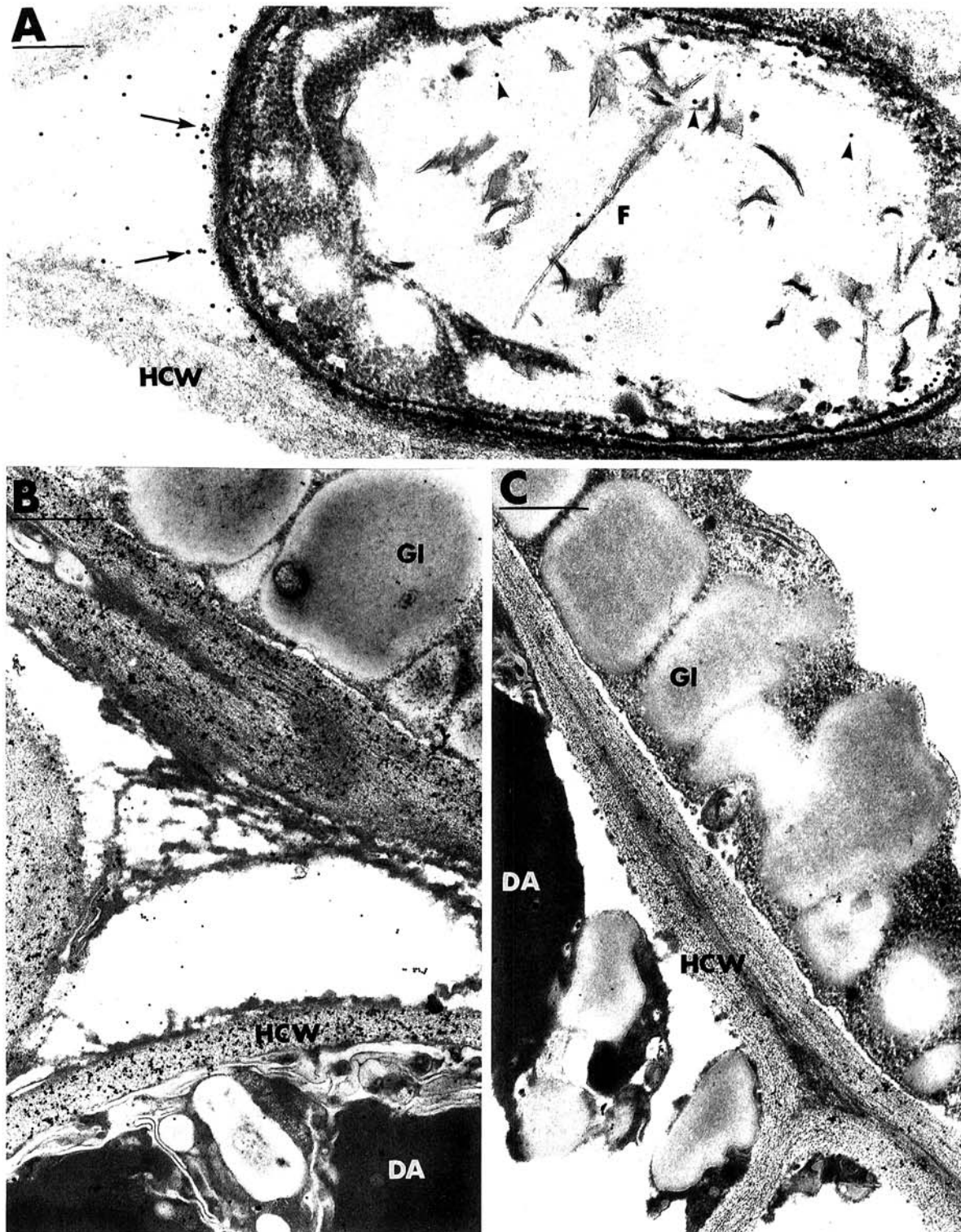

Fig. 6. Transmission electron micrographs of tomato root tissues from chitosan-treated seeds (1 mg/ml) grown in chitosan-amended substrate (1 mg/ml) and inoculated with *Fusarium oxysporum* f. sp. *radicis-lycopersici*. **A,** labeling of chitin with the wheat germ agglutinin/ovomucoid-gold complex. The distribution of gold particles is irregular over the wall of a disorganized fungal cell (F). Some gold particles are seen in the cell lumen (arrowheads) while others are apparently released from the cell wall (arrows). (×50,000, bar = 0.25 μm) **B,** labeling of cellulose with the gold-complexed exoglucanase. An intense labeling occurs over the host cell walls (HCW). The globules (Gl) and the dense aggregates (DA) formed in response to infection are unlabeled. (×30,000, bar = 0.5 μm) **C,** labeling of pectic substances with the *Aplysia* gonad lectin–gold complex. Gold particles are associated with the host cell wall. Labeling is absent over the globules (Gl) and the dense aggregates (DA). (×30,000, bar = 0.5 μm)

radicle. Provided that contact is effective, one may assume that chitosan diffusion is facilitated by the presence of nondifferentiated tissues in the germinating embryos. Whatever the mode of chitosan interaction with the embryos, our results convincingly show that seedling protection against FORL attack is significantly improved by a combination of seed coating and substrate amendment. Even though it is clear that some chitosan molecules exposed at the seed surface are taken up by the germinating embryos (since a reduction of disease incidence was monitored with plants grown in nonamended substrate), our observations suggest, at least with the interaction under study, that the minimal amount of oligosaccharides required to elicit persistent and stable biological control cannot be provided by seed coating only. Immunocytochemical studies with antichitosan antibodies are presently
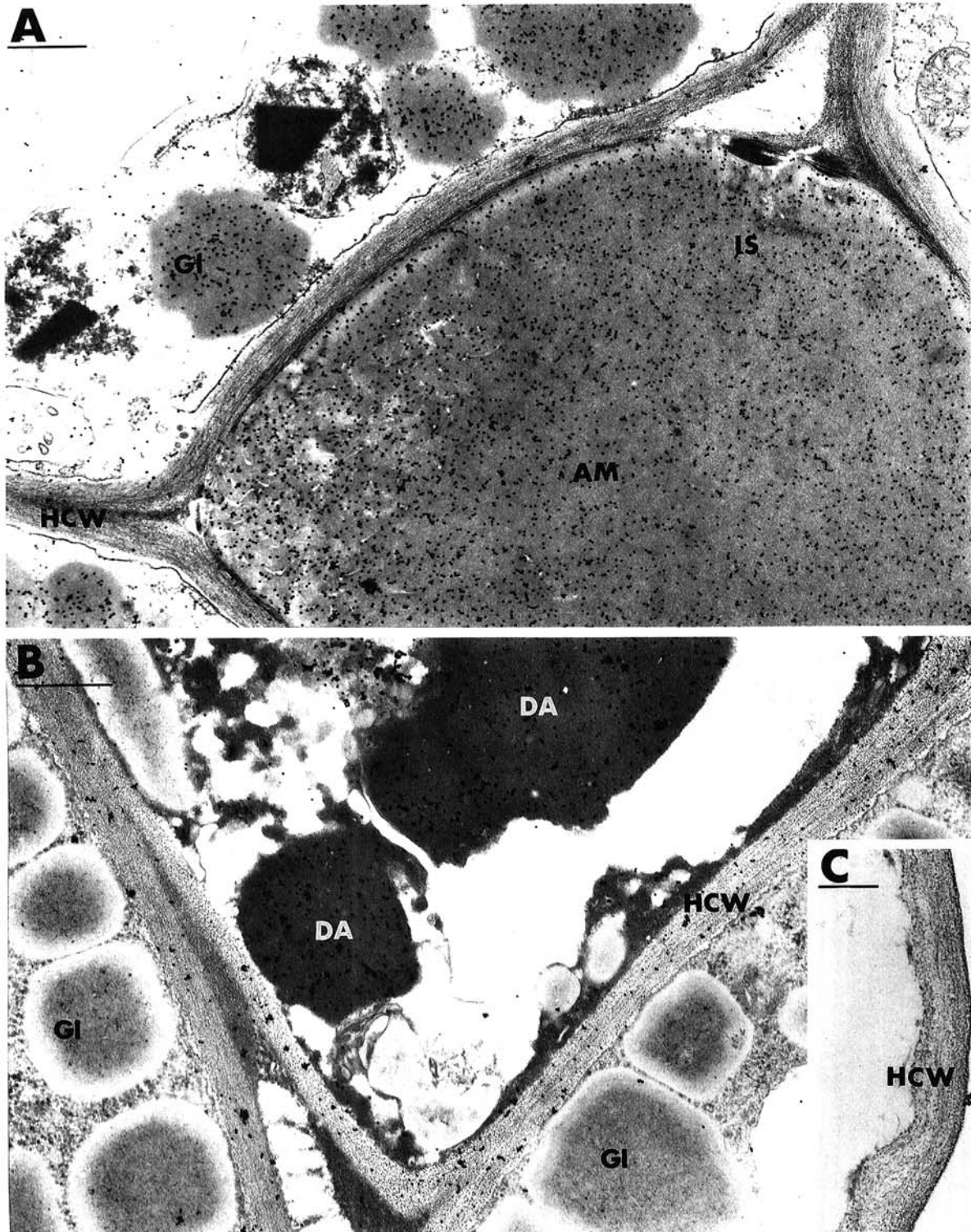

Fig. 7. Transmission electron micrographs of tomato root tissues from chitosan-treated seeds (1 mg/ml) grown in chitosan-amended substrate (1 mg/ml) and inoculated with *Fusarium oxysporum* f. sp. *radicis-lycopersici*. **A,** labeling of β-1,3-glucans with a purified tobacco β-1,3 glucanase complexed to gold. The globules (Gl) as well as the material (AM) filling the intercellular spaces are specifically and intensely labeled. Scattered gold particles are seen over the host cell wall (HCW). (× 24,000, bar = 0.5 μm) **B and C,** labeling of phenolic and ligninlike compounds with a purified laccase complexed to gold. **B,** the globules (Gl) are unlabeled while the dense aggregates (DA) are heavily labeled. The host cell wall (HCW) is decorated by a significant number of gold particles. (×30,000, bar = 0.5 μm.) **C,** in roots from infected, control plants, the host cell wall is unlabeled. (×40,000, bar = 0.25 μm)

being conducted on chitosan-treated tomato seeds at different stages of germination. These investigations should provide information about the spatiotemporal distribution of chitosan molecules in the early stages of seedling emergence.

Relatively high concentrations of chitosan were found to be necessary to achieve full seedling protection without causing phytotoxicity. Since only chitosan oligomers with a degree of polymerization between 7 and 10 are effective elicitors (25), it is likely that the potentially active oligomers available in this heterogeneous solution are in a low proportion. This may explain why a high dosage of this solution was required to elicit a host response.

Reduction of disease incidence in tomato seedlings produced by chitosan-coated seeds and grown on amended substrate was associated with marked host metabolic changes. The main facets of this altered metabolism concerned the induction of a response resembling hypersensitivity at sites of fungal entry and the abnormal accumulation of substances in the noncolonized inner tissues. Examination of the spatial distribution of these host reactions revealed that both the intensity and the magnitude of the response decreased at the endodermis level to become hardly discernible in the vascular stele. Because FORL is a vascular wilt pathogen that normally penetrates the vascular bundles in the tomato roots and spreads along the xylem vessels in the direction of water flow (Fig. 3A) (12), it is not surprising that structural and chemical barriers are laid down in the outer tissues to halt lateral fungal ingress toward the stele. Unexpected, however, was the absence of vascular reactions, such as gelation or tyloses, known to be typical defense strategies elaborated by genetically resistant tomato cultivars to attack by FORL (8,12). In light of these observations, one may suggest that the chitosan-mediated reactions observed in susceptible tomato plants reflect a generalized response rather than a specific induction of resistance against a given pathogen. It would now be interesting to determine whether chitosan treatment has the capacity to confer resistance against a wide range of tomato pathogens including not only root fungi but also foliar pathogens such as Botrytis cinerea.

Although indirect evidence for the production of chitinases was provided by the altered pattern of chitin distribution over the cell walls of the invading hyphae, the finding that chitin molecules still occurred over the walls of disorganized fungal cells, often reduced to empty shells, was taken as an indication that chitinases were probably not the first antimicrobial compounds involved in the process of fungal growth inhibition in planta. By contrast, the heavy accumulation of densely stained deposits, frequently encircling pathogen hyphae in the colonized cells and also accumulating in the noninfected inner cortex, appeared to be an early feature of the host defense strategy. The strong electron density of this material suggested that it could be enriched with phenolic compounds, especially with phenols containing O-dihydroxy groups known to readily interact with osmium tetroxide (OsO4) and to appear highly opaque under the electron microscope (36). This hypothesis was confirmed by the labeling pattern obtained after incubation of the sections with a gold-complexed laccase.

Laccases are copper-containing polyphenol oxidases, mainly produced by wood-rotting fungi (32), which play a key role in lignin breakdown (11) in addition to being involved in the oxidation and polymerization of endogenous plant phenols (29). Because of their multifaceted function, laccases have several potential substrates including mono- and diphenols as well as lignin-related compounds (29). The present cytochemical study provides the first evidence that a purified laccase, once complexed to gold, can be a powerful alternative for accurately localizing phenols in plant tissues. Although the chemical nature of the phenolic compounds involved cannot be defined exactly by the use of the laccase-gold complex alone, the high electron density of the labeled aggregates suggests that at least some of the detected compounds are O-dihydroxy group–containing phenols (36). In light of the labeling pattern obtained with the gold-complexed laccase, it seems logical to assume that chitosan treatment triggers either the de novo synthesis of phenolic compounds, or the polymerization of pre-existing free, soluble phenols, or both. Several

lines of evidence have shown that the free radicals formed during oxidative polymerization reactions are highly toxic and can readily induce fungal cell breakdown through their interaction with the plasma membrane (40). Whether the formation of such polymerized phenols in the outer root cortex is involved in both the restriction of fungal growth and the noticeable degradation of the invading hyphae remains to be further examined. The observation that these compounds often interacted physically with the cell walls of hyphae apparently suffering from some damage speaks in favor of a direct fungitoxic activity. A similar conclusion was drawn by Cherif et al (14) in the case of silicon-treated cucumber plants. The authors reported that the increased production in the root tissues of dense deposits, likely enriched with phenolic compounds, paralleled fungal cell breakdown. Interestingly, laccase-binding molecules were also detected in the host cell walls, thus indicating the infusion of phenolic or ligninlike compounds. Since a similar wall labeling was not seen in nontreated infected plants, one may assume that the deposition of these molecules in the cell walls of elicitor-treated plants is involved in the resistance process, probably by strenghtening the wall architecture.

In agreement with our previous results (10), the present data clearly indicate that the expression of defense reactions in chitosan-treated plants occurs with a much higher magnitude upon fungal attack. Striking differences in the extent of phenol accumulation were observed among chitosan-treated plants, whether they were infected or not by FORL. This suggests that contact with the pathogen is essential for signaling the plant to mobilize its defense strategy. However, the absence of phenolic deposition in nontreated infected plants demonstrates that defense mechanisms cannot be stimulated by the pathogen alone. Thus, it seems likely that chitosan sensitizes the plant to respond more rapidly to a potential attack without causing extensive accumulation of new products and drastic changes in the metabolism.

In addition to phenolic compounds, the present cytochemical investigation revealed a massive accumulation of β-1,3-glucans in the reacting host cells. In a recent paper, we demonstrated that a β-1,3-glucanase, purified from tobacco plants reacting hypersensitively to TMV, was a powerful tool for localizing β-1,3-glucans in plant tissues (3). The present cytochemical observations bring new insights into the value of this probe for accurately delineating the sites of β-1,3-glucan accumulation during the process of induced resistance. The high specificity of the labeling pattern obtained with the gold-complexed laccase and β-1,3-glucanase allowed clear distinction between the deposits formed in response to infection. Interestingly, β-1,3-glucan deposition was found to occur predominantly in the noncolonized inner cortex whereas phenolic accumulation appeared to be more largely distributed in all reacting cells, whether they were colonized or not. This variation in the spatial distribution of the two types of substances indicates that their involvement in the defense strategy elaborated by the plant differs but is probably complementary. According to their pattern of distribution, phenolic compounds may be the first defensive line designed to directly inhibit fungal growth by creating a fungitoxic environment in addition to rendering the plant cell walls impervious to microbial toxins and enzymes. The restriction of β-1,3-glucans in noninfected areas suggests that they act as a second mechanical barrier laid down for halting potential penetration by fungal cells that would have escaped the primary line of defense. It thus appears likely that deposition of phenolic or ligninlike compounds plays a key role in the establishment of effective resistance to fungal colonization. This conclusion agrees with the observations reported by others on the secondary importance in the plant defense process of deposits rich in β-1,3-glucan (15,43). Similarly, Perumalla and Heath (31), studying the inhibitory effect of 2-deoxy-D-glucose on callose synthesis, found that callose was not the main factor involved in pathogen restriction in nonhost bean plants. Also, in these experiments it appeared that deposition of phenolics and silicon was mainly responsible for the observed resistance.

The results presented here show that exogenously applied chitosan induces a set of plant defense reactions that culminate in the creation of a toxic environment adversely affecting the patho-

gen causing fungal-growth inhibition. These observations are consistent with the proposed role of chitosan as an active signaling molecule and shed more light on the potential of induced resistance as a valuable alternative means of disease control.

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