Ultrastructural and Cytochemical Aspects of Chitosan on *Fusarium oxysporum* f. sp. *radicis-lycopersici*, Agent of Tomato Crown and Root Rot

Nicole Benhamou

Département de Phytologie, Faculté des Sciences de l'Agriculture et de l'Alimentation, Université Laval, Sainte-Foy, Québec, Canada G1K 7P4.

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ABSTRACT

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The effect of chitosan on the growth, morphology, and ultrastructure of *Fusarium oxysporum* f. sp. *radicis-lycopersici* was investigated. Chitosan was found to inhibit the radial growth of *F. o. radicis-lycopersici* with an optimal effect at concentrations ranging from 3 to 6 mg/ml. Light microscope observations showed that chitosan induced morphological changes, including hyphal swelling and distortion. Ultrastructural changes observed in chitosan-treated cells included alteration of the plasma membrane and pronounced aggregation of the cytoplasm. An additional reaction was the abnormal deposition of an amorphous material that contained large amounts of chitin, as shown by its strong affinity to wheat germ agglutinin. Chitosan stimulated the activities of both β-1,3-glucanase and chitosanase but did not affect chitinase and chitin deacetylase activities. The possible modes of action of chitosan are discussed relative to morphological and ultrastructural alterations, abnormal depositions of wall-like material, and the enhanced enzymatic activities.

Fusarium oxysporum Schlechtend.:Fr. f. sp. *radicis-lycopersici* W. R. Jarvis & Shoemaker (22) is a widespread soilborne pathogen that may be responsible for severe tomato yield losses in greenhouse soils and hydroponic systems (20,23). This pathogen is unique among *Fusarium* spp., in that it readily recolonizes steam-sterilized soils by microconidia that are dispersed aerially (30). As with other vascular wilt pathogens (4), *F. o. radicis-lycopersici* gains entry into roots through wounds and colonizes in the direction of xylem vessels, where it multiplies abundantly (9). Fungal ingress from the epidermis to the vascular stele is usually accompanied by considerable cell damage and irreversible cell wall alterations, leading most often to root tissue maceration (9).

Repeated attempts to control Fusarium crown and root rot by application of fungicides such as benomyl and thiram have failed, apparently because of problems of phytotoxicity (21). Despite satisfactory results obtained through the application of captafol to steamed soils (29), this control measure is no longer available, because this fungicide has been withdrawn from the market. Another possible method of disease control is the use of resistant cultivars. However, despite progress in the development of resistant tomato cultivars (7), there are currently no high-yielding cultivars with complete resistance to Fusarium crown and root rot. In recent years, attention has been directed toward alternative methods for controlling this disease. These include antagonism through the use of microorganisms such as *Trichoderma* spp. (10,27), allelopathy based on the antimicrobial effect of phenols released from lettuce residues incorporated into infested soils (19), and cross-protection by preinoculating tomato plants with avirulent isolates of *Fusarium* spp. (26). These approaches have, so far, obtained low degrees of success but remain potential avenues for Fusarium wilt control.

A growing body of evidence from some laboratories supports the concept that chitosan, the deacetylated form of chitin, may be valuable in control of fungal diseases (17). Chitosan, a polycationic polymer of β-1,4-linked D-glucosamine residues, is a component of the walls of a wide range of fungi, including *Fusarium* spp. (6). It has been convincingly demonstrated that chitosan oligosaccharides, released from fungal cell walls during plant–fungus interactions, were elicitors of plant defense mechanisms (24,33). In addition to stimulating the plant to defend itself through the activation of resistance genes, chitosan has been shown to inhibit germination and growth of some pathogenic fungi (25). Mechanisms by which chitosan may activate host genes and inhibit fungal growth are not fully elucidated.

As a preface to in situ investigations of the effect of external applications of chitosan on *Fusarium*-infected tomato roots, the present study was undertaken to gain insight into the antifungal properties of chitosan on *F. o. radicis-lycopersici*, with emphasis on the cellular and molecular events associated with fungal growth inhibition in vitro.

MATERIALS AND METHODS

Fungal culture and growth conditions. The isolate of *F. o. radicis-lycopersici* (supplied by P. O. Thibodeau, Service de la Recherche en Defense des Cultures, MAPAQ) was recovered from an infected tomato plant and grown on potato-dextrose agar (PDA) at 25 C. It was periodically inoculated into and isolated from ripe tomato fruits.

Preparation of purified chitosan. Crab-shell chitosan was purchased from Sigma Chemical Co. (St. Louis, MO). This commercial preparation was ground to a fine powder by extended grinding in a mortar, washed repeatedly in distilled water, pelleted by low-speed centrifugation, and air-dried. Sheets of chitosan were dissolved in 0.25 N HCl, and the insoluble material was removed by centrifugation at 10,000 rpm for 10 min. Chitosan was precipitated by neutralization with 2.5 N NaOH (pH 9.8), collected by centrifugation, and thoroughly washed with sterile distilled water to remove salts. Purified chitosan was lyophilized and kept in a desiccator at room temperature until use.

Antifungal assays. Antifungal assays were carried out on PDA plates amended with chitosan at different concentrations. Lyophilized chitosan was dissolved in 0.25 N HCl under continuous stirring, and the pH was adjusted to 5.5–6.0 with 2 N NaOH. The viscous stock solution (10 mg/ml) was autoclaved and added to sterile, molten PDA to obtain chitosan concentrations of 1, 2, 3, 4, or 6 mg/ml. Aliquots of 20 ml of the suspension were immediately dispensed into petri plates. Five PDA plates per chitosan concentration were seeded with a 6-mm-diameter

TABLE 1. Effect of chitosan on inhibition of radial growth of *Fusarium oxysporum* f. sp. *radicis-lycopersici*

Chitosan concentration (mg/ml)	Inhibition of radial growth (%)					
	Days after inoculation					
	1[a]	2	3	4	5	6
0	0	0	0	0	0	0
1.0	100	76.2 (±0.2)[b]	74.3 (±0.6)	61.6 (±0.1)	55.3 (±0.8)	51.5 (±0.4)
2.0	100	88.4 (±0.3)	87.8 (±0.5)	79.2 (±0.3)	79.3 (±0.7)	79.4 (±0.2)
3.0	100	96.1 (±0.1)	96.9 (±0.5)	97.4 (±0.5)	98.6 (±0.2)	98.7 (±0.3)
4.0	100	97.7 (±0.6)	97.1 (±0.2)	97.9 (±0.6)	98.7 (±0.1)	98.9 (±0.1)
6.0	100	99.4 (±0.7)	99.8 (±0.8)	99.9 (±0.4)	99.9 (±0.1)	99.9 (±0.1)

[a] By 1 day after inoculation, no fungal growth was observed in chitosan-amended plates.
[b] Values in parentheses are standard errors of the mean.

mycelial plug taken from the margin of a 5-day-old *F. o. radicis-lycopersici* culture. The experiment was repeated four times. Inoculated plates were incubated at 25 C in the dark, and fungal growth was recorded at 1-day intervals until the control (0 mg/ml of chitosan) reached the edge of the plate. Growth inhibition is expressed as the percentage of inhibition of radial growth relative to the control. Means and standard deviations were estimated from the observation of 20 plates per chitosan concentration.

Effect of chitosan on the morphology of *F. o. radicis-lycopersici*. Cultures of 5-day-old *F. o. radicis-lycopersici* were flooded with sterile distilled water and gently shaken to remove the microconidia. Microconidia were washed twice with sterile distilled water by low-speed centrifugation, and the spore suspension was finally adjusted to 1×10^5 conidia per milliliter with sterile distilled water. Two 50-μl aliquots of the spore suspension were deposited on the surface of microscope slides covered with a thin (5-mm-thick) layer of 1% (w/v) water agar, pH 5.5, amended with chitosan at concentrations ranging from 0 to 6 mg/ml. Inoculated slides were kept in a moist chamber at 25 C. One day after inoculation, slides were stained with 1% aqueous toluidine blue and examined by light microscopy to assess spore germination and morphological changes of young, emerging hyphae. The experiment was repeated twice for each chitosan concentration.

Transmission electron microscopy. Mycelial samples, collected from the margin of 6-day-old colonies grown on chitosan-amended or chitosan-free PDA, 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 postfixed with 1% (w/v) osmium tetroxide in the same buffer for 1 h at 4 C. Samples were dehydrated in a graded ethanol series and embedded in Epon 812. Ultrathin sections, collected on formvar-coated nickel grids, were either directly stained with uranyl acetate and lead citrate or processed for cytochemical labeling prior to examination with a JEOL 1200 EX electron microscope at 80 kv.

Preparation of the colloidal gold probe. Colloidal gold with particles averaging 15 nm in diameter was prepared according to Frens (13). For localization of *N*-acetylglucosamine residues (chitin), wheat germ agglutinin, a lectin with *N*-acetylglucosamine binding specificity (5) was used in a two-step procedure. This lectin was not directly complexed to gold, because of its low molecular weight. Ovomucoid, a glycoprotein from egg white bearing a sugar specific for wheat germ agglutinin, was complexed to gold at pH 5.4 (6) and used as a second-step reagent.

Cytochemical labeling. For the indirect labeling of chitin, ultrathin sections were incubated on a drop of phosphate-buffered saline (PBS), pH 7.4, for 5 min, then transferred to a drop of wheat germ agglutinin (25 μg/ml in PBS, pH 7.4) for 1 h at room temperature in a moist chamber. After washing with PBS, sections were incubated on a drop of gold-complexed ovomucoid (1:20 in PBS containing 0.02% [w/v] of polyethylene glycol [PEG] 20,000) for 30 min at room temperature. They were washed with PBS, rinsed with distilled water, and air-dried prior to staining with uranyl acetate and lead citrate.

Specificity of the labeling was assessed by 1) incubation with wheat germ agglutinin to which *N-N'-N''*-triacetylchitotriose (1 mg/ml in PBS) was added; 2) incubation with wheat germ agglutinin, followed by unlabeled ovomucoid, and then ovomucoid-gold complex; and 3) incubation with the gold suspension alone.

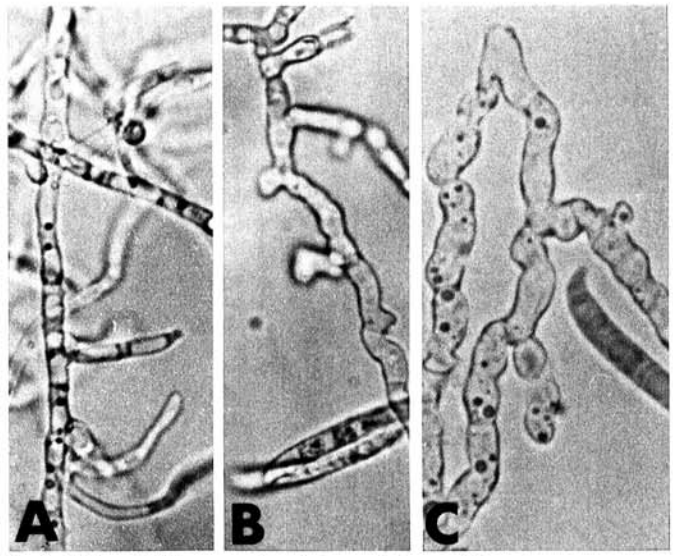

Fig. 1. Effect of chitosan on hyphal morphology of *Fusarium oxysporum* f. sp. *radicis-lycopersici*. **A,** Control; **B,** chitosan at 1 mg/ml; **C,** chitosan at 3 mg/ml.

Quantification of labeling. Density of labeling over the walls of fungal cells untreated or treated with chitosan was compared by determining the number of gold particles per μm^2. Area determinations were carried out by the point-counting method (35), using negatives of electron micrographs projected on a lattice. The amount of labeling over a specified area was estimated by counting the number of gold particles on a photographic enlargement. The density of labeling was calculated as follows: $Ns = Ni/Sa$, where Ns represents the number of gold particles per unit surface, Ni is the number of gold particles, and Sa is a specified area.

Polyacrylamide gel electrophoresis. Mycelial samples were ground to homogenization at 4 C in 50 mM sodium phosphate buffer, pH 5.0. The homogenate was centrifuged at 15,000 rpm for 15 min at 4 C, and the supernatant was used as crude preparation for biochemical investigations.

Detection of chitinase, chitin deacetylase, and chitosanase activities after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to recently described procedures (15,31,32). Similarly, detection of β-1,3-glucanase activity after native PAGE was performed according to Côté et al (11).

RESULTS

Effect of chitosan on percent inhibition of radial growth of *F. o. radicis-lycopersici*. Chitosan significantly reduced the radial growth of *F. o. radicis-lycopersici*, with a marked effect at concentrations ranging from 3 to 6 mg/ml (Table 1). Growth in-

hibition ranging from 51 to 76% was obtained with chitosan at lower concentrations (1 and 2 mg/ml). By 6–7 days after inoculation, fungal growth on chitosan-amended plates was halted, whereas the fungus grown on control plates developed actively.

Morphological changes. Inhibition of fungal growth in vitro was further complemented by light microscope investigations (Fig. 1). When deposited on chitosan-free agar, spores of *F. o. radicis-lycopersici* germinated and gave rise to a regularly septate my-

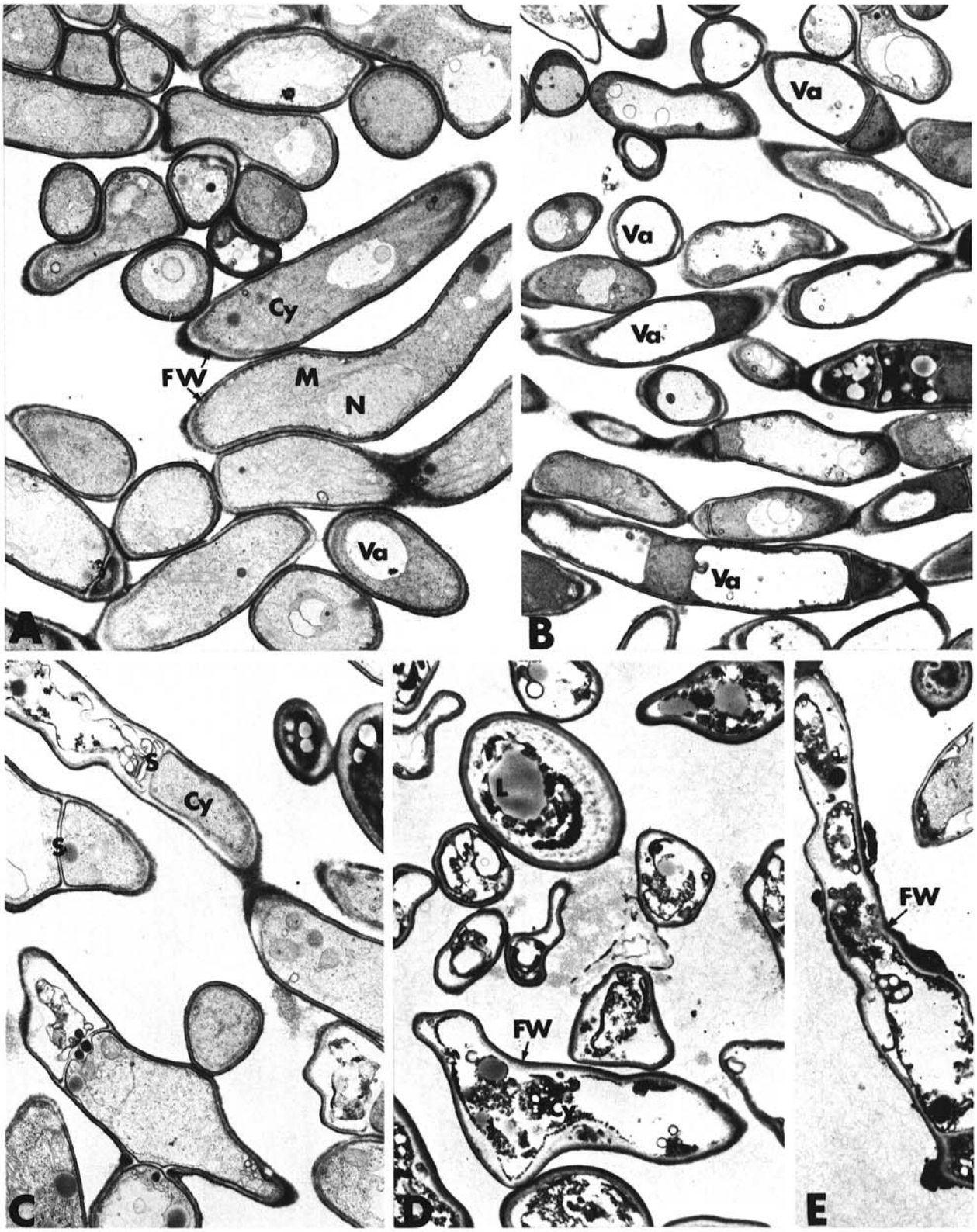

Fig. 2. Transmission electron micrographs of *Fusarium oxysporum* f. sp. *radicis-lycopersici* grown on potato-dextrose agar unamended and amended with chitosan. **A,** Chitosan at 1 mg/ml (control). Cells are surrounded by a thin cell wall and contain a dense cytoplasm with organelles such as nucleus and mitochondria (×7,500). **B,** Chitosan at 1 mg/ml. An intense vacuolation is observed (×5,400). **C,** Chitosan at 2 mg/ml. Alterations characterized by retraction of the plasma membrane and cytoplasm aggregation occur in one portion of fungal hyphae (×7,500). **D,** Chitosan at 3 mg/ml. Hyphae are severely damaged. The cytoplasm is reduced to strands of electron-dense material (×7,500). **E,** Chitosan at 4 mg/ml. Hyphae are markedly contorted and highly altered (×7,500). Cy = cytoplasm; FW = fungal wall; L = lipid body; M = mitochondrion; N = nucleus; S = septum; Va = vacuole.

celium in which hyphae were often branched at diverging angles (Fig. 1A). The average hyphal diameter was 3 μm. When chitosan was added to the agar, morphological changes were noticeable (Fig. 1B and C). At all chitosan concentrations, hyphae grew in abnormal shapes. A greater degree of contortion was observed at higher chitosan concentrations (Fig. 1B and C). One of the most striking features was a marked hyphal swelling. A twofold increase in hyphal diameter was observed with 1 mg/ml of chitosan (Fig. 1B) as compared to the control. Higher concentrations of chitosan induced significant increase in hyphal swelling, leading, in some cases, to cell lysis. Interestingly, conidial morphology did not appear to be affected by treatments with chitosan (Fig. 1C).

Ultrastructural changes. Examination of hyphae of *F. o. radicis-lycopersici* by transmission electron microscopy at low magnifications confirmed that fungal cells subjected to chitosan were structurally damaged (Fig. 2B–E). Under chitosan-free conditions, hyphae were delimited by a thin, electron-opaque wall and contained a dense cytoplasm in which a large number of organelles, including nuclei, mitochondria, and endoplasmic reticulum, were found (Fig. 2A).

Observations of hyphae of *F. o. radicis-lycopersici* on chitosan-amended media showed various degrees of cell alteration, ranging from an increased vacuolation (Fig. 2B) to a nearly complete loss of cellular integrity (Fig. 2D). Chitosan at 1 mg/ml induced intense cytoplasmic vacuolation (Fig. 2B). Centrally located vacuoles expanded to such an extent that cytoplasm and organelles became gradually restricted to the periphery of the cells. This chitosan concentration caused slight morphological changes (Fig. 2B). At 2 mg/ml of chitosan, hyphal cells were abnormally shaped and markedly damaged (Fig. 2C). In most cases, cells were partially damaged, as judged by the occurrence of apparently normal hyphal portions (Fig. 2C) separated from highly altered areas by septa. Fungal cells, grown on PDA amended with higher concentrations of chitosan (3–6 mg/ml), were highly distorted and severely damaged (Fig. 2D and E). The cytoplasm was usually reduced to fine strands of electron-dense material, and organelles such as mitochondria were no longer discernible (Fig. 2D). Hyphal enlargement was occasionally seen.

Observations at higher magnifications provided a more detailed picture of the cellular disorganization induced by chitosan. Hyphal cells grown under control conditions were regularly septate, with

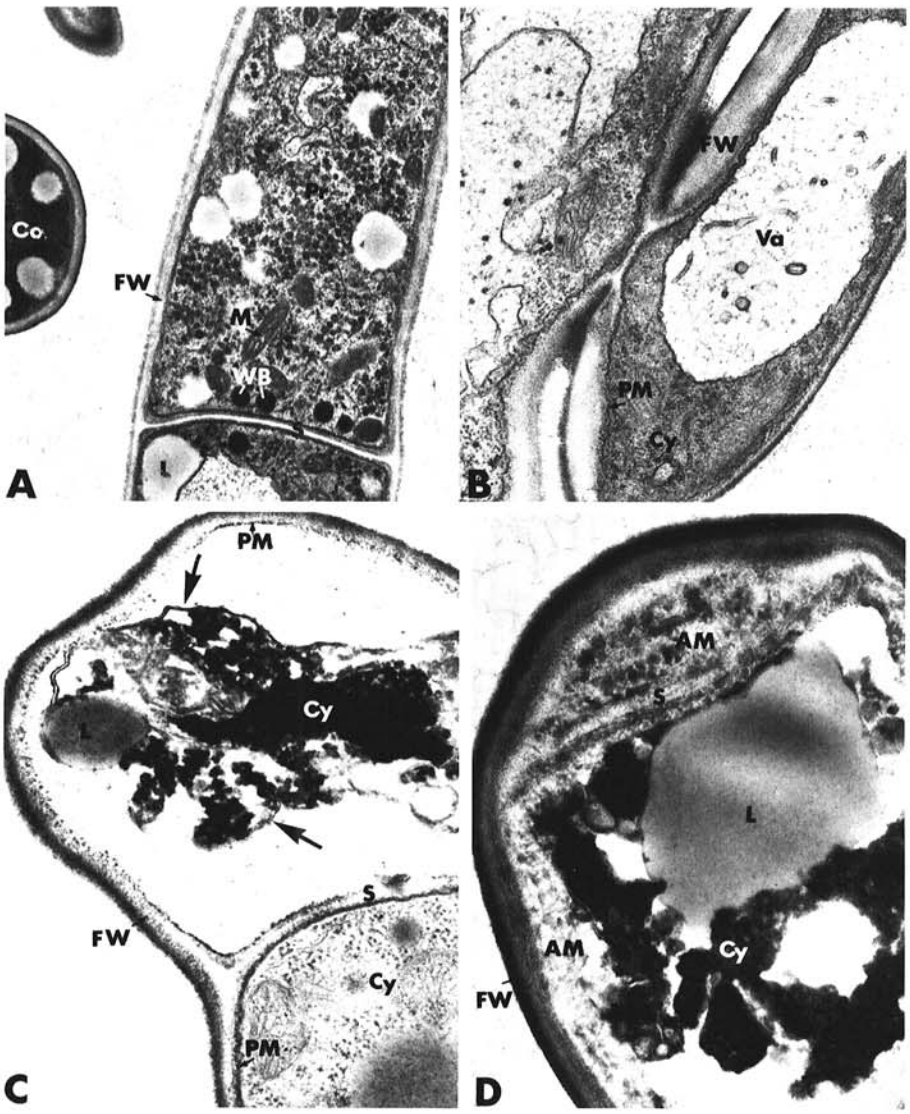

Fig. 3. Transmission electron micrographs of *Fusarium oxysporum* f. sp. *radicis-lycopersici* hyphae grown on potato-dextrose agar unamended or amended with chitosan. **A,** Chitosan at 0 mg/ml (control). A regularly septate hyphal cell contains a polyribosome-rich cytoplasm in which numerous organelles such as mitochondria are embedded. The fungal wall is thin (×27,000). **B,** Chitosan at 1 mg/ml. An increased vacuolation is observed (×27,000). **C,** Chitosan at 2 mg/ml. One portion of a fungal cell shows pronounced alteration characterized by a marked cytoplasm aggregation. The plasma membrane is damaged (arrows) (×40,000). **D,** Chitosan at 3 mg/ml. An abnormal deposition of amorphous material occurs in areas adjacent to the cell wall (×45,000). AM = amorphous material; Cy = cytoplasm; FW = fungal wall; M = mitochondrion; PM = plasma membrane; S = septum; Va = vacuole.

Woronin bodies typically associated with septa (Fig. 3A). The plasma membrane was closely appressed against the thin cell wall, and the cytoplasm appeared metabolically active, as judged by the amount of polyribosomes and organelles. Local retraction of the plasma membrane accompanied by cell wall swelling were typical features of fungal cells grown on PDA amended with chitosan at 1 mg/ml (Fig. 3B). Large, centrally located vacuoles containing small, electron-opaque inclusions embedded in a fibril-

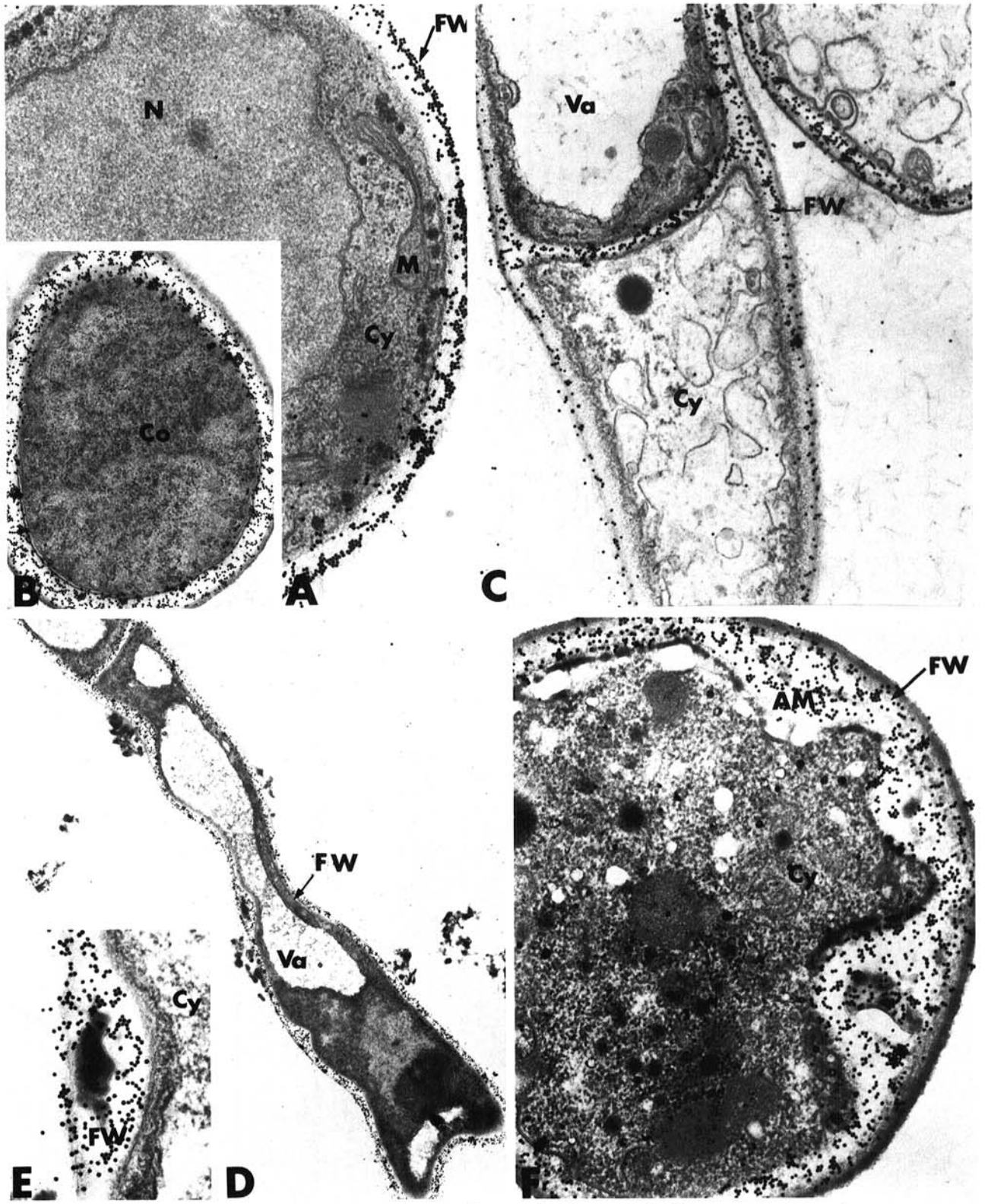

Fig. 4. Transmission electron micrographs of *Fusarium oxysporum* f. sp. *radicis-lycopersici* hyphae grown on potato-dextrose agar unamended or amended with chitosan. Labeling of chitin with wheat germ agglutinin–ovomucoid-gold complex. **A and B,** Chitosan at 0 mg/ml (control). Gold particles are deposited over the hyphal wall, mainly over the outermost wall layers (arrows). **B,** Labeling is evenly distributed over the wall of conidium (**A,** ×36,000; **B,** ×27,000). **C,** Chitosan at 1 mg/ml. Gold particles are specifically distributed over the wall of vacuolated hyphal cells (×36,000). **D,** Chitosan at 2 mg/ml. The wall of a highly contorted hyphal cell is labeled, even in E, areas showing signs of alteration (**D,** × 11,800; **E,** ×45,000). **F,** Chitosan at 3 mg/ml. The amorphous material formed between the wall and the retracted cytoplasm is intensely labeled (×36,000). AM = amorphous material; Co = conidium; Cy = cytoplasm; FW = fungal wall; M = mitochondrion; N = nucleus; S = septum; Va = vacuole.

lar network were frequently observed (Fig. 3B). In addition, loss of cytoplasmic integrity and involution of vacuolar membranes occurred in some fungal cells. There was an apparent retraction of the plasma membrane in cells exposed to 2 mg/ml of chitosan (Fig. 3C, arrows). In contrast, in apparently intact hyphal portions the plasma membrane was regularly appressed against the cell wall (Fig. 3C). Although altered hyphal portions were swollen, obvious structural modifications of cell walls were not visible. Fungal cells subjected to higher concentrations of chitosan (3–4 mg/ml) were heavily damaged, as judged by the marked cytoplasmic aggregation (Fig. 3D). Lipid bodies were often the only recognizable structures. An interesting feature of these fungal cells was the apparent deposition of an amorphous material between the cell wall and the retracted cytoplasm (Fig. 3D).

Cytochemical localization of chitin. Application of the wheat germ agglutinin–ovomucoid-gold complex to sections of fungal colonies grown on chitosan-free PDA resulted in an intense deposition of gold particles over cell walls of both hyphae (Fig. 4A) and spores (Fig. 4B). Cytoplasm and organelles were nearly free of labeling. A close examination of the labeling pattern over hyphal cell walls showed that gold particles accumulated preferentially over the outermost wall layers (Fig. 4A, arrows). In contrast, conidial walls were evenly labeled (Fig. 4B).

When wheat germ agglutinin–ovomucoid-gold complex was applied to ultrathin sections of fungal colonies grown on chitosan-amended PDA, labeling was also restricted to the cell surface (Fig. 4C, D, and F; Fig. 6). In all cases, vacuoles and disorganized cytoplasm were unlabeled. Examination of *F. o. radicis-lycopersici* hyphae subjected to chitosan at 1 mg/ml revealed that gold particles were specifically associated with the walls of highly vacuolated cells (Fig. 4C). However, labeling intensity decreased with an increase in chitosan concentration (Table 2). Fungal cells subjected to higher concentrations of chitosan (2, 3, and 4 mg/ml) exhibited an unusual labeling pattern (Fig. 4D and F). In such cells, the gradual retraction and aggregation of the cytoplasm was most often accompanied by the formation of an amorphous material in the so-called paramural spaces (Fig. 4E and F). This amorphous material could be easily distinguished from the cell wall by its loosened texture and its lower electron-density. Following incubation with wheat germ agglutinin–ovomucoid-gold complex, a heavy deposition of gold particles was observed over this newly formed material (Figs. 4D and 5). Although labeling still occurred over the walls of these highly altered fungal cells, gold particles were less numerous than in the control (Table 2). Labeling appeared mainly concentrated over the amorphous material, which infringed on the aggregated cytoplasmic remnants (Fig. 5A and C). In some cells, polymorphic structures, resembling wall invaginations, were intensely labeled (Fig. 5B). Specificity of the labeling obtained with the wheat germ agglutinin–ovomucoid-gold complex was assessed by the negative results obtained with all control tests, including incubation with wheat germ agglutinin previously absorbed with *N-N'-N"*-triacetylchitotriose (not shown).

Detection of enzymatic activities in extracts of *F. o. radicis-lycopersici*. Analysis of mycelial extracts for chitinase after SDS-PAGE showed that chitosan at 4 and 6 mg/ml slightly stimulated the activity of this hydrolytic enzyme in *F. o. radicis-lycopersici* (not shown). Activity of chitin deacetylase, the enzyme responsible for the conversion of chitin into chitosan, was not detected in

TABLE 2. Effect of chitosan concentration on density of labeling with wheat germ agglutinin (WGA)–ovomucoid-gold complex on *Fusarium oxysporum* f. sp. *radicis-lycopersici*

Chitosan concentration (mg/ml)	WGA–ovomucoid-gold density[a]	
	Cell wall	Amorphous material
0	302.4 (±12.6)[b]	Material absent
1.0	193.5 (±32.4)	Material absent
2.0	178.8 (±41.1)	41.4 (±16.2)
3.0	151.3 (±11.5)	228.2 (±35.3)
4.0	164.5 (±18.3)	241.3 (±25.4)
6.0	150.9 (±16.2)	283.6 (±18.9)

[a] Densities were determined by counting number of gold particles over specified areas of cell walls on 20 micrographs.
[b] Values in the parentheses are standard errors of the mean.

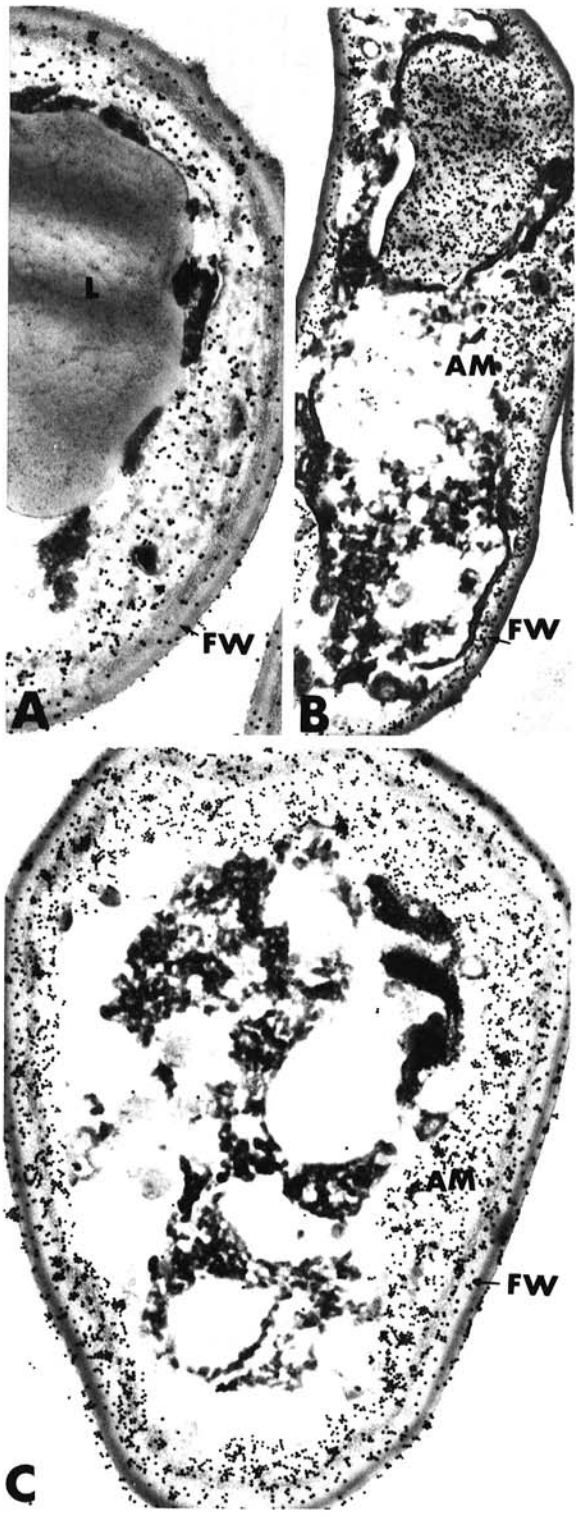

Fig. 5. Transmission electron micrographs of *Fusarium oxysporum* f. sp. *radicis-lycopersici* hyphae grown on potato-dextrose agar unamended or amended with chitosan. Labeling of chitin with wheat germ agglutinin–ovomucoid-gold complex. **A,** Chitosan at 4 mg/ml. The fungal cell wall appears less intensely labeled than that of the control (Fig. 4A). Gold particles are specifically deposited over the amorphous material (×36,000). **B,** Chitosan at 4 mg/ml. A wall-like invagination is heavily labeled (×27,000). **C,** Chitosan at 6 mg/ml. A severely damaged hyphal cell exhibits an intense labeling of the newly formed wall-like material (×36,000). AM = amorphous material; FW = fungal wall; L = lipid body.

mycelial extracts from either control or chitosan-treated *F. o. radicis-lycopersici* (not shown).

In contrast, chitosan was found to significantly stimulate the activity of both β-1,3-glucanase and chitosanase (Figs. 6 and 7). Extracts from mycelium treated with chitosan showed one major band with β-1,3-glucanase activity (Fig. 6, lanes B–D). This protein band, which was very weak in the control, showed a marked increase in activity following treatment with chitosan at 2 mg/ml. Similarly, a substantial increase in chitosanase activity was observed upon chitosan treatment, as estimated by the appearance of two bands at about 38 kDa in extracts exposed to chitosan at 4 and 6 mg/ml (Fig. 7).

DISCUSSION

Although root-infecting pathogens cause important and destructive plant diseases, there are no effective chemical control methods for a number of them, including several *Fusarium* spp. (20). Recently, considerable attention has been paid to the development of alternative methods of disease management (19,26). Several lines of evidence have suggested that natural compounds, such as chitin and chitosan, could promote the plant to defend itself and could also interfere with growth of the pathogen (17,24,25,33). The demonstration that chitosan could inhibit the growth of several pathogenic fungi (18,25) suggests that this polycationic compound could be of value for biotechnological applications (17). The present study was undertaken to gain a better insight into the cellular and molecular events induced in *F. o. radicis-lycopersici* following exposure to various concentrations of chitosan. Our observations demonstrate that chitosan is not only effective in halting fungal growth but also induces marked morphological changes, structural alterations, and molecular disorganization in the pathogen.

Plate assays showed that *F. o. radicis-lycopersici* was highly sensitive to chitosan. An inhibition of radial growth was observed at all concentrations, but the optimal antifungal activity that yielded more than 99% inhibition was obtained with concentrations ranging from 3 to 6 mg/ml. Such high chitosan concentrations can be considered nonphysiological, and it is likely that the effective amount of chitosan would have been several times less, provided that insoluble oligomers could have been removed from the suspension. At any rate, only those small oligomers that become soluble are accessible to the fungus. The low level of soluble oligomers in the preparations used in this study may explain the reason why high concentrations of chitosan were required. This drastic effect of chitosan on *F. o. radicis-lycopersici* correlates well with the current concept that all fungi, except those containing chitosan as a major wall compound, are vulnerable to chitosan (25). Although chitosan has been reported to occur in the walls of *Fusarium* hyphae and spores (16), it is considered to be a minor wall component as compared to chitin and glucans (2,3).

Light and electron microscope investigations revealed that growth inhibition of *F. o. radicis-lycopersici* as a response to chitosan was accompanied by marked cellular changes. These changes included hyphal swelling, increased vacuolation, retraction and alteration of the plasma membrane, cytoplasm aggregation, and abnormal cell wall deposition. Whether such disturbances in the overall fungal cell organization are related to a direct effect of chitosan is unknown. However, considering the polycationic properties of chitosan (25), chitosan-induced alterations of the plasma membrane may be largely responsible for the observed morphological and structural changes. Because of the alternating orientation of positively charged glucosamine units along the polymer, chitosan may readily interfere with negatively charged residues of macromolecules exposed at the fungal cell surface. It is known that changes in the lipid or phospholipid composition of the fungal plasma membrane lead to abnormal membrane permeability by altering the fluidity properties (34). Therefore, one may speculate that chitosan-induced alterations in the permeability of the plasma membrane in *F. o. radicis-lycopersici* cells have promoted internal osmotic imbalances, leading to the observed disturbances such as cytoplasm disorganization and aggregation. Support for this speculation is provided by the finding that leakage of proteinaceous and UV-absorbing material from *Pythium paroecandrum* was induced by chitosan (25). In line with this concept, our ultrastructural observations have shown that plasma membrane alterations occurred in cells showing obvious signs of disorganization. Although several lines of evidence indicate that a positive correlation exists between interaction of chitosan with the plasma membrane and subsequent cellular changes, the possibility that chitosan may enter the fungal

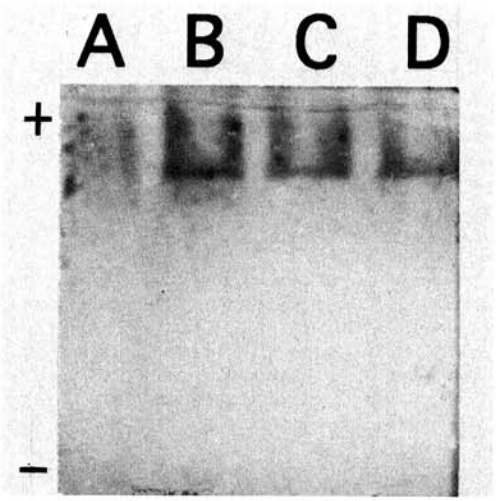

Fig. 6. β-1,3-Glucanase activity after native polyacrylamide gel electrophoresis (PAGE) of extracts from mycelium of *Fusarium oxysporum* f. sp. *radicis-lycopersici* grown on potato-dextrose agar **A,** unamended, or amended with chitosan at **B,** 1 mg/ml, **C,** 2 mg/ml, and **D,** 4 mg/ml. Extracts were subjected to native PAGE in a 15% (w/v) polyacrylamide gel containing 1.5 mg/ml of laminarin (β-1,3-glucan) as substrate. After electrophoresis, the gel was incubated in 50 mM acetate buffer, pH 5.0, for 45 min at 37 C. The gel was stained with aniline blue, and lytic zones were revealed by UV transillumination (11). An increase in β-1,3-glucanase activity is observed in chitosan-treated samples (lanes B–D) as compared to the control (lane A).

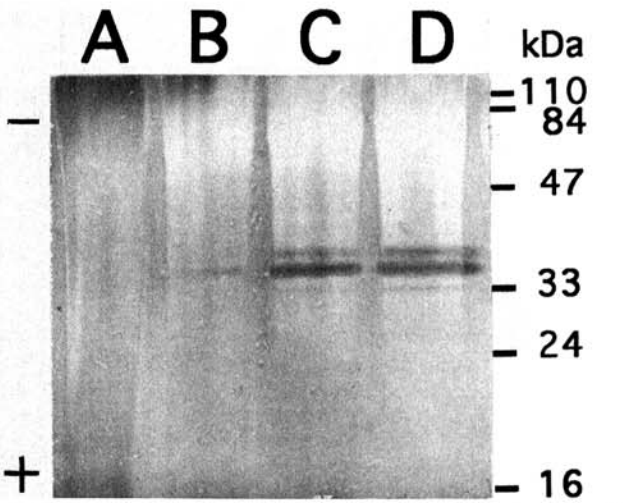

Fig. 7. Chitosanase activity after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of extracts from mycelium of *Fusarium oxysporum* f. sp. *radicis-lycopersici* grown on potato-dextrose agar **A,** unamended, or amended with chitosan at **B,** 1 mg/ml, **C,** 2 mg/ml, and **D,** 4 mg/ml. Extracts were subjected to SDS-PAGE in a 15% (w/v) polyacrylamide gel containing chitosan from *Mucor mucedo* at a final concentration of 0.05%. After electrophoresis, the gel was incubated for 18 h at 37 C in buffered Triton X-100. Chitosanase activity was detected by Coomassie brilliant blue R-250 staining (15). Molecular mass markers are indicated on the right. A striking increase in chitosanase activity is observed upon chitosan treatment (lanes B–D).

cell through loosened walls and interact with nuclear DNA, as suggested by Hadwiger et al (16), cannot be ruled out. Whether chitosan has dual modes of action is still a matter of speculation, which stresses the importance of obtaining additional information to improve interpretation of ultrastructural results.

Our cytological observations have shown that chitosan-induced alterations in *F. o. radicis-lycopersici* were associated with the new deposition of an amorphous material in paramural spaces. The occurrence of large amounts of chitin in these abnormal deposits, as revealed by cytochemical tests with wheat germ agglutinin, raises the question of how the fungal cell can organize the deposition of wall-like material in unusual cell areas. At least two models may be advanced. First, the amorphous material may directly originate from the adjacent cell wall through the stretching of preexisting polymers. This hypothesis implies that hydrolytic enzymes are produced by the fungus to loosen the wall structure while allowing the release of chitin macromolecules. Support for this concept is provided by the finding that the activities of both β-1,3-glucanase and chitosanase were stimulated by chitosan treatment. In light of these results, one may speculate that alteration of β-1,3-glucans and chitosan could affect the structural integrity of the wall, leading to the release of some molecules caused by an increased wall permeability. The induction of cytological alterations by chitosan cannot be attributed to a direct effect on wall chitin, since chitinase and chitin deacetylase activities were not detected on gel assays (1). In this context, the observed decrease in the amount of wall-bound chitin upon chitosan exposure may indicate a release rather than a hydrolysis or a conversion of chitin.

In the second model, the amorphous material may be laid down as newly synthesized material. Although there are still some gaps in our understanding of the mechanisms by which cell walls are formed in fungal hyphae, published reports suggest that chitin synthetase is evenly distributed in the plasma membrane and can be activated by cytoplasmic proteases to synthesize the chitin chains (8). Membrane-bound proteins (i.e., enzymes) are known to be linked to lipids which influence their biological activity (28). Alterations in the lipid content of plasma membrane induced by chitosan may well be responsible for a deregulation of membrane-bound enzymes. Disturbances in the regulation of enzymes involved in the synthesis of wall compounds (i.e., chitin synthetase) may explain the accumulation of chitin at sites where deposition of wall polymers normally does not occur. A similar conclusion was reached by Fuller et al (14), who studied the effect of the fungicide cyproconazole on cells of *Sclerotium rolfsii*. The authors attributed the formation of abnormal wall deposits in *S. rolfsii* hyphae to chemical changes induced in the plasma membrane by the fungicide. The exact mechanism involved in the formation of abnormal chitin-rich deposits in chitosan-treated cells of *F. o. radicis-lycopersici* is still open to question. Whether this process implies a de novo synthesis of chitin, or simply relies on the release of preexisting wall-bound chitin, remains to be further investigated at different levels, including the analysis of chitin synthetase activity in chitosan-treated cells. As was recently pointed out (12), it is likely that the inhibitory effect of chitosan on fungal growth is derived from more than one single mechanism. It would be interesting to determine the sequence of molecular events leading to effective growth inhibition and cellular alterations. In conclusion, our results demonstrate that *F. o. radicis-lycopersici* is highly vulnerable to chitosan and suggest that this polycationic polymer, readily obtained from the conversion of chitin from crustacean shells, may have important applications in the biological control of Fusarium crown and root rot of tomato.

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