Plant Physiol. (1980) 66, 205–211
0032-0889/80/66/0205/07/$00.00/0

Chitosan as a Component of Pea-*Fusarium solani* Interactions[1,2]

Received for publication September 10, 1979 and in revised form March 3, 1980

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ABSTRACT

Chitosan, a polymer of β-1,4-linked glucosamine residues with a strong affinity for DNA, was implicated in the pea pod-*Fusarium solani* interaction as an elicitor of phytoalexin production, an inhibitor of fungal growth and a chemical which can protect pea tissue from infection by *F. solani* f. sp. *pisi*. Purified *Fusarium* fungal cell walls can elicit phytoalexin production in pea pod tissue. Enzymes from acetone powders of pea tissue release eliciting components from the *F. solani* f. sp. *phaseoli* cell walls. Hydrochloric acid-hydrolyzed *F. solani* cell walls are about 20% glucosamine. The actual chitosan content of *F. solani* cell walls is about 1%. However, chitosan assays and histochemical observations indicate that chitosan content of *F. solani* spores and adjacent pea cells increases following inoculation. Dormant *F. solani* spores also accumulate chitosan. Concentrations of nitrous acid-cleaved chitosan as low as 0.9 microgram per milliliter and 3 micrograms per milliliter elicit phytoalexin induction and inhibit germination of *F. solani* macroconidia, respectively. When chitosan is applied to pea pod tissue with or prior to *F. solani* f. sp. *pisi*, the tissue is protected from infection.

Investigations of the incompatible interaction between *Fusarium solani* f. sp. *phaseoli* and pea pod tissue (*Pisum sativum*) indicate that increasing RNA synthesis and alterations in the conformation of the pea nucleus are detectable within minutes after inoculation (16). In the compatible interaction between *F. solani* f. sp. *pisi* and pea pod tissue, which results in susceptibility, RNA synthesis is unchanged. However, there are major alterations in the pea nucleus and nuclear membrane. The components involved with the spontaneous communication of these effects have not been identified. There are, however, numerous pure chemicals and chromatographically characterized cell fractions which can substitute for the fungal cell in eliciting phytoalexin production (2, 4, 8, 9, 12, 17–19), a biochemical monitor of the host response, which starts within 6 h in most legumes (13, 20). Many fractions of the fungal cell can elicit phytoalexin production in peas (R. Dobson and L. A. Hadwiger, unpublished); however, those eliciting components derived from the fungal cell wall are regarded as functionally important because of their immediate proximity to the plant cell. We have demonstrated that the fungal cell wall must physically contact the plant cell to induce, maximally, phytoalexin synthesis (15). Conversely, the plant cell must contact the fungus to suppress fungal growth. In either case, contact phenomena appear to initiate the enzymic processing of fungal walls or wall precursors.

The fungal cell wall is a complex structure containing varying types and percentages of carbohydrates, protein, lipids, etc. (5, 24). There is evidence that phytoalexin elicitors derived from the fungus or fungal cell wall can be carbohydrate (4), protein (8), or a combination thereof (2, 9). We have assayed the production of phytoalexin and disease-resistant responses attributable to fungal carbohydrates by applying *Fusarium* cell walls, chitin, chitosan, cellulose, and other suspected fungal cell wall components to pea pod endocarps. Chitosan was found (14) to be the most active ingredient contained within fungal cell walls. Thus, its role in the host-parasite interaction between *F. solani* and pea pod tissue was examined in detail.

Chitosan (β-1,4-linked glucosamine) is a deacetylated derivative of chitin and is a component of the cell walls of many fungi (5). In *Mucor*, chitosan reportedly is formed subsequent to chitin (3). There are no reports on the interconversion of these two compounds during infection by plant pathogenic fungi.

In this study, we have discovered that chitosan is a compound of *Fusarium* cells which both induces pisatin synthesis and inhibits fungal germination and growth. The histological localization of chitosan during infection suggests that chitosan plays a prominent role in the host-parasite interaction. Finally, chitosan, when applied with the inoculum, can protect the pea tissue from *F. solani* f. sp. *pisi*, a pathogen of peas.

MATERIALS AND METHODS

Shrimp shell chitosan and chitin were purchased from Sigma. Snow crab chitosan was generously supplied by Madera Products Inc., Albany, Ore. These commercial preparations were dispersed into 80-mesh fragments with a Wiley mill and further dispersed in water to particles predominantly 5 μm or smaller by extended grinding in a mortar. The suspension of chitosan (or chitin) was washed repeatedly in 10 × volumes of H_2O, pelleted by centrifugation at 10,000 rpm, and then air-dried. The air-dried sheets of chitosan were resuspended in sterile H_2O with a tissue grinder just prior to use. Some chitosan was also further purified by dissolving the 80-mesh particles in 0.1 N acetic acid, removing insoluble material by centrifugation, precipitating chitosan by neutralization, and finally washing the precipitate extensively with sterile H_2O.

The Madera snow crab chitosan is 80% deacetylated and contains 7.49% nitrogen. The shrimp shell chitosan when hydrolyzed 3 h with 6 N HCl, gave an 86% yield of glucosamine. There was no other ninhydrin-positive hydrolysis product. An analysis by Sigma (private communication) indicated that traces of glucose were present in the shrimp chitosan. The shrimp chitosan was utilized throughout this study. Although the more acetylated snow crab chitosan had less biological activity, it was utilized for comparative purposes to indicate more than one preparation of chitosan had phytoalexin-inducing potential.

Shrimp chitosan was further fragmented with the nitrous acid treatment described by Crosby (7). The shrimp chitosan (15 g) was dissolved in 600 ml of 1% acetic acid. Sodium nitrite (1%) was slowly dissolved (final content, 1% w/v) with the chitosan while mixing. This chitosan solution was stirred for 1 h at 22 C,

[1] This work was supported in part by National Science Foundation Grant PCM-7712924.

[2] Scientific Paper No. 5440, College of Agriculture Research Center, Washington State University, Pullman, Washington.

neutralized with 1 N NaOH, and dialyzed against three, 20 × volumes of deionized H_2O for 3 days at 4 C. The suspension of crystallized chitosan particles was utilized directly after a brief dispersion in a tissue grinder or was frozen with liquid N_2 and stored at −80 C.

Pisatin Assay. Suspensions of chitosan fragments from 80-mesh powder preparations or from the nitrous acid cleavage process were applied to the exposed endocarp surface of freshly excised immature pea pods. Pisatin of each 0.5-g lot of pea pods was extracted overnight with 10 ml of hexane. The pisatin in the residue of the hexane extract was quantitated on the basis of its A_{309} in ethanol. Fractionation of the hexane extract on TLC developed with chloroform indicates that pisatin is the only compound whose A can be detected at 309 nm.

Induction of Resistance to *F. solani* f. sp. *pisi* in Pea Pods with Chitosan or the Incompatible Pathogen *F. solani* f. sp. *phaseoli*. Shrimp chitosan or spore suspensions of f. sp. *phaseoli* (2.4×10^6 spores/ml) were applied to pea endocarp tissue before, with, or after application of the pathogen f. sp. *pisi* (2.3×10^6 spores/ml). The growth of both fungi on uniform-sized epidermal sections of endocarp tissue was observed cytologically at 24 and 48 h. The two fungi can be distinguished with reasonable certainty even after 24 h on the pea pod. However, the growth of all spores on pod tissue was used as a measure of induced resistance when both organisms were on the same tissue. The growth of the 50:50 mixture was compared with each pathogen growing alone on the pod tissue.

Fungal Wall Preparation. Macroconidia of *F. solani* were harvested from a pea pod-supplemented PDA medium. The fungal spores were grown in Vogel's (27) medium (supplemented with 500 mg/l casein hydrolysate) until the mycelia were three to five times as long as the macroconidia. These germinated spores were suspended in a cold lysis buffer (11) (0.3 M sucrose, 40 mM $NaHSO_3$, 25 mM Tris-HCl, 10 mM $MgSO_4$, 0.5 mM EDTA, and 0.3% Nonidet P-40 by volume, final pH adjusted to 7.4) and homogenized in a VirTis blender at 35,000 rpm for 5 min at 0 C. The cell wall fragments then were pelleted at 5,000 rpm for 5 s. This pellet then was dispersed in 10% Triton X-100 and sonicated for four 2-min periods in an ice bath. Again the cell wall pellet was recovered by centrifugation at 5,000 rpm for 5 s, dispersed in water, and resonicated. This last step was repeated until the walls appeared empty and clean in the light microscope at × 450 magnification and until there was no detergent foaming.

Cell Wall Preparations by Enzyme Digestion. The carbohydrates of the cell walls prepared above were further purified by digesting the walls 24 h with 1 mg/ml ribonuclease I (Sigma) and 24 h with 1 mg/ml type I papain (Sigma). The cell wall residue was redispersed in sterile H_2O and centrifuged repeatedly until the enzyme protein was removed. A final sonication of the cells yielded a totally amorphous material designated as "cell wall carbohydrate."

Chitosan Assay. A method for the direct quantitation of the hexosamine-containing polymers within fungal cell walls has been reported (10). Nitrous acid treatment specifically depolymerizes polymers containing hexosamines with a primary amino group (10). The nitrous acid treatment discriminates between chitin and chitosan because nitrous acid only affects deamination and depolymerization if the polymer carries free amino groups. This process, which degrades the glucosamine residues of chitosan to 2,5-anhydromannose, does not affect a pure polymer of *N*-acetylglucosamine (chitin) but probably degrades any glucosamine-rich segments of chitin (10). The conversion of chitosan to 2,5-anhydromannose was utilized in conjunction with an assay for the 2,5-anhydrohexoses devised by Tsuji *et al.* (26). In this assay, 2,5-anhydrohexose reacts with MBTH[3] and ferric chloride to yield an intense blue-purple color which is assayed at 650 nm. The procedure followed was precisely that of Ride and Drysdale (22). Chitin and chitosan were utilized as standards. Free mannose hydrochloride and *i*-erythritol reacted with the nitrous acid-MBTH stain but to a lesser extent than did chitosan. The color reaction was negative for untreated glucosamine and for nitrous acid-MBTH-treated myoinositol, mannitol, galactose, sorbose, anhydroglucitol, arabitol, galacturonic acid, xylose, trehalose dihydrate, and arabinose.

Glucosamine Assay. The glucosamine content of 6 N HCl-hydrolyzed shrimp chitosan purchased from Sigma and *F. solani* cell walls was quantitated by the Washington State University Bioanalytical Laboratory on an amino acid analyzer.

Estimation of Chitosan in *Fusarium* Macroconidia. The chitosan content of *Fusarium* macroconidia accessible to the nitrous acid treatment was directly estimated using the nitrous acid-MBTH technique described above. *F. solani* spore suspensions (1.7×10^7 spores/1.5 ml) were applied to the endocarps of 3 g pea pods, dispersed in droplets in a Petri plate, or cultured in 50 ml Vogel's medium in a shake culture. The spores were recovered from the pods and Petri plates with a camel's hair brush in a total volume of 50 ml sterile H_2O. The macroconidia were pelleted at 10,000 rpm. The spore pellet was resuspended in 1.5 ml H_2O counted and was analyzed for chitosan according to Ride and Drysdale (22).

Histological Localization of Chitosan in Plant and Fungal Cells. The hexosamine-specific color reaction (22) described above was also utilized to detect histologically the accumulation of glucosamine-containing polymers in fungal cells and in healthy and infected plant cells. The entire pea pod or spore preparation was processed as described above, except that the incubation period with MBTH was extended to 1 h at 22 C, and the samples were not boiled. Tissue sections were prepared by hand with a razor blade and were viewed in the light microscope.

Hexosamine-containing polymers were digested from these tissues and were also concentrated and chromatographed on Whatman 3MM paper employing the three chromatographic solvent systems described by Browder and Beevers (6). The developed chromatographic sheets then were sprayed with the MBTH and $FeCl_3$ solutions used to detect the characteristic blue compounds. The blue compounds recovered always co-chromatographed with the derivative compounds resulting from the nitrous acid processing of glucosamine or shrimp chitosan.

DNA Chitosan Binding Evaluations. DNA binding to chitosan was examined using a revision of the technique of Yu *et al.* (28). Highly polymerized calf thymus DNA (Sigma) was dissolved in phosphate buffer to make a 0.1 mg/ml solution and sheared by sonication for three 2-min intervals. The DNA solution was submerged in ice water during sonication to prevent insoluble material. The supernatant was adjusted to an $A_{260} = 1.0$ and dialyzed against phosphate buffer prior to use. Columns (6.5 × 1 cm) containing 250 mg highly washed cellulose chitosan and chitin were packed to a column bed volume of 1 ml. The column was equilibrated with phosphate buffer. DNA (110 μg/1.5 ml) was applied to the column at room temperature. The DNA was eluted with a discontinuous NaCl gradient produced by manually applying 1.0-ml and subsequently 2.5-ml increments of increasingly higher molarities of NaCl in phosphate buffer.

Cooke microdilution plates and Cooke microdiluters were utilized serially to dilute suspensions of chitosan to determine the end point concentrations which precipitate highly polymerized calf thymus DNA within 24 h at 22 C. Freshly prepared concentrations of DNA and chitosan near the precipitation end point, but with no immediate detectable precipitation, were used to follow the melting-temperature profiles of DNA. DNA melting-temperature profiles were simultaneously recorded in a Gilford spectrophotometer. The temperature of the cuvettes was pro-

[3] Abbreviation: MBTH: 3-methyl-2-benzothiazolone hydrazone hydrochloride.

grammed to increase 1 C/min. The temperature in the cooling cycle decreased 10 C/min.

RESULTS AND DISCUSSION

Chitosan was implicated in the pea pod-*F. solani* interaction as an elicitor of phytoalexin production, an inhibitor of fungal growth, and a chemical which can protect pea tissue from infection by a pathogenic fungus.

Phytoalexin-inducing Components in *F. solani* Cell Walls. Water-soluble phytoalexin-inducing components are efficiently removed from *F. solani* cell wall fractions during the rigorous cell wall purification process. Water extracts of these fungal cell walls (170 μl packed cell walls/ml H_2O) do not induce pisatin in 24 h.

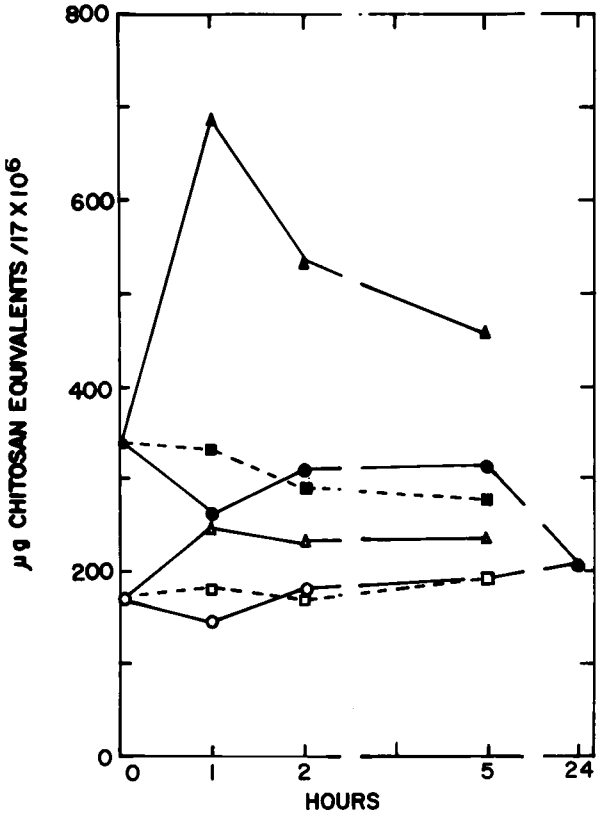

FIG. 1. Chitosan recovered from *F. solani* f. sp. *phaseoli* and f. sp. *pisi* macroconidia following contact with pea pod surface. *F. solani* f. sp. *pisi* (△) and *F. solani* f. sp. *phaseoli* (▲) macroconidia were recovered from the pod at varying intervals following inoculation and assayed for chitosan (see under "Materials and Methods"). Chitosan content of f. sp. *pisi* (○) and f. sp. *phaseoli* (●) grown on the surface of a glass Petri plate. Chitosan content of f. sp. *pisi* (□) and f. sp. *phaseoli* (■) grown in Vogel's (27) medium in shake culture.

Dilute acetic acid (0.1 N) extracts of fungal wall induce 31 μg pisatin/g pod in 24 h. However, the acetic acid-soluble extract of fungal cell walls, which had been incubated 4 h with crude pea enzyme, induce 108 μg pisatin. Surprisingly, the crude pea plant enzyme preparation after 4 h of self-digestion releases enough acetic acid-soluble material to induce 20 μg of pisatin in 24 h. These results suggest that phytoalexin inducers can be released from fungal cell walls (or plant material) by crude plant enzyme preparations. We demonstrated (15) that *F. solani* f. sp. *phaseoli* spores neither induce host responses nor stop growing on pea pod surfaces unless the spore makes direct contact with the plant cell. Further, *Fusarium* macroconidia germinated in liquid culture medium do not release optimal amounts of phytoalexin elicitor (9) until the fungal growth rate begins to decline (7 days), whereas the same spores applied directly to pea tissue can start inducing phytoalexin production within 6 h (25). There seems to be a required processing of fungal phytoalexin elicitors which may be accomplished by enzymes present in the plant cell or, in the case of elicitors produced in shake culture, by enzymes released from fungal cells as the mycelia age or lyse. Hydrolytic enzymes may be required to release and cleave-to-size macromolecules. The chitosan in the fungal cell may be one such macromolecule.

Chitinous Compounds in *F. solani* Walls. The purified and acid-hydrolyzed cell walls of *F. solani* f. sp. *pisi*, a pathogen of peas, and *F. solani* f. sp. *phaseoli*, a pathogen of beans, were found to contain 20.6 ± 1.5% and 19.7 ± 0.4% glucosamine, respectively. The chitosan detectable in nonhydrolyzed fungal walls, using the nitrous acid-MBTH method (22), was 1.2% for *F. solani* f. sp. *pisi* and 1.5% for *F. solani* f. sp. *phaseoli*. Thus, it appears that chitin is a major cell wall component, and chitosan is present only in small quantities. Since the method for quantitating chitosan also detects segments of glucosamine polymers within chitin, the actual percentage of pure chitosan may be lower.

Chitosan Content of Macroconidia following Contact with Plant Tissue. Chitosan effectively mimics the *Fusarium* macroconidium in inducing host responses, such as phytoalexin production; thus, if this compound is centrally involved with the host-parasite interaction, it should be readily detectable in infecting *Fusarium* macroconidia. *F. solani* f. sp. *phaseoli* spores contain approximately twice as much assayable chitosan as *F. solani* f. sp. *pisi* prior to contact with the host tissue (Fig. 1). Chitosan content of *F. solani* f. sp. *phaseoli* increased up to 100% within 2 h after contacting plant tissue, whereas the chitosan content of *F. solani* f. sp. *pisi* (10.3 μg/10^6 spores) increased up to 43% within 2 h after contacting the plant tissue. The precision of this chitosan analysis of macroconidia is influenced by the following factors: *F. solani* f. sp. *phaseoli* spores are slightly larger (1 μm in length) than f. sp. *pisi*; some of the material staining positively for chitosan when observed histologically appears to be loosely attached to the fungal cell surface and is probably lost during spore recovery; conversely, much of the stain-specific material remains in the spore and is not detected in the assay. Deacetylated segments of chitin (which is rarely fully acetylated) also react positively to the chitosan assay.

Table I. *Pisatin-inducing Potential of Chitosan Compared with Cell Walls of F. solani*

Treatment compound	Pisatin Produced in 24 h at Following Applied Concentrations (μg/ml)									
	2,000	1,000	500	250	125	62.5	31	15.6	7	3
	μg/g pod tissue									
Chitosan[a]	495	325	301	388	88	34	56	52	16	
Cell walls[b] of *F. solani*										
f. sp. *pisi*	106	83	80	36	21	24	2	6		
f. sp. *phaseoli*	237	130	83	74	36	35	35	9	0	tr.

[a] Chitosan was washed thoroughly and finely ground in a mortar. Chitosan suspended in 250 ml H_2O was applied to endocarps of 0.5 g immature pea pod tissue.

[b] Purified *F. solani* cell walls were dried, weighed, and resuspended in 250 ml sterile H_2O.

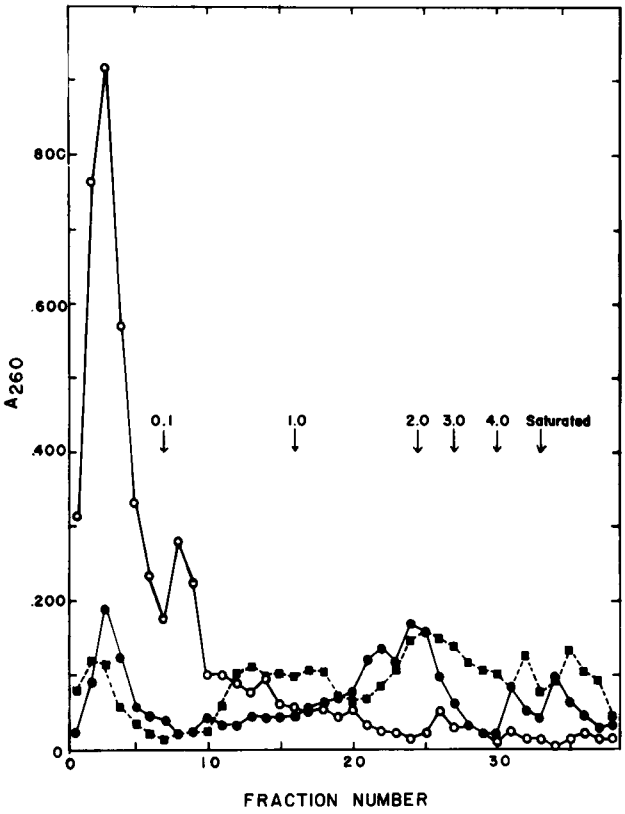

FIG. 2. Affinity of sonicated calf thymus DNA on cellulose, chitin, or chitosan columns. Solutions of sonicated DNA (110 μg/1.5 ml) were applied to columns (6.5 × 1 cm) containing 1-ml bed volumes of cellulose (○), chitin (■) or chitosan (●). The DNA was eluted with NaCl solutions in phosphate buffer (pH 6.7). One-ml volumes of increasing concentrations of NaCl solutions were added manually with each concentration being 0.1 M higher than the previous one until the NaCl concentration reached 2.0 M.

Chitosan and Fungal Cell Walls as Elicitors of Pisatin Production. Chitosan effectively induces phytoalexin pisatin when applied to pea pod endocarps (Table I). Pisatin induction was readily detectable 24 h after pods were treated with only 7 μg milled 80 mesh or with 0.9 μg nitrous acid-cleaved chitosan/ml. Chitosan treatments of 2 mg/ml induced pisatin in quantities comprising more than 0.2% of the tissue dry weight. The pisatin-inducing potential of chitosan was considerably greater than that of purified cell walls of *F. solani*. In comparable tests, other carbohydrates, such as cellulose, cellibiose, laminarin, polygalacturonic acid, inulin, carrageenan, amylopectin, amylose, chitin, acid hydrolysis products of chitin, *N*-acetylglucosamine, glucosamine, and mannosamine, were not effective pisatin inducers. Small but detectable quantities of pisatin are induced with chitin or mannosamine if these compounds are applied at concentrations in excess of 2 mg/ml.

DNA Specificity of Chitosan. Since chitosan mimics the action of fungi in inducing phytoalexin production and many of the compounds known to induce pisatin are highly DNA-specific (12, 17, 18, 23), the affinity of chitosan for DNA was evaluated with comparable affinity columns prepared from cellulose and chitosan. Short fragments of calf thymus DNA were differentially retained by the chitosan column at salt concentrations up to 4.0 M. These same fragments were completely recovered from cellulose columns eluted with 100 mM NaCl (Fig. 2).

Chitosan's affinity for DNA can also be visualized directly. Calf thymus DNA dissolved in 0.005 N Tris (pH 7.5) is precipitated from solution by adding equal concentrations of milled 80-meshed shrimp chitosan. Calf thymus DNA solutions (250 μg DNA/ml 0.005 N Tris) are progressively precipitated with nitrous acid-cleaved chitosan suspensions from 118 to 250 μg/ml. The nature of the attachment of the insoluble chitosan fragments to native DNA or DNA-methyl green (Sigma D-2376) can be observed directly with the fluorescence microscope (Fig. 3). The association of chitosan with DNA slightly distorts the melting temperature profile of calf thymus DNA (Fig. 4).

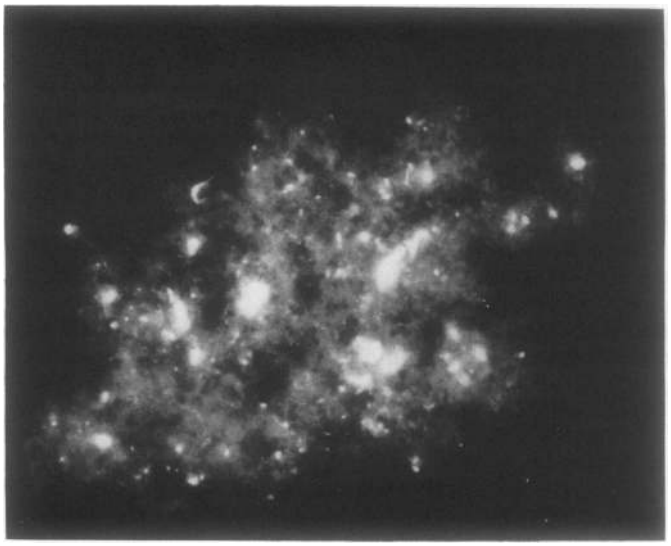

FIG. 3. Fluorescence microscope photograph of DNA-methyl green precipitated by chitosan. The fluorescence of a nitrous acid-cleaved chitosan crystal observed as the dull fibrous background has complexed (24 h) with and precipitated the blue fluorescing (intense white) DNA. As the content of DNA increases, the entire crystal becomes associated with precipitated DNA. (× 450 magnification, filter BG-3 between the light source and sample, filter 47 between the objective and image plane).

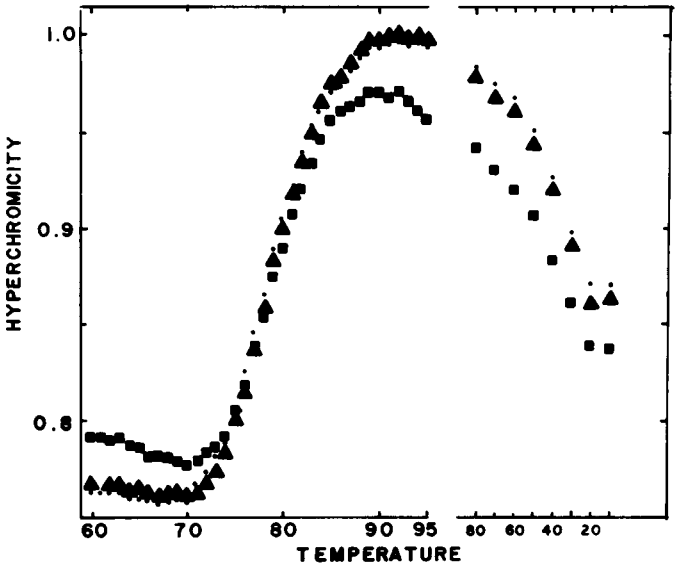

FIG. 4. Melting temperature profile of calf thymus DNA in the presence of varying concentrations of nitrous acid-cleaved shrimp chitosan. Highly polymerized calf thymus (50 μg/ml 5 mM Tris, pH 7.5) was mixed with 25 (▲), 12.5 (●), or 0.0 (■) μg chitosan and immediately heated at a rate of 1 C increase/min, and finally cooled at 10 C decrease/min.

Chitosan as an Inhibitor of *F. solani*. Chitosan (milled to ~5-μm particles) inhibits the germination and growth of *F. solani* f. sp. *phaseoli* and f. sp. *pisi* at 31 and 62 μg/ml, respectively (Table II).

Table II. *Comparison of Chitosan and Chitin as Inhibitors of F. solani*

About 24 propagules were added to dilution chambers in 0.025 ml Vogel's medium (27). Suspensions of the additive were applied to wells in 2-fold dilutions ranging from 1,000 to 7 μg/ml. Growth was recorded at 24 and 66 h. The concentrations given in the table are the minimal concentrations which give complete inhibition.

Additive to medium	Minimal Growth-inhibiting Concentration			
	F. solani f. sp. *phaseoli*		*F. solani* f. sp. *pisi*	
	Within 24 h	Within 66 h	Within 24 h	Within 66 h
	μg/ml			
None				
Chitosan	31	62	62	125
Chitin	1,000	>1,000	1,000	>1,000
Cell walls of f. sp. *pisi*	1,000	>1,000	1,000	>1,000
Cell walls of f. sp. *phaseoli*	1,000	>1,000	500	>1,000

Table III. *Induced Resistance Due to F. solani f. sp. phaseoli*

Pea pods 2 cm in length were split and inoculated with 10 μl/pod half of *F. solani* f. sp. *phaseoli* (2.4×10^6 spores/ml) and *F. solani* f. sp. *pisi* (2.3×10^6 spores/ml) following the schedule in the table. To be considered positive for growth, the germ tube had to be at least 5 μm in length. Per cent growth was determined by randomly counting four samples of 100 spores each and averaging the growth numbers.

F. solani f. sp. Inoculum schedule	Per Cent Growth			
	24 h		48 h	
		SE $\bar{X}$		SE $\bar{X}$
phaseoli 0 time	0	0	0	0
pisi 0 time	20	1.8	50	2.6
phaseoli and *pisi* mixed 0 time	15	1.3	25	2.7
phaseoli minus 1 h, *pisi* 0 time	4	0.8	7	0.8
phaseoli minus 3 h, *pisi* 0 time	2	2.3	9	1.4
phaseoli minus 24 h, *pisi* 0 time	0	0	0	0
pisi minus 1 h, *phaseoli* 0 time	10	1.8	20	0.8
pisi minus 3 h, *phaseoli* 0 time	5	2.6	20	1.4

Table IV. *Effect of Chitosan on the Colonization of F. solani in Pea Endocarp Tissue*

Pathogen[a]	Carbohydrate Applied[b]	Time Carbohydrate Applied	Reaction[c]	
			5 Days	7 Days
F. solani f. sp. *pisi*	None		Sus	Sus
F. solani f. sp. *pisi*	Chitosan	zero time	Res	Res
F. solani f. sp. *pisi*	Chitosan	minus 4 h	Res	Res
F. solani f. sp. *pisi*	Chitosan	minus 12 h	Res	Res
F. solani f. sp. *phaseoli*	None		Res	Res
F. solani f. sp. *phaseoli*	Chitosan	minus 4 h	Res	Res
F. solani f. sp. *phaseoli*	Chitosan	minus 12 h	Res	Res

[a] Five μl of a 3×10^6 spore/ml solution was applied to the endocarp of each immature pea pod half.

[b] Ten mg/ml suspensions of 80-mesh chitosan.

[c] Reaction: Sus = susceptible, Res = resistant.

A fungistatic effect of chitosan on form *phaseoli* is observed at 15 μg/ml. Form *phaseoli* appears to be somewhat more sensitive to native chitosan than form *pisi*. However, the effective level of chitosan required completely to inhibit growth is dependent on the particle size of chitosan and the spore density. Well dispersed chitosan particles 5 μm or less in diameter are more inhibitory to growth of fungi than 80-mesh particles (1). Chitosan levels required to completely inhibit growth of *F. solani* were reduced significantly by chemically cleaving chitosan with the nitrous acid treatment of Crosby (7). The growth of both forms of *F. solani* conidia was completely inhibited for 48 h with 3 to 7 μg nitrous acid-cleaved chitosan/ml.

F. solani spores grown in chitosan-supplemented Vogel's medium in shake culture efficiently recover and clump the milled chitosan particles within the medium. When viewed in the light microscope, the chitosan particles appear to become immediately attached to all available surfaces of the spore. Thus, as the number of spores increase, there are fewer particles of chitosan/spore. Chitosan particles can also "cross-link" spores.

Effect of Chitosan on the Colonization of *F. solani* in Pea Endocarp Tissue. The application of *F. solani* f. sp. *phaseoli*, which is nonpathogenic on peas, to pea tissue with or prior to the pathogen *F. solani* f. sp. *pisi* generates a state of resistance against both fungi (Table III). To determine if chitosan can mimic the non-pathogen in protecting pea tissue from *F. solani* f. sp. *pisi*, it was applied to the pea endocarp surface with or prior to this pathogen (Table IV). Chitosan (10 mg/ml) applications protect pea endocarp tissue from the pathogen for at least 17 days after inoculation if applied with or prior to the pathogen. This long-term protection can be obtained with chitosan concentrations as low as 500 μg/ml. Short-term protection (24 h) is possible with 30 to 500 μg/ml of chitosan. Nitrous acid-cleaved chitosan applied 24 h in advance of *F. solani* f. sp. *pisi* induced resistance to this pathogen at concentrations as low as 2 μg/ml. The hexosamine polymers applied at these low concentration rendered the entire endocarp surface resistant. Inasmuch as these levels of chitosan activity will directly inhibit fungal growth in liquid culture, we presently do not know if the protective effect is involved with the action of chitosan on the plant host. However, chitosan applied alone has the potential to elicit observable host responses such as phytoalexin production and the "hypersensitive response" (yellow-green lesions around the chitosan particles). These "lesions" appear similar to lesions caused by *F. solani* f. sp. *phaseoli* inoculations which induces protection against *F. solani* f. sp. *pisi*.

Histological Localization of Chitosan in the Host-Parasite Interaction. The nitrous acid-MBTH assay of Ride and Drysdale (22) can be utilized histologically to localize chitosan in tissue sections because it develops a blue, stable color. Within 24 h after tissue was treated with *F. solani* f. sp. *phaseoli* (Fig. 5F), *F. solani* f. sp. *pisi* (Fig. 5G), or chitosan (Fig. 5H), there is an accumulation of material within the adjacent plant cells which reacts positively with the chitosan stain, which suggests that the plant tissue accumulates some hexosamine polymers. A dense accumulation of chitosan-positive stain in the pea tissue occurs adjacent to the fungal macroconidia. After 48 h, accumulations of stain were observed in the germ tubes of the macroconidia of both *F. solani* f. sp. *phaseoli* and f. sp. *pisi* spores in which growth was terminated (Fig. 6). The accumulation of chitosan (in multiple preparations and observations of more than 300 lesions) appears to be greater and more readily dispersed in adjacent plant cells of the compatible reaction than those of the incompatible reaction at 48 h (Fig. 6, B and C).

Chitosan Content in Dormant Spores. Chitosan is both a natural component of *Fusarium* and an effective inhibitor of macroconidia germination; therefore, chitosan content of dormant macroconidia and chlamydospores was also estimated cytologically with the chitosan stain. Figure 5E shows the localized accumulation of chitosan within a chlamydospore. The unstained controls (not shown) were colorless. Dormant macroconidia produced in shake culture (Fig. 5J) also stain positively for chitosan. Macroconidia in fresh media, which were stained as germination was initiating, show a clearing of the light background of chitosan stain in the

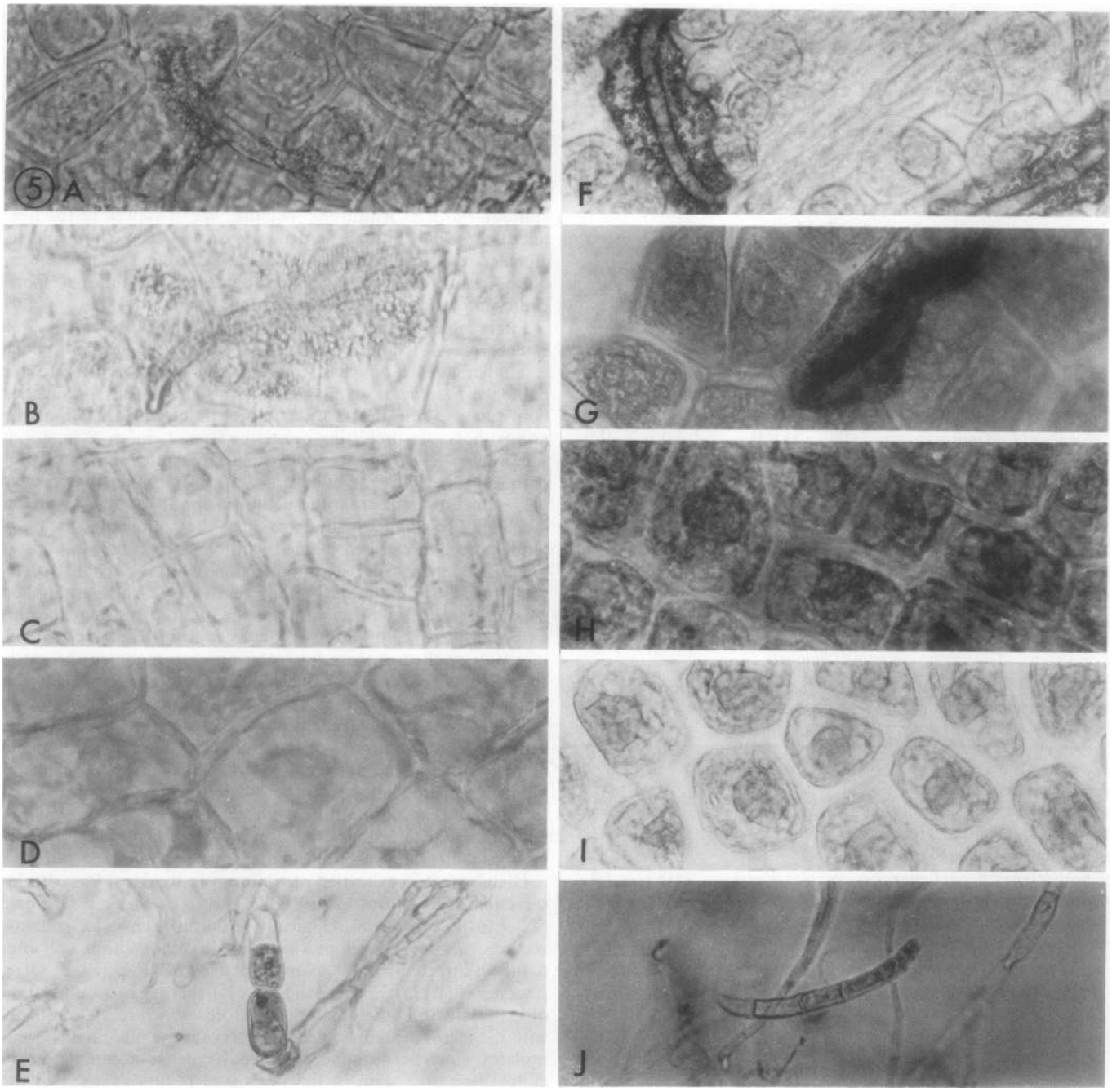

FIG. 5. Light microscope (× 450) photographs showing accumulations of chitosan in infected tissue and in dormant spores. Fresh sections of pea pod tissue were compared 24 h following treatment, *F. solani* f. sp. *phaseoli* (A), *F. solani* f. sp. *pisi* (B), 1 mg/ml chitosan (C), and H_2O (D), with their respective chitosan-stained (see under "Materials and Methods") sections (F, G, H, and I). A chlamydospore (E) and a dormant macroconidia (J) also accumulate chitosan-positive stain.

vicinity of the germinating tip.

CONCLUSIONS

Our observations suggest that the chitosan produced by the fungus in the host-parasite interaction both enters the plant cells and accumulates within the fungal cell. The action of chitosan in initiating phytoalexin production, protecting pea tissue from *F. solani* f. sp. *pisi*, and/or directly terminating fungal growth indicates it may have a central role in disease resistance.

Part or all of these functional attributes may well be involved in total disease resistance. Inasmuch as nitrous acid-cleaved chitosan molecules are fungicidal at hormonal levels, this finding suggests that the potential to cause a hyperaccumulation of chitosan within or around the fungus may be more important in resistance than the host tissue's accumulation of pisatin which is a relatively weak antifungal agent. The induction of pisatin by compounds which readily associate with DNA is accompanied by increases in the level of phenylalanine ammonia lyase (18) and a differential rate of synthesis for many of the pea proteins (12). This differential rate of protein synthesis also occurs when nitrous acid-cleaved chitosan or *F. solani* spores are the inducers (W. Wagoner, and L. Hadwiger, unpublished). Thus, the action of chitosan on the host which is most beneficial to disease resistance may be one of increasing or maintaining certain enzymes possibly those with the potential to hydrolyze fungal cell walls (21).

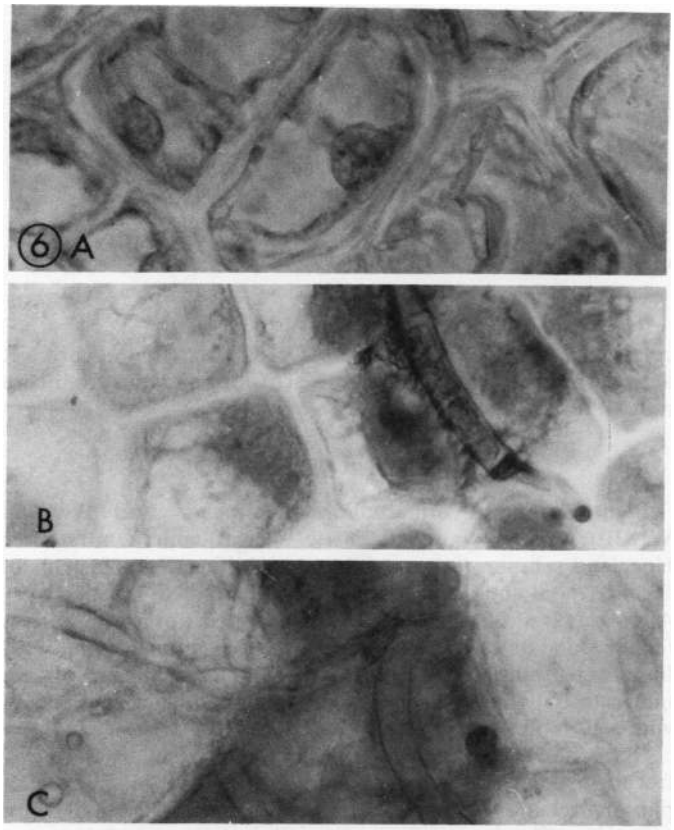

FIG. 6. Light microscope (× 450) photographs showing chitosan accumulation (darkly stained regions) in pea pod endocarp tissue 48 h after treatment with water (A) inoculation with *F. solani* f. sp. *phaseoli* (B), or *F. solani* f. sp. *pisi* (C).

The ultimate effect of chitosan on the specificity of the disease resistance response could depend on numerous interactions. The availability of chitosan may be influenced by many factors in the host or pathogen, including its rate of synthesis or degradation, its hydrolytic release from the fungal walls, and/or the rate at which it is transported across the host-parasite interface.

The large accumulation of chitosan in dormant chlamydospores suggests that chitosan may function in the natural onset of dormancy. These observations point to the potential importance of hexosamine polymers as regulatory compounds. Finally, since chitosan inhibits the growth of many fungi it may also have potential as a commercial fungicide (1).

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