Plant Physiol. (1981) 67, 170–175
0032-0889/81/67/0170/06/$00.50/0

Localization of Fungal Components in the Pea-*Fusarium* Interaction Detected Immunochemically with Anti-chitosan and Anti-fungal Cell Wall Antisera[1, 2]

Received for publication May 14, 1980 and in revised form July 22, 1980

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ABSTRACT

Antisera specific for purified cell walls of *Fusarium solani* f. sp. *pisi* and *phaseoli* and of shrimp shell chitosan were utilized as immunochemical probes to determine the location of fungal components in the pea-*Fusarium* interaction.

Within 15 minutes after inoculation, fungal cell wall components appear to enter the plant cell and to accumulate inside the plant cell wall as fungal growth on the plant tissue is inhibited. The accumulation patterns of chitosan and all components containing hexosamine polymers resembled those of the fungal wall components.

Chitosan is present on, and is released from, the outer surface of the fungal spore. Within 15 minutes after applying [^{3}H]chitosan to the surface of the plant tissue, the label is readily detectable within the plant cytoplasm and conspicuously detectable within the plant nucleus. It is proposed that the potential for transport of chitosan between the spores of *Fusarium solani* and pea cells, in addition to its potential to inhibit fungal growth and elicit disease resistance responses, suggests chitosan has a major regulatory role in this host-parasite interaction.

Chitosan, a polymer of β-1,4-linked glucosamine, has been implicated in pea-*Fusarium solani* interactions as an elicitor of phytoalexin production, an inhibitor of fungal growth (2), and a chemical which can protect pea tissue from infection by *Fusarium solani* f. sp. *pisi*, a pathogen of peas (10). A histochemical analysis (10) utilizing a hexosamine-specific stain (18) indicated there are significant accumulations of chitosan in fungal spores following inoculation, especially in germ tubes in which growth has been terminated following contact with plant tissue. Chitosan was also detected in plant cells adjacent to the germinating fungal spore. Finally, the accumulation of chitosan in dormant fungal spores suggested that this accumulation serves as a natural dormancy factor.

The objectives of this paper are to examine by immunochemical techniques the localization of chitosan and other fungal wall components in the pea-*Fusarium* interaction. The accumulation of these compounds was observed within the period when resistance is first expressed against *F. solani* f. sp. *phaseoli* (24).

MATERIALS AND METHODS

F. solani f. sp. *pisi*, strain P-A (American Type Culture Collection 38136) and *F. solani* f. sp. *phaseoli*, strain W-8 (American Type Culture Collection 38135), were obtained from R. J. Cook and D. J. Burke, respectively. The *Pisum sativum* pods were from the Alaska-type variety "Dot."

Reagents and Serum. Normal sheep serum, 3,3′-diaminobenzidine (DAB), and peroxidase antiperoxidase were obtained from Cappel Laboratories, Inc., Cochranville, PA. FITC[3] and RITC were obtained from Sigma.

Shrimp chitosan was tritiated by New England Nuclear using the tritium gas exposure labeling technique. Eighty-mesh chitosan was ground to a fine powder and washed repeatedly in sterile distilled H_2O. Fifty mg of this material was dissolved in dilute acetic acid prior to tritiation. Following tritiation, the chitosan was crystallized from the mother liquor by adjusting the pH to 8.0 with NaOH. The crystals were washed repeatedly in sterile H_2O, redissolved in acetic acid, and reprecipitated with NaOH. A final water wash was employed to remove Na-acetate.

Pea Endocarp Inoculation Procedure. Macroconidia of *F. solani* f. sp. *pisi* (virulent) or *F. solani* f. sp. *phaseoli* (avirulent) were suspended in sterile H_2O at concentrations of 3.0×10^6 spores/ml. Immature pea pods were halved and the freshly exposed endocarp tissue was inoculated with 25 μl of the appropriate spore suspension and allowed to incubate at 25 C in a moist chamber for the desired length of time. At the termination of incubation, endocarp sections were examined using electron, fluorescent, or light microscopic techniques. Additional sets of pods were inoculated and examined at comparable intervals to check the virulence of the cultures. The virulent form *pisi* is compatible with pea tissue and, within 7 days, causes coalescing lesions (susceptible reaction). The avirulent form *phaseoli* is incompatible and causes pin-point lesions (resistant reaction).

Preparation of Anti-chitosan Antisera. Milled shrimp chitosan, previously described (10), at a concentration of 1 mg/ml PBS (pH 7.2) (0.1% NaCl, 5 mM Na-phosphate), was mixed with an equal volume of incomplete adjuvant and utilized as the antigen source. The antigen was injected at four sites (50 μl/site) into 8-week-old New Zealand Rabbits. This was done weekly for 3 weeks with a subsequent booster injection given 2 weeks later and another injection given 1 week prior to bleeding. Freshly drawn blood was allowed to stand for 1 h at 25 C for clot contraction. The serum fraction containing antibodies was freed from remaining red blood cells by centrifugation and stored at −70 C.

Preparation of Anti-cell Wall Antisera. Cell walls of *F. solani* f.

[1] This work was supported in part by National Science Foundation Grant 7712924 and Sea Grant R/X-8. This is scientific paper No. 5639, College of Agriculture Research Center, Washington State University.

[2] This paper is dedicated to the memory of Noe Higinbotham, our esteemed colleague at Washington State University.

[3] Abbreviations: FITC, fluorescein isothiocyanate; RITC, rhodamine isothiocyanate; PBS, phosphate-buffered saline; MBTH, 3-methyl-2-benzothiazolone hydrazone hydrochloride.

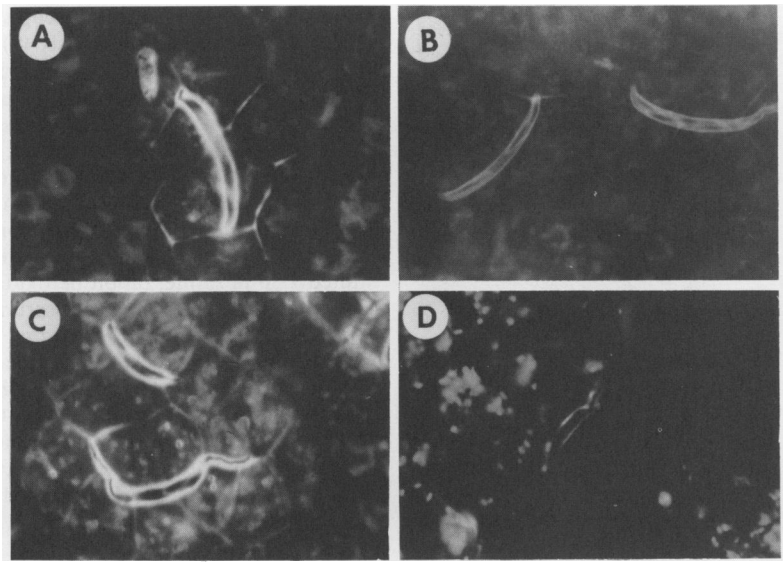

FIG. 1. Immunofluorescent photographs of the pea-*Fusarium* interaction using FITC-conjugated rabbit antisera. All photographs (5-s exposures) are of 5-h treatments with one of the *F. solani* f. sp. on pea endocarp tissue. The fluorescence (bright yellow to yellow-green) has been rated from 0 (no fluorescence) to +4 (very bright fluorescence). A, *F. solani* f. sp. *phaseoli*-infected tissue stained with FITC-conjugated chitosan antiserum. Fluorescence was consistently +4. B, *F. solani* f. sp. *phaseoli* stained with FITC-conjugated normal rabbit antisera. Fluorescence was consistently +1 or less. C, *F. solani* f. sp. *pisi*-infected tissue stained with FITC-conjugated chitosan antisera. Fluorescence was consistently +4 in fungal cell and +3 to +4 in surrounding plant tissue. D, *F. solani* f. sp. *pisi* stained with RITC counter stain and overlayed with glycerol. The fluorescence seen in the photograph is bright red, indicating that it is the rhodamine. There was a small amount of yellow fluorescence (0 to +1) seen in the outline of the fungal cell wall.

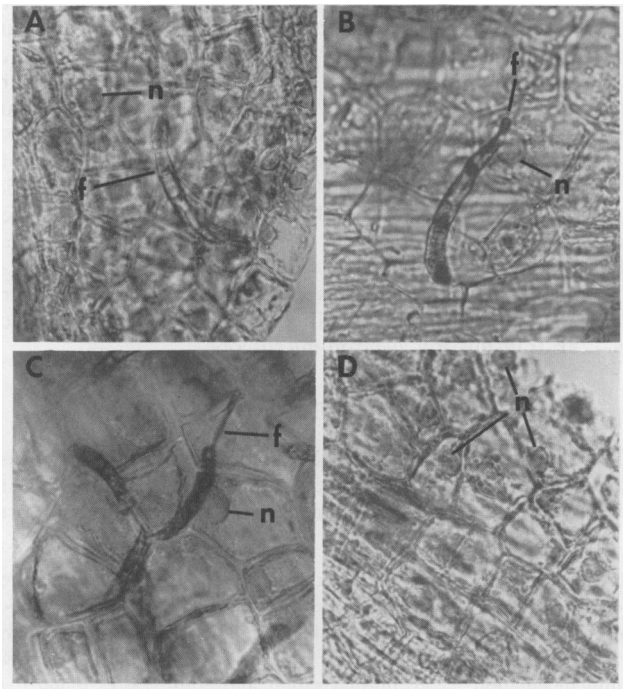

FIG. 2. Nitrous acid-MBTH-stained surface sections of pea endocarp tissue infected with *F. solani* macroconidia. A, *F. solani* f. sp. *phaseoli* 1 h after inoculation; B, *F. solani* f. sp. *phaseoli* 5 h after inoculation; C, *F. solani* f. sp. *pisi* 12 h after inoculation; D, H_2O-treated control. f, fungus; n, plant nucleus.

sp. *pisi* or f. sp. *phaseoli* were purified as previously described (16). Suspensions of the walls (1 mg/ml PBS) were mixed with an equal volume of complete adjuvant and administered as described above.

Determination of Titer. The titer was determined by the method of Parker and Grove (17) with the following exception. Milled shrimp chitosan was dissolved in concentrated acetic acid, precipitated by neutralization with 1 N NaOH, and centrifuged at 6000 rpm for 10 min. The supernatant was discarded, and the chitosan was washed two times with distilled H_2O. After air drying, this chitosan preparation was finely ground in a glass mortar and suspended in Tris buffer (pH 7.2) at a concentration of 1 mg/ml.

Serial dilutions were carried out following the method of Malajezuk *et al.* (15) except that dilution plates were incubated at 25 C for 2 h, after which agglutination was observed under a dissecting scope.

Preparation of Fluorescent Antibody. The methods used in the separation of the γ-globulin, protein determination, and conjugation of the antisera to FITC were precisely those of Schmidt *et al.* (21).

Immunofluorescent Staining of Fresh Tissue. Following the treatment period, thin sections were made from the inoculated endocarp surfaces using a razor blade. The tissue sections were placed, endocarp surface up, on a small cork plate and held flat with pins. The sections were flooded with RITC as prepared by Bohlool and Schmidt (4) and allowed to incubate 15 min at 25 C in a moist chamber. Three to 4 drops of chitosan-conjugated FITC (1:20 dilution in PBS) antiserum were placed over the rhodamine layer and allowed to incubate 20 min at 25 C. The sections next were washed by floating the cork plate, endocarp surface down, in 15 ml PBS for 5 min and then rinsed twice in H_2O for 10 min. Sections then were placed with the inoculated side of the endocarp tissue facing up on a microscope slide, overlayed with a drop of glycerol, and covered with a coverslip. Preparations were examined immediately under the UV microscope. Several dilutions of conjugated antisera were tested; the 1:20 dilution of the conjugated antibodies gave the brightest reaction with the least amount of nonspecific background staining.

Histological Examinations of Hexosamine Polymers in Pea-Fusarium Interaction. The nitrous acid-MBTH assays of Ride and Drysdale (18) and Tsuji *et al.* (25) were utilized histologically to observe localization of hexosamine polymers (chitosan-like material) in tissue sections as previously described (10).

Embedding and Immunochemical Staining of Sections for

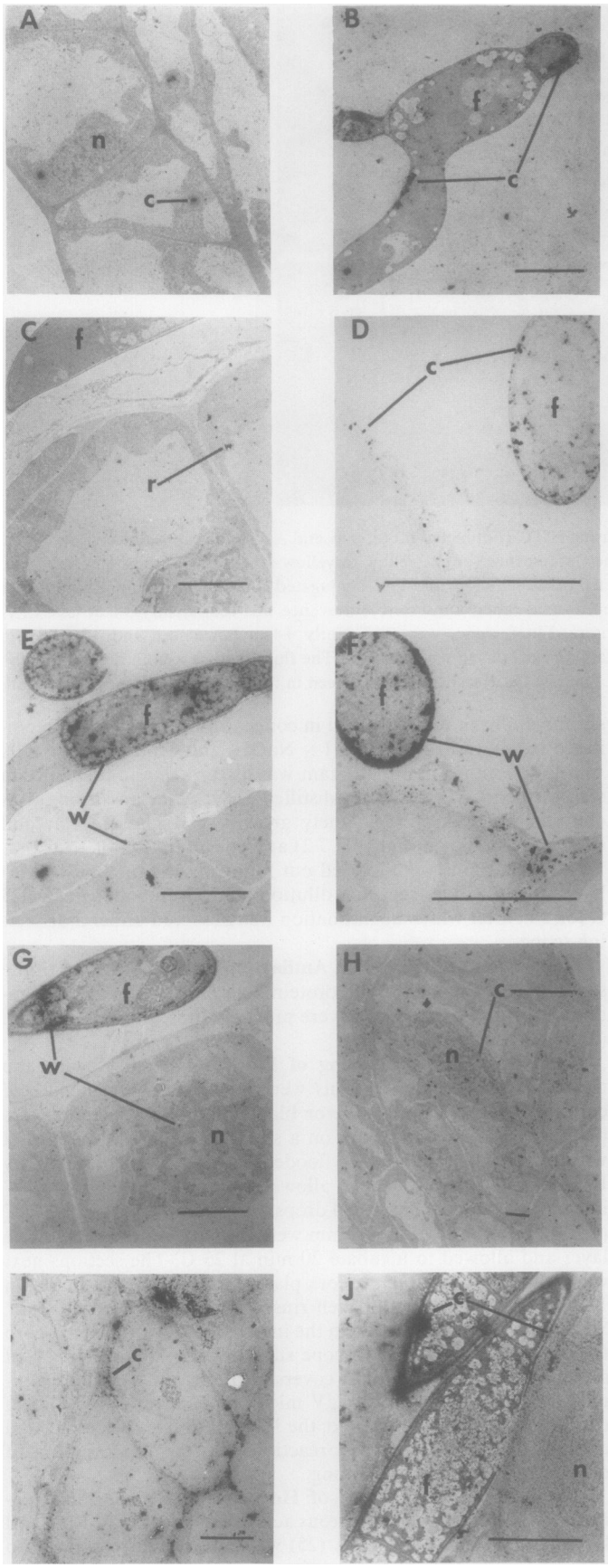

FIG. 3. Electron micrographs of *Fusarium* macroconidia and infected pea endocarp tissue, immunochemically labeled with chitosan or *F. solani* cell wall antisera preparations. A, endocarp tissue, 1 h H_2O-treated non-inoculated control; label: immunospecific chitosan antiserum preparation. B, *F. solani* f. sp. *phaseoli* macroconidia germinated in liquid culture; label: immunospecific chitosan antiserum preparation. C, *F. solani* f. sp. *phaseoli*, 15-min inoculation; label: normal rabbit antiserum. D, *F. solani* f. sp. *phaseoli*, 15-min inoculation; label: immunospecific chitosan antiserum. E, *F. solani* f. sp. *phaseoli*, 15-min inoculation; label: immunospecific f. sp. *phaseoli* cell wall antiserum. F, *F. solani* f. sp. *phaseoli*, 15-min inoculation; label: immunospecific f. sp. *pisi* cell wall antiserum preparation. G, *F. solani* f. sp. *pisi*, 15-min inoculation; label: immunospecific f. sp. *phaseoli* cell wall antiserum preparation. H, *F. solani* f. sp. *pisi*, 15-min inoculation (only endocarp tissue visible); label: immunospecific chitosan antiserum preparation. I, chitosan-treated endocarp tissue, 7-h inoculation; label: immunospecific chitosan antiserum preparation. J, *F. solani* f. sp. *phaseoli*, 5.5-h inoculation; label: immunospecific chitosan antiserum preparation. c, chitosan; w, *F. solani* cell wall; f, fungus; n, plant nucleus; r, normal rabbit antiserum. Bar = 5 μm.

Transmission Electron Microscopy. The embedding procedure used was as described previously (9). The postembedding immunocytochemical staining was as described by Sternberger (23). The embedded 100- to 150-μm sections of control and inoculated pea pod tissue on nickel grids received the immunostaining ingredients in the following order: 5% peroxide; saline; normal rabbit antiserum, 1:30 dilution in saline; anti-chitosan rabbit antiserum diluted 1:100 in 10% normal rabbit serum in saline; saline; normal rabbit antiserum 1:30 in saline; anti-rabbit immunoglobulin G sheep antiserum diluted 1:10 in saline; saline; normal rabbit antiserum diluted 1:30 in saline; rabbit peroxidase antiperoxidase antiserum diluted 1:50 in 1% normal antiserum in saline; 50 mM Tris (pH 7.6); 0.0125% 3,3′-diaminobenzidine in 0.0025% H_2O_2 and 50 mM Tris; distilled H_2O; 4% OsO_4; distilled H_2O.

Autoradiography and Electron Microscopy. Pea pod endocarp tissue was treated with 1 μCi [^{3}H]chitosan/pod half. Following the uptake period, the pod half was washed three times in 50 ml sterile H_2O and immediately immersed in Karnofsky's fixative (14). After 2 h at 4 C, the pieces were washed in 0.2 M cacodylate buffer, fixed in 2% OsO_4 for 1.5 h and washed successively in 0.2 M cacodylate buffer and distilled H_2O. The pieces were transferred to 15% ethanol and then to a 30% ethanolic solution containing 1% uranyl acetate for 3 h. The pieces then were dehydrated via a graded ethanol series and subjected to an ascending series of solutions containing propylene oxide and Spurr's resin (13). After polymerization, the tissue pieces were sectioned on a Porter-Blum MT2-B ultramicrotome equipped with a diamond knife. The microtome sections were floated on 300-mesh copper grids.

The commercial autoradiographic emulsion, Kodak NTE, was processed in the dark according to Salpeter and Bachmann (19) prior to use. One part emulsion was added to 5 parts H_2O and dissolved at 70 C. Following centrifugation for 5 min in a clinical rotor (preheated to 70 C), the bottom of the centrifuge tube, containing closely packed silver halide granules, was chilled, and the supernatant fraction cntaining excess water and gelatin was discarded. One part concentrated silver granules was diluted with 8 parts water and heated to 70 C. A monolayer of this emulsion was applied to the grids with a platinum loop. The period of latent image formation was typically 4 weeks at 4 C in an atmosphere of helium. Grids were developed with Dektol for 1 min at 24 C, rinsed 1 min in water, and rinsed 2 min in rapid fixer. After a final water rinse, grids were air-dried and examined with a Hitachi HN-125E transmission electron microscope.

RESULTS AND DISCUSSION

The specific localization of FITC-labeled anti-chitosan antiserum in the pea-*Fusarium* interaction is shown in Figure 1. Fluorescence is rated from 0 (no fluorescence) to +4 (brightest fluores-

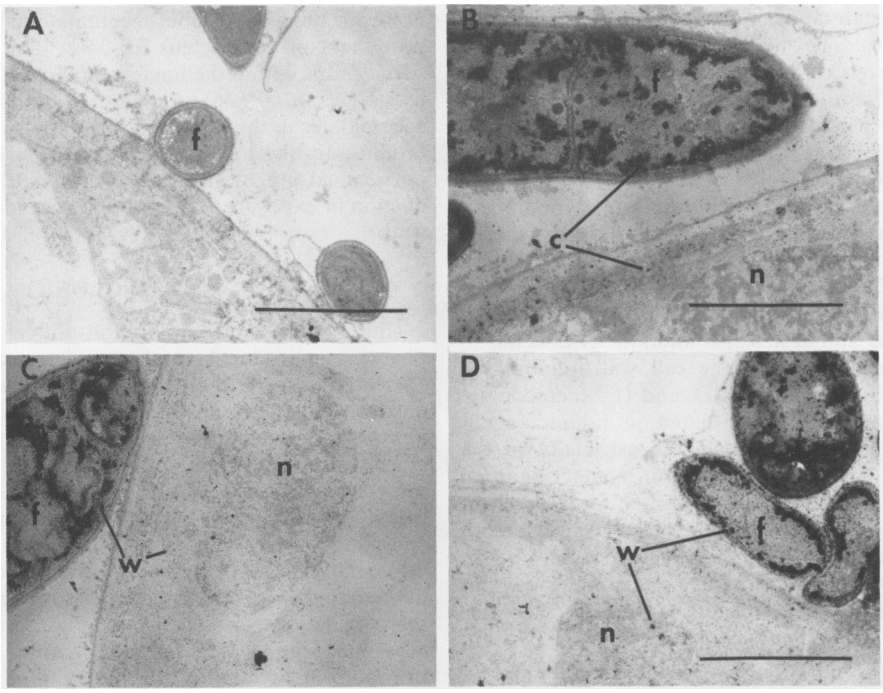

FIG. 4. Electron micrographs of pea endocarp tissue inoculated for 36 h with *F. solani* f. sp. *pisi* and labeled with immunospecific antisera. A, normal rabbt antisera preparation control. B, immunospecific chitosan antiserum preparation; C, immunospecific *F. solani* f. sp. *phaseoli* cell wall antiserum preparation. D, immunospecific *F. solani* f. sp. *pisi* cell wall antiserum preparation. c, chitosan; w, *F. solani* f. sp. cell wall antiserum; f, fungus; n, plant nucleus. Bar = 5 μm.

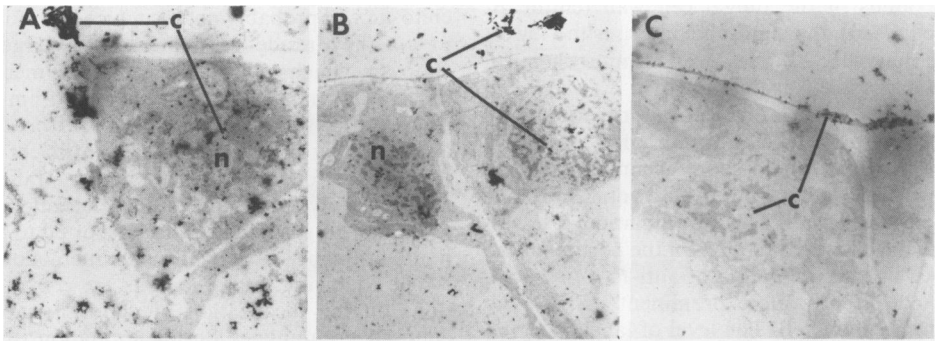

FIG. 5. Autoradiographs of microtome cross-sections of surface pea endocarp cells treated with [^{3}H]chitosan (1 μCi/pod half). A, [^{3}H]chitosan-uptake period, 15 min; B, [^{3}H]chitosan-uptake period, 6 h. C, [^{3}H]chitosan-uptake period, 24 h. n, plant nucleus; f, fungus; c, chitosan.

cence). Within 5 h after inoculation, intense fluorescence (Fig. 1, A and C) is observed at the outer edge of the infecting macroconidiospore, and fluorescence (+3 to +4) is observed in the plant tissue surrounding the macroconidia. This is more noticeable in the compatible reaction (Fig. 1C). The antigen recognized by this fluorescence-labeled technique appears to accumulate most readily along the plant cell walls; however, in the incompatible reaction, it can often be observed in the nucleus of the plant cell. The plant nucleus also receives fluorescent antisera in the compatible reaction; the nucleus is usually less conspicuous.

In contrast, the control preparations of inoculated tissue without immunochemical treatment (Fig. 1D) or stained with FITC-conjugated normal rabbit serum (Fig. 1B) exhibited a very low level of fluorescence (+1). The fluorescence obtained with the normal rabbit serum was consistently lower than the fluorescence (+4) in the inoculated tissue stained with FITC-conjugated chitosan antisera. Autofluorescence of the inoculated tissue was essentially quenched with the RITC treatment up to 6 h after inoculation (Fig. 1D), except for a small amount in the fungal macroconidia (the autofluorescence of the hypersensitivity response intensifies after 18 h and obscures that of the fluorescent antisera). This accumulation of chitosan is typically more extensive in the compatible rather than in the incompatible, reaction within the first 5 h after inoculation.

A hexosamine-specific stain (10) suitable for conventional light microscopy has also been employed to follow the localization of hexosamine polymers in the first 12 h after infection (Fig. 2). Again, hexosamine-containing compounds can be detected at the outer edge of the macroconidia, in the plant nuclei, and in the spore vicinity (Fig. 2, A–C). Within the first hour (Fig. 2A), it is not always possible to associate the blue hexosamine stain (which on the photo appears as a darkened area) with the infecting fungus since many of the fungal spores are detached in the nitrous acid treatment. At 5 h (Fig. 2B) and 12 h (Fig. 2C) after inoculation, however, the spores remain more securely attached and dark stained then can be seen within the fungal spore and in the adjacent tissue. In the incompatible interaction, large accumulations of hexosamine occur in both the germinated fungal spores and in the tips of germinated spores that have been inhibited. In the compatible interaction, macroconidia also accumulate hexos-

amine but, within 12 h, stain appears to leach out of the successfully growing germ tubes. The nuclei of healthy tissue contain lower, but detectable, levels of blue stain, which indicates a low level of endogenous hexosamine polymers. Hexosamine molecules reportedly are present in pea tissue (5).

The electron microscope was utilized (Figs. 3 and 4) to detect label, immunospecific for chitosan or cell walls of *F. solani*, in cross-sections of infected tissue. Protochloroplasts of noninoculated tissue (Fig. 3A) have an affinity for antiserum prepared against chitosan and have no detectable affinity for normal serum. Thus, one must ignore these organelles during analysis for localization of the immunospecific label of pea cell sections obtained from infected tissue. The anti-chitosan immunolabel attaches to regions at the spore surface, within the cell wall, and in the protoplasm of the fungal cell (Fig. 3, B, D, and I). Macroconidia of *F. solani* f. sp. *phaseoli* that germinated in liquid Vogel's medium (26) (Fig. 3B) are sparsely labeled, especially in the rapidly growing germ tubes. Following 15 min contact between host and the incompatible pathogens, the anti-chitosan label increases in the fungal hyphae and within the cell wall of the plant (Fig. 3, D, and H). After 15 min, the chitosan-specific label in plants infected with *F. solani* f. sp. *pisi* can be observed throughout several surface plant cell layers, being present in the cell walls, cytoplasm, and nuclei of the pea endocarp cells (Fig. 3H). Within 7 h after milled 80-mesh chitosan is applied to the endocarp, a heavy accumulation of the chitosan-specific label is distributed at the host-parasite interface and throughout the endocarp cell walls and cytoplasm (Fig. 3, I and J). It appears that chitosan accumulates in the outer portion of the fungal cell (Fig. 3J) and, presumably, is transmitted into the plant cells almost immediately after inoculation.

Of the major compounds of the fungal cell wall (chitin, β-glucans, protein, and chitosan), the chitosan comprises the lowest percentage of the total mass (16, 22). However, immunochemical labeling with antiserum prepared against purified *F. solani* cell walls shows (Fig. 3, E–G) a pattern of localization very closely related to that observed with the anti-chitosan label. Figure 3E indicates that the incompatible fungal spore accumulates label, prepared against its own cell walls, within the cytoplasm of the spore as well as inside the cell wall. The accumulation is less in the tip of the spore which is germinating than in the portion of the spore which is inactive. The accumulation of anti-*F. solani* f. sp. *pisi* cell wall label is consistently more prominent in both the fungal wall and within the plant cell. The level of label observed in the upper cells of multiple endocarp sections suggests that this fungal material is being readily conveyed to plant cells within the first hour of infection. Many, but not all, of the compatible fungal spores of *F. solani* f. sp. *pisi* resume active growth on the endocarp tissue within 24 to 48 h when one or both tips of the macroconidia escape the influence of plant cells. Figure 4, B, C, and D, shows fungal spore tips which do not resume growth after 36 h. This lack of growth is associated with an accumulation of material which accumulates label from anti-chitosan, anti-*F. solani* f. sp. *phaseoli*, and anti-*F. solani* f. sp. *pisi* walls. Again, this suggests that chitosan or similar hexosamine molecules are among the components accumulating in portions of the pathogen in which growth is suppressed.

The plant cell wall is regarded as a barrier to high mol wt compounds, such as chitosan (6). Pea tissue possesses enzymes capable of cleaving chitosan (16) molecules to mol wt which could conceivably pass through the cell wall barrier. [^{3}H]Chitosan was applied to healthy pea tissue to determine if a portion of the molecule actually enters the plant cell. Figure 5 indicates that [^{3}H]chitosan or a chitosan degradation product readily enters the plant cell. The localization pattern of [^{3}H]chitosan label within the cells generally resembles that detectable with anti-chitosan antisera. Much of the label appears to be distributed in the cytoplasm; however, some of the label accumulates in the nucleus and remains prominent in the nucleus for 24 h. The ratio of the number of silver grains within the nucleus to those detected in the cytoplasm was 1.42 (SD = 0.36). A minimal amount of [^{3}H]chitosan label accumulates in the plant cell wall. The heavier wall accumulation of chitosan-like material detected with the anti-chitosan immunochemical preparation may be an indication of enhanced production or exposure of inherent hexosamine polymers in the plant wall.

CONCLUSION

Chitosan is known to inhibit fungal growth and to induce phytoalexin production and disease protection in pea tissue (2, 10, 16). Specific light, fluorescence, and electron microscope analyses indicate that chitosan or a similar hexosamine polymer is an important component in pea-*Fusarium* interactions.

The specific information derived from these sensitive techniques must be interpreted cautiously and in relationship to the appropriate controls. The following technique weakness should be considered. A low level of autofluorescence exists during fluorescent analysis of infected endocarp tissue. An inherent level of hexosamine is present in pea tissue (5) and may fluctuate during the inoculation periods examined with the anti-chitosan antisera label preparation. The autoradiography analyses may trace the transport of metabolic breakdown products of chitosan rather than high mol wt hexosamine polymers. Conversely, the following points are included to elicit confidence in the three major techniques used: (*a*) the nitrous acid-MBTH technique is specific for hexosamine (7, 18, 25) and, since the preparative processing steps deplete pea tissues of low mol wt hexosamine, this stain detects primarily hexosamine polymers; (*b*) the antisera developed against chitosan will probably not recognize low mol wt glucosamine units unless they reside as haptens in a larger complex; (*c*) [^{3}H]-chitosan, which is a high mol wt polymer when applied to the tissue, is taken up in large quantities within 15 min and, therefore little time is available for the metabolic breakdown to hexosamine and assembly into a molecule of sufficient size to be retained in the tissue throughout the sample preparation solvent changes. It appears likely that chitosan-like molecules are both accumulated in the fungal germ tube and transported to plant cells in the early hours of the pea-*Fusarium solani* interaction.

The following interpretations are consistent with the observations on the localization of chitosan in infected tissue. Since chitosan accumulates during formation of dormant spores (10) and inhibits fungal growth at extremely low levels, it may be suppressing growth in *Fusarium* as a natural dormancy factor. There is indeed an accumulation of chitosan within the fungal cell, especially in regions just inside the fungal cell wall in organelles resembling chitosomes (3). The compatible *Fusarium* macroconidia appear to have the potential to prevent or lessen chitosan accumulations in the growing tip of those spores which successfully germinate and grow on pea tissue.

The *Fusarium* spores release chitosan-like material, some of which appears to be rapidly taken up by the endocarp cells and localized within the cell walls, cytoplasm, and nucleus. Thus, most parts of the cell are potential target sites for cellular incompatibility reactions. This liberal distribution might well be expected when the source is external to the cell. For example, when [^{3}H]chitosan is applied to the pea tissue, the large crystals remain outside the cell. The [^{3}H]chitosan which enters the cell is presumably of lower mol wt and is first distributed by cytoplasmic streaming. A significant portion of the label accumulates in the nucleus.

The nuclear localization of [^{3}H]chitosan and fungal wall components suggests a potential site for the initiation of cell compatibility or incompatibility interactions which is an alternative to the cell surface-cell surface sites (1) most commonly examined in host-parasite interactions. The possible direct involvement of the plant

nucleus is compatible with our recent studies on phytoalexin induction which indicate that this response is induced by compounds which initially and specifically modify the pea DNA (8, 11, 12, 20).

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