Cell Death and Membrane Leakage Not Associated with the Induction of Disease Resistance in Peas by Chitosan or *Fusarium solani* f. sp. *phaseoli*

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ABSTRACT

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No correlation was found between disease resistance and host cell death and membrane leakage in the interactions between pea endocarp tissue and *Fusarium solani* f. sp. *pisi* (compatible) or *F. s.* f. sp. *phaseoli* (incompatible). Initially, the pea endocarp tissue responds to both the compatible and incompatible pathogens. After inoculation, both fungi germinated, but their growth was suppressed within 5 hr. However, within 24 hr, the compatible, but not the incompatible, pathogen resumed active growth. Host cell viability as measured by the vital stains, fluorescein diacetate (FDA) and phenosafranin, was still apparent in the incompatible interaction beyond 17 hr postinoculation. At this time, some host cells were also beginning to develop into a hypersensitive response, and cell viability was absent in cells near multiple conidial attachment sites. Viability was reduced more rapidly in the compatible interaction. Fungal cell viability in the presence of chitosan was related to both the chitosan concentration and the length of the exposure. The initial growth inhibitory effect of chitosan is probably not due to a reduction in fungal cell viability since the fungal tissue retained the ability for regrowth and remained FDA fluorescent following exposure to growth suppressing levels of chitosan. Changes in conductivity due to release of electrolytes from damaged plant tissue after fungal inoculation or chitosan treatment did not correlate with disease resistance. However, a sharp increase in electrolyte leakage was observed after 48 hr in the compatible interaction. Chitosan also failed to enhance the release of electrolytes from formae speciales of *F. solani* at concentrations which suppress their germination and growth.

Additional key words: hypersensitive response.

The cell walls of *Fusarium solani* and many other fungi contain polymers of β-1,4-linked glucosamine called chitosan (4,8). Hexosamine-containing oligomers are released from the fungal cell wall during the interaction between pea tissue and *F. solani* formae speciales. These fragments both penetrate the plant cell and accumulate within the fungal cell (9). Chitosan applied externally to *F. solani* macroconidia inhibits germination and growth (1), whereas chitosan applied to pea endocarp tissue activates the disease resistance responses as does inoculation with the incompatible pathogen (8). These responses include the activation of "disease resistance response" genes (17), which have been assayed by hybridization with specific cDNAs cloned from pea mRNAs (6) and by translation of accumulated mRNAs from induced tissue into proteins using both in vivo and in vitro techniques (11).

The mode by which chitosan can both induce plant genes and suppress fungal growth is not completely understood. It is now known that the effective hexosamine polymer size for both functions is a heptamer (or larger) (14) and that the polymer has alternating positive charges along its length as a consequence of alternating orientations of the glucosamine residues. Such positively charged molecules are known to influence both cell membranes (19) and nucleic acid conformation (20). It has been demonstrated that chitosan can localize within plant nuclei, cell walls, and membranes (9). Chitosan can attach in vitro to DNA molecules and change their physical properties (8,9). Chitosan inhibits the accumulation and synthesis of RNA in *F. solani* (13).

The present study was conducted to determine the influence of chitosan and fungal plant pathogens on cell membranes as measured by ion leakage and cell viability using the vital stains,

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fluorescein diacetate (FDA), and phenosafranin. An assessment of the membrane leakage and the viability of plant and fungal cells within the period in which the host resistance responses are initiated is useful in understanding the mechanisms by which resistance is induced. It is also important to know whether chitosan functions in the host tissue directly by activating a response in the contacted cell or indirectly by killing the host cell and triggering the release of a secondary messenger(s) into adjoining cells as proposed for other host-parasite systems (2).

MATERIALS AND METHODS

Organisms and reagents. *F. s.* f. sp. *pisi* strain P-A (American Type Culture Collection 38136), a compatible pathogen of peas, and *F. s.* f. sp. *phaseoli* strain W8 (American Type Culture Collection 38135), an incompatible pathogen of peas, were used throughout this study and were maintained on pea shoot-amended PDA plates. Pea pods were produced on greenhouse grown *Pisum sativum* cultivar "Alaska."

Crab shell and shrimp shell chitosans were obtained from Bioshell, Inc., Albany, OR, and Sigma Chemical Company, St. Louis, MO, respectively.

FDA and phenosafranin were obtained from Sigma.

Inoculation and cell viability assays. *F. solani* macroconidia (1×10^7 per milliliter) of either *F. s.* f. sp. *phaseoli*, a bean pathogen that is nonpathogenic to peas, or *F. s.* f. sp. *pisi*, a pea pathogen, chitosan (5–1,000 μg per milliliter) or water were applied to pea pod endocarp surface as previously described (8). Plant and fungal cell viability following inoculation was examined using the vital stain, FDA (5). Tissues were stained for 30 sec by immersing the treated endocarp surface into 500 μl of a 1% FDA solution (5 μl of 1 mg of FDA per milliliter of acetone freshly added to 500 μl H_2O). Cellular fluorescence was examined immediately using an Olympus HBT microscope equipped with an epifluorescent [BH2-DMUV] light source filtered through barium filters [L420 and Y455] and exicter filters UGl(U) and BP-405(V). Samples were photographed using Kodak Ektachrome 160 ASA tungsten film. Pea cell viability was also evaluated by staining pea pods directly with a 0.1% phenosafranin solution and then viewing within 5 min under visible light.

Cell viability after chitosan treatments. Native shrimp chitosan and HCl-cleaved chitosan [HCl-cleaved chitosan: treated with 6 N HCl for 4 hr at 53 C (14)] at concentrations that inhibit fungal growth and elicit phytoalexin production were evaluated for their ability to reduce pea pod cell viability. Pod halves were treated with 25-μl aliquots of chitosan (pH 6.4) and individual pod halves were assayed for viability. Reduced cell viability was indicated by a reduction in cellular fluorescence following staining with FDA (see above). Native chitosan contained partially acetylated (10–20%) glucosamine residues within polymers in excess of 1×10^6 mol wt (1). HCl-cleaved chitosan contained only deactylated glucosamine polymers, most of which were heptamer or larger in size (14).

$^{35}SO_4$ ion leakage assay. Immature pea pods (2 cm length) were split and immediately treated with 5 μl of a $^{35}SO_4$ solution (8.9 μCi). The pods were incubated for 40 min, during which all of the visible surface liquid containing isotope was incorporated by the tissue. The tissue was then washed with 1 ml of sterile water to remove excess surface isotope. The pod halves were blotted dry with Kimwipes, then added to 1-ml volumes of the following treatments: water, chitosan (1,000, 250, or 60 μg per milliliter). Aliquots of three samples per treatment time period were removed at 0.5-, 1.5-, 3.5-, and 6-hr intervals and counted in a Packard scintillation counter Model Tri Carb 4000 to determine radioactivity released.

Ion leakage assayed by conductivity readings. Immature pea pods in lots of 0.5 g were treated with water, *F. s.* f. sp. *phaseoli* (1×10^7 macroconidia per milliliter), *F. s.* f. sp. *pisi* (1×10^7 macroconidia per milliliter), or chitosan (1,000, 500, or 100 μg per milliliter) as described above. At various time intervals 10 ml of sterile double distilled H_2O was added to the petri plates and mixed thoroughly. The wash water was read directly in a Hach Conductivity meter.

Effect of chitosan on ion leakage from *F. solani* as measured by changes in conductivity. Shake cultures of Vogel's minimal medium were inoculated with 1×10^6 macroconidia of *F. s.* f. sp. *phaseoli* or *F. s.* f. sp. *pisi* and incubated at 22 C on an orbital shaker for 24 hr. The mycelia were retained on a sterile 5.0 μm Millipore filter, washed twice with sterile glass distilled water and transferred to a chitosan dilution series. Ten-milliliter aliquots from each treatment were removed at 1, 2, 4, 6, and 24 hr, the mycelia were removed by filtration as above and the resulting cell free filtrate was used for conductivity measurements.

Effect of chitosan on *Fusarium solani* macroconidial germination and viability. Erlenmeyer flasks containing a dilution series of chitosan in 50 ml of Vogel's minimal medium were inoculated with 4×10^4 macroconidia of either *F. s.* f. sp. *phaseoli* or *F. s.* f. sp. *pisi* and incubated on an orbital shaker at 22 C. One-milliliter aliquots of the growth suspended germlings were removed at 1-, 6-, and 24-hr intervals and centrifuged in an Eppendorf microfuge. The pellets were washed twice in sterile water and the final pellet was resuspended in 10 ml of sterile water. A 1-ml aliquot from the washed conidial suspension was inoculated uniformly onto PDA plates amended with pea seedlings (2 g/L). Growth was scored at 72 hr.

***Fusarium solani* germling viability following chitosan treatment.** Aliquots of 25 μl, each containing 200 germlings developed from macroconidia inoculated in Vogel's minimal medium for 2, 5.5, or 15 hr were applied uniformly to microdilution plate wells in which crab shell chitosan (pH 6.1) had been serially diluted (1,000-0.5 μg/ml). Fungal growth was recorded at 24 and 48 hr. Conidial and hyphal viability were monitored by FDA staining.

Cell viability of macroconidia exposed continuously to a range of growth suppressing concentrations of chitosan was assayed by FDA staining. Aliquots were aseptically removed at 1, 6, and 24 hr and stained with an equal volume of 0.05% FDA. Stained slides were incubated at 22 C in the dark for 30 sec before viewing.

RESULTS

Effect of host-parasite interactions on the viability of plant and fungal cells. Because treatment with chitosan or inoculation with *F. s.* f. sp. *phaseoli* can induce resistance responses in peas characterized by inhibition of the growth of *F. s.* f. sp. *pisi*, the influence of these treatments on the viability of pea endocarp cells and fungal germlings was assessed.

FDA staining indicated that there was no appreciable decrease in the viability of the pea cells within the first 6 hr after inoculation with *F. s.* f. sp. *phaseoli*. Six hours is the approximate time known to be required for the complete suppression of growth of *F. s.* f. sp. *phaseoli* (Fig. 1B) and partial suppression of the compatible, *F. s.* f. sp. *pisi*. Water-treated control and chitosan-treated pea tissue also remained viable through this period (Fig. 1A and D, respectively). Fluorescence persisted even in endocarp cells, which had macroconidia directly attached, whereas the macroconidial inoculum of *F. s.* f. sp. *phaseoli* fluoresced at 6 hr even though growth had ceased. Fungal cell fluorescence was reduced at 11 hr and was negligible at 20 hr. The pea cells continued to fluoresce 8–12 hr after treatments with *F. s.* f. sp. *phaseoli* or *F. s.* f. sp. *pisi*, although fluorescence was reduced more quickly by the compatible pathogen. Also, fluorescence was reduced more rapidly in regions where multiple incompatible or compatible macroconidia were attached. Seventeen hours after treatment with *F. s.* f. sp. *phaseoli*, the pea endocarp surface cells fluoresced except in areas where multiple macroconidia were attached (Fig. 1C) and in those cells that developed into the hypersensitive reaction (which appears at 18–24 hr). This attenuated fluorescence indicated loss of cell viability. Twenty-four to 30 hr after inoculation with *F. s.* f. sp. *pisi* a much broader region of nonfluorescent surface cells surrounded macroconidial attachment sites.

Effect of chitosan on the viability of *F. solani* germlings. The germination and growth of formae speciales of *F. solani* is inhibited in the presence of chitosan. To determine if this inhibition is associated with changes in cell viability, *F. solani* macroconidia

TABLE 1. Chitosan concentrations that completely inhibit growth of macroconidia of *Fusarium solani* after germination periods of 0, 2, 5.5, and 15 hr

Germination period before chitosan treatment[b] (hr)	Chitosan concentration[a]	
	F.s. f.sp. *phaseoli* (μg/ml)	*F.s.* f.sp. *pisi* (μg/ml)
0	4	4
2	8	16
5.5	8	16
15	31	125

[a] Minimum chitosan concentration completely inhibiting growth of *F. solani* formae speciales.

[b] Fungal macroconidia of either formae speciales were suspended in Vogel's media as previously described (8) and incubated on a rotary shaker in continuous light for the given time periods. After germination, 25-μl aliquots, each containing >200 germlings, were applied uniformly to microdilution plate wells in which crab shell chitosan had been serially diluted (1,000–0.5 μg/ml). Inhibition of fungal growth was scored at 24 and 48 hr.

were allowed to germinate for 0, 2, 5.5, or 15 hr before treatment with chitosan. Chitosan was antifungal to both *F. s.* f. sp. *phaseoli* and *F. s.* f. sp. *pisi* at concentrations as low as 4 μg/ml, when applied directly to macroconidia (0 hr) (Table 1).

Macroconidia of both formae speciales did not germinate and showed only weak FDA fluorescence after exposure to chitosan concentrations of 1 mg/ml for less than 1 hr. The apical and foot cells of the macroconidia of *F. s.* f. sp. *pisi* were significantly more fluorescent than the other cells in the macroconidia. The FDA fluorescence retained by both formae speciales increased slightly as the chitosan treatment level decreased until at 4 μg of chitosan per milliliter it was indistinguishable from that of the control.

Chitosan treatments inhibited growth of *F. s.* f. sp. *phaseoli* and *F. s.* f. sp. *pisi* after a 2-hr germination period in nonchitosan containing Vogel's medium at concentrations equal to or greater than 8 and 16 μg/ml, respectively (Table 1). Macroconidia, germ tubes, and hyphae of *F. s.* f. sp. *phaseoli* and *F. s.* f. sp. *pisi* continued fluorescencing intensely when exposed to chitosan concentrations of 4 and 8 μg/ml, respectively. At chitosan concentrations greater than 16 and 31 μg/ml for *F. s.* f. sp. *phaseoli*

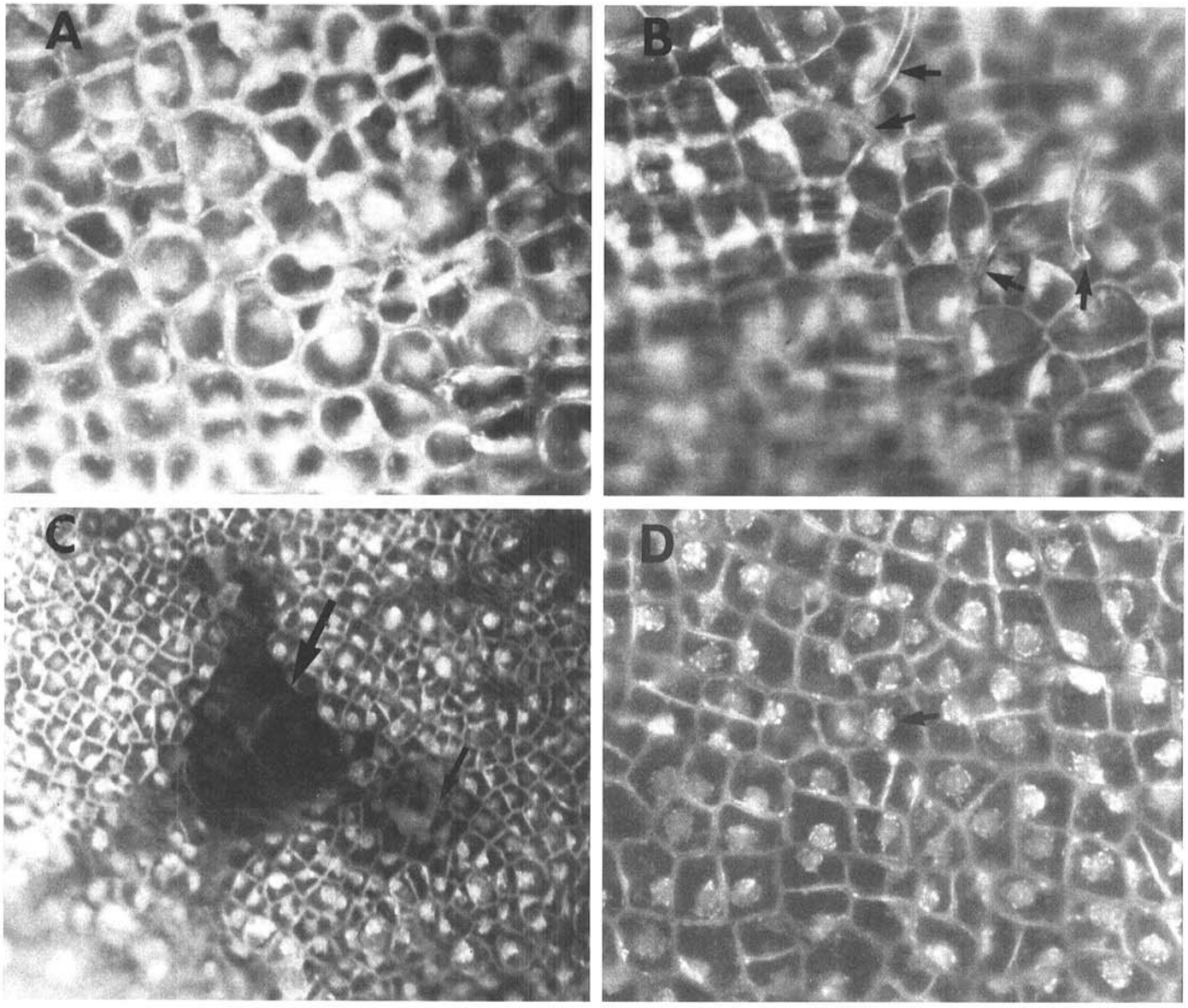

Fig. 1. Fluorescein diacetate related fluorescence in pea endocarp cells 17 hr after H_2O treatment **(A);** 6 hr after inoculation with *Fusarium solani* f. sp. *phaseoli* (incompatible nonpathogen) **B.** 17 hr after inoculation with *F. s.* f. sp. *phaseoli* **C;** or 8 hr after inoculation with HCL-cleaved chitosan (1,000 μg/ml) **(D).** Note that fluorescence of the control tissue remains intense for 17 hr. Pea cells in direct contact with *F. s.* f. sp. *phaseoli* (B, small arrows) or with chitosan (D) retain some fluorescence for 6 hr. This fluorescence diminished within 17 hr in chitosan treatment or in areas in direct contact with multiple incompatible, nonpathogen macroconidia (C, large arrow) but not in areas in direct contact with a single macroconidium (C, small arrow).

and *F. s.* f. sp. *pisi*, respectively, cellular fluorescence remained within the cytoplasm but was not associated with the wall membrane. As the germination period increased to 15 hr the concentration of chitosan required to suppress fungal growth increased to 31 and 125 μg/ml for *F. s.* f. sp. *phaseoli* and *F. s.* f. sp. *pisi*, respectively, whereas FDA fluorescence was attenuated in both formae speciales at chitosan concentrations greater than 16 and 31 μg/ml, respectively.

The reduction of FDA fluorescence in *F. solani* macroconidia appears to be related to both the concentration of chitosan as well as the length of time the propagules are exposed to the chitosan (Table 2). Macroconidia of both formae speciales displayed very low fluorescence immediately after addition of 1,000 μg/ml chitosan. As the concentration of the chitosan treatment decreased there was a correspondingly greater retention of FDA fluorescence in the macroconidia. Fluorescence of macroconidia treated with 40 μg/ml and below was indistinguishable from the control even though this treatment completely suppressed fungal growth (Table 2). Because fluorescence also remained intense for at least 6 hr at chitosan concentrations up to 50 μg/ml and total inhibition of growth is possible with 4 μg/ml chitosan, the initial growth inhibitory effect of chitosan is probably not due to a reduction of cellular viability.

Cell viability of *F. solani* macroconidia recovering from chitosan induced growth suppression. As indicated above, *F. solani* macroconidial germination can be inhibited by chitosan at 4 μg/ml. These inhibited spores often resume growth when isolated from the pea tissue and placed in fresh growth media or when the pea pod tissue deteriorates due to extensive senescence. Therefore, the exact chitosan level and exposure period that allows recovery was defined to determine if loss of cellular viability is associated with growth suppression.

The data in Table 3 indicate that *F. s.* f. sp. *phaseoli* cannot recover from a 1-hr exposure to chitosan at concentrations equal to or greater than 75 μg/ml even though some cellular fluorescence is retained. Macroconidia of *F. s.* f. sp. *pisi* are somewhat more tolerant to chitosan and a percentage can resume growth following a 24-hr exposure to chitosan concentrations as high as 100 μg/ml. These results demonstrate that spores with chitosan-induced low fluorescence are still sufficiently viable to resume growth in the absence of chitosan.

Cell viability assessed by phenosafranin staining. Phenosafranin staining provides a different assessment of cellular viability. This dye quickly stains injured and dead pea pod cells such as those that were damaged by the spatula used to separate the pod halves. However, the treated endocarp cells described above, which exhibited low levels of FDA fluorescence, did not stain with phenosafranin (data not shown). Although phenosafranin efficiently stained all cells discolored by the hypersensitive reaction at 17–24 hr (Fig. 2E), it failed to stain cells that were actively resisting the nonpathogen (Fig. 2A) during the first 6 hr after inoculation. After 6 hr there was a preferential nuclear staining within cells adjacent to macroconidia (Figs. 2B and C). Nuclear staining could be obtained within 3 hr when an excessive chitosan concentration (2,000 μg/ml) was applied to the endocarp cells (Fig 2D). These results suggest that both chitosan and fungal cells can influence the uptake of the phenosafranin stain by pea endocarp cells. Such staining may indicate effects on the cell membrane and/or nuclear structure; however, there was no indication that the induction of resistance required cell death. Pods treated with other compounds capable of inducing phytoalexin production [e.g., Actinomycin D (1–10 μg/ml)] also rapidly take up phenosafranin into their nuclei (data not shown).

Effect of chitosan and inoculum treatments on ion leakage of pea pods as determined by conductivity. Conductivity measurements of 10 ml of water washes of the half-gram lots of treated pea halves at various time intervals after treatment with the compatible or incompatible fungi are presented in Figure 3. No significant increase in the release of conductive material attributable to inoculation with either of the formae speciales of *F. solani* was observed within 24 hr. After this period there was a sharp increase in electrolyte leakage in the compatible interaction that was associated with accelerated fungal growth and a discoloration and softening of the pod tissue due to hydrolysis associated with infection.

There was no increase in release of electrolytes from chitosan treated tissues (Fig. 4). The conductivity external to the pea pod tissue was due to the residual conductivity of the chitosan treatment. This conductivity was reduced with time because of the uptake or localization of the chitosan into or onto the pod tissue reduced the inherent conductivity of the treatment solution. Therefore another approach was necessary to adequately assess the possible chitosan induced ion leakage.

Alternatively, $^{35}SO_4$ uptake and release from pea pods was used to estimate changes in the cell membrane ion transport. Microcuries (8.9) of $^{35}SO_4$ were applied to a uniform set of pod halves in an attempt to saturate intercellular pools. Chitosan and

TABLE 2. The effect of chitosan on the viability of macroconidia of *Fusarium solani* as assayed by FDA fluorescence

Chitosan concentration (μg/ml)	Length of chitosan treatment (hr)							
	F.s. f. sp. *phaseoli*				*F.s.* f. sp. *pisi*			
	0	1	6	24	0	1	6	24
0	h[a]	h	h	h	h	h	h	h
1	h	h	h	h	h	h	h	h
10	h	h	h	h	h	h	h	h
20	h	h	h	h	h	h	h	h
30	h	h	h	h	h	h	h	h
40	h	h	h	h	h	h	h	h
50	w	w	w	w	h	h	w	w
75	w	w	w	w	w	w	w	w
100	w	w	w	w	w	w	w	w
125	w	w	w	w	w	w	w	w
150	w	w	w	w	w	w	w	w
250	w	w	w	···	w	w	w	···
500	l	l	···	···	l	l	···	···
1,000	l	···	···	···	l	···	···	···

[a] Fluorescence intensities of macronconidial cells: h = highly fluorescent, no major difference from H_2O control; w = weakly fluorescent; l = very low fluorescence (almost invisible); (···) = nonfluorescent. Results represent the average of 100 randomly selected macroconidia per treatment and time period of three replications.

TABLE 3. Chitosan concentrations and treatment periods that permanently inhibit or allow regrowth of macroconidia of *Fusarium solani* after removal of exogenous chitosan from the medium

Chitosan concentration (μg/ml)	Length of chitosan treatment (hr)					
	F.s. f. sp. *phaseoli*			*F.s.* f. sp. *pisi*		
	1	6	24	1	6	24
0	+[a,b]	+	+	+	+	+
1	+	+	+	+	+	+
10[c]	+	+	+	+	+	+
20	+	+	+	+	+	+
30	+	+	+	+	+	+
40	+	+	+	+	+	+
50	+(22)	0	0	+(22)	+(20)	+(6)
75	0	0	0	+(17)	+(18)	+(3)
100	0	0	0	+(16)	+(3)	+(2)
125	0	0	0	0	0	0
250	0	0	0	0	0	0
500	0	0	0	0	0	0
1,000	0	0	0	0	0	0

[a] + = recovered growth of more than 200 colonies or actual number of colonies in parenthesis. 0 = no growth after transfer to chitosan-free PDA medium.

[b] Results represent the average value for 100 macroconidia at each concentration and time. The experiment was repeated at least three times.

[c] Enclosed zone indicates the range of chitosan concentrations allowing fungal growth if the chitosan is removed from the growth medium at the designated time but at which no growth occurs if the chitosan is not removed from the growth medium.

the water control treatment could then be compared directly on the basis of their efficiency in releasing $^{35}SO_4$ from the endocarp tissue. Chitosan treatments (1,000, 250, or 60 μg/ml) failed to induce a measurable increase in the release of $^{35}SO_4$ labeled material over that of the water control from pea pod cells (data not shown). This is another indication that the effect of chitosan, which results in the induction of a resistance response within 6 hr, is apparently unrelated to its effect on cell permeability.

Electrolyte release from fungal mycelia after chitosan treatments. Chitosan treatments (1 and 100 μg/ml) which were below and above the minimum level required to suppress the growth of *F. s.* f. sp. *phaseoli* and *F. s.* f. sp. *pisi* did not measurably enhance electrolyte release (Fig. 5) beyond the level of conductivity of the chitosan solution alone. Conductivity was actually reduced in both formae speciales following heating of the 100 μg/ml chitosan treatments (Fig. 5), which may relate to the high agglutinating and chelating properties of chitosan. Both formae speciales showed a significant decrease in electrolyte leakage through the various time periods normally associated temporally with the growth inhibition when, in the actual host-parasite interaction, the fungi are in contact with the pea pod tissue.

DISCUSSION

The vital staining of pea tissue using FDA and phenosafranin after inoculation with *F. solani* formae speciales or treatment with chitosan indicated the reduction and eventual loss of viability in both plant and fungal cells. However, because complete viability reduction occurred subsequent to the period that is crucial for the resistance response of the plant tissue, loss of viability may be more of a consequence than a cause of the active resistance response of the pea tissue. In pea pod tissue, the hypersensitive discoloration of cells in the vicinity of the inoculum does not appear to be directly associated with disease resistance because it develops more than 9 hr after the expression of resistance. Hypersensitive cells were often several cells or more removed from the nonhypersensitive cell directly in contact with the macroconidia whose growth had been suppressed. This implies that the hypersensitive cell death of endocarp cells is apparently not required for the induction of disease resistance in peas. Király et al (15) also observed that hypersensitive cell death is only a consequence of, not the cause of, resistance in potato to *Phytophthora infestans*, beans to *Uromyces vulgaris*, and wheat to *Puccinia graminis*. They were able to induce

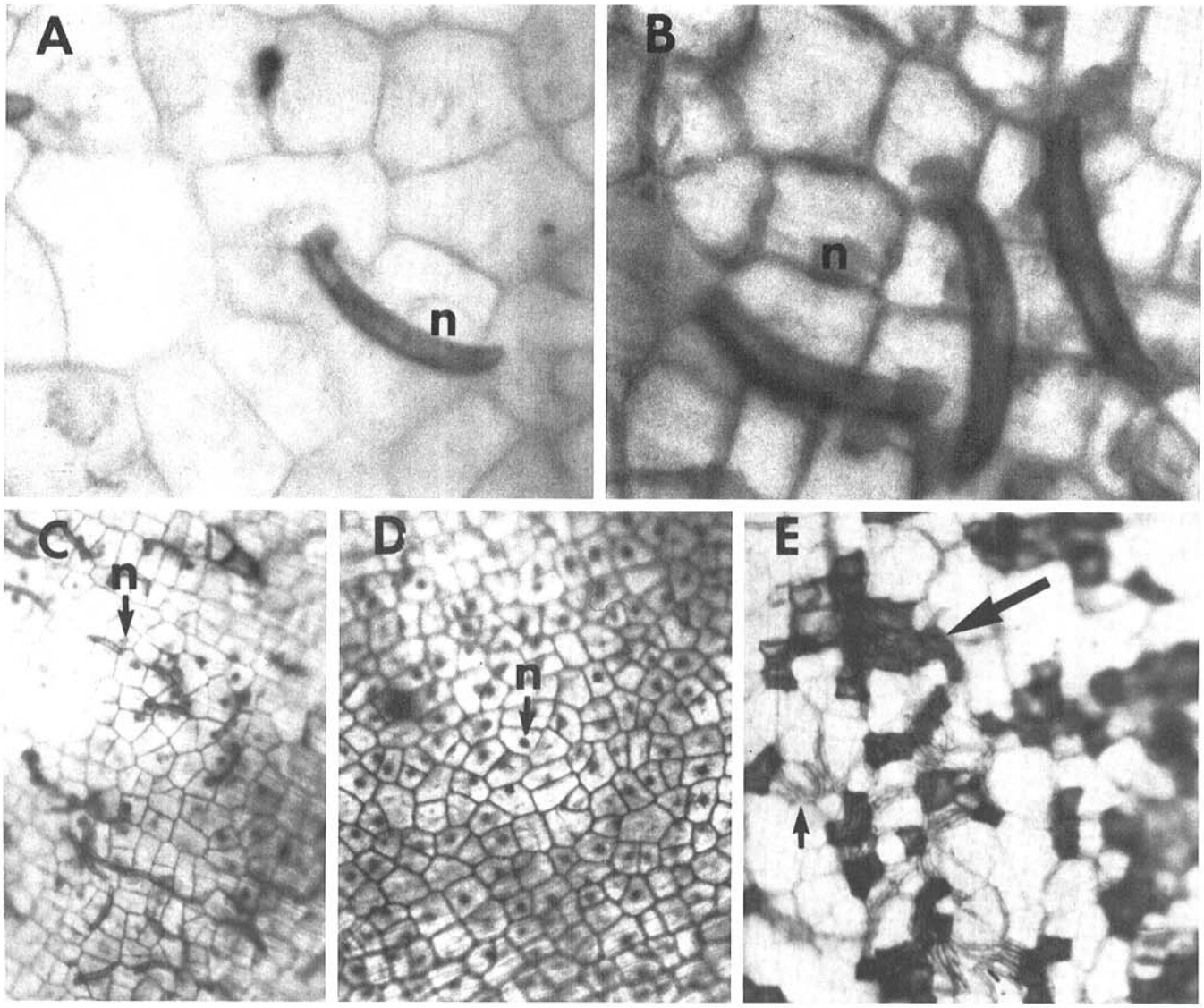

Fig. 2. Cell viability in pea endocarp cells indicated by phenosafranin staining 6 hr **(A)** or 19 hr **(B)** after inoculation with *Fusarium solani* f. sp. *phaseoli*, 7 hr after inoculation with *F. solani* f. sp. *pisi* **(C)** 3 hr after treatment with native shrimp chitosan (2 mg/ml) **(D)**, and 31 hr after inoculation with *F. s.* f. sp. *pisi* (E). Note that staining indicates no complete cell death before 24 hr. Dead cells appear solid black (large arrows). Small arrows (2E) indicate macroconidia in contact with unstained plant cells. Enhanced cellular and nuclear staining is observed within 3 hr in pods receiving excessively high levels of chitosan or within 7 hr following inoculation with the pathogen and (within 19 hr) following inoculation with the incompatible, nonpathogenic *F. s.* f. sp. *phaseoli*.

a hypersensitive necrosis in potato tuber, wheat leaf, and bean leaf tissues infected with compatible races of the pathogens when the pathogens were inhibited from further growth in the host tissues. They concluded that the observed hypersensitive reaction in the incompatible host resulted from an unknown defense reaction in which the pathogens are damaged as a consequence of the host resistance. The damaged pathogens then released an "endotoxin," which induced the hypersensitive necrosis.

In the pea-*F. solani* interaction, cell death of the incompatible pathogen was not essential for the expression of the host resistance as suggested by Király et al (15). However chitosan, which is rapidly released from the fungal cell wall (9) and can induce an authentic disease resistance response and potentially reduces cell viability in the host tissue, may be acting somewhat analogous to the proposed pathogen-released endotoxin of Király et al (15).

In other host-parasite systems [e.g., *Phaseolus vulgaris–Colletotrichum lindemuthianum* (2,3)], it has been proposed that on infection the host cells release (or activate) a constitutive elicitor that induces the synthesis of phytoalexins first in the infected cells, then in adjacent cells as the elicitor migrates. The phytoalexins then are transported back into the resisting infected cell and thereby inhibit the pathogen's growth (2,3). From the results presented in this paper and elsewhere (8,10) it appears that a disease resistance mechanism as described by Bailey (2) does not function in the pea-*F. solani* host-parasite system. Growth of both the compatible and incompatible *F. solani* formae speciales is suppressed within 5 hr after inoculation of the pea tissue (6). The compatible fungus (*F. s.* f. sp. *pisi*) resumes growth on the pea tissue by 24 hr despite high levels of phytoalexin accumulation and widespread hypersensitive cell death, whereas growth of the incompatible fungus (*F. s.* f. sp. *phaseoli*) remains inhibited. This growth inhibition is not associated with hypersensitive cell death.

Because of its polycationic nature, chitosan may potentially influence multiple cellular sites, including the cell membrane and nuclear structure. Presently, much of the reported data is compatible with the hypothesis that chitosan interacts with plant nuclear DNA, thereby initiating the activation of host disease resistance response genes (6–14).

Other reports propose that chitosan may function by damaging the host cell membranes (21,22) thereby initiating the host's response. Such membrane damage is purported to be due to the chitosan's polycationic character because the change in membrane permeability was also observed following treatment of tissue culture cells with poly-L-lysine and other polycationic compounds (21). These effects could be reduced by addition of divalent cations. It appeared that chitosan induced "pores" in the membranes

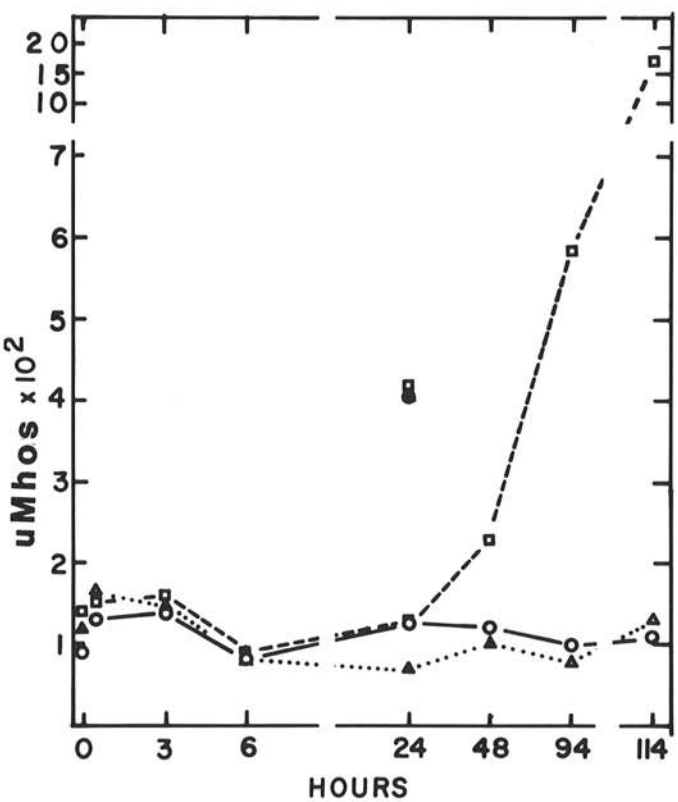

Fig.3. Conductivity of electrolytes in the water wash of pea pod halves treated with H_2O (circles), *Fusarium solani* f. sp. *phaseoli* (1×10^7 macroconidia per milliliter (triangles), or *F. s.* f. sp. *pisi* (1×10^7 macroconidia per milliliter) (squares). Conductivity recovered after boiling of the samples after the 24-hr incubation period are indicated by symbols without connecting lines.

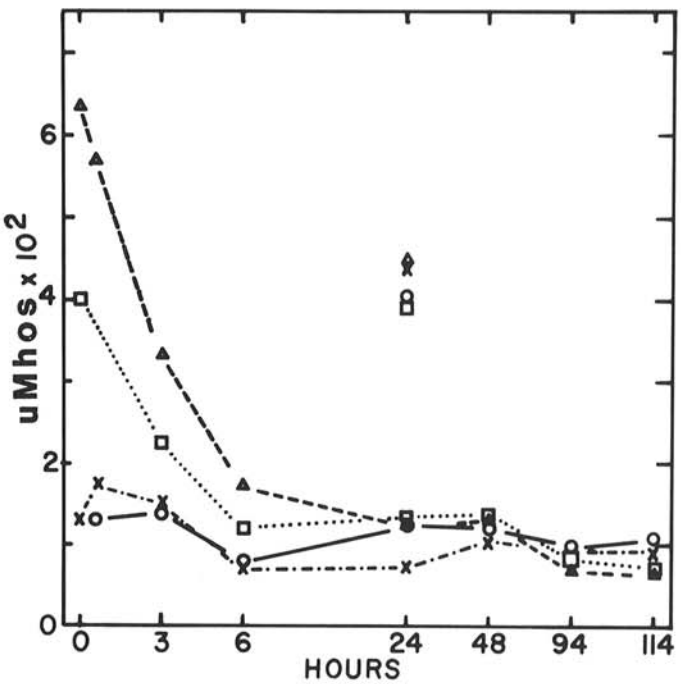

Fig. 4. Conductivity of electrolytes in the water wash of pea pod halves treated with H_2O (circles); chitosan: (1,000 μg/ml) (triangles), (500 μg/ml) (squares), or (100 μg/ml) (X's). Symbols without lines at the 24-hr time period indicate the electrolytes released following boiling.

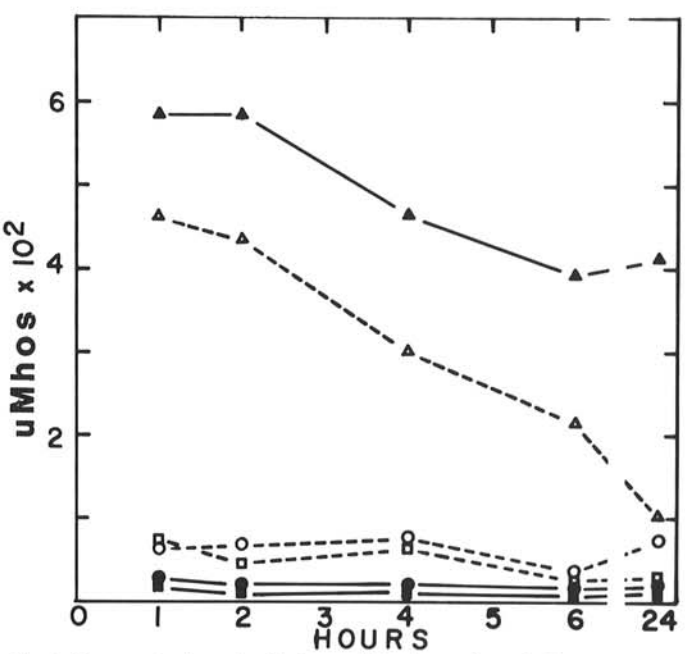

Fig. 5. Changes in electrolyte leakage of *F. s.* f. sp. *phaseoli* after treatment with chitosan (1 μg/ml) (closed squares); chitosan (1 μg/ml), then 40 C for 30 min (open squares); chitosan (100 μg/ml) (closed triangles); chitosan (100 μg/ml), then 40 C for 30 min (open triangles); H_2O (closed circles); H_2O, then 40 C for 30 min (open circles).

because solutes released from the chitosan-treated suspension cultures appear to be mainly of low molecular weight. Reuvini et al (16) demonstrated that poly-L-lysine also induced pore formation selectively in the plasmlemma of *Nicotiana tabacum* cv. *xanthi* suspension culture cells.

Using spin label and fluorescent lipid-soluble probes and isolated microsomal membranes from primary leaves of *Phaseolus vulgaris*, Roberts et al (18) demonstrated that the polyamines spermidine, spermine, and, to a lesser extent, putrescine at physiologic concentrations, reduced membrane fluidity (e.g., stabilized the membranes) in a manner similar to that caused by calcium. They attribute the polyamine-induced membrane stabilization to rigidification at the bilayer surface (18).

Studies of chitosan's effects on plant membranes have until now been done only with suspension culture cells. Our present study using intact tissue indicates that chitosan's effects on the host tissue occur well in advance of any detectable membrane damage. Electrolyte leakage, as measured by conductivity, from chitosan-treated pea endocarp tissue was not statistically different from that of the water control tissue (Fig. 4). Release of ^{35}S-labelled material from pea endocarp tissue after chitosan treatment also showed that chitosan-induced membrane leakage was minimal when compared with the water control.

The lack of chitosan-induced membrane leakage and the described staining properties of the two vital stains, FDA and phenosafranin, strongly suggest that the host tissue is intact and generally undamaged when the host resistance response is initiated. The fact, however, that pod endocarp tissue adjacent to macroconidia or tissues treated with excessive chitosan or Actinomycin D exhibit a preferential staining of their nuclear material by phenosafranin indicate that the nuclear material is somehow influenced by these treatments. These observations suggest at least two possibilities: 1) the treatments alter the cell membrane allowing the stain to penetrate the nucleus of the plant; or 2) the inductive compound alters the host's nuclear structure enabling the phenosafranin to selectively localize within the nucleus.

The failure of chitosan to reduce the intensity of the vital staining of fungal tissue at concentrations that have been shown to inhibit germination and subsequent growth suggests that chitosan does not function by killing the fungal tissues (Tables 2 and 3), except at excessive concentrations.

Because it is possible to detect changes in the host cell's nuclear structure (7), the entrance of chitosan into pea cells adjacent to those directly in contact with fungal material (9), and the activation of the pea resistance response genes (23) all within 2 hr after inoculation of the pea tissue with the incompatible nonpathogenic *F. solani* f. sp. *phaseoli*; we propose that initiation of the disease resistance response is associated with induced structural changes in the host's nuclear material. The delayed hypersensitive cell death and host membrane deterioration are possibly consequences of the activated resistance response.

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