

Chitosan Effects on Blackmold Rot and Pathogenic Factors Produced by *Alternaria alternata* in Postharvest Tomatoes

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ABSTRACT. Stem scar application of chitosan inhibited growth and production of pathogenic factors by blackmold rot [*Alternaria alternata* (Fr.:Fr.) Keissl.] in challenged tomato (*Lycopersicon esculentum* Mill.) fruit stored at 20 °C for 28 days. Blackmold lesions were visible within 4 days of inoculation in control fruit, compared with >7 days in chitosan-treated fruit. Macerating enzyme activity (polygalacturonase, pectate lyase, and cellulase) in the tissue in the vicinity of the lesions was <50% in chitosan-treated fruit compared with control fruit. Chitosan also inhibited production of oxalic and fumaric acids (chelating agents) and host-specific toxins such as alternariol and alternariol monomethylether by the fungus. The pH of the infected tissue decreased from 4.7 to 4.0 in the control fruit, the optimum for polygalacturonase activity, while the pH of chitosan-treated fruit remained at 4.6. In addition, chitosan also induced production of rishitin (a phytoalexin) in tomato tissue. Such chitosan–pathogen–host interactions may be exploited in the control of postharvest pathogens of fresh fruit and vegetables.

Alternaria alternata is a causal agent of blackmold rot of tomato (*Lycopersicon esculentum*) fruit, a disease frequently causing substantial postharvest losses. The occurrence of *Alternaria* Nees in a wide variety of fruit and vegetables under diverse conditions of cultivation, handling, and storage suggests that the losses caused by *Alternaria* are comparable to other mold genera such as *Aspergillus* P. Mich ex Link, *Penicillium* Link and *Fusarium* Link (Stinson et al., 1980). The fungus tends to proliferate in tomato fruit and other produce affected by sun scald, chilling injury, blossom-end rot, faulty blossom scars, and growth cracks (Pearson and Hall, 1975). The fungus grows well at low temperatures, and is associated with extensive spoilage of cold-stored fruit and vegetables (Stinson et al., 1980).

Alternaria produces a number of toxins as pathogenicity factors, among them alternariol (AL) and alternariol monomethylether (AME) are major ones, since these are produced by most *Alternaria* species in large quantities (Heisler et al., 1980). Toxins of *Alternaria* have been detected as natural contaminants of tomato fruit and tomato products (Stack et al., 1985), apples [*Malus sylvestris* (L.) Mill var. *domestica* (Borkh) Mansf.] (Stinson et al., 1981) and olives (*Olea europaea* L.) (Visconti et al., 1986). In addition, the fungus also produces cell wall-degrading enzymes and organic acids (El-Shaieb and Malibari, 1995; Hirsch et al., 1995). It has been suggested that organic acids produced by pathogens can damage host tissues by lowering pH of the tissue and acting synergistically with pectic enzymes in the digestion of Ca-pectate (Margo et al., 1984). Control of blackmold rot of tomato can be achieved by pre- and postharvest antifungal treatments, since the fungus can infect the fruit in the field and become latent in green tomato, and resume growth as the fruit ripens. The use of fungicides on fruit needs

strict control due to potential health risks, and none of them are approved for postharvest use. Hence, there is a need to exploit natural antifungal substances and host defenses for the control of postharvest pathogens.

Chitosan (β ,1-4, linked glucosamine polymer), the deacetylated form of chitin, has been shown to be antifungal against a wide range of fungi (El Ghaouth et al., 1992a). Chitosan induces chitinases in plant tissue (El Ghaouth et al., 1992b; Mauch et al., 1984), structural defense barriers in bell pepper fruit [*Capsicum annuum* L. var. *annuum* (Grossum Group)] (El Ghaouth et al., 1994), and elicits the production of phytoalexin in pea pods (*Pisum sativum* L.) (Kendra and Hadwiger, 1984). Chitosan coating of strawberries (*Fragaria ×ananassa* Duchesne) and tomato fruit reduces the severity of infection by *Botrytis cinerea* (Pers.) Fr. (gray mold) and *Rhizopus stolonifer* (Ehrenb.) Fr. (*Rhizopus* rot) and delays ripening (El Ghaouth et al., 1991; El Ghaouth et al., 1992c). Chitosan interferes with macerating enzyme production in *B. cinerea* when applied to the stem scars of bell pepper fruit (El Ghaouth et al., 1997). Our previous results showed that chitosan affects growth, morphology, and toxin production by *A. alternata* (Reddy et al., 1998), and multiplication and secretion of macerating enzymes by *Erwinia carotovora* ssp. *carotovora* (Jones) Bergey et al. (bacterial soft rot), in inoculated potato tissue (*Solanum tuberosum* L.) (Reddy et al., 1997). These observations revealed that chitosan, in addition to its direct antimicrobial activity, also interferes with pathogenic factors and induces host defenses. We report herein the mechanisms of chitosan action in controlling the progress of blackmold rot in postharvest tomato fruit.

Materials and Methods

MATERIALS. Shrimp shell chitosan was purchased from Nova-Chem Ltd. (Dartmouth, Nova Scotia, Canada) and ground into a fine powder. The purified chitosan was prepared by dissolving chitosan in 0.25 mol·L⁻¹ HCl, and the undissolved particles were removed by centrifugation (15 min at 10,000 g_n). The solution was then neutralized with 2.5 mol·L⁻¹ NaOH to a pH of 8.0 to precipitate the chitosan. The precipitated chitosan was recovered

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by filtration and washed extensively with deionized water to remove salts, and subsequently lyophilized. Chitosan solution ($10 \text{ g}\cdot\text{L}^{-1}$) was prepared by dissolving chitosan in $0.05 \text{ mol}\cdot\text{L}^{-1}$ HCl and pH was adjusted to 5.6. Mature green 'Trust' tomato fruit were harvested in the Spring 1997 from the greenhouse at Laval University and sorted for uniformity of size and absence of blemishes. Selected fruit were surface sterilized by immersion in $13 \text{ mmol}\cdot\text{L}^{-1}$ NaOCl for 3 min, rinsed in sterile deionized water and dried in ambient air. The fruit were then randomly divided into 20 fruit lots. *Alternaria alternata* was isolated from infected tomato and cultures were maintained on potato dextrose agar (PDA) at $22 \text{ }^\circ\text{C}$. Spore suspensions were obtained by flooding cultures of 10-d-old *A. alternata* with sterile distilled water containing $1 \text{ mL}\cdot\text{L}^{-1}$ Tween 80. Spore counts were determined with a haemocytometer, and the spore concentration was adjusted with sterile distilled water to 2.5×10^5 conidia/mL.

CONTROL OF BLACKMOLD ROT. Fruit stem scars were treated individually either with chitosan solution or sterile deionized water. On each stem scar, $400 \mu\text{L}$ of chitosan solution ($10 \text{ g}\cdot\text{L}^{-1}$) or sterile water was applied and were dried under ambient conditions for 1 h. After air drying, fruit were wound-inoculated 1 cm away from the stem scar by pipetting $50 \mu\text{L}$ of spore suspension and were subsequently placed on wire mesh platforms in plastic containers and stored in the dark at $20 \text{ }^\circ\text{C}$. A layer of water was placed at the bottom of the container to maintain high humidity and containers were closed with perforated lids to eliminate any accumulation of CO_2 . Fruit were evaluated daily for lesions over a period of 28 d, and pericarp tissue samples from the edge of the lesions were drawn at various storage intervals after inoculation for assaying cell wall-degrading enzymes, toxins, and organic acids produced by the fungus and phytoalexin elicited by chitosan treatment. Disease severity of inoculated fruit was followed during storage in both control and chitosan-treated fruit in three replications, each replication consisting of five fruit stored in separate containers. For evaluating pathogenesis and disease resistance, there were 15 fruit for each treatment (inoculated and noninoculated controls, and inoculated and noninoculated chitosan treatment), and storage interval (0, 7, 14, 21, or 28 d). Analysis of enzyme activity and phytoalexin level were performed on separate lots of five fruit, while toxin and acid content as well as pH were determined on the same lot of five fruit. Fruit were arranged in a randomized complete block design, and the experiment was conducted twice. Data were pooled and analysis of variance was applied to the different experimental data (SAS Institute, 1988) and the standard error of the mean was determined. Means were compared using the Fisher's protected least significant difference (LSD) at $P < 0.05$.

TISSUE SAMPLES AND pH. Pericarp tissues were sampled from inoculated fruit by excising the tissue from the inoculation site up to 1 to 2 cm beyond the edge of the expanding lesion. Tissues were sampled from noninoculated fruit around the stem scar from the edge up to a span of 1.5 to 2.0 cm. The pH of the tissue suspended in distilled water was measured using a pH meter (Model 50, Fisher Scientific, Montreal, Canada).

ENZYME PREPARATION AND ASSAY. Pericarp tissue samples (3 g) were homogenized in 12 mL buffer solution ($10 \text{ mmol}\cdot\text{L}^{-1}$ PIPES buffer (pH 6.5) and $5 \text{ mmol}\cdot\text{L}^{-1}$ thiourea) using a micromill (Bel-Art Products, Pequannock, N.J.) for 1 min and the homogenate was centrifuged at $20,000 g_n$ for 30 min. The pellet was washed twice by redissolving it in 2 mL of the buffer solution and centrifuging at $20,000 g_n$ for 10 min. The combined supernatants constituted the crude extract. All extraction procedures were performed at $4 \text{ }^\circ\text{C}$. Protein determinations were carried out according to the method of Bradford (1976).

The method of Ayers et al. (1966) was followed to determine polygalacturonase (PG) and pectate lyase (PL) activities. The reaction mixture consisted of 3 mL $10 \text{ g}\cdot\text{L}^{-1}$ polygalacturonic acid (Sigma Chemical Co., St. Louis, Mo.), $2.5 \text{ mL } 0.1 \text{ mol}\cdot\text{L}^{-1}$ Tris/HCl pH 8.0, 0.5 mL of enzyme extract, and CaCl_2 adjusted to $0.8 \text{ mmol}\cdot\text{L}^{-1}$ in the mixture. The mixture was incubated at $30 \text{ }^\circ\text{C}$ for 4 h, and the excess substrate and enzymes were precipitated by adding $0.6 \text{ mL } 90 \text{ g}\cdot\text{L}^{-1}$ ZnSO_4 and $0.6 \text{ mL } 0.5 \text{ mol}\cdot\text{L}^{-1}$ NaOH. After vigorous mixing, the precipitate was sedimented by centrifugation at $30,000 g_n$ for 10 min. To 1 mL of the supernatant, $0.6 \text{ mL } 0.04 \text{ mol}\cdot\text{L}^{-1}$ thiobarbituric acid, $0.3 \text{ mL } 1 \text{ mol}\cdot\text{L}^{-1}$ HCl and 0.1 mL distilled water were added, and the mixture was heated in a boiling water bath for 30 min to develop the color. After cooling, the absorbance of the solutions were measured at 515 nm for PG and 550 nm for PL using a spectrophotometer (model 8451A; Hewlett Packard, Kirkland, Quebec, Canada).

Cellulase activity was determined using CM cellulose-RBB (remazol brilliant blue) as substrate (Wolf and Wirth, 1989). The reaction mixtures contained $0.3 \text{ mL } (1 \text{ g}\cdot\text{L}^{-1})$ substrate solution, $0.6 \text{ mL } 0.1 \text{ mol}\cdot\text{L}^{-1}$ sodium acetate buffer (pH 4.8) and 0.3 mL crude extract. The reaction was terminated after 4 h at $25 \text{ }^\circ\text{C}$ by adding $0.3 \text{ mL } 0.5 \text{ mol}\cdot\text{L}^{-1}$ HCl. After cooling in an ice-water bath for 15 min, the mixture was centrifuged at $14,000 g_n$ for 5 min and the absorbance of the dye covalently linked to the degraded polysaccharide was measured at 600 nm using a spectrophotometer (model 8451A; Hewlett Packard).

EXTRACTION OF TOXINS AND ORGANIC ACIDS. Pericarp tissue samples (3 g) were homogenized in methanol (1 g of tissue : 3 mL of methanol) and centrifuged at $1,000 g_n$ for 5 min. The residue was reextracted twice in the same manner and the pH of the combined supernatants was adjusted to 8.0 with $1.27 \text{ mol}\cdot\text{L}^{-1}$ NH_4HCO_3 . An equivalent volume of water was added to the extract and the aliquot was extracted twice with 200 mL of chloroform each time. The combined chloroform extracts were reduced in volume to $\approx 80 \text{ mL}$ under vacuum (8.93 kPa) and dried overnight over anhydrous Na_2SO_4 . The filtered extract was dried completely under reduced pressure before derivatization for toxin analysis. Organic acids were extracted from the water extract. The samples were dried overnight over anhydrous Na_2SO_4 and partitioned twice with diethyl ether (10 mL followed by 5 mL). The samples were evaporated under nitrogen and subsequently transferred to analytical glass vials and further evaporated to dryness under nitrogen. Both dried fractions for toxins and organic acids were derivatized by adding $100 \mu\text{L}$ dimethyl formamide and $50 \mu\text{L}$ of the reagent methyl *tert*-butyl silyl trifluoroacetamide (MTBSTFA) and the reaction mixture was incubated at $65 \text{ }^\circ\text{C}$ for 2 h before analysis by gas chromatography (GC).

EXTRACTION OF PHYTOALEXINS. Pericarp tissue samples (10 g) were homogenized in methanol (1 g of tissue : 3 mL methanol) and centrifuged at $1,000 g_n$ for 5 min (Hildenbrand and Ninnemann, 1994). The residue was reextracted twice in the same manner and the combined supernatants were dried under vacuum at $40 \text{ }^\circ\text{C}$. The residue was dissolved in 100 mL aqueous methanol ($14.8 \text{ mol}\cdot\text{L}^{-1}$) and partitioned three times with 100 mL chloroform, allowing the phases to separate for 1 h. The combined chloroform fractions were reduced in volume to $\approx 80 \text{ mL}$ under vacuum and dried overnight by adding anhydrous Na_2SO_4 . The filtered extract was evaporated under reduced pressure and redissolved in 1.5 mL dichloromethane. The samples were transferred to analytical glass vials, evaporated further under nitrogen, and derivatized before analysis by GC.

GAS CHROMATOGRAPHY. Alternariol, AME, oxalic and fumaric acids, and rishitin were assayed by GC (model 5890; Hewlett

Table 1. Blackmold rot development in chitosan-treated and control tomato fruit.

Treatment	Days after inoculation			
	7	14	21	28
	Lesion diam (cm) ^z			
Water control	0.9 ± 0.2	4.2 ± 0.9	8.3 ± 1.5	10.6 ± 2.0
Chitosan	0 ± 0	2.1 ± 0.4	3.3 ± 0.6	3.8 ± 0.7

^zEach value represents the mean of four samples per treatment ± SE.

Packard, Avondale, Pa) equipped with a flame ionization detector. The chromatographic separation was carried out on a capillary column (model DB5-30M; Hewlett Packard,) 0.25 mm i.d. × 30 m long and 0.25 μm film thickness. Hydrogen was used as the carrier gas at a linear velocity of 35.5 cm·s⁻¹ at 200 °C. The detector temperature was 325 °C, and the injector temperature was 300 °C. The oven temperature was programmed from 150 °C for 2 min, then to 300 °C at 5 °C·min⁻¹, and held at 300 °C for 5 min. The injections were performed with an auto sampler (model 7673; Hewlett Packard). The data were processed using Hewlett Packard HP3365 software. Under these conditions, the order of elution was oxalic and fumaric acids, AME, rishitin and AL. Standards of toxins and organic acids were used for identification (Sigma), while rishitin was identified by comparing with the standard obtained from Susan McCormick, National Center for Agricultural Utilization Research (NCAUR), U.S. Dept. of Agriculture, Agricultural Research Service, Peoria, Ill.

Results

EFFECT OF CHITOSAN ON BLACKMOLD ROT DEVELOPMENT. Blackmold rot developed at a higher rate ($P < 0.05$) in the inoculated control fruit compared with chitosan-treated fruit (Table 1). In the inoculated control fruit, lesions were visible within 4 d after inoculation and increased significantly with storage time, while in the chitosan-treated fruit, lesions were visible only after 7 d of storage. The size of the lesion in chitosan-treated fruit was less than one fourth of the fruit surface after 28 d of storage compared with total rot of the fruit in the control lot. Control of lesion development in chitosan-treated fruit indicates that chitosan had an inhibitory effect on pathogenic factors of *A. alternata*.

MACERATING ENZYMES. Analysis of macerating enzymatic activity in extracts of tissue around the lesions in both chitosan and nonchitosan-treated fruit challenged with the pathogen showed lower activities ($P < 0.05$) of polygalacturonase, pectate lyase, and cellulase in chitosan-treated fruit compared with inoculated control fruit (Fig. 1). Among the enzymes, polygalacturonase activity was the highest in the infected tissue followed by pectate lyase and cellulase in both inoculated control and chitosan-treated fruit. Maximum polygalacturonase activity was recorded after 21 d of storage, followed by a decline. Maximum cellulase activity was recorded at 14 d of storage. In general, the activities of all cell wall-degrading enzymes remained 50% lower in chitosan-treated fruit compared with inoculated control fruit.

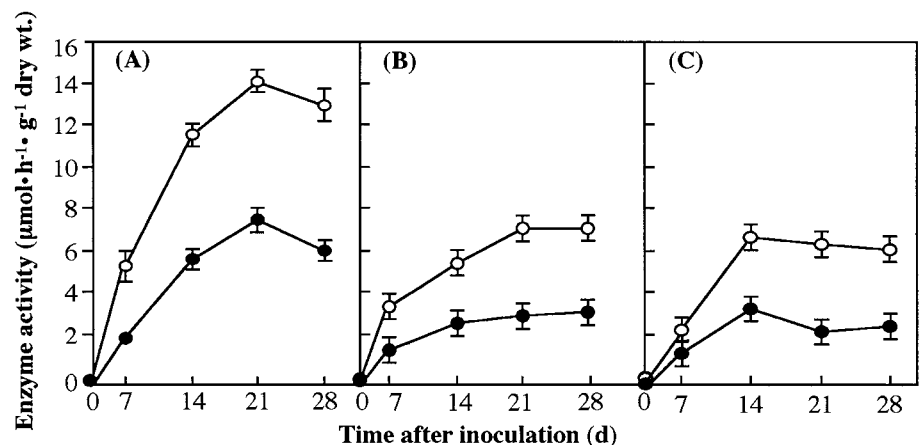
ORGANIC ACIDS. Oxalic and fumaric acid production by *A. alternata* increased with storage in both control as well as chitosan-treated fruit challenged with the pathogen

(Fig. 2). However, the levels of acids were lower ($P < 0.05$) in chitosan-treated fruit compared with control fruit. Oxalic acid level was higher in both tissues compared with fumaric acid throughout the storage period. Only small amounts of oxalic acid (12 mg·g⁻¹) and fumaric acid (8 mg·g⁻¹) were detected in healthy tomato tissue up to a period of 14 d. After 28 d storage, the amount of oxalic acid produced by the fungus in chitosan-treated fruit was <25% of the quantity produced in the control fruit, but the inhibition was relatively small for fumaric acid. The levels of both acids increased and the pH of the tissue decreased in the inoculated control fruit throughout the storage period. The pH decreased from 4.7 to 4.0 from day 7 to day 28 in the inoculated control, but it remained at ≈4.6 in chitosan treated fruit (Fig. 3). In healthy fruit, only a slight decrease in pH was noticed from day 7 to day 28. These results show that organic acids secreted by the fungus in inoculated control fruit decreased the tissue pH to 4.0 which is optimum for PG activity. Enzymatic activities in tissue extracts from inoculated control fruit were tested in vitro at pH ranging from 4.0 to 4.7. The activities were not significantly different in this pH range, indicating that this narrow decrease in pH at the site of infection may not have an influence on enzymatic activity.

HOST SPECIFIC TOXINS. Chitosan treatment of tomato fruit also interfered with production of toxins by the fungus. Although the toxin concentration of tissue increased from day 7 to day 28 in both chitosan-treated and control fruit challenged with the pathogen, it was lower ($P < 0.05$) in chitosan-treated fruit. Alternariol content varied from 1 to 2.3 mg·g⁻¹ of infected tissue in the controls compared with 0 to 1.3 mg·g⁻¹ in chitosan-treated fruit from day 7 to day 28 (Fig. 4A). A similar trend was observed in the alternariol monomethylether content of the infected tissue (Fig. 4B).

PHYTOALEXIN. Chitosan treatment also induced production of rishitin, a sesquiterpenoid phytoalexin of tomato (Fig. 5). The highest amounts of rishitin were present in chitosan-treated fruit challenged with the pathogen. In both chitosan-treated and control fruit challenged with the pathogen, rishitin levels increased up to 21 d of storage beyond which it decreased. Rishitin concentration was higher ($P < 0.05$) in chitosan-treated fruit as well as fruit challenged with the pathogen following chitosan treatment compared with inoculated control fruit. Rishitin concentration continued to increase in chitosan-treated fruit challenged with the pathogen up to 21 d after treatment.

Fig. 1. Changes in the activities of macerating enzymes in tomato fruit treated with (●) or without (○) chitosan, and challenged with *A. alternata*. (A) Polygalacturonase, (B) pectate lyase, and (C) cellulase. Vertical bars represent SEs of five samples per treatment.



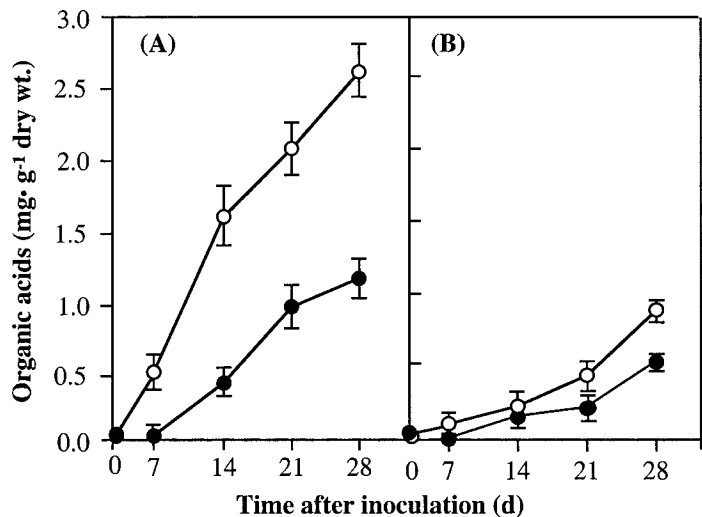


Fig. 2. Changes in (A) oxalic acid and (B) fumaric acid content in tomato fruit treated with (●) or without (○) chitosan, and challenged with *A. alternata*. Vertical bars represent ses of five samples per treatment.

Discussion

Stem scar application of chitosan to tomato fruit inhibited the progress of blackmold rot by restricting growth and secretion of pathogenic factors by *A. alternata*. Inhibition of fungal growth as evidenced by reduced lesion size in chitosan treatments shows that chitosan was directly antifungal. In addition, chitosan interfered with production of fungal virulence factors such as cell wall degrading enzymes, organic acids, and host specific toxins. Several studies have confirmed direct antifungal action of chitosan (Allan and Hadwiger, 1979; El Ghaouth et al., 1991, 1992a). Although information is available on the antimicrobial effects of chitosan, it is imperative to know the actual mechanism and its interference in pathogenesis of disease causing organisms in plants. Our previous studies showed that chitosan treatment at sublethal concentrations interferes with production of host specific toxins such as AL and AME by *A. alternata* more than the fungal biomass (Reddy et al., 1998) and macerating enzymes by *E. carotovora* in potato (Reddy et al., 1997). Many studies have confirmed the antimicrobial effect of chitosan when it is in direct contact with the target organism. In the present study, we inoculated the pathogen at sites adjacent to treated stem scars of the fruit in order to gain some understanding of any systemic action of chitosan, and observed inhibition of the pathogen growth even though the sites of chitosan treatment and inoculation were not the same. This suggests that chitosan acts in some systemic manner which involves either a direct effect or induction of defense reactions in the host. This is possible by translocation of chitosan oligosaccharides by enzymatic cleavage of chitosan polymer and/or by a mechanism involving signal transduction. Chitosan is unusually susceptible to a variety of enzymes such as proteases, cellulases, pectic enzymes, and lipases (Pantaleone et al., 1992). It is then possible that the applied chitosan polymer is cleaved by plant enzymes when in contact with the tissue; and chitosan oligosaccharides translocate from the application site to the neighboring cells, and inhibit fungal growth at sites away from the site of application. This hypothesis is supported by the observation of Hadwiger and Beckman (1980) that chitosan accumulates in plant cells adjacent to the sites of chitosan treatment in pea pod-*Fusarium solani* interactions. The second scenario may also be at

work. We detected rishitin at sites away from the stem scar where chitosan was applied. Since phytoalexins cannot translocate but their signals can (Kuc, 1987), we hypothesize that rishitin accumulation elsewhere than the site of treatment is mediated by signal molecules. The finding of Doares et al. (1995) that chitosan oligosaccharides activate plant defense genes by signal transduction involving jasmonic acid similar to wound response, sustains this hypothesis.

Secretion of cell-wall degrading enzymes, organic acids, and host specific toxins have long been recognized to be of prime importance in necrotrophic pathogenesis (Ikotun, 1984; Schafer, 1994). Pectic substances play an important role in maintaining the architecture of plant cell walls. Depolymerization of pectin has been shown to be a prerequisite for degradation of other cell wall polymers (Bauer et al., 1977). Decreased enzymatic activity in chitosan-treated fruit at the infection site of the fungus suggests that chitosan treatment affected the capability of *A. alternata* to produce macerating enzymes. Bennett et al. (1993) showed that polygalacturonase-solubilized pectic fragments from tomato tissue may act as signal molecules which specifically induce toxin production of *A. alternata*. This can account for the increased toxin production in nonchitosan-treated fruit. On the other hand, in chitosan-treated fruit, the solubilization capacity of PG produced in the presence of chitosan could also be lower as was shown earlier with the potato-*Erwinia* system (Reddy et al., 1997). Hence, the required signals to induce toxin production by the pathogen may not have been generated resulting in decreased virulence of the fungus. Cellulase activity was also observed, but was lower than PG and PL, and its activity was also inhibited by chitosan treatment.

Secretion of organic acids by the pathogens facilitates its rapid colonization of the tissue, and is a determinant in its virulence. Oxalic and fumaric acids can chelate calcium from the middle lamella of plant cell walls rendering pectic substances more susceptible for hydrolysis by pectolytic enzymes secreted by the fungus (Margo et al., 1984), and destabilizing the cell membranes causing

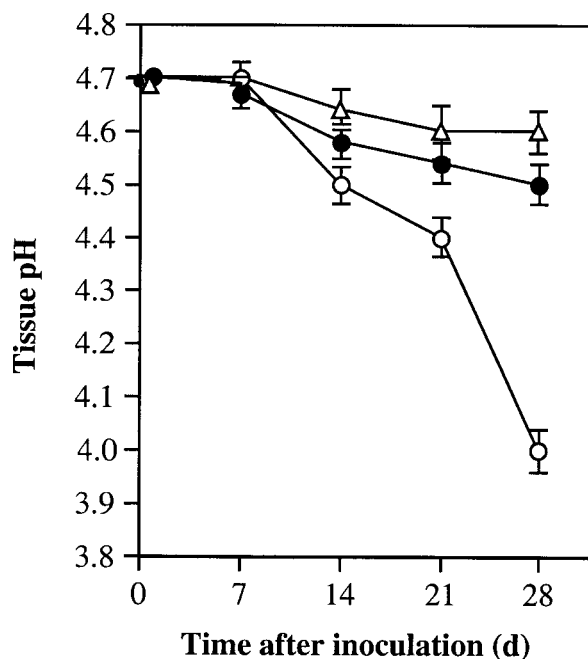


Fig. 3. Changes in pH of tomato fruit treated with (●) or without (○) chitosan, and challenged with *A. alternata*, and in nontreated, noninoculated fruit (D). Vertical bars represent ses of five samples per treatment.

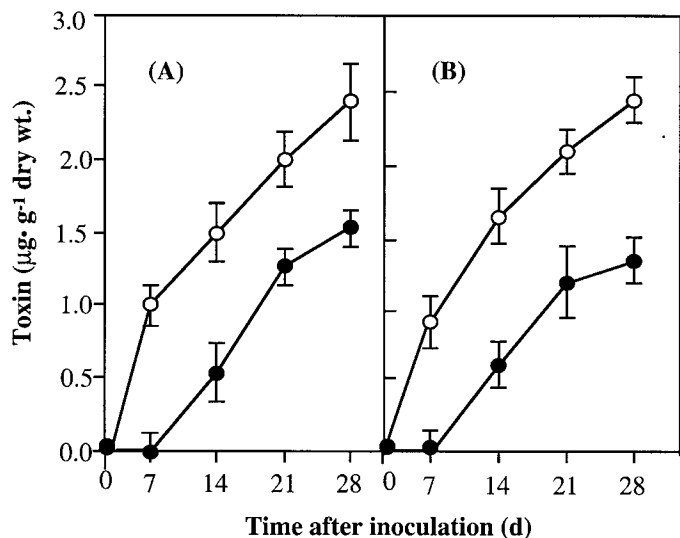


Fig. 4. Changes in phytotoxins in tomato fruit treated with (●) or without (○) chitosan, and challenged with *A. alternata*. (A) Alternariol and (B) alternariol monomethylether. Vertical bars represent ses of five samples per treatment.

cellular leakage (Pooviah, 1986). In addition, acidification of the tissue at the infection site would also tend to neutralize the inhibitory effect of tomatine, a preformed inhibitor in tomato. Tomatine is a weak base and exhibits maximum fungistatic activity in its nonionized form, while its protonated form is less effective. Fungi such as *B. cinerea* which are able to decrease the pH at the infection site are virulent, whereas those which increase the pH such as *Monilia fructigena* Pers.: Fr (brown rot fungus) are inhibited by tomatine (Schlösser, 1975). Thus higher production of organic acids in inoculated control fruit than in chitosan-treated fruit promotes rapid growth of the fungus in control fruit as indicated by the larger lesion size, and actively growing fungus produces more acids enabling it to colonize the tissue further. Bateman and Beer (1965) found a synergistic interaction between oxalate and polygalacturonase in *Sclerotium rolfsii* Sacc. infected plants which may account for the rapid collapse and death of infected tissue. A mixture of endopolygalacturonase and $25 \text{ mmol}\cdot\text{L}^{-1}$ oxalic acid macerated and killed sweet potato [*Ipomea batatas* (L.) Lam.] tissue faster than either of them individually (Ikotun, 1984). Oxalic and fumaric acids act synergistically with cell wall degrading enzymes, especially pectolytic enzymes (Hancock, 1972; Ikotun, 1984; Maxwell and Lumsden, 1970). In the inoculated control, the tissue pH decreased to 4.0 from 4.7 within 14 d after inoculation presumably due to increases in oxalic and fumaric acid content. This would lead to protonation of pectic substances of the cell wall and may contribute to loosening of the wall, facilitating its digestion by cell wall degrading enzymes. Our study demonstrates that a lower level of organic acid production by the pathogen in the presence of chitosan, rendered the fungus less virulent. It would be interesting to see the capacity of the fungus to produce virulence factors with repetitive exposure to chitosan which could be used in evolving hypovirulent strains of the fungus.

Alternaria toxins are known to cause severe chlorosis and necrosis in plant tissue and plant flaccidity with limited water loss (Otani et al., 1995). Results herein demonstrate that lesion expansion and production of AL and AME by the fungus was inhibited by chitosan application concomitant to mycelial growth. However, the specific concentration of toxin was higher in infected tissue from inoculated control fruit compared with chitosan-treated fruit, indi-

cating that chitosan, in addition to inhibiting fungal growth impaired the production of phytotoxic fungal metabolites by the fungus which is in agreement with our previous results (Reddy et al., 1998).

Chitosan treatment also enhanced production of rishitin, a major antifungal phytoalexin in tomato. Several studies have shown the ability of chitosan to induce host defenses. Chitosan treatment of potato slices protects the tissue against soft rot caused by *E. carotovora* by inducing rishitin and phytoalexin (Reddy et al., unpublished). The reduction in the rishitin content after 21 d could be due to decreased capacity of the host to produce rishitin with progress of tissue ripening, or catabolism by the pathogen or even by the host, since they can also be toxic to the host (Darvill and Albersheim, 1984).

One trait that may potentially determine either pathogenicity or virulence towards a specific host is the ability of the fungus to tolerate fungistatic or toxic compounds produced by the host such as phytoalexins (VanEtten et al., 1989). Higher tolerance to the host phytoalexin is correlated to higher virulence on that host (Bowyer et al., 1995; Miao and VanEtten, 1992). Though rishitin was induced in response to infection in inoculated control fruit, the fungus grew without any inhibition due to either tolerance of the fungus to low concentrations of phytoalexin or delayed formation of phytoalexin, as is the case in many host-pathogen interactions which makes this defense less effective (Darvill and Albersheim, 1984). However, accumulation of phytoalexin induced by chitosan treatment before colonization of the tissue by the pathogen, could provide an advantage to the tissue in fighting the infection. Further accumulation of rishitin in chitosan-treated fruit in response to inoculation may reinforce the defense, thereby protecting the tissue. In addition to rishitin, involvement of preformed inhibitors such as tomatine present in high concentrations in the peel of green tomatoes can also contribute to the resistance (Swinburne, 1983), however it degrades with maturity of tissue. Tomatine can be detoxified by tomatinase produced by specific pathogens (Osburn et al., 1995), or rendered ineffective by acidification of the tissue by the pathogen (Schlösser, 1975). The early appearance and fast expansion of lesions observed

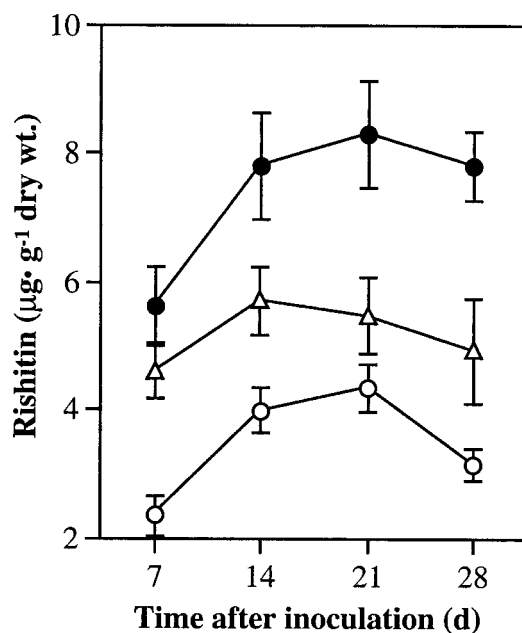


Fig. 5. Changes in rishitin content of tomato fruit treated with (●) or without (○) chitosan, and in fruit treated with chitosan but noninoculated (△). Vertical bars represent ses of five samples per treatment.

in inoculated control fruit may be due to detoxification mechanisms adapted by the fast growing fungus. But with chitosan treatment the fungus remains quiescent or exhibits slower progress of infection due to either loss of virulence of the fungus or preformed antimicrobial substances, and induced antimicrobial compounds such as rishitin (Reddy et al., unpublished) and phenolic compounds (Dornenburg and Knorr, 1997; Pearce et al., 1998; Reddy et al., 1999) in the tissue.

In conclusion, the present investigation demonstrates that stem scar application of chitosan inhibits development of blackmold rot of tomatoes and reduces production of pathogenic factors by the fungus, such as cell wall-degrading enzymes, organic acids, and host specific toxins responsible for fungal penetration and host tissue damage. Chitosan also induces phytoalexin production in the host. Such interactions of chitosan with the pathogen and the host suggest that chitosan could be a useful natural material for postharvest preservation of fruit and vegetables.

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