Chitosan Treatment of Wheat Seeds Induces Resistance to *Fusarium graminearum* and Improves Seed Quality

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Chitosan treatment (2−8 mg/mL) of wheat seeds significantly improved seed germination to recommended seed certification standards (>85%) and vigor at concentrations >4 mg/mL, in two cultivars of spring wheat (Norseman and Max), by controlling seed-borne *Fusarium graminearum* infection. The germination was <80% in the control and >85% in benomyl- and chitosan-treated seeds. Seed-borne *F. graminearum* was reduced to >50% at higher chitosan treatments compared to the control. Synthesis of phenolic acids was stimulated in primary leaves following chitosan treatment, and levels of these phenolic acids, especially ferulic acid, increased significantly with increasing chitosan concentration. Lignin content of primary leaves also showed a similar pattern. The synthesis of precursors of lignin such as *p*-coumaric, ferulic, and sinapic acids and phenolic acids having antimicrobial activity such as benzoic, *p*-coumaric, caffeic, protocatechuic, chlorogenic, ferulic, and gallic acids was also stimulated by chitosan treatment. The induction of phenolic acids and lignin was significantly lower in cv. Max compared to Norseman. Chitosan also inhibited fungal transmission to the primary roots of germinating seedlings. Results suggest that chitosan controlled seed-borne *F. graminearum* infection and increased the resistance in seedlings by stimulating the accumulation of phenolics and lignin. Thus, chitosan has a potential for improvement of seed quality and enhancement of crop yields as well as increased value of stored grains for food and feed.

Keywords: *Fusarium graminearum; wheat seed; chitosan; induced resistance; phenolic acids; lignin; seed quality*

INTRODUCTION

Fusarium head blight (FHB) of wheat (*Triticum aestivum* L.) affects all wheat classes, and *Fusarium graminearum* Schwabe is the principal pathogen in Canada (Gilbert et al., 1993, 1994). This disease causes losses in both yield and quality (Clear and Patrick, 1990). A yield loss of 30−70% has been attributed to FHB in spring wheat from Atlantic provinces of Canada (Martin and Johnston, 1982), and losses due to reduced yield and quality were estimated at $CDN 75 million in 1993 (Gilbert et al., 1994). The fungus is seed borne (Wang and Miller, 1988), and the seeds affected by this epidemic had reduced germination and vigor and produced seedlings with poor emergence (Gilbert and Tekauz, 1995; Gilbert et al., 1997). Furthermore, the fungus is capable of producing trichothecene mycotoxins, which can induce food-borne intoxication in humans and livestock (Ueno, 1987). Appearance of FHB in wheat is a cause for concern, especially for grain growers, livestock producers, and cereal processors.

Seed treatment with fungicides such as imazalil, TCMTB, iprodione, carboxin, thiram, and gauzantine has significantly reduced the amount of seed-borne *F. graminearum* infection (Mihuta-Grim and Forster, 1989). The fungicide propiconazole reduced FHB by 41% and increased the yield by 34% (Martin and Johnston, 1982), and seed quality with respect to size and color was also improved. However, fungicide treatments are discouraged due to toxic residues and development of resistance in pathogens, and none of them have been recommended to treat the grain meant for consumption. Thus, there is an urgent need for safer methods of pathogen control. Manipulation of natural defenses of host could provide a viable alternative (Sequeira, 1990). Host resistance can be enhanced by treatment with elicitors such as salicylic acid (Gaffney et al., 1993) or oligosaccharides and glycoproteins originating from either fungal cell walls (Anderson, 1988) or host cell walls (Ryan and Farmer, 1991).

Chitosan, a polycationic polymer of β-1,4 linked D-glucosamine, is a bioactive agent known to be antifungal (El Ghaouth et al., 1992; Hirano and Nagao, 1989). There is a growing interest in the use of chitosan to provide a level of protection against plant pathogens. Chitosan has been utilized in soil amendment, in seed, and as a foliar treatment to control pathogens (Hadwiger, 1994). The incidence of *Fusarium* crown rot in tomato was reduced by soil amendment and seed treatment with chitosan, and one mechanism appears to be the elicitation of pathogenesis-related proteins in the host (Benhamou et al., 1994). When applied on wounded wheat leaves, chitosan induced lignification and consequently restricted the growth of nonpathogenic fungi in wheat (Barber and Ride, 1988). Chitosan inhibited the growth of *Aspergillus flavus* and aflatoxin production in liquid culture, preharvest maize, and

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groundnut, and it also enhanced phytoalexin production in germinating peanut (Cuero et al., 1991a,b). Our previous study (Bhaskara Reddy et al., 1998) showed that chitosan inhibited growth and toxin production by *Alternaria alternata* f. sp. *lycopersici* in culture.

The objectives of this study were to determine the effects of a chitosan coating of wheat seeds on *F. graminearum* infection, germination, and vigor and also to evaluate the physiological basis of resistance of chitosan-coated seeds.

MATERIALS AND METHODS

Seed Material and Determination of Initial Seed Infection. Spring wheat samples (*Triticum aestivum* L.) of cv. Norseman and Max susceptible to *F. graminearum* were obtained from local growers. Four hundred seeds in four replicates of 100 each were surface sterilized in 0.3% NaOCl solution for 2 min, rinsed several times with sterile water, dried in ambient air, and placed on potato dextrose agar (PDA). The agar plates were placed under continuous cool white light for 7 days at 20 °C, after which time seed-borne *F. graminearum* growing from the seed was identified using standard keys (Nelson et al., 1983).

Preparation of Chitosan. Shrimp shell chitosan was purchased from Nova-Chem (Halifax, NS, Canada) and ground to a fine powder. Purified chitosan was prepared by dissolving chitosan in 0.25 N HCl, and the undissolved particles were removed by centrifugation (15 min, 10000*g*). The viscous solution was then neutralized with 2.5 N NaOH to pH 9.8 to precipitate the chitosan. The precipitated chitosan was recovered by filtration, washed extensively with deionized water to remove salts, and lyophilized. Chitosan stock solution (10 mg/mL) was prepared by dissolving chitosan in 0.05 N HCl, and the pH was adjusted to 5.6. The stock solution was autoclaved and subsequently added to sterile, distilled water to obtain desired final chitosan concentrations of 2, 4, 6, and 8 mg/mL.

Seed Treatment. Seeds were surface sterilized for 2 min by immersion in 0.3% NaOCl, rinsed in sterile distilled water, and immersed into chitosan solutions (pH 5.6) at concentrations ranging from 0 to 8 mg/mL. After gentle stirring for 15 min, the excess solution was drained off and seeds were air-dried in a sterile cabinet and kept in a desiccator until further use. Seeds were also treated with sterile water alone or with 0.02 N HCl in sterile water as controls and with benomyl [0.5 g of active ingredient (ai)/kg of seed] for comparison. For each treatment 400 seeds in four replicates of 100 each were treated separately.

Assessment of Seed Infection, Germination, and Vigor Index. Seed-borne natural infection of chitosan-treated seed was determined as described above. For determining seed germination and vigor, replicates of 100 seeds each were placed in germination pouches (Mega International, Minneapolis, MN) covered with plastic bags. Each pouch was wetted with 10 mL of distilled water and arranged in wired boxes and kept at 20 °C in the laboratory for 10 days. The percentage of germination and lengths of primary roots and shoots were determined on the 11th day. A seed was considered to have germinated if both roots and shoots were present. The vigor index was calculated by multiplying the percentage germination by the mean root and shoot length (centimeters) (Abdul-Baki and Anderson, 1973). The primary roots of the seedlings were also examined for the incidence of *F. graminearum* infection under the microscope as well as by culturing the fungus on PDA, from infected root lesions, and the incidence of disease was expressed as the number of infected roots.

Extraction of Free and Bound Phenolic Acids from Wheat Leaves. Phenolic acids were extracted according to the method described by Southerton and Deverall (1990a). Leaf material from 10-day-old seedlings was collected by cutting 2 cm segments from the region between 1 and 7 cm from the apex of the leaf. Leaf samples weighing 400 mg from each treatment were wrapped in aluminum foil, frozen at −20 °C, and stored in airtight bags until further use. Before extraction, the samples were lyophilized (Freeze-Dry System, Labconco Corp., Kansas City, MO).

Free phenolic acids were extracted from the lyophilized samples. Samples were ground to a fine powder in a mortar using Celite as abrasive. Methanol (5 mL) was added to the leaf powder in 15 mL centrifuge tubes, and the tubes were allowed to stand for 30 min, after which time they were centrifuged at 100*g* for 10 min. The supernatant was retained, and the residue was washed with 5 mL of methanol. After centrifugation of the wash solution, the combined supernatants were evaporated to dryness at 40 °C on a rotary evaporator and redissolved in 5 mL of hot (≈80 °C) distilled water. After centrifugation and cooling to room temperature, the supernatant was transferred to a test tube, and an equal volume of 0.2 M acetate buffer (pH 4.5) containing 0.1 mg/mL β-glucosidase (Sigma Chemical Co., St. Louis, MO) was added. The solution was incubated at 37 °C for 16 h to liberate phenolic acids from glucosides. To release phenolic acids from esters, 2 mL of degassed 3 M NaOH was added, and the mixture was further degassed by bubbling nitrogen through the solution; the test tubes were incubated at 25 °C for 16 h under nitrogen atmosphere.

Bound phenolic acids were extracted from the residues remaining after methanol extraction for free phenolic acids. The methanol was evaporated from the residues and 10 mL of 0.5 M NaOH was added to the centrifuge tubes. The solution was degassed by bubbling nitrogen through the solution and the tubes incubated at 25 °C for 16 h under nitrogen atmosphere.

Gas Chromatography. Phenolic acids were assayed by GC using a 5% phenylmethylsilicone fused silica capillary column (Hewlett-Packard, model DB5-30M, 30 m × 0.25 mm, film thickness = 0.25 μm) with an oven temperature programmed as follows: from 150 °C (isothermal for 2 min) to 300 °C at 5 °C min^{-1}; an isothermal period of 3 min at 300 °C. Injector and detector temperatures were 300 and 325 °C, respectively. Samples of 1.0 μL were injected with a split ratio of 1:50. The linear velocity of the carrier gas (hydrogen) was 35 cm s^{-1} at 100 °C isothermal. The samples were derivatized by adding 50 μL of *N*,*N*-dimethylformamide (DMF) and 50 μL of *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluroacetamide (MTBSTFA) and kept at 65 °C for 1 h before the analysis.

Quantitative Determination of Phenolic Acids. Phenolic acids were estimated by measuring peak areas. Calibration curves of standard phenolic acids relative to an internal standard (*n*-hexacosane) were used for quantitative determination. The concentration of all acids used in the analysis or reproducibility was 100 μg/mL. Standard curves were linear in the range 0−100 μg/mL. The phenolic acids in the samples were identified by comparing their retention times with those of authentic compounds (Sigma Chemical Co.).

Analysis of Lignin Content. For lignin analysis the procedure of Hammerschmidt (1984) was followed. One hundred milligrams of the wheat tissue from the foot region (≈2.5 cm portion of the primary shoot attached to the seed) of 10-day-old seedlings was taken from each treatment and placed in 5 mL of methanol. The tissue was extracted during 48 h with four changes of methanol. After methanolic extraction, the tissue was ground and placed in a test tube containing 0.5 mL of thioglycolic acid (Sigma Chemical Co.) and 5 mL of 2 N HCl. The tubes were capped with glass marbles and heated at 95 °C for 4 h. After cooling, the solids were collected by centrifugation and the supernatant was discarded. The solids were washed by resuspension in 5 mL of water followed by centrifugation. The supernatant was discarded, and the solids were incubated in 5 mL of 0.5 N NaOH for 18 h to solubilize the ligninthioglycolic acid (LTGA). After this extraction, the solids were removed by centrifugation (two washes, with 2 mL of 0.5 N NaOH each time). The NaOH extracts and water washes were combined and poured into a 15 mL conical centrifuge tube, and 1 mL of concentrated HCl was added. The acidified solution was held at 4 °C for 4 h to aid in LTGA precipitation. The precipitated LTGA was collected by centrifugation (500*g*, 15 min). The pellet was washed twice by

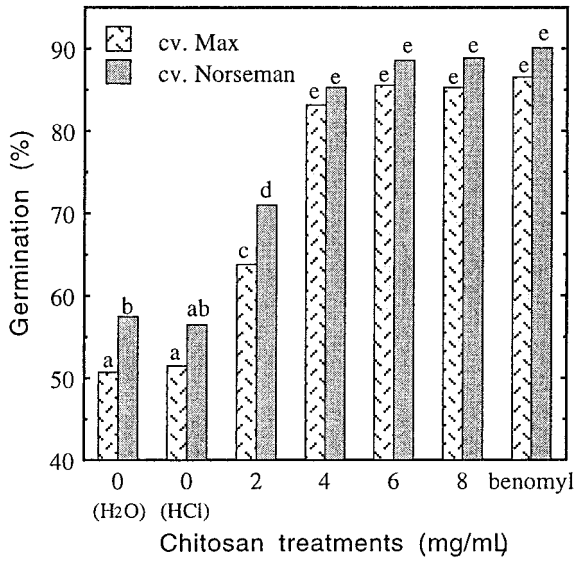

Figure 1. Effect of chitosan treatment on germination of seeds of two wheat cultivars evaluated 10 days after germination at 20 °C. Histograms with the same letter are not significantly different ($P < 0.05$) between both cultivars and chitosan concentrations based on Fisher's (protected) LSD test.

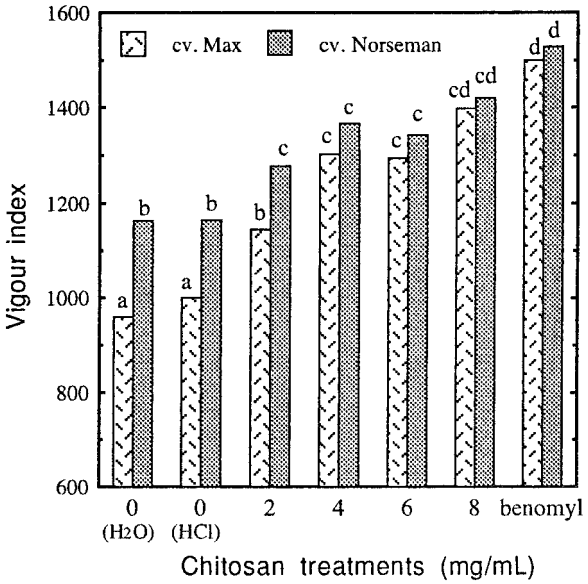

Figure 2. Effect of chitosan seed treatment on vigor of the seedlings of two wheat cultivars evaluated 10 days after germination at 20 °C. Histograms with the same letter are not significantly different ($P < 0.05$) between both cultivars and chitosan concentrations based on Fisher's (protected) LSD test.

resuspension and centrifugation in 0.1 N HCl (2 mL per wash). The final pellet was dissolved in 0.5 N NaOH to a final volume of 2.5 mL. The final solution was centrifuged (10000*g*, 3 min) to remove any insoluble material prior to measurement of the absorbance of the solution at 280 nm.

Statistical Analysis. Each treatment was carried out in triplicates in a completely randomized design, and the experiments were repeated twice. As the variances were homogeneous between the two repetetions, the data were pooled, and the analysis of variance was computed by the program Super ANOVA (Abacus Concepts, Inc.) with cultivar and treatment as fixed variables. The means were compared between the cultivars and the treatments, and least significant differences were calculated by Fisher's (protected) LSD ($P < 0.05$).

RESULTS

Seed Germination and Seedling Vigor. The germination and vigor increased significantly ($P < 0.05$) for both cultivars from 2 to 4 mg/mL chitosan concentration compared to the control, and no further increase was noticed at higher chitosan concentrations (6 and 8 mg/mL) (Figures 1 and 2). Germination of untreated seeds was 57% in cv. Norseman and 50% in Max, which were lower than the certified germination standards for wheat (85%). The germination in chitosan-treated seed varied from 70 to 90% in cv. Norseman. Although the germination at 2 mg/mL treatment was significantly higher compared to the control, it was <85%. The germination was >85% with benomyl treatment for both cultivars and was comparable to 4−8 mg/mL chitosan treatments. No significant difference ($P < 0.05$) in germination was observed between untreated seeds and seeds treated with 0.02 N HCl. Chitosan treatment was also a significant factor in improving seed vigor at concentrations >4 mg/mL. The vigor index was 1163 for control seeds in cv. Norsemen, but it reached 1440 at 8 mg/mL chitosan treatment and 1529 for benomyl-treated seeds. Similarly, it was 958 for control seeds in cv. Max and increased to 1398 at 8 mg/mL chitosan treatment and to 1500 for benomyl-treated seeds (Figure 2). In general, germination and vigor were lower in seeds of cv. Max compared to cv. Norsemen.

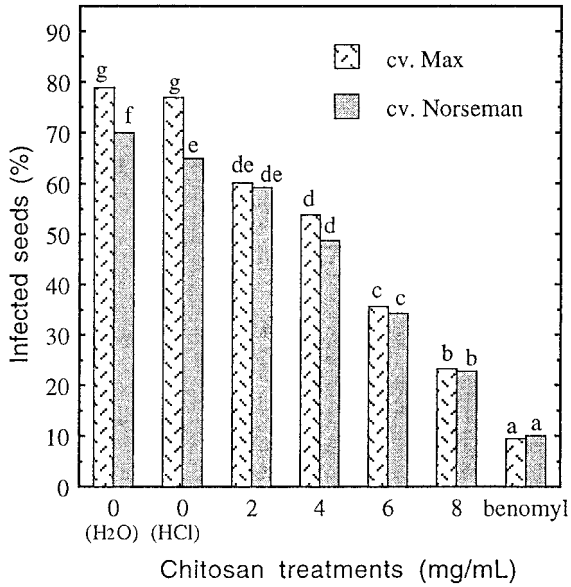

Figure 3. Effect of chitosan treatment on seed-borne infection of *F. graminearum* in two wheat cultivars as assessed by PDA method (see Materials and Methods). Histograms with the same letter are not significantly different ($P < 0.05$) between both cultivars and chitosan concentrations based on Fisher's (protected) LSD test.

Seed and Primary Root Infection. The seed-borne *F. graminearum* infection in seeds of cv. Norseman was 70 and 65% in control and HCl-treated seeds, respectively (Figure 3). Chitosan treatments significantly ($P < 0.05$) reduced the seed-borne infection, which ranged from 59 to 23% from 2 mg to 8 mg/mL treatments, and it was only 10% in benomyl-treated seeds. The initial infection in cv. Max was higher than in Norseman; it was 78% in control seeds of cv. Max and ranged from 60 to 23% from the lower to higher chitosan concentrations. Benomyl-treated seeds recorded an infection level of only 9% (Figure 3). Typical root symptoms of *Fusarium* were characterized by the formation of brownish

Table 1. Effect of Chitosan Seed Coating on Colonization of Primary Roots by *F. graminearum*

treatment (mg/ mL)	infected roots[a] (%)	
	cv. Norseman	cv. Max
chitosan, 0	40.6 ± 2.6 f (70)[b]	39.3 ± 6.1 f (77)
2	27. 5 ± 5.4 e (38)	31.3 ± 2.1 e (49)
4	18.4 ± 4.2 d (21)	20.7 ± 2.5 d (25)
6	10.3 ± 3.7 bc (12)	12.3 ± 5.5 c (15)
8	6.6 ± 2.2 ab (7)	8.3 ± 4.1 b (9)
benomyl	2.7 ± 2.5 a (2)	4.3 ± 2.0 a (3)
HCl	38.6 ± 2.0 f (68)	36.5 ± 4.0 f (71)

[a] Means followed by the same letter are not statistically different at 0.05 probability level based on the Fisher's (protected) LSD test. [b] Numbers in parentheses represent percentage of infected primary roots with seed germination corrected to 100%.

or reddish brown lesions, and these seedlings showed symptoms of root rot as well. Microscopic examination and subsequent culture of root symptoms revealed infection by *F. graminearum*. Chitosan seed coating resulted in significant reduction of primary root infections (Table 1), which suggests that the spread of seed-borne *Fusarium* to the seedling parts was impeded.

Free and Bound Phenolic Acids. Six cinnamic acids, eight benzoic acid derivatives, and one cinnamic acid ester (chlorogenic acid) were identified by GC in the extracts from the treated and untreated leaves of two cultivars in both free and bound forms (Tables 2−5). There is no difference in the levels of total phenolic acid titer between the control and HCl treatments, but the level was lower with benomyl treatment compared to control. There was significant interaction ($P < 0.05$) between cultivar and chitosan concentration on the accumulation of free and bound phenolic acids. The accumulation of both free and bound phenolic acids was higher in cv. Norseman than in cv. Max. The increase in total free phenolic acid titer with increase in chitosan concentration was higher up to 4 mg/mL chitosan concentration in both cultivars, but a sharp increase in total bound phenolic acids was found at 2 mg/mL chitosan concentration. A >50% increase in total free phenolic acid titer was observed at 8 mg/mL treatment in both cultivars compared to the control, and the increase was >40% in the total bound phenolic acid titer. The levels of both total phenolic acids and total cinnamic acid derivatives were higher in the bound form in both cultivars, but the levels of benzoic acid derivatives were higher in the free form. In both cultivars, the levels of cinnamic acids were higher than the benzoic acid derivatives. Ferulic acid titer was far higher among all of the phenolic acids in both free and bound forms in both cultivars. It represented >50 and >60% of free and bound cinnamic acids and >30 and >40% of total bound cinnamic and phenolic acids in both cultivars, respectively. An increase of >80% in free ferulic acid was observed at 8 mg/mL chitosan treatment in both cultivars, but the increase was lower (35−55%) in the bound form. Furthermore, an increase of >50% in free *p*-coumaric, caffeic, benzoic, 4-hydroxybenzoic, protocatechuic, gallic, and chlorogenic acids were also recorded at 8 mg/mL chitosan concentration in cv. Norseman, but their increases in the bound form was lower. The increases in these phenolic acids at 8 mg/mL chitosan concentration was also comparable in cv. Max. The increases in some of the phenolic acids such as sinapic, vanillic, and salicylic were smaller in both free and bound form with increases in chitosan concentration in both cultivars. The pattern was similar for free syringic acid, but it was found in smaller quantities in the bound form and its increase with chitosan concentration was also small.

Lignin. Lignin deposition increased significantly ($P < 0.05$) with increases in chitosan treatment in both cultivars (Figure 4), and it was again lower in cv. Max compared to Norseman. The lignin content increased by >50% in seedlings of both cultivars from seed treated with 6 and 8 mg/mL chitosan concentrations compared to respective controls. No significant difference was observed in lignin content between the control, HCl, and benomyl treatments, which were significantly lower compared to chitosan treatments.

DISCUSSION

Seed and grain qualities are recognized as major factors that determine the success or failure of a crop and consumption value of grains. This study demonstrates that seed treatment with chitosan controls natural seed-borne *F. graminearum* infection, contributing to the improvement of seed quality. Chitosan is known to improve the yield and quality of wheat and reduce mycotoxin production in maize (Hadwiger et al., 1984; Cuero et al., 1991a). Chitosan treatment is likely to provide a temporary barrier between the soil-borne organisms in contact with the seed. This is necessary when the seed is sown in infested soil. Generally, soil is the principal reservoir of *F. graminearum* in addition to the seed-borne infection. Wheat seed that was only lightly infected with *F. graminearum* exhibited impaired germination and extensive fungal invasion of the scutellum and embryonic axis during imbibition (Bechtel et al., 1985). It is evident from this study that chitosan-treated seed maintained higher germination by controlling fungal growth. McMullen and Stack (1987) reported significant improvement in emergence following fungicide seed treatment of spring wheat that was moderately damaged by FHB; however, phytotoxicity symptoms were associated with fungicide treatments (Piening et al., 1983), in addition to other concerns of toxic residues and development of resistance of pathogens to fungicides. Chitosan seed treatment even at the highest level (8 mg/mL) did not show any symptoms of phytotoxicity as we did not observe any seedling abnormality other than the one associated with fungal infection, and no adverse effects of chitosan as a plant protection agent have been shown yet.

Several lines of experimental evidence have shown that seed treatments with bacterial or fungal antagonists are effective in protecting germinating embryos and seedlings from the damaging action of root pathogens by antibiosis, mycoparasitism, and competition. Evidently such mechanisms do not likely account for the restriction of pathogen in chitosan-treated seed, but antifungal properties of chitosan may partly be responsible for a reduction of the pathogen growth at the seed surface. Our results indicate that induction of phenolic acids and lignification of cells is one of the mechanisms by which chitosan contributes to seedling protection. Several possibilities of imbibition of chitosan by the seed are explored. It is possible that imbibition of seeds

Table 2. Free Phenolic Acids in Primary Leaves of Wheat Cv. Norseman after 10 Days of Growth following Seed Treatment with Chitosan

phenolic acid, cinnamic/*benzoic* acid derivatives	phenolic content of primary leaves[a] (mg g^{-1} of dry weight)						
			chitosan concentration (mg/ mL)				
	control[b]	HCl	2	4	6	8	benomyl
trans-cinnamic	56.4 c	53.2 b	55.4 bc	62.6 d	65.2 d	72.2 e	45.6 a
benzoic	*42.2 b*	*44.4 b*	*52.6 c*	*62.2 d*	*65.6 e*	*68.4 e*	*38.4 a*
p-coumaric	35.6 a	38.5 ab	52. 3 b	60.5 c	62.3 c	68.5 d	33.7 a
4-hydroxybenzoic	*38.2 b*	*45.3 c*	*52.6 d*	*58.5 e*	*60.6 e*	*65.2 f*	*28.2 a*
caffeic	32.6 b	35.4 c	42.6 d	45.4 d	53.6 e	58.5 f	25.6 a
protocatechuic	*42.6 b*	*45.2 c*	*46.2 c*	*52.2 d*	*63.6 e*	*65.2 e*	*38.6 a*
chlorogenic	30.2 ab	33.2 b	30.6 ab	42.5 c	45.6 d	52.2 e	28.6 a
ferulic	311.5 ab	317.6 b	368.6 c	544.5 d	556.5 e	568.6 f	306.3 a
vanillic	*93.4 ab*	*85.0 a*	*95.3 b*	*98.5 b*	*102.6 c*	*102.8 c*	*88.6 a*
sinapic	105.6 b	96.3 a	125.3 c	134.5 d	142.6 e	144.6 e	103.4 ab
syringic	*85.6 a*	*82.7 a*	*96.3 b*	*106.5 c*	*114.5 cd*	*118.4 d*	*82.3 a*
o-coumaric	36.5 c	33.2 b	35.2 bc	38.6 c	42.2 d	52.6 e	30.6 a
salicylic	*82.6 b*	*78.2 a*	*85.6 c*	*92.2 d*	*98.4 e*	*96.2 e*	*80.4 ab*
3,4,5-trihydroxycinnamic[b]	nd[c]	nd	nd	nd	nd	nd	nd
gallic	*38.6 b*	*35.2 ab*	*42.2 c*	*52.6 d*	*63.6 e*	*65.2 e*	*32.6 a*
2,5-dihydroxycinnamic[b]	nd	nd	nd	nd	nd	nd	nd
gentisic	*52.3 b*	*55.6 c*	*53.6 bc*	*62.3 d*	*66.8 e*	*65.6 e*	*45.2 a*
total soluble acids (%)	1084 (100)	1079 (99)	1234 (113)	1513 (139)	1604 (148)	1664 (153)	1008 (93)
total cinnamic acid derivatives	578.2	574.2	679.4	886.1	922.4	965.0	545.2
total benzoic acid derivatives	*475.5*	*471.6*	*524.4*	*585.0*	*635.7*	*647.0*	*434.3*

[a] Means followed by the same letter for a given phenolic acid are not significantly different at 0.05 probability level based on the Fisher's (protected) LSD test. Figures in parentheses are means expressed as precentage of the amount of the total soluble acids relative to the control. [b] Control seeds were treated with sterile water. Free phenolic acids are phenolic acids liberated from methanol soluble esters and glycosides by hydrolysis with NaOH. [c] nd, not determined.

Table 3. Bound Phenolic Acids in Primary Leaves of Wheat Cv. Norseman after 10 Days of Growth following Seed Treatment with Chitosan

phenolic acid, phenolic/*benzoic* acid derivatives	phenolic content of primary leaves[a] (mg g^{-1} of dry weight)						
			chitosan concentration (mg/ mL)				
	control[b]	HCl	2	4	6	8	benomyl
trans-cinnamic	48.2	46.2	52.4	68.6	72.4	70.6	35.4
benzoic	*53.6 b*	*52.4 b*	*56.8 c*	*63.2 d*	*72.6 e*	*70.4 e*	*48.2 a*
p-coumaric	76.6 a	83.3 ab	93.6 b	105.5 c	123.3 d	138.7 e	76.6 a
4-hydroxybenzoic	*45.2 bc*	*43.6 b*	*48.2 c*	*52.3 d*	*62.6 e*	*64.2 e*	*38.6 a*
caffeic	42.6 b	44.2 bc	46.8 c	50.2 d	62.6 e	65.6 f	38.2 a
protocatechuic	*48.6 b*	*45.2 ab*	*48.6 b*	*52.2 c*	*63.2 d*	*66.2 e*	*42.2 a*
chlorogenic	36.2 c	30.6 a	33.6 b	42.6 d	45.2 e	45.6 e	32.3 ab
ferulic	482.3 ab	488.6 b	664.3 c	705.2 d	727.7 e	744.2 f	478.2 a
vanillic	*57.3 b*	*51.6 a*	*75.6 c*	*79.2 c*	*89.2 d*	*91.6 d*	*48.2 a*
sinapic	84.6 b	82.3 b	97.2 c	103.7 c	114.2 d	118.3 d	75.3 a
syringic	*36.3 b*	*35.4 b*	*36.4 b*	*38.2 b*	*40.3 bc*	*50.3 c*	*27.4 a*
o-coumaric	42.6 b	43.2 b	45.6 c	52.3 d	58.6 e	60.2 e	38.2 a
salicylic	*72.3 b*	*70.6 ab*	*72.6 b*	*78.2 cd*	*80.2 d*	*85.6 e*	*68.6 a*
3,4,5-trihydroxycinnamic[b]	nd[c]	nd	nd	nd	nd	nd	nd
gallic	*43.2 c*	*38.6 b*	*45.2 c*	*50.3 d*	*56.2 e*	*62.2 f*	*35.6 a*
2,5-dihydroxycinnamic[b]	nd	nd	nd	nd	nd	nd	nd
gentisic	*62.6 b*	*65.2 bc*	*66.2 c*	*65.6 bc*	*72.2 d*	*75.6 e*	*58.2 a*
total insoluble acids (%)	1232 (100)	1221 (99)	1483 (120)	1607 (130)	1740 (141)	1808 (147)	1141 (93)
total cinnamic acid derivatives	776.9	787.8	999.9	1085.5	1158.8	1197.6	741.9
total benzoic acid derivatives	*419.1*	*402.6*	*449.6*	*479.2*	*536.5*	*566.1*	*367.0*

[a] Means followed by the same letter for a given phenolic acid are not significantly different at 0.05 probability level based on the Fisher's (protected) LSD test. Figures in *parentheses* are means expressed as precentage of the amount of the total insoluble acids relative to the control. [b] Control seeds were treated with sterile water. Bound phenolic acids are phenolic acids liberated from methanol insoluble esters by hydrolysis with NaOH. [c] nd, not determined.

following planting in a wet substrate likely affects the seed envelope permeability, causing microscopic ruptures through which chitosan oligosaccharides can diffuse. The other possibility is that interaction with the chitosan molecule might be initiated upon seed germination. This obviously implies surface interaction between the emerging seedling and the seed coat, which could lead to the transfer of chitosan from seed coat to both plumule and the radicle. Hadwiger (1994) showed that radiolabeled chitosan-H^3 applied to wheat seeds was detectable in leaves of seedling plants. The other possibility is the presence of enzymes in germinating wheat capable of releasing chitosan oligomers. Detection of chitinase activities in wheat germ and leaves and *N*-acetyl-β-D-glucosaminidase activity in the aleurone layers of resting wheat seeds suggests that chitosan can be degraded into oligosaccharides (Molano et al., 1979; Barber and Ride, 1988; Carratu et al., 1985).

The lignification-eliciting activity of the oligomers broadly matches their binding specificity for wheat germ

Table 4. Free Phenolic Acids in Primary Leaves of Wheat Cv. Max after 10 Days of Growth following Seed Treatment with Chitosan

phenolic acid, cinnamic/*benzoic* acid derivatives	phenolic content of primary leaves[a] (mg g^{-1} of dry weight)						
			chitosan concentration (mg/ mL)				
	control[b]	HCl	2	4	6	8	benomyl
trans-cinnamic	53.4 b	50.2 b	52.2 c	62.2 c	63.2 c	60.4 c	45.6 a
benzoic	*33.4 b*	*35.6 b*	*32.2 b*	*45.6 c*	*53.2 d*	*55.6 d*	*28.6 a*
p-coumaric	44.4 b	40.3 ab	54.6 c	53.3 c	61.2 d	65.6 d	35.2 a
4-hydroxybenzoic	*32.2 b*	*35.6 b*	*42.3 c*	*46.2 c*	*52.2 d*	*54.6 d*	*28.2 a*
caffeic	28.5 a	25.4 a	28.5 a	33.4 b	42.4 c	40.6 c	26.4 a
protocatechuic	*35.6 ab*	*38.2 b*	*42.6 c*	*45.2 cd*	*52.6 d*	*56.2 e*	*32.2 a*
chlorogenic	25.6 a	28.2 ab	30.2 b	35.6 c	38.2 d	42.6 e	25.2 a
ferulic	244.2 ab	249.2 b	291.6 c	425.2 d	458.3 e	462.3 e	239.3 a
vanillic	*85.3 a*	*84.2 a*	*92.6 b*	*97.2 b*	*113.3 c*	*123.3 d*	*81.3 a*
sinapic	95.2 b	91.2 b	104.3 c	113.3 d	115.2 d	115.3 d	67.6 a
syringic	*71.2 b*	*65.6 ab*	*79.2 c*	*80.2 c*	*95.3 d*	*104.2 e*	*60.2 a*
o-coumaric	35.2 ab	36.2 b	38.6 b	45.3 c	46.2 c	50.6 d	32.5 a
salicylic	*52.2 bc*	*50.2 b*	*54.6 c*	*58.2 d*	*62.6 ef*	*65.2 f*	*45.2 a*
3,4,5-trihydroxycinnamic[b]	nd[c]	nd	nd	nd	nd	nd	nd
gallic	*35.6 b*	*38.2 b*	*42.2 c*	*46.8 d*	*52.3 e*	*58.6 f*	*30.2 a*
2,5-dihydroxycinnamic[b]	nd	nd	nd	nd	nd	nd	nd
gentisic	*38.2 b*	*35.6 ab*	*42.6 c*	*48.2 d*	*52.6 ef*	*55.6 f*	*32.6 a*
total soluble acids (%)	909 (100)	904 (99)	1029 (113)	1235 (136)	1306 (144)	1411 (155)	810 (89)
total cinnamic acid derivatives	501.0	492.5	569.8	732.7	786.5	794.8	446.6
total benzoic acid derivatives	*383.7*	*383.2*	*428.3*	*467.6*	*432.1*	*573.3*	*338.5*

[a] Means followed by the same letter for a given phenolic acid are not significantly different at 0.05 probability level based on the Fisher's (protected) LSD test. Figures in parentheses are means expressed as precentage of the amount of the total soluble acids relative to the control. [b] Control seeds were treated with sterile water. Free phenolic acids are phenolic acids liberated from methanol soluble esters and glycosides by hydrolysis with NaOH. [c] nd, not determined.

Table 5. Bound Phenolic Acids in Primary Leaves of Wheat Cv. Max after 10 Days of Growth following Seed Treatment with Chitosan

phenolic acid, cinnamic/*benzoic* acid derivatives	phenolic content of primary leaves[a] (mg g^{-1} of dry weight)						
			chitosan concentration (mg/ mL)				
	control[b]	HCl	2	4	6	8	benomyl
trans-cinnamic	63.2 ab	60.4 a	65.6 b	64.2 b	72.6 c	75.2 c	60.6 a
benzoic	*48.6 b*	*45.3 a*	*50.6 bc*	*53.2 c*	*61.2 d*	*65.6 e*	*43.2 a*
p-coumaric	58.6 a	65.2 b	104.2 c	112.2 d	115.2 d	110.2 d	64.2 b
4-hydroxybenzoic	*43.2 b*	*45.6 b*	*52.1 c*	*54.6 c*	*58.2 d*	*62.3 d*	*38.6 a*
caffeic	35.2 b	33.2 b	42.6 c	48.4 d	45.6 cd	53.6 e	28.4 a
protocatechuic	*42.5 b*	*45.6 bc*	*48.3 c*	*52.4 d*	*62.4 e*	*65.6 e*	*35.2 a*
chlorogenic	33.2 b	35.6 b	36.2 b	42.6 c	45.6 c	52.2 d	28.6 a
ferulic	479.2 b	499.6 c	555.5 d	610.2 e	666.2 f	663.2 f	465.6 a
vanillic	*53.3 b*	*45.2 a*	*68.2 c*	*69.2 c*	*80.2 d*	*99.2 e*	*45.3 a*
sinapic	82.6 b	78.3a	86.6 b	95.2 c	109.3 d	110.2 d	75.2 a
syringic	*33.6 b*	*32.2 b*	*34.6 b*	*36.2 b*	*39.2 bc*	*44.2 c*	*26.2 a*
o-coumaric	42.6 b	45.6 b	45.2 b	52.2 c	55.6 cd	58.2 d	38.6 a
salicylic	*52.6 b*	*55.6 b*	*55.2 b*	*62.2 c*	*65.6 c*	*70.2 d*	*48.6 a*
3,4,5-trihydroxycinnamic[b]	nd[c]	nd	nd	nd	nd	nd	nd
gallic	*45.2 ab*	*46.8 b*	*45.6 ab*	*52.2 c*	*56.8 d*	*60.2 e*	*42.2 a*
2,5-dihydroxycinnamic[b]	nd	nd	nd	nd	nd	nd	nd
gentisic	*32.6 a*	*35.2 a*	*40.2 b*	*45.6 c*	*53.6 d*	*55.6 d*	*32.2 a*
total insoluble acids (%)	1146 (100)	1169 (102)	1331 (116)	1450 (126)	1587 (138)	1645 (143)	1073 (93)
total cinnamic acid derivatives	761.4	782.3	899.7	982.4	1064.5	1070.6	732.6
total benzoic acid derivatives	*351.6*	*351.5*	*394.8*	*425.6*	*477.2*	*522.9*	*311.9*

[a] Means followed by the same letter for a given phenolic acid are not significantly different at 0.05 probability level based on the Fisher's (protected) LSD test. Figures in parentheses are means expressed as precentage of the amount of the total insoluble acids relative to the control. [b] Controls were treated with sterile water. Bound phenolic acids are those liberated from methanol insoluble esters by hydrolysis with NaOH. [c] nd, not determined.

agglutinin (Goldstein et al., 1975), which indicates that binding of chitosan to germinating embryos is necessary to elicit defense reactions. Chitosan, as a highly positively charged polymer, is likely to have an affinity for negatively charged molecules throughout the plant tissue (Hadwiger et al., 1989). It has been demonstrated that chitosan adsorbs to the plant cell wall (Hadwiger et al., 1981) and interacts with plant cell membrane for its biological action (Kauss et al., 1990). Considering its polycationic nature, chitosan may readily interact with negatively charged residues of macromolecules exposed at the cell surface, thus causing important changes in the membrane composition (El Ghaouth et al., 1992; Leuba and Stossel, 1986). In addition, chitosan may also interact with cellular DNA of the plant and mimic the gene activating action of a plant pathogenic fungus inducing defense reactions in the plant (Hadwiger et al., 1984, 1989). However, the possibility that chitosan itself directly interacts with fungal hyphae cannot be ruled out. Whatever the mode of chitosan action, our results

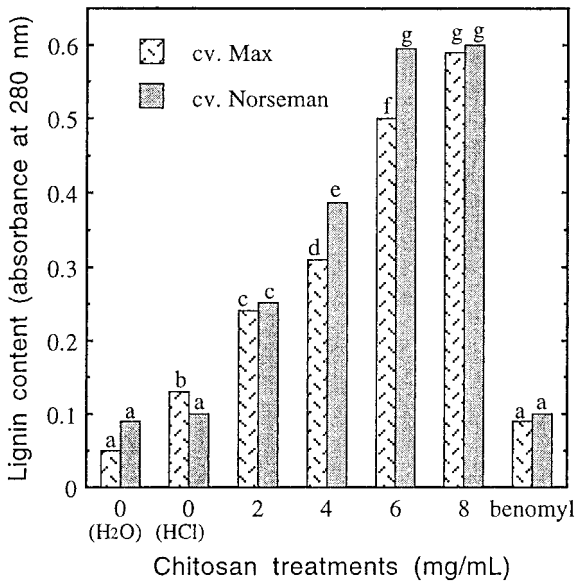

Figure 4. Effect of chitosan seed treatment on lignin content in the foot region of the seedlings of two wheat cultivars evaluated 10 days after germination at 20 °C. Histograms with the same letter are not significantly different ($P < 0.05$) between both cultivars and chitosan concentrations based on Fisher's (protected) LSD test.

convincingly show that the successful suppression of the fungal growth by chitosan treatment is likely to benefit the growth and, subsequently, the yield of treated crops.

Resistance of chitosan-treated seed to *F. graminearum* was characterized by the apparent failure of the fungus to penetrate into the seed tissue, even though there was some growth on the surface. Significant increase in lignin deposition following chitosan treatment and decrease in tissue infection suggest a role for lignification in the active defense of germinating wheat seed against *F. graminearum*. Ride and co-workers (Ride, 1978; Ride and Pearce, 1979) have clearly demonstrated that lignified cell walls are more resistant to cellulases and pectinases. Biochemical studies have shown increased levels of a number of key enzymes of the phenylpropanoid pathway in wheat leaves during the expression of resistance to rust fungi (Moerschbacher et al., 1989; Southerton and Deverall, 1990b). Lignin synthesized via this pathway has been implicated in the resistance of wheat to the fungal pathogens *Puccinia graminis* f.sp. *tritici* (Tiburzy and Reisener, 1990) and *P. recondita* f.sp. *tritici* (Southerton and Deverall, 1990c). Treatment of wheat seeds with chitosan prior to planting suppressed lodging of the stem caused by *Pseudocercosporella herpotrichoides* due to lignin deposition as lignin is considered to be a cell- or stem-strengthening component, and the yields increased by ~10% (Hadwiger et al., 1984).

Many of the phenolic acids are known to have antifungal activity (Nicholson et al., 1989). The cinnamic acid derivatives having antimicrobial activity include caffeic, *p*-coumaric, and ferulic acids, and the benzoic acid derivatives include benzoic, protocatechuic, and gallic acids (Beuchat, 1994; Walker, 1994). Chlorogenic acid may be the most important antimicrobial acid (Dixon and Paiva, 1995). Ferulic, *p*-coumaric, and sinapic acids are precursors of lignin (Vance et al., 1980). Appreciable amounts of phenolic acids were present in the control seeds, presumably elicited by seed-borne infection. However, the levels were lower in benomyl-treated seeds compared to control, because benomyl effectively reduced the level of seed-borne infection. Chitosan also reduced seed-borne infection while accumulating phenolic acids in proportion to its concentration. This suggests that chitosan interacts with the plant tissue and elicits host defense in addition to its direct antifungal activity. Chitosan treatment increased antimicrobial cinnamic acids (caffeic, *p*-coumaric, and ferulic), benzoic acid derivatives (benzoic, protoctechuic, and gallic), and ester (chlorogenic acid). The accumulation of various cinnamic acid derivatives and their corresponding benzoic acid derivatives suggests that chitosan activates the phenylpropanoid pathway. Recently, Dornenburg and Knorr (1997) showed that chitosan induced the activity of phenylalanine ammonia-lyase in potato cell cultures, the key enzyme in the phenylpropanoid pathway. Furthermore, the increases in the levels of lignin precursors (ferulic and *p*-coumaric acids) were also significant with the exception of sinapic acid, although the latter was present in high levels in the naturally infected control tissue. This suggests that lignin induced in response to infection is likely to be copolymers of coniferyl and sinapyl alcohols with a small proportion of *p*-coumaryl alcohol, whereas chitosan-induced lignin may likely be formed predominantly from coniferyl alcohol.

Higgins (1982) reported that hyphal tips of *Cladosporium fulvum* near wall appositions in resistant tomato leaves often were discolored yellow, suggestive of phenol deposition. Although we detected *Fusarium* in root samples of chitosan-treated seed, it is possible that it was restricted only to epidermis and cortex and not xylem. Benhamou et al. (1994) reported that in chitosan-treated tomato seeds, *Fusarium* was restricted to the surface of root tissue. Although a trace of mycelium was observed on the germinating seed in the present study, it failed to penetrate the growing radicle, probably due to deposition of phenols at the hyphal tips in addition to lignification of host cells. Our results clearly demonstrate a positive relation between phenolic acid accumulation and reduction in pathogen incidence. Chitosan also appears to have sensitized the tissue that it was able to respond more rapidly to fungal attack. The concentration and speed at which defense compounds accumulate against infection determine the host susceptibility or resistance against pathogenic infection (Zook and Kuc, 1991). Thus, chitosan seed treatment has the potential to inhibit the growth of fungal pathogens in the vicinity of the seed by direct contact and to enhance plant response by entering into the emerging seedlings.

It has been shown that percentage of infected kernels correlated highly with the concentration of deoxynivalenol (DON), a mycotoxin produced by *F. graminearum* (Wong et al., 1995). The relationship between growth of *F. graminearum* and water potential of wheat kernels is another interesting area to discuss as seed moisture content plays an important role in the growth of mycotoxigenic fungi. Polymeric coatings have been investigated to retard imbibition rate when seeds are placed in a moisture-saturated atmosphere and to avoid chilling injury in large-seeded legumes and cotton, which was attributed to rapid water uptake when seeds of low moisture content are sown in a cold and wet soil (Herner, 1986). Chitosan by its film-forming nature and as a physical barrier can no doubt play an important role in seed moisture content control, which would

influence germination rate and plant performance, in addition to its antifungal and bioeliciting properties.

In conclusion, chitosan treatment controlled natural seed-borne *F. graminearum* infection and increased seed germination and vigor; it also elicited phenolic acid production and lignin deposition in germinating seedlings. Thus, there is a high potential for chitosan in developing strategies for the management of seed-borne pathogens and reducing risks of mycotoxin in foods and feedstocks.

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