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Resistance Induction Against White Rot of Tuber Mustard Using Chitosans and Acetyl Salicylic Acid

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Abstract

Introduction: White rot, caused by *Sclerotinia sclerotiorum*, has recently become a serious threat to tuber mustard cultivation in Hangzhou, China. The objective of this study was to evaluate the inhibitory effect of acetyl salicylic acid (ASA) and three different chitosans (A, B and C) against mustard white rot. The degree of N-deacetylation and the molecular weight of chitosans A, B and C were 85%-1129 kDa, 95%-521 kDa and 75%-607 kDa, respectively.

Materials and Methods: The inhibitory effect of chitosans with different concentrations against the mycelia growth and sclerotia formation of 3 isolates of the pathogen was determined in vitro. In addition, the efficacy of chitosans and ASA against mustard white rot was assessed during in vivo tests. After protein extraction, effects of chitosans and ASA on resistance related enzymes including chitinase, β-1,3-glucanase, phenylalanine ammonia lyase, polyphenol oxidase (PPO) and peroxidase (POD) were evaluated.

Results: The chitosans reduced the mycelia growth and sclerotia formation of the pathogen. The chitosans showed significant antifungal effect against the disease in vivo. The chitosans and ASA markedly reduced the severity of the disease over time. Moreover, the chitosans and ASA markedly enhanced the level of most of the resistant related enzymes after 3 and 6 days. The chitosan B was found to have the best effect against tested pathogen isolates.

Conclusions: The chitosan with the lowest molecular weight was found to be more effective against the disease. In addition, chitosans and ASA were able to significantly increase resistance-related enzymes over time indicating that they can be considered as resistant inducers against mustard white rot.

Keywords: *Brassica juncea*, Chitinases, Peroxidase, POD, Sclerotinia Sclerotiorum

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Introduction

Tuber mustard, *Brassica juncea* var. *tumida*, is an economically and nutritionally important Brassica plant which is grown in many fields of Hangzhou, capital of Zhejiang province, in south east China. White rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, has recently become a serious threat to tuber mustard cultivation in Hangzhou and other mustard growing parts of the Zhejiang province. The pathogen is a soil borne ubiquitous fungus which can infect over 400 dicotyledonous plant species.[1] Initial symptoms of white rot appear as water soaked spots on leaves or stems. Later, the lesions on the leaf extend to petiole and infect the stem. Lesions on the stem appear as a pale grey to white lesions on the stem at or above the soil surface. As the disease advances, it spreads to the upper branches. Finally, stem girdles at the point of infection, leading to wilting and the death of the plant. Black sclerotial bodies are produced on or inside the hollow stem. Even though no study has been carried out to evaluate yield loss caused by *S. sclerotiorum* in Hangzhou, Sclerotinia rot of mustard is reported to cause yield losses up to 40 percent in India.[2] Although some control methods such as biological control,[3-5] biofumigation[6,7] and application of plant crude extracts[8,9] have been effective against Sclerotinia diseases, the majority of local mustard growers prefer the application of fungicides mostly iprodione and dichloran.[10] However, due to increasing concerns about the environment, human health and the development of fungicide resistance several research have been conducted in the Zhejiang province to introduce sustainable control methods to farmers in order to reduce fungicides applied in mustard fields.

Chitin is the most abundant natural amino polysaccharide on the earth and chitosan (β-1,4-linked glucosamine) is

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a deacetylated derivative of chitin, although the degree of the N-deacetylation is almost never complete.[11] There are many studies showing that chitosan is able to induce a considerable defensive reaction in plants against several pathogens including *S. sclerotiorum*.[12,13] The N-deacetylation and molecular weight are two key characteristics of chitosans for particular functions.[14] In addition to inducing systemic acquired resistance, chitosans have shown significant antifungal activities against numerous plant pathogens.[13-15]

Salycilic acid (SA) is a phenolic plant hormone which plays an important role in regulating defenses in plants against biotrophic and hemibiotrophic pathogens.[16] As reported by Gaffney et al,[17] transgenic plants which express the bacterial *NahG* gene resulting in the change of SA into catechol are more susceptible to several pathogens. Delaney et al[18] showed that the endogenous level of SA and/or its conjugates increase in the plants infected by phytopathogens which lead to higher expression of pathogenesis related genes. No research has been conducted to assess acetyl salicylic acid (ASA) and compare chemically-different chitosans against mustard white rot.

Therefore, the objective of this study was to evaluate the inhibitory effect of ASA and 3 chitosans with different N-deacetylation and molecular weight against 3 isolates of *S. sclerotiorum*. Enzymatic analysis was carried out to assess the activity of 3 important enzymes involved in the resistance induction against phytopathogens.

Materials and Methods

Origin of *Sclerotinia sclerotiorum* and Chemicals

Three isolates of *S. sclerotiorum* were chosen from the isolates collected from infected tuber mustard fields in Hangzhou. These isolates had already shown high aggressiveness against mustard plants under greenhouse conditions (data not published). The isolates were designated as 1, 2 and 3, respectively. They were routinely cultured on Potato Dextrose Agar (PDA, infusion of 200 g of potato, 20 g of dextrose, and 15 g of agar for 1 L of medium) and stored at 4(±1)°C until used.[5] Three different kinds of chitosan produced from crab shells were obtained from Sigma-Aldrich (St Louis, MO, USA) and were named as chitosan A, chitosan B and chitosan C, respectively. The N-deacetylation and molecular weight of chitosans A, B and C were 85%-1129 kDa, 95%-521 kDa and 75%-607 kDa, respectively. In order to prepare 2 g/L solution of different chitosans, 2 g of each chitosan powder was dissolved in 80 mL of deionized water to which 2.5 mL of 10 N acetic acid had been added. After stirring (300 rpm) for 12 hours at room temperature, the pH was adjusted to 5.7 using 1 N NaOH. The solution was then made up to 100 mL with deionized water and autoclaved at 121°C for 20 minutes. The 0.5 and 5 g/L solutions were also made using this method.[13] In addition, ASA ($C_9H_8O_4$, heavy metals <20 ppm, purity >98%) was purchased from the Sangon Chemical Company (Shanghai, China).

In Vitro Effect of Chitosans Against *Sclerotinia sclerotiorum*

The isolates of *S. sclerotiorum* were grown on Carrot Dextrose Agar (CDA, infusion of 200 g of carrot, 20 g of dextrose, and 15 g of agar for 1 L of medium; final pH at 25 °C=5.6±0.2) and were used for *in vitro* and *in vivo* experiments.

Two drops (20 µL) of each chitosan (A, B and C) at the concentrations of 0.5, 2 and 5 g/L were swabbed on the surface of CDA in 90 mm Petri plates using a glass L-shaped rod. A 5 mm mycelial plug taken from the leading edge of a 3 day mycelium was then centrally inoculated facing down onto each CDA medium.[13] There were two sets of controls in this test: sterile deionized water (C1) and 100 mL autoclaved deionized water containing 2.5 mL of 10 N acetic acid and adjusted to pH 5.6 using 1 N NaOH (C2). Two drops of C1 and C2 were used in controls. The Petri plates were sealed and incubated at 21-22°C. The percent inhibition of *S. sclerotiorum* radial growth in each treatment was measured 5 days after inoculation using the formula $I=100-(T\times100/C)$, where: I= percent growth inhibition, T= colony diameter in treatment and C= colony diameter in control. In addition, the percent inhibition of sclerotia formation was calculated after 15 days according to the formula $S=100-(Ts\times100/Cs)$, where: S= percent inhibition of sclerotia formation, Ts= the number of sclerotia in treatment and Cs= the number of sclerotia in control.[8] This experiment was repeated 3 times with 5 repetitions for each treatment, and the effects of different treatments were determined in a completely randomized design.

Antifungal Effect of Chitosans Against Mustard White Rot

Tuber mustard (*B. juncea* var. *tumida*) seeds were bought from Feng Seeds company (Ningbo, Zhejiang province, China). After disinfection in 2% hypochlorite sodium for 2 minutes, the seeds were washed with autoclaved tap water and dried on sterile filter papers. The seeds were sown in each plastic pot filled with field soil pasteurized at 75 ± 5 C for 1 hour, topped with 1 cm of vermiculite and were watered as needed for 70 days.

The plants were sprayed with 3 treatments (chitosans A, B and C) at concentrations 0.5, 2 and 5 g/L, so that the plants were coated all over. There were 2 sets of controls: sterile deionized water (C1) and 100 mL autoclaved deionized water containing 2.5 mL of 10 N acetic acid and adjusted to pH 5.6 using 1 N NaOH (C2).[13]

Four hours after spraying, the leaves of the plants were cut off the stems for evaluation of disease severity. A 5 mm mycelial plug was placed at the center of each leaf with the mycelium towards leaf surface. The leaves were placed in 200 mm Petri plates on 2 filter papers soaked with sterile water. These filter papers were used to provide necessary humidity in the plates.[9] The plates were remained at 20-22°C. The infection (water soaked) radius on leaves was determined 7 days after inoculation. This experiment was repeated 4 times within 4 consecutive weeks for 3 isolates in a completely randomized block design.

Evaluation of Chitosans and Acetyl Salicylic Acid Against Mustard White Rot Over Time

This experiment was conducted to assess the development of systemic resistance caused by ASA and chitosans against mustard white rot over time.[13] In this test, 70-day mustard plants were sprayed with ASA dissolved in sterile deionized

water at concentrations 100, 300 and 600 mg/L. In addition, chitosans A, B and C at different concentrations (0.5, 2 and 5 g/L) were sprayed on mustard plants. There were two sets of controls (C1 and C2) as mentioned above. After leaving plants in laboratory conditions (24-26°C) for seven days, the leaves were cut off the plants and were inoculated as explained above and remained at 20-21°C. The infection radius on leaves was determined 7 days after inoculation with pathogen isolates. This experiment was repeated 3 times within 3 weeks for 3 isolates in a completely randomized block design.

Protein Extraction

One, 3 and 6 days after spraying with chitosans (5 g/L) and ASA (600 mg/L), 70-day mustard leaves were cut off the plants. These concentrations had already shown the best inhibitory effect against the disease at *in vivo* tests. According to Roberti et al,[19] the leaves were weighed and ground in liquid nitrogen to fine powder in a pre-chilled mortar and pestle. Using 20 mM sodium acetate buffer (pH=5.2, 1 mL for 1 g fresh weight) containing polyvinylpolypyrrolidone (1 %, Sangon Chemical Company, Shanghai, China), total proteins were extracted and incubated at 4°C for 1.5 minutes under continuous gentle stirring. Afterwards, the extracts were centrifuged twice at 4°C at 8000 g for 25 minutes. In the next step, the supernatant was filtered using a Syringe Filter Unit (GV Millex®, Millipore, USA). The filtered material was then concentrated and desalted using a Centrifugal Filter Unit (Ultrafree®, Millipore, USA). The protein concentrations were evaluated by the protein-dye binding technique.[20,21]

Assessment of Chitinase Activity

One, 3 and 6 days after treatment with chitosans (5 g/L) and ASA (600 mg/L), the activities of β-N-acetyl hexosaminidase, endochitinase and chitin 1,4-β-chitobiosidase were evaluated with 3 replications.[21,22] Chitinase evaluation were based on colorimetric assessment of *p*-nitrophenyl which was cleaved from the chitin-analogous substrates, *p*-nitrophenyl-N-acetyl-β-D-glucosaminide, *p*-nitrophenyl-β-D-N-N,N″-triacetylchitotriose and *p*-nitrophenyl-β-D-N,N′-diacetylchitobiose, respectively (all provided from Sangon Chemical Company, Shanghai, China). In this experiment, 40 µL of stock of each substrate solution (3 mg L^{-1}) were dissolved in 40 mM acetate buffer (pH = 5, 1 mL for 1 g fresh weight) and added to 40 µL of the protein extracted from each sample. After incubation for 2.5 hours in a laboratory bain-marie at 38°C, 0.06 of Na_2Ca_3 (0.3 M) was added and the reaction was stopped. The absorbance was determined at 405 nm. Finally, the chitinase activity was calculated using an absorption coefficient for the p-nitrophenyl of 19 mM cm^{-1}.

Assessment of β -1,3-Glucanase Activity

The activity β-1,3-Glucanase was determined with 3 replications by calculating the production rate of reducing sugars, using laminarin (Sangon Chemical Company, Shanghai, China) as the substrate according to Kauffmann et al.[23] In this experiment, the protein used in each replication of each treatment was extracted from 3 plants. The reaction mixture included 0.5 mL of sodium acetate buffer (0.1 M, pH=5.3), containing 85 µL of the protein extract and 1 mg L^{-1} laminarin. After incubating for 2.5 hours at 38°C, alkaline copper reagent (0.3 mL) was added and the combination was heated for 25 minutes at 100°C. After cooling, Nelson's chromogenic reagent (0.2 mL) was added and absorbance was determined at 650 nm. The enzyme standards, substrate blanks and glucose were included. One unit of enzyme activity was considered as the amount of enzyme releasing 1 mg of glucose per min.

Evaluation of Peroxidase Activity

According to Caruso et al,[24] the activity of peroxidase (POD) was assayed with 3 replications by measuring the absorbance increase at 470 nm at 30°C due to the tetraguaiacol formation from 0.47 % guaiacol (v/v) and 14 mM H_2O_2 in 55 mM Tris buffer (pH 7.5). For each sample, the crude extract of protein (7 mg of total protein) was added to final volume (2 mL) of the reaction mixture. In this experiment, the protein used in each replication of each treatment was extracted from 3 plants. The activity of POD was measured using a coefficient of absorption for the tetraguaiacol of 27.6 mM cm^{-1} at 470 nm.

Spectrophotometry Analysis of Phenylalanine Ammonia Lyase, Polyphenol Oxidase and Peroxidase

The 70-day plants were sprayed with different chitosans and ASA at the concentration of 5g/L and 500 mg/L, respectively. The enzymatic analyses were done 2 and 4 days after inoculation with the pathogen. Activity assessments of PAL,[25] polyphenol oxidase (PPO)[26] and POD[27] were carried out. These experiments were repeated two times and there were 3 replicates in each treatment. There were 3 sets of controls: plant leaves sprayed with sterile deionized water (C1), plant leaves sprayed with 100 mL autoclaved deionized water containing 2.5 ml of 10 N acetic acid and adjusted to pH 5.6 using 1 N NaOH (C2) and plant leaves inoculated with the pathogen (isolate 1) (C3).

All enzyme extraction procedures were conducted at 4°C. To assess the activity of PAL,[25] 1 g of leaves tissue obtained from 3 plants for each replication was macerated and mixed with 2 mL extracting buffer [0.2 M boric acid buffer containing 10% (w/v), polyvinylpolypyrrolidone (PVPP), 1 mM EDTA and 50 mM β mercaptoethanol, pH 8.8]. For PPO and POD, 2 g of leaves tissue obtained from 3 plants for each replication was macerated with 10 mL of 100 mM sodium phosphate buffer (pH 6.4) containing PVPP (0.2 g), homogenized and centrifuged at 8000 g at 4°C for 30 minutes, and the supernatant was collected and used for enzyme assay.

In order to determine PAL activity, the enzyme extract (300 µL) was incubated with 1 mL 0.02 M L-phenylalanine and 2 mL of the PAL extracting buffer at 24°C for 2 minutes, and absorbance at 290 nm was measured in an ultraviolet spectrophotometer. The PAL activity was expressed as U290, where U290=0.01ΔOD290/mg protein/min.

In order to determine the PPO activity,[26] 100 µL of enzyme extract was incubated with 2 ml of 0.05 M phosphate buffer (pH 7.0) and 0.5 mL of 0.5 M catechol at 24°C for 2 minutes, and the absorbance at 398 nm was measured with an ultraviolet spectrophotometer. The PPO activity was expressed as U398,

where U398 = 0.01ΔOD398/mg protein/min.
The POD activity was determined using guaiacol as substrate.[27] The reaction mixture consisted of 0.1 mL of crude extract and 2 mL of guaiacol (8 mM in 100 mM sodium phosphate buffer, pH 6.4) and was incubated for 30 minutes at 30°C. The increase in absorbance at 460 nm was measured after 1 mL H_2O_2 (24 mM) was added. The activity of POD was expressed as U460, where U460 = 0.01ΔOD460/mg protein/min.

Data Analysis

The means of treatments recorded in percent were transformed in Sin^{1-} percentage transformation.[28] The effects of different treatments in each experiment were determined by the analysis of variance (ANOVA) using SAS software (SAS 8.2, 1999-2001; SAS Institute Inc., Cary, NC) in completely randomized design tests. Before running the statistics, the homogeneity of the variance was proved using Hartley's F_{max} test. Means of treatments were separated using Fisher LSD test.

Results

In Vitro Evaluation of Chitosans Against Radial Growth and Sclerotia Formation

After 5 days of incubation, 3 chitosans at different concentrations were significantly able to decrease mycelial growth ($P<0.05$) of the pathogen, and there was a positive correlation between the concentrations of chitosans and the growth inhibition (Figure 1, Table 1). In this test, C1 and C2 differed in their effect on the pathogen isolates. The number of sclerotia ($P<0.05$) was significantly reduced by all chitosans at the concentration 5 g/L. In addition, a positive correlation was observed between the concentrations of chitosans and the sclerotia formation (Table 2).

Chitosan A reduced the mycelial growth of all isolates at the concentration of 0.5 g/L with statistically similar performances. However, chitosan A at concentration 2 and 5 g/L inhibited isolate 1 more than isolates 2 and 3. No inhibitory effect was observed in sclerotia formation at concentration 0.5 g/L. However, chitosan A at concentration 5 g/L inhibited sclerotia formation of isolate 1 more than isolates 2 and 3 (Tables 1 and 2).

Chitosan B reduced the mycelial growth of all isolates at concentration 0.5 and 5 g/L with statistically similar performances. A at concentration 2 g/L, mycelial growth of isolate 1 was more inhibited than isolates 2 and 3. No inhibitory effect was observed in sclerotia formation at concentration 0.5 g/L. However chitosan B at concentration 2 and 5 g/L reduced sclerotia formation of isolate 1 more than other isolates (Tables 1 and 2).

Chitosan C decreased the mycelial growth of all 3 isolates at the concentration of 0.5 g/L at a statistically similar level. However, the mycelial growth of isolate 1 was more inhibited than other isolates at concentration 2 and 5 g/L. The chitosan C at the concentration of 0.5 g/L did not show any inhibitory effect against sclerotia formation for all 3 isolates. At concentration 2 g/L, chitosan C reduced sclerotia formation of only isolate 1. However, chitosan C at the concentration of 5 g/L reduced sclerotia formation of isolate 1 more than other isolates.

Antifungal Effect of Chitosans Against Mustard White Rot

After seven days of inoculation with the pathogen, the results showed that 3 chitosans at concentrations 2 and 5 g/L were able to significantly reduce ($P<0.05$) infection radius (Table 3, Figure 1). Furthermore, there was a positive correlation between inhibitory efficacy and chitosans concentrations. In this test, C1 and C2 differed in their effect on the pathogen isolates.

In the plants inoculated with isolates 1 and 2, the highest inhibitory effect was observed in chitosan B (5 g/L) followed by chitosan B (2 g/L) as well as chitosans A and C (5 g/L) with statistically similar performances (Table 3).

For isolate 3, chitosan B (5 g/L) was found to be the most effective treatment against the disease followed by chitosan B (2 g/L). The next treatments in order of superiority were chitosans A and C (2 g/L). No inhibitory efficacy was observed in chitosans A and C (0.5 g/L) compared to the controls (Table 3).

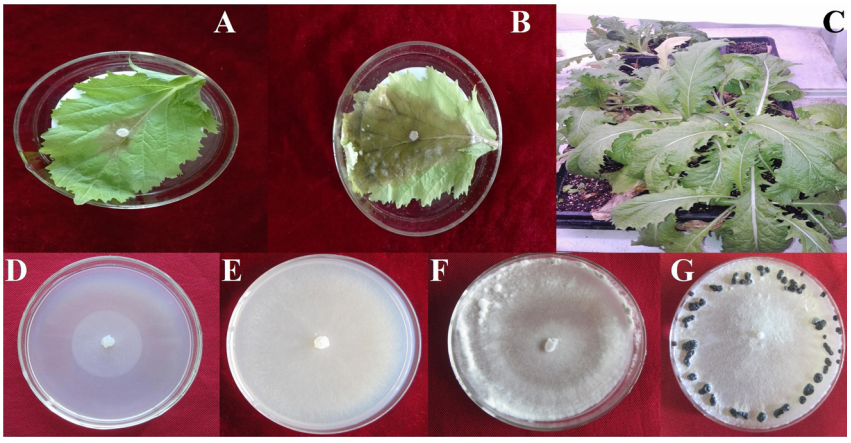

Figure 1. Inhibitory Effect of Chitosan B (5 g/L) Against Tuber Mustard White Rot Caused by *Sclerotinia sclerotiorum* (isolate 1). Compared with control (B), the infection radius caused by the pathogen was meaningfully reduced on plant leaves after seven days (A) cut off from 70-day plant (C). Compared with control (E), the radial growth of the pathogen (D) was meaningfully reduced after 5 days. In addition, the sclerotia formation (F) was significantly inhibited after 15 days compared to the control (G).

Table 1. Inhibitory Effect of 3 Chitosans at Different Concentrations Against Radial Growth of *Sclerotinia sclerotiorum* (3 isolates) on Carrot Dextrose Agar 5 Days After Inoculation at 21-22°C

	Radial Growth Inhibition (%)[a]								
	Chitosan A			Chitosan B			Chitosan C		
	0.5 g/L	2 g/L	5 g/L	0.5 g/L	2 g/L	5 g/L	0.5 g/L	2 g/L	5 g/L
Controls	0	0	0	0	0	0	0	0	0
Isolate 1	10.3±2.4[a]	41.2±9.7[b]	53.8±14.7[b]	27.8±5.4[a]	52.1±13.6[b]	85.1±17.3[a]	9.3±2.6[a]	42.8±9.2[b]	55.2±16.8[b]
Isolate 2	11.5±3.2[a]	32.8±6.1[a]	42.9±8.6[a]	26.5±7.2[a]	43.2±10.5[a]	83.9±12.6[a]	11.2±3.9[a]	31.5±7.6[a]	44.7±10.3[a]
Isolate 3	10.5±2.8[a]	34.5±7.3[a]	48.6±7.5[ab]	27.1±4.9[a]	41.8±7.3[a]	84.1±17.5[a]	10.8±2.4[a]	33.9±5.3[a]	43.9±14.6[a]

[a] Within columns, means followed by a common letter do not differ significantly at the $P<0.05$ level of confidence according to Fisher's test. Values in the table indicate means ± standard error. This experiment was repeated 3 times and each treatment was replicated 5 times.

Table 2. Inhibition Percentage of Sclerotia Formation of *Sclerotinia sclerotiorum* (3 Isolates) by 3 Chitosans at Different Concentrations on Carrot Dextrose Agar 15 Days After Inoculation at 21-22°C

	Percent inhibition of Sclerotia Formation[a]								
	Chitosan A			Chitosan B			Chitosan C		
	0.5 g/L	2 g/L	5 g/L	0.5 g/L	2 g/L	5 g/L	0.5 g/L	2 g/L	5 g/L
Controls	0	0	0	0	0	0	0	0	0
Isolate 1	0	8.5±1.3[a]	24.1±5.3[b]	0	32.8±5.7[b]	62.5±18.3[b]	0	12.9±4.6[a]	51.2±17.8[b]
Isolate 2	0	0	11.6±7.2[a]	0	20.3±4.5[a]	49.8±15.7[a]	0	0	43.5±11.9[a]
Isolate 3	0	0	13.1±4.8[a]	0	21.9±6.1[a]	48.9±11.6[a]	0	0	41.7±16.7[a]

[a] As same as noted for Table 1.

Efficacy of Chitosans and Acetyl Salicylic Acid Against Mustard White Rot Over Time

The results after seven days of inoculation with the pathogen isolates showed that all the 3 chitosans at concentrations 2 and 5 g/L as well as ASA (300 and 600 mg/L) reduced ($P<0.05$) infection radius on mustard leaves (Table 3).

In the plants inoculated with isolate 1, the highest efficacy was observed in chitosan B (5 g/L) followed by chitosan B (2 g/L) and chitosan C (5 g/L) with statistically similar results.

For isolate 2, chitosan B (5 g/L) showed the best inhibitory effect followed by chitosan B (2 g/L), chitosan C (5 g/L) and ASA (600 mg/L) with a same statistical level (Table 4).

For isolate 3, chitosan B (5 g/L) was the best effective treatment which was followed by chitosan B (2 g/L), chitosan C (5 g/L) and ASA (300 and 600 mg/L) with statistically similar performances (Table 4).

Table 3. Antifungal Efficacy of 3 Chitosans at Different Concentrations Against Tuber Mustard White rot after 7 Days of Inoculation by 3 Isolates of *Sclerotinia sclerotiorum*

		Infection Radius (cm)[a]		
	Concentration	Isolate 1	Isolate 2	Isolate 3
Control 1		9.7±0.3[a]	8.5±1.4[a]	9.8±0.2[a]
Control 2		9.1±0.5[a]	8.3±0.9[a]	9.4±0.7[a]
Chitosan A	0.5 g/L	9.5±0.4[a]	8.8±1.5[a]	9.5±0.4[a]
	2 g/L	7.1±1.3[b]	5.5±1.2[b]	6.9±1.3[b]
	5 g/L	2.3±0.8[c]	2.8±0.5[c]	3.1±0.9[c]
Chitosan B	0.5 g/L	4.1±1.7[bc]	3.4±1.1[bc]	3.2±0.7[c]
	2 g/L	2.6±0.5[c]	2.9±0.7[c]	2.1±0.5[cd]
	5 g/L	1.3±0.8[cd]	0.9±0.1[d]	1.1±0.3[d]
Chitosan C	0.5 g/L	9.2±0.7[a]	8.5±0.9[a]	9.4±0.5[a]
	2 g/L	7.2±1.9[b]	3.5±1.2[bc]	6.5±1.8[b]
	5 g/L	2.8±.06[c]	2.7±0.8[c]	3.5±0.8[c]

[a] This experiment was repeated 4 times. Within columns, means followed by a common letter do not differ significantly at the $P<0.05$ level of confidence according to Fisher exact test. Values in the table indicate means ± standard error.

Activity Assessment of Chitinases, β-1, 3-Glucanase and Peroxidase

The results showed that level of most enzymes significantly increased ($P>0.05$) after 3 and 6 days compared with controls (Table 5). The highest level of β-N-acetyl hexosaminidase was observed in chitosans A, B and C after 6 days (Table 5). In addition, the most activity of endochitinase was observed in chitosan B after 3 and 6 days. The chitosans B and C increased the activity of Chitin 1,4-β-chitobiosidase after 6 days more

Table 4. Resistance Induction Against Tuber Mustard White Rot Caused by 3 Isolates of *Sclerotinia sclerotiorum* After 7 Days of Spraying 3 Chitosans and Acetyl Salicylic Acid at Different Concentrations on Plants

		Infection Radius (cm)[a]		
	Concentration	Isolate 1	Isolate 2	Isolate 3
Control 1		9.4±0.5[a]	8.7±1.2[a]	9.2±0.7[a]
Control 2		9.5±0.2[a]	8.9±0.7[a]	9.5±0.3[a]
Chitosan A	0.5 g/L	9.1±0.8[a]	8.5±1.4[a]	9.1±0.8[a]
	2 g/L	5.3±1.2[b]	5.6±0.9[b]	5.5±1.2[b]
	5 g/L	5.7±0.9[b]	5.1±0.8[b]	5.3±0.4[b]
Chitosan B	0.5 g/L	9.5±0.4[a]	7.5±1.2[ab]	7.2±1.1[ab]
	2 g/L	2.5±0.7[c]	2.8±0.5[c]	2.6±0.9[c]
	5 g/L	1.2±0.3[cd]	0.5±0.1[d]	0.7±0.1[d]
Chitosan C	0.5 g/L	9.3±0.5[a]	7.4±1.6[ab]	9.3±1.5[a]
	2 g/L	3.7±0.4[bc]	5.4±1.1[b]	5.2±1.2[b]
	5 g/L	2.7±0.6[c]	2.5±0.3[c]	2.4±0.8[c]
Acetyl salicylic acid	100 mg/L	9.3±0.5[a]	8.8±1.7[a]	9.6±1.7[a]
	300 mg/L	5.5±1.2[b]	3.7±1.6[bc]	2.8±0.3[c]
	600 mg/L	3.9±1.8[bc]	2.7±0.5[c]	2.5±0.4[c]

[a] All the means within a column followed by the same letter are not significantly different at the $P<0.05$ level of confidence according to Fisher exact test. This experiment was repeated 3 times within 3 weeks.

Table 5. Effect of 3 Chitosans (5 g/L) and Acetyl Salicylic Acid (600 mg/L) on Resistant Related Enzymes in Tuber Mustard Leaves After 1, 3 and 6 Days of Spraying on 70-Day Plants

	Enzyme Activity After One Day (U/mg Protein)*				
	β-N-acetyl Hexosaminidase	Endochitinase	Chitin 1,4-β-Chitobiosidase	β -1,3-Glucanase	POD
Control 1	0.00534 [a]	0.00331 [a]	0.00242 [a]	1.83 [a]	1.35 [a]
Control 2	0.00539 [a]	0.00335 [a]	0.00249 [a]	1.79 [a]	1.38 [a]
Chitosan A	0.00536 [a]	0.00341 [a]	0.00342 [b]	1.85 [a]	1.34 [a]
Chitosan B	0.00655 [b]	0.00462 [b]	0.00351 [b]	2.56 [b]	2.59 [b]
Chitosan C	0.00541 [a]	0.00339 [a]	0.00348 [b]	1.84 [a]	2.61 [b]
ASA	0.00543 [a]	0.00342 [a]	0.00251 [a]	1.75 [a]	1.33 [a]
	Enzyme Activity After 3 Day (U/mg Protein)*				
	β-N-acetyl Hexosaminidase	Endochitinase	Chitin 1,4-β-Chitobiosidase	β -1,3-Glucanase	POD
Control 1	0.00536 [a]	0.00335 [a]	0.00245 [a]	1.85 [a]	1.36 [a]
Control 2	0.00541 [a]	0.00339 [a]	0.00243 [a]	1.72 [a]	1.37 [a]
Chitosan A	0.00683 [b]	0.00483 [b]	0.00352 [b]	2.63 [b]	1.38 [a]
Chitosan B	0.00691 [b]	0.00672 [c]	0.00345 [b]	3.95 [c]	2.57 [b]
Chitosan C	0.00602 [ab]	0.00473 [b]	0.00349 [b]	3.11 [bc]	2.61 [b]
ASA	0.00685 [b]	0.00479 [b]	0.00248 [a]	2.66 [b]	2.67 [b]
	Enzyme Activity After 6 Day (U/mg Protein)*				
	β-N-acetyl Hexosaminidase	Endochitinase	Chitin 1,4-β-Chitobiosidase	β -1,3-Glucanase	POD
Control 1	0.00542 [a]	0.00337 [a]	0.00251 [a]	1.78 [a]	1.34 [a]
Control 2	0.00537 [a]	0.00341 [a]	0.00249 [a]	1.86 [a]	1.33 [a]
Chitosan A	0.00722 [c]	0.00491 [b]	0.00346 [b]	2.65 [b]	2.63 [b]
Chitosan B	0.00729 [c]	0.00683 [c]	0.00563 [c]	4.09 [c]	3.89 [c]
Chitosan C	0.00733 [c]	0.00595 [bc]	0.00559 [c]	2.59 [b]	3.93 [c]
ASA	0.00688 [b]	0.00485 [b]	0.00436 [bc]	2.76 [b]	2.71 [b]

* All the means within a column followed by the same letter are not significantly different at the P>0.05 level of confidence according to Fisher exact test. U: one unit of enzyme activity was defined as the amount of enzyme which releases 1 µmol of substrate per min.

than the other treatments compared to the controls. The chitosan B (after 3 and 6 days) and chitosan C (after 6 days) were the most effective treatments in increasing the activity of β-1,3-glucanase with a same statistical level. The highest POD activity was found to be in chitosans B and C after 6 days (Table 5).

Activity of PAL, PPO and POD

After 2 days, the highest level of PPO was observed in chitosan C followed by other chitosans as well as ASA with similar statistical results (Table 6). All 3 chitosans significantly increased the POD activity in a statistically same level. However no change was observed in the PAL activity in plant leaves due to 3 chitosans and ASA compared to the controls. After 4 days, chitosan B increased the activity of PPO in the highest level. The highest level of POD was observed in chitosan C followed by chitosan B. Also, the ASA significantly increased the PAL level more than the other treatments. The 3 chitosans were the next treatments in order of superiority with similar statistical results (Table 6).

Discussion

The *S. sclerotiorum* is a ubiquitous phytopathogen which infects many dicotyledonous plants including Brassica crops. Although many research have been conducted to assess control measures against Sclerotinia diseases on different crops,[4-10] no research has been found about nonchemical methods against tuber mustard white rot.

The *in vitro* antifungal effect of tested chitosans and ASA was evaluated in previous research.[13] In this study, chitosans

Table 6. Effect of 3 Chitosans (5 g/L) and Acetyl Salicylic Acid (600 mg/L) on Phenylalanine Ammonia Lyase, Polyphenoloxidase and Peroxidase in Tuber Mustard Leaves After 2 and 4 Days of Inoculation With *Sclerotinia sclerotiorum* (Isolate 1)

	Enzyme Activity					
	PPO		POD		PAL	
	After 2 Days	After 4 Days	After 2 Days	After 4 Days	After 2 Days	After 4 Days
Control 1	68.3±9.2 [a]	67.5±12.8 [a]	5.6±1.2 [a]	5.1±2.3 [a]	9.8±2.3 [a]	10.3±1.7 [a]
Control 2	72.4±12.9 [a]	74.3±20.4 [a]	4.9±0.8 [a]	5.4±1.8 [a]	10.2±1.5 [a]	9.6±2.4 [a]
Control 3	69.7±11.1 [a]	72.8±19.3 [a]	5.3±1.6 [a]	5.6±2.7 [a]	11.3±2.7 [a]	11.5±1.6 [a]
Chitosan A	156.5±27.4 [b]	165.2±18.7 [b]	12.8±2.7 [b]	13.9±4.5 [b]	10.5±1.6 [a]	25.9±5.8 [b]
Chitosan B	285.9±36.2 [c]	428.4±51.5 [d]	13.2±4.5 [b]	18.8±2.1 [bc]	11.1±3.2 [a]	27.1±6.3 [b]
Chitosan C	161.7±19.4 [b]	371.2±44.9 [cd]	11.7±3.9 [b]	25.4±5.6 [c]	9.9±2.4 [a]	28.4±3.7 [b]
ASA	153.5±28.6 [b]	267.5±32.8 [c]	8.2±1.6 [ab]	12.7±5.4 [b]	11.4±3.1 [a]	45.9±5.2 [c]

All the means within a column followed by the same letter are not significantly different at the *P*<0.05 level of confidence according to Fisher's test. The PPO activity was expressed as U398, where U398=0.01ΔOD398/mg protein/min. These experiments were repeated two times and there were three replicates in each treatment. There were three sets of controls: plant leaves sprayed with sterile deionized water (Control 1), plant leaves sprayed with 100 mL autoclaved deionized water containing 2.5 ml of 10 N acetic acid and adjusted to pH 5.6 using 1 N NaOH (Control 2) and plant leaves inoculated with the pathogen (isolate 1) (Control 3). The activity of POD was expressed as U460, where U460=0.01ΔOD460/mg protein/min. The PAL activity was expressed as U290, where U290=0.01ΔOD290/mg protein/min.

showed significant inhibitory effect against the disease. Previous studies have shown that chitosans, as nontoxic and inexpensive biopolymers, are able to markedly reduce the severity of different plant pathogens.[13-29] In this research, 3 different chitosans designated as A, B and C were assessed against the pathogen. As a result, chitosan B had the highest DN and the least molecular weight compared to chitosans A and C. Although all tested chitosans showed inhibitory efficacy against mustard white rot, chitosan B was found to have the best effect against tested pathogen isolates. These results were in agreement with previous researches which showed that the fungicidal effect of chitosan rises when the DN increases.[30,31] It may be due to lower numbers of acetyl groups, more deacetylated chitosans possess a higher polycationic character and develop polyelectrolyte complexes with negatively charge carboxyl groups present in the cell walls of fungi.[32] In this study, chitosan with lowest molecular weight (chitosan B) showed the best effect against *S. sclerotiorum*. Numerous studies have shown that low molecular weight chitosans are more effective against fungal phytopathogens.[33,34] However some researches have shown that high molecular weight chitosans are more effective against the mycelial growth of some plant pathogenic fungi.[35,36] Therefore, it is essential to study the exact role of physiochemical properties, as well as molecular and ultrastructural mechanisms, of chitosans involved in its antifungal activity. Previous studies have shown that isolates of *S. sclerotiorum* show different reaction to different biocontrol agents and environmental conditions.[37,38] Therefore, the effect of chitosans and ASA was assessed against 3 isolates of the pathogen. In this research, ASA significantly decreased infection radius of the pathogen isolates on plant leaves. Although SA has been able to affect fungal development, spore viability of *Saccharomyces cerevisiae*,[39] conidial germination of *Sphaerotheca fuliginea*[40] and blastospore germination of *Paecilomyces fumosoroseus*,[41] some researchers have suggested that SA does not have direct antifungal activity.[42] The chitosans and ASA applied seven days before inoculation with pathogen were still able to reduce infection levels indicating that chitosans and ASA may be able to induce an acquired resistance induction. The resistance against plant pathogens has been reported to be associated with the changes of the activity of 3 enzymes including PPO, POD and PAL[13] which have been assessed in this study. The results of this study showed that the activity of the 3 above mentioned enzymes increases due to the application of different chitosans and ASA after two and 4 days. However no change was found in PAL level after 2 days. The chitinase has reported to have an important role in resistance induction against Sclerotinia diseases. A previous research has reported increased resistance against carrot rot in transgenic carrots expressing chitinase *chit42* from *Trichoderma harzianum*.[43]

Conclusions

In conclusion, this research showed that chitosans and ASA have the potential to reduce the severity of mustard white rot. In general, the chitosan with lowest molecular weight was found to be more effective against the disease. In addition, chitosans and ASA were able to significantly increase resistance-related enzymes over time indicating that they can be considered as resistant inducers against mustard white rot.

Authors' Contributions

All authors equally contributed to the current study.

Conflict of Interest Disclosures

The authors declare they have no conflicts of interest.

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