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ORIGINAL PAPER

Effects of postharvest oligochitosan treatment on anthracnose disease in citrus (*Citrus sinensis* L. Osbeck) fruit

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Abstract Citrus fruit (*Citrus sinensis* L. Osbeck) is susceptible to infection by *Colletotrichum gloeosporioides* during poststorage, which rapidly decreases sensory and nutritional quality of the fruit. The ability of oligochitosan treatment to control *C. gloeosporioides* of citrus fruit during storage was examined, and possible underlying mechanisms were discussed. Disease incidence and lesion diameter were lower in oligochitosan-treated fruits compared with their respective controls. The fruits dipped in oligochitosan showed increased contents of lignin, hydroxyproline-rich glycoprotein (HRGP), hydrogen peroxide (H_2O_2), ascorbate, glutathione, total phenol, and flavonoid compounds. In addition, enzymatic activities of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), peroxidase (POD, EC 1.11.1.7), polyphenoloxidase (PPO, EC 1.14.18.1), ascorbate peroxidase (APX, EC 1.11.1.11), and β-1, 3-glucanase (GLU, EC 3.2.1.39) also increased in citrus fruit peels, all of which were correlated with the onset of induced disease resistance. These results indicated that oligochitosan treatment can induce disease resistance of citrus fruit to *C. gloeosporioides* Penz. Oligochitosan can be a potential alternative to conventional control methods of postharvest anthracnose in citrus fruit.

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Keywords Oligochitosan · Citrus fruit (*Citrus sinensis* L. Osbeck) · Induced disease resistance · Reactive oxygen species (ROS) metabolism · Defense enzyme

Introduction

Citrus fruit (*Citrus sinensis* L. Osbeck) is cultivated worldwide to produce fresh and processed products. *Penicillium digitatum* (green mold) and *Penicillium italicum* (blue mold) are the major pathogens that infect citrus fruits after harvest, which can cause serious annual losses [1]. Anthracnose, another postharvest disease caused by *Colletotrichum gloeosporioides* Penz., can lead to considerable economic losses, particularly with prolonged storage, and can often be ignored. Traditionally, synthetic fungicides are primarily used to control this disease. However, to fulfill consumer's demand on lesser use of chemicals on fruits, studies must focus on the development of natural compounds that can be utilized as alternative antimicrobials and preservatives.

Oligochitosan, prepared by the enzymatic hydrolysis of deacetylated chitosan polymers, possesses versatile functional properties that are useful in the field of agriculture and has been considered as a potential plant disease vaccine [2]. Oligochitosan can elicit multiple plant defenses against various kinds of biotic and abiotic stresses in several plants, such as rice, tobacco, tomato, peach, pear, jujube, grape berry, citrus, apple, and litchi [3–14]. Oligochitosan specifically binds to the cell walls and membranes of plants and subsequently penetrates the cell membrane and putatively binds to intracellular targets [15]. During this process, oligochitosan can promote the accumulation of volatile components exhibiting antibacterial activity and the accumulation of those defense-related enzymes, such as phenylalanine ammonialyase (PAL), chitinase (CHI),

β-1, 3-glucanase (GLU), peroxidase (POD), and polyphenoloxidase (PPO), as well as defense-related compounds, such as lignin [10], phytoalexins, and phenolic secondary metabolites [16]. In addition, the direct and broad-spectrum antimicrobial activity of oligochitosan [14, 16, 17] and its growth-promoting properties and antioxidant effects also should be considered [13, 18]. According to some published papers, Ca^{2+}, reactive oxygen species (ROS), nitric oxide (NO), jasmonic acid (JA), and salicylic acid (SA) are all involved in the oligochitosan-activated signaling pathway [6, 8, 9, 19, 20]. An oligochitosan-induced protein kinase (OIPK) has been previously isolated and identified in tobacco [21, 22]. The above findings suggest that oligochitosan is a promising alternative to synthetic fungicides to control postharvest diseases in horticultural products.

A previous study demonstrated the antifungal activity of chitosan against *P. digitatum*, *P. italicum*, *Botrydiplodia lecanidion*, and *Botrytis cinerea* in Tankan citrus fruit (*Citrus tankan* Hayata) [5]. Our previous work also indicated that chitosan treatment can induce disease resistance in navel orange [1]. However, limited information is available regarding the control effects of postharvest oligochitosan treatment on anthracnose and its related mechanisms in citrus fruit. This study investigated whether or not oligochitosan influences anthracnose development in postharvest citrus fruit (*C. sinensis* L. Osbeck). This study also elucidated the mechanisms underlying this process.

Materials and methods

Fruits and oligochitosan treatment

Citrus fruits (*C. sinensis* L. Osbeck cv. Jincheng 447[#]) were harvested early in the morning from the Beibei District of Chongqing City, China, and then transported immediately to the laboratory. All fruits were uniform in size and color and had no physical injuries or infections.

Citrus fruits were washed with tap water and dried overnight at room temperature. Subsequently, the fruits were dipped into 1.5 % (g/g) oligochitosan (molecular weight: 1,500–2,000 Da, purchased from Jinan Haidebei Marine Bioengineering Co., Ltd., Shandong, China) for 1 min and dried in air, and sterile distilled water was used as a control treatment. Fruits were individually packaged with plastic bags and incubated at 20 °C, 85–90 % RH for pathogen inoculation and biochemical analysis [23].

Pathogen preparation and inoculation

C. gloeosporioides Penz. was isolated from an infected citrus fruit showing a typical anthracnose symptoms and then incubated in potato dextrose agar (PDA) medium at 28 °C. We observed that the orange fruit was infected with this microorganism and typical anthracnose symptoms reappeared. The isolates were initially identified morphologically, physiologically, and pathogenically as *C. gloeosporioides* Penz. strains [24]. Fungal spores were then obtained by flooding the 12-day-old cultures with sterile distilled water containing 0.05 % (v/v) Tween 80. Spore suspensions were filtered using four layers of sterilized cheesecloth to remove adhering mycelia. The spore concentration was adjusted to 10^5 spores mL^{-1} with sterile distilled water with the aid of a hemocytometer before use [25].

The oligochitosan-treated and control fruits were surface-sterilized with 70 % ethanol, washed with tap water, and dried in air, and then wounded using a sterile syringe at three points [4 mm (depth) × 4 mm (width)] at the equator of each fruit. Approximately 15 μL of the conidial suspension of *C. gloeosporioides* Penz. containing 1×10^5 spores mL^{-1} was pipetted into each wound. The same volume of sterile distilled water was applied on the controls. The fruits were individually packaged in plastic bags 4 h after inoculation and incubated at 20 °C, 85–90 % RH for 32 days. Disease incidence and lesion diameter were measured according to the method of Qin et al. [26]. A fruit was considered decayed when the visible rot zone outside the wounded area on the fruit measured >1 mm in width. The lesion diameter was recorded at 20, 24, 28, and 32 days after treatment, whereas those for disease incidence were recorded at 4, 8, 12, and 24 days after treatment. Each treatment was performed in three replicates with ten fruit per replicate, and the experiment was conducted twice.

Sample collecting for biochemical determinations and enzyme analysis

Peel samples were taken from the equinoctial tissue of the oligochitosan-treated and control fruits at same time intervals (0, 7, 14, 21, 28, 35, and 42 days after treatment). Each sample was packed in aluminum foil individually and frozen in liquid nitrogen immediately, and then kept at −80 °C for the biochemical determinations and enzyme analysis. Each experiment involved 70 individuals with three replicates arranged in a completely randomized design.

Measurements of lignins and HRGPs contents

Lignin contents were determined according to the method of Morrison [27] with some modifications. Sample tissue (1 g) was thoroughly homogenized with 5 mL of 95 % ethanol. The homogenate was centrifuged at 4,000×*g* for 10 min. The resulting cell wall pellet was washed thrice with 3 mL of 95 % ethanol and thrice with 3 mL of ethanol: hexane (1:2, V:V). The precipitate was then dried at

105 °C, suspended in 3 mL of 25 % acetobrom-glacial acetic acid, and incubated at 70 °C in water for 30 min. The reaction was terminated with 0.9 mL of 2 mol L^{-1} NaOH, metered with glacial acetic acid to 10 mL and centrifuged at 4,000×*g* for 10 min at 4 °C. The supernatant was collected, and the absorbance was measured at 280 nm, with lignin contents expressed as OD_{280} g^{-1} fresh weight (FW).

Hydroxyproline-rich glycoprotein (HRGP) contents were assessed according to the method of Kivirikko et al. [28], with some modifications. To prepare the cell wall, the rinds (1 g) were homogenized using a pestle and mortar at 4 °C in 5 mL of 100 mM phosphate buffer at pH 7.2. The suspension of broken cells was centrifuged at 4,000×*g* for 7 min. The cell walls were repeatedly washed four times with 2 mL of 100 mmol L^{-1} phosphate buffer (pH 7.2), once with 2 mL Triton X-100 (0.5 %), thrice with 2 mL of distilled water, once with 2 mL sodium salts (1 mol L^{-1}), thrice with 2 mL of distilled water, and once with 2 mL of acetone. The resulting extraction (CWM) was air dried at 60 °C. In general, HRGP content is positively related to hydroxyproline (Hyp). Therefore, the relative content of cell wall HRGP was determined by analyzing the Hyp content in the cell wall hydrolysate. Content units were expressed as μg HRGPs per mg of cell wall.

Measurements of total phenolics and flavonoids contents

Total phenolic and flavonoid compounds were measured according to the method of Liu et al. [7]. One gram of sample tissue was homogenized with 20 mL of ice-cold 1 % HCl-methanol solution and centrifuged at 15,000×*g* for 15 min at 4 °C. The supernatant was collected, and the absorbance at 280 and 325 nm was measured, with phenolic compounds expressed as OD_{280} g^{-1} FW and flavonoid compounds expressed as OD_{325} g^{-1} FW.

Measurement of activities of defense-related enzymes

Phenylalanine ammonialyase activity was assayed according to the method of Yao and Tian [29] with some modifications. Sample tissue (5 g) was homogenized with 5 mL of ice-cold extraction solution to stabilize the enzyme. The homogenate was centrifuged at 12,000×*g* at 4 °C for 30 min. The supernatant was collected as enzyme extraction to assay the activities of PAL. PAL activity was expressed as ΔOD_{290} g^{-1} FW.

For CHI and GLU activities assay, 1 g of sample tissue was thoroughly homogenized with 4 mL of 200 mmol L^{-1} sodium acetate buffer (pH 5.2, containing 5 mmol L^{-1} *β*-mercaptoethanol, 1 mmol L^{-1} EDTA), 1 mmol L^{-1} PEG 6000, and 8 % polyvinylpolypyrrolidone at 4 °C. The homogenate was centrifuged at 12,000×*g* for 30 min at 4 °C, and the supernatant was collected for the enzymatic assay. CHI activity was measured according to the method of Cao et al. [30]. CHI activity was expressed as units g^{-1} FW. One unit was defined as the enzyme activity catalyzing the formation of 1×10^{-9} μg of *N*-acetyl-*D*-glucosamine per second. GLU activity was determined according to the method of Abeles and Forrence [31], with some modifications. Enzymatic activity was expressed units g^{-1} FW, where one unit was defined as the reducing sugar equivalent to 10^{-9} mol of glucose produced per second per g FW.

Measurements of H_2O_2 content and activities of CAT and SOD

H_2O_2 levels were estimated by forming titanium–hydro peroxide complexes according to the method of Patterson et al. [32]. The H_2O_2 content was expressed as micromole per gram FW.

For the catalase (CAT) activity assay, 2 g of sample tissue was homogenized with 6 mL of 50 mmol L^{-1} sodium phosphate buffer, pH 7.5, containing 2 % (w/v) polyvinylpyrrolidone (PVP) and 5 mmol L^{-1} dithiothreitol (DTT). The homogenate was centrifuged at 12,000×*g* at 4 °C for 30 min, and the supernatant was collected for the enzymatic assay. CAT activity was determined according to the method described by Havir and Mchale [33].

For superoxide dismutase (SOD) activity assay, 2 g of sample tissue was homogenized with 6 mL of ice-cold 50 mmol L^{-1} sodium phosphate buffer (pH 7.8) and centrifuged at 4 °C for 30 min at 15,000×*g*, after which the supernatant was collected. SOD activity was assayed by recording the decrease in optical density of nitro blue tetrazolium dye by enzyme according to the method of Prochazkova et al. [34].

Measurement of enzyme activities and non-enzyme components of the AsA–GSH cycle

For ascorbate peroxidase (APX) and glutathione reductase (GR) activity assays, 2 g of sample tissue was homogenized with 6 mL of 50 mmol L^{-1} sodium phosphate buffer, pH 7.5, containing 2 % (w/v) PVP and 5 mmol L^{-1} DTT. The homogenate was centrifuged at 12,000×*g* at 4 °C for 30 min, and the supernatant was collected for the enzymatic assay. Enzymatic activities of APX and GR were determined according to the methods described by Nakano and Asada [35].

For the ascorbate (AsA) assay, 5 g of sample tissue was homogenized with 5 mL of 2 % (w/v) oxalic acid. The homogenate was centrifuged at 12,000×*g* at 4 °C for 10 min, and 6 mL of the supernatant was titrated with standard 2, 6-dichlorophenol indophenol according to the method of El Bulk et al. [36].

For the glutathione (GSH) assay, 2 g of sample tissue was homogenized with 6 mL of 5 % (w/v) pre-chilled trichloroacetic acid (containing 5 mmol L^{-1} EDTA-Na_2) and centrifuged at 12,000×*g* for 30 min at 4 °C. The supernatant was used for GSH determination according to the methods described by Brehe and Bruch [37] with some modifications.

Measurements of activities of POD and PPO

Peroxidase and PPO activities were analyzed using the method of Zauberman et al. [38]. Sample tissue (1.0 g) was mixed with 6 mL of ice-cold sodium phosphate buffer (100 mmol L^{-1}, pH 6.8). The results were expressed as units per gram FW. One unit of POD activity was defined as the amount of enzyme that caused one unit of change in absorbance per minute at 470 nm. One unit of PPO activity was defined as the amount of enzyme that caused one unit of change in absorbance per minute at 420 nm.

Statistical analysis

All statistical analyses were performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± SD. Differences among oligochitosan treatment and control fruit of the same day were compared using Student's *t* test. A *P* value of <0.05 was considered statistically significant.

Results

Effect of oligochitosan treatment on disease symptoms of citrus fruits

The effects of oligochitosan on the disease symptoms of citrus fruits in response to infection by *C. gloeosporioides* Penz. are illustrated in Fig. 1A. The lesion diameters in treated fruits were 46.49, 54.44, and 53.40 % lower than those in control fruits at the 24th, 28th, and 32nd days after inoculation, respectively ($P < 0.05$). The disease incidences in control fruits were 4.9 and 3 times higher than those in treated fruits at the 8th and 12th day after inoculation, respectively (Fig. 1B). In addition, complete decay was observed in control fruits at 24 days, whereas complete decay was observed in oligochitosan-treated fruits at 36 days after incubation (data not shown). These results indicate that disease resistance in citrus fruits was significantly enhanced by oligochitosan treatment.

Effects of oligochitosan treatment on HRGP and lignin content in citrus fruits

Hydroxyproline-rich glycoproteins are one of the major types of proteins found in plant cell walls. Figure 2A shows that oligochitosan treatment can promote the accumulation of HRGPs in citrus fruit peels. HRGP contents in treated fruits were higher than those in the controls during the whole storage period, except at the 21st day of treatment. HRGP contents were 49.79 and 73.42 % higher, respectively, than the control fruits, at the 7th and 42nd day after treatment. Interestingly, at the 28th and 35th day after treatment, the HRGP contents in control fruits sharply decreased, which cannot be accurately determined using the method described above.

Oligochitosan can induce the production of lignin, another important structure-related compound. During the entire sample period, lignin contents in control fruits considerably decreased (Fig. 2B), whereas lignin contents in oligochitosan-treated fruits decreased during the first 14 days and then increased again. Oligochitosan can significantly promote the accumulation of lignin in citrus fruit peels during the later time of the sampling period

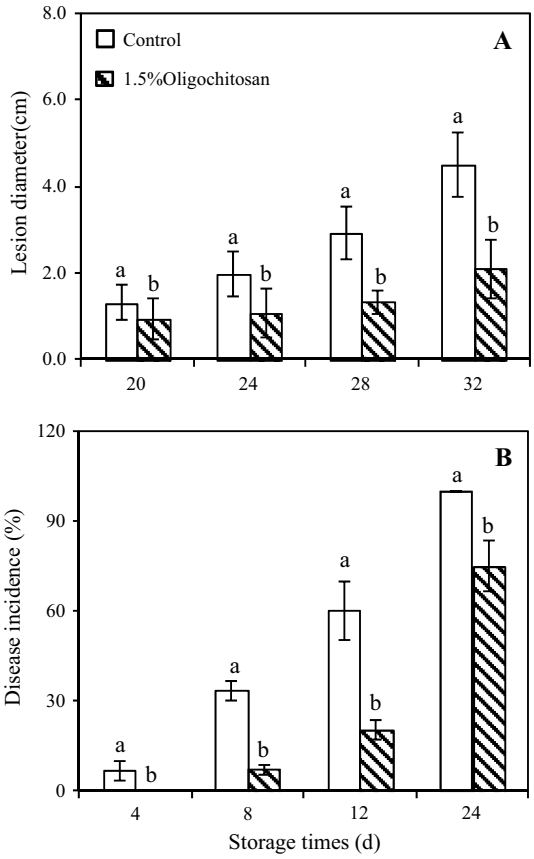

Fig. 1 Effects of oligochitosan treatment on lesion diameters (**A**) and disease incidence (**B**) in citrus fruits inoculated with *C. gloeosporioides* Penz. Citrus fruits (*Citrus sinensis* L. Osbeck) were immersed in 1.5 % (g/g) oligochitosan for 1 min, with sterile distilled water as the control. *Each column* represents the mean of three replicates. *Bars* indicate the standard errors. Lesion diameter (**A**) and disease incidence (**B**) marked with *different letters* (on the same day) are significantly different (LSD test, $P = 0.05$)

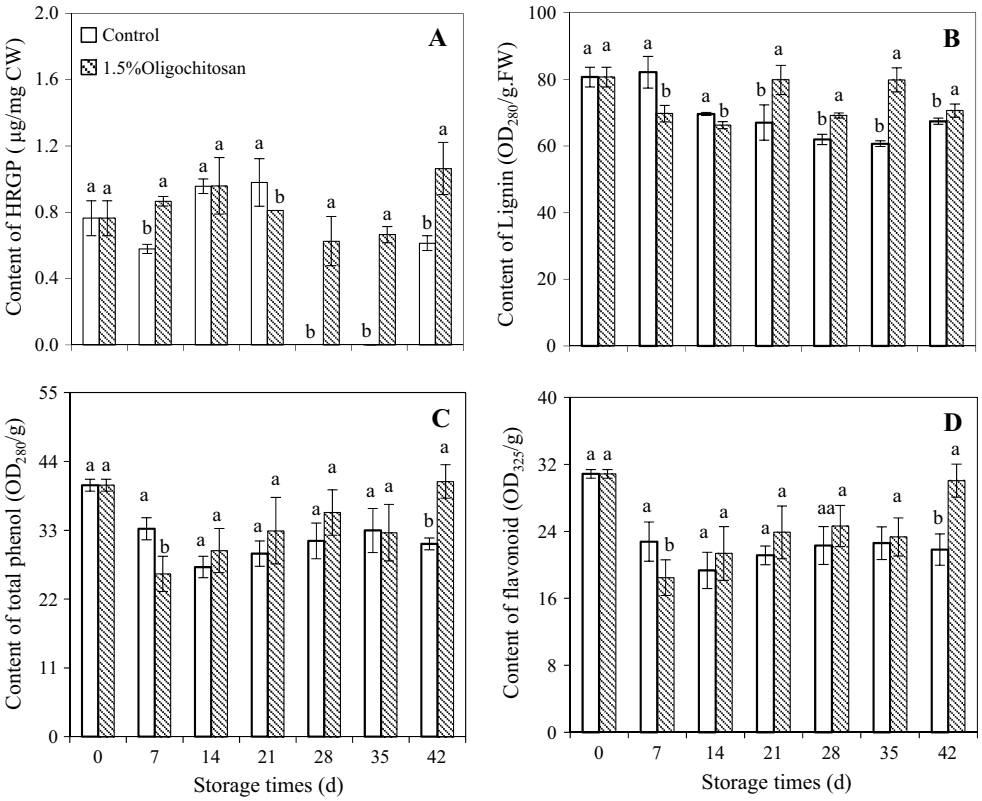

Fig. 2 Effects of oligochitosan treatment on contents of HRGP (**A**), lignin (**B**), total phenol (**C**) and flavonoid (**D**) in citrus fruit peels. Citrus fruits (*Citrus sinensis* L. Osbeck) were immersed in 1.5 % (g/g) oligochitosan for 1 min, with sterile distilled water as the control. *Each column* represents the mean of three replicates. *Bars* indicate the standard errors. Contents of HRGP (**A**), lignin (**B**), total phenol (**C**) and flavonoid (**D**) marked with *different letters* (on the same day) are significantly different (LSD test, $P = 0.05$)

($P < 0.05$). The lignin content was 31.50 % higher in oligochitosan-treated fruits than in control fruits at 35th days after treatment.

Effects of oligochitosan treatment on accumulation of total phenol and flavonoid in citrus fruits

The total phenol content in citrus fruit peels decreased during the early time of storage, followed by a slow increase. Oligochitosan stimulated the accumulation of total phenols in citrus fruit peels after the 14th day of storage. At the 42nd day, the total phenol content was 32.37 % higher than that in control fruits ($P < 0.05$) (Fig. 2C). The effects of oligochitosan on content of flavonoids were similar with its effects on total phenol content (Fig. 2D). The flavonoid content was 37.75 % higher in treated fruits than in control fruits at the 42nd day after treatment.

Effects of oligochitosan treatment on activities of defensive enzymes in citrus fruits

Chitinase and GLU, which belong to the PR-2 and PR-3 families, catalyze the hydrolysis of chitin and β-1, 3-glucan (major components of fungal cell walls), respectively [39]. The activity of GLU was significantly enhanced by oligochitosan during the early time of storage compared with that in control fruits (Fig. 3A). GLU activity in oligochitosan-treated fruit reached the peak at the 14th day after treatment. GLU activities in treated fruits were 74.58 and 29.38 % higher than in control fruits at the 7th and 14th day after storage. No significant differences in the GLU activities between two groups were observed afterward ($P < 0.05$). Oligochitosan did not show significant effects on CHI activity (Fig. 3B).

Phenylalanine ammonialyase activity in oligochitosan-treated fruit reached its peak at the 21st and 42nd days after treatment, whereas PAL activity in control fruit reached its peak at the 14th and 35th days in control fruits. However, the peak value of PAL activity sharply decreased upon treatment with oligochitosan (Fig. 3C).

Effects of oligochitosan treatment on level of H_2O_2 and activities of SOD and CAT in citrus fruits

H_2O_2 is the most important ROS that can cross-membranes and directly function in cell signaling. CAT and SOD are two essential enzymes involved in H_2O_2 metabolism [40]. Figure 4A shows that H_2O_2 levels in the fruits rapidly increased and reached their peaks 7 days after storage. Afterward, H_2O_2 contents in control fruits gradually declined. H_2O_2 levels were significantly increased by oligochitosan treatment during anaphase storage, and subsequent decrease in H_2O_2 levels was in the range of 20.82–36.59 % for treated fruits and 62.38 % for control fruits.

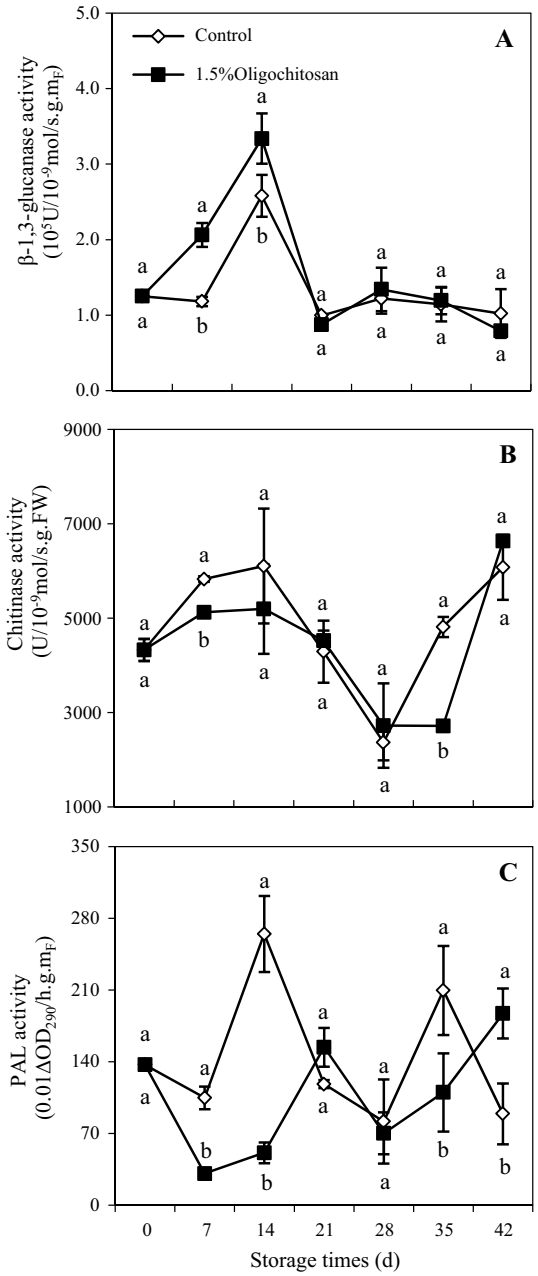

Fig. 3 Effects of oligochitosan treatment on activities of β-1, 3-glucanase (**A**), Chitinase (**B**) and PAL (**C**) in citrus fruit peels. Citrus fruits (*Citrus sinensis* L. Osbeck) were immersed in 1.5 % (g/g) oligochitosan for 1 min, with sterile distilled water as the control. *Each column* represents the mean of three replicates. *Bars* indicate the standard errors. Activities of β-1, 3-glucanase (**A**), Chitinase (**B**) and PAL (**C**) marked with *different letters* (on the same day) are significantly different (LSD test, $P = 0.05$)

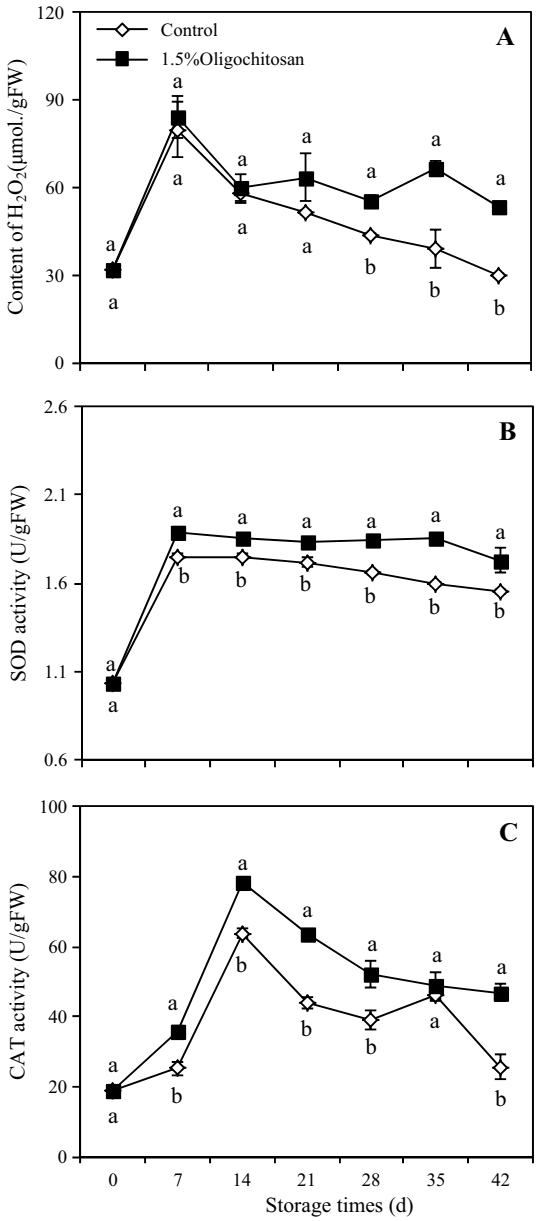

Fig. 4 Effects of oligochitosan treatment on level of H_2O_2 (**A**) and activities of SOD (**B**) and CAT (**C**) in citrus fruit peels. Citrus fruits (*Citrus sinensis* L. Osbeck) were immersed in 1.5 % (g/g) oligochitosan for 1 min, with sterile distilled water as the control. *Bars* indicate the standard errors. *Each column* represents the mean of three replicates. Contents of H_2O_2 (**A**) and activities of SOD (**B**) and CAT (**C**) marked with *different letters* (on the same day) are significantly different (LSD test, $P = 0.05$)

Superoxide dismutase activities in both control and oligochitosan-treated fruits rapidly increased and reached their peak at 7 days, followed by a gradual decrease with prolonged storage period. SOD activity in oligochitosan-treated fruits was 16.25 % higher than in control fruits at the 35th day of storage (Fig. 4B). CAT activities in both groups rapidly increased and reached their peak at 14 days, followed by a gradual decrease. Oligochitosan treatment promoted the increase in CAT activity during the entire storage period. Figure 4C clearly showed significant differences in the enzymatic activities between control and oligochitosan-treated fruits ($P < 0.05$). CAT activities in

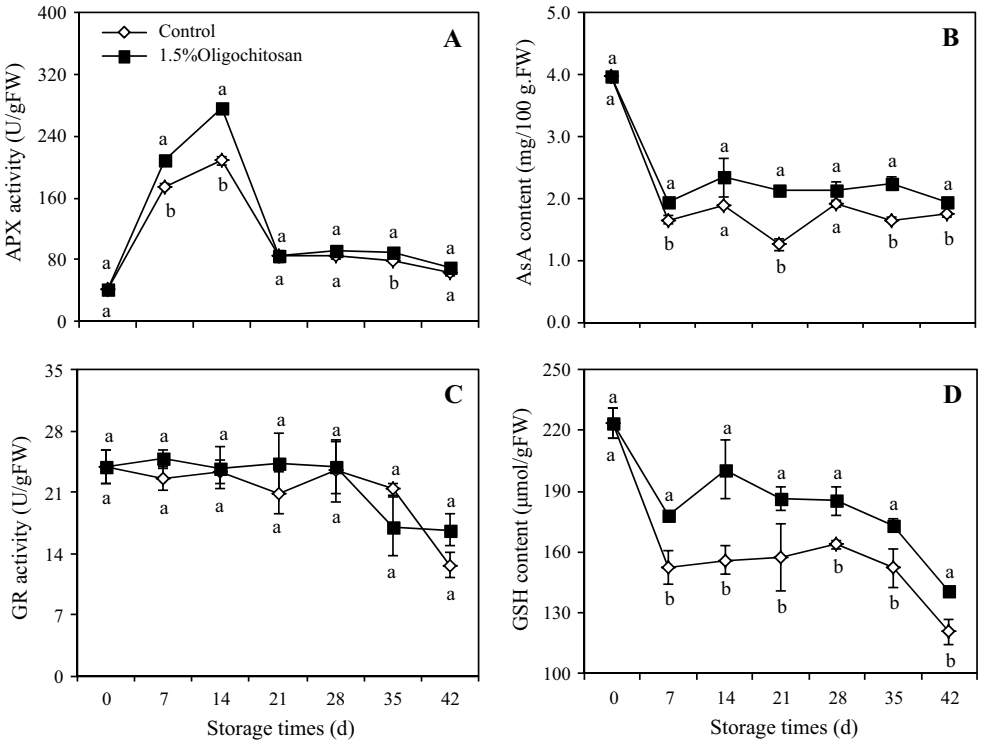

Fig. 5 Effects of oligochitosan treatment on enzymes and non-enzyme components in the AsA–GSH cycle in citrus fruit peels. Citrus fruits (*Citrus sinensis* L. Osbeck) were immersed in 1.5 % (g/g) oligochitosan for 1 min, with sterile distilled water as the control. *Bars* indicate the standard errors. *Each column* represents the mean of three replicates. Activities of APX (**A**) and GR (**C**) and contents of ASA (**B**) and GSH (**D**) marked with *different letters* (on the same day) are significantly different (LSD test, $P = 0.05$)

oligochitosan-treated fruits were 40.84, 45.67, and 82.52 % higher than the values for control fruits, at the 7th, 21st, and 42nd days after treatment.

Effects of oligochitosan treatment on enzymes and non-enzyme components in the AsA–GSH cycle in citrus fruits

APX activities in both groups rapidly increased and reached their peaks at the 14th day, followed by a sharp decrease in activity (Fig. 5A). The peak value in oligochitosan-treated fruits was 32.74 % higher than that in control fruits. AsA contents in both groups declined during storage, and oligochitosan treatment significantly inhibited the decrease in AsA contents. Figure 5B showed that AsA contents in the treated fruits were 69.25 and 35.45 % higher at the 21st and 35th days of storage compared with control fruits, respectively.

In the control fruits, GR activity slightly changed within the first 28 days, after which a remarkable decrease was observed (Fig. 5C). The results suggested that oligochitosan led to a slight increase in GR activity, except on the 35th day, but the differences between these two groups were not statistically significant ($P < 0.05$). GSH contents in all fruits decreased during storage, but the decrease rates in the oligochitosan-treated fruits were slower (Fig. 5D). On the first 7 days, GSH contents in control fruits decreased by 31.69 and by 20.31 % in oligochitosan-treated fruits. At the 14th day of storage, GSH content was 28.47 % higher in oligochitosan-treated fruits than in control fruits.

Effects of oligochitosan treatment on the activity of POD and PPO in citrus fruits

POD activity in control fruits declined gradually during the entire storage period, although it was markedly enhanced by oligochitosan treatment ($P < 0.05$). As shown in Fig. 6A, POD activity in the oligochitosan-treated fruits increased by 23.7 % after 7 days of storage, followed by a decline on the 21st day. POD activity in oligochitosan-treated fruits was approximately 46.78 and 50.04 % higher than those in control fruits at the 35th and 42nd days after storage, respectively (Fig. 6A).

PPO activity in control fruits showed a slightly increasing trend, reached their peak at the 14 day, and decreased rapidly (Fig. 6B). Oligochitosan treatment significantly enhanced PPO activity on the 7th, 14th, and 28th days after storage, and the peak value (day 14) was 40.13 % higher than those in control fruits, and PPO activity was 90.35 % higher than that in control fruits at the 28th day after treatment.

Discussion

Among all the natural elicitor compounds that have been previously reported or applied, oligochitosan offers a great potential as a water-soluble, non-toxic, biodegradable, and biocompatible substance that possesses versatile functional properties for agricultural applications [1]. Previous studies

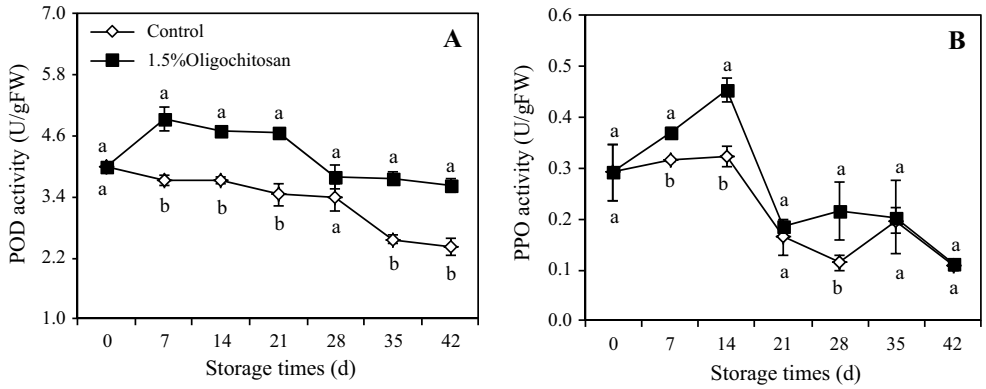

Fig. 6 Effects of oligochitosan treatment on activities of POD (**A**) and PPO (**B**) in citrus fruit peels. Citrus fruits (*Citrus sinensis* L. Osbeck) were immersed in 1.5 % (g/g) oligochitosan for 1 min, with sterile distilled water as the control. *Bars* indicate the standard errors. *Each column* represents the mean of three replicates. Activities of POD (**A**) and PPO (**B**) marked with *different letters* (on the same day) are significantly different (LSD test, $P = 0.05$)

have demonstrated that oligochitosan can reduce brown rot caused by *Monilinia fructicola* in peach [14] and inhibit mycelial growth and spore germination of *Alternaria kikuchiana* Tanaka and *Physalospora piricola* Nose in pear [11]. In the present study, disease incidence and lesion diameter were lower in oligochitosan-treated fruits compared with the respective controls. These results suggested that disease resistance against *C. gloeosporioides* Penz. in citrus fruits was remarkably enhanced by oligochitosan treatment when stored at ambient temperature ($P < 0.05$).

Reactive oxygen species are defense signaling molecules, in which H_2O_2 is considered to be the most important species. Rapid generation of H_2O_2 is one of the earliest events correlated with plant resistance to pathogens [41]. Defense response and reduction in stomatal aperture induced by oligochitosan are connected with the H_2O_2 signaling pathway [8]. Apoptosis-like cell death in tobacco cells induced by oligochitosan is also independent of H_2O_2 signaling pathway [20]. In this paper, oligochitosan was found to significantly increase H_2O_2 contents in citrus fruits after 21 days of storage ($P < 0.05$). Therefore, enhancing ROS generation may also be part of the mechanism of oligochitosan-induced disease resistance in citrus fruits.

Considering that ROS markedly contributes to the enhancement of disease resistance in plant tissues, high concentrations of ROS can cause cellular damage and membrane lipid peroxidation [42]. Under physiological conditions, ROS can be scavenged off, and oxidative damage can be minimized because of the existence of antioxidant enzymes and non-enzyme antioxidants, such as SOD, CAT, POD, APX, GR, AsA, and GSH. Activities of SOD, CAT, and POD, as well as the balance of AsA–GSH cycle in cells are crucial for determining the steady-state levels of superoxide radicals and H_2O_2 [43]. SOD catalyzes the conversion of O^{2-} to H_2O_2, which is subsequently decomposed into H_2O and O_2 by CAT or POD [44]. In the present study, SOD and CAT activities were found to increase in oligochitosan-treated fruits during storage. APX activity in citrus fruits was significantly increased by oligochitosan treatment during early storage, and AsA and GSH contents during storage were markedly reduced. However, no differences in GR activity were observed in both groups. The obtained results were consistent with experimental results previously reported on jujube fruits [13].

Hydroxyproline-rich glycoproteins can constitute a network with the cellulose of cell walls or form a cross-linked mesh through covalent linkage with pectin or ionic complexes [45]. They provide deposition sites for the lignin in the papilla, which serves as a structural barrier to aid in resistance against pathogens [46]. Oligochitosan induces the deposition of lignin [47]. As precursors of lignin, phenolic compounds that display antimicrobial activity, such as benzoic, have been previously reported [48]. In addition, some phenolic compounds are components of primary cell walls. The oxidative cross-linking of some phenolics limits wall extensibility and could have important functions in resistance to fungal pathogens [49].

Moreover, H_2O_2 may be involved in oxidation of phenols during defense reactions. Cooper and Varner [50] demonstrated that cell wall HRGPs became insoluble in carrot cell wall mediated by POD and H_2O_2. POD and H_2O_2 also have important functions in the process of oxidative cross-linking of the cell wall. Results from the present study indicated that oligochitosan treatment can significantly promote the accumulation of HRGP and lignin in citrus fruit peels ($P < 0.05$) and that the total phenol contents increased to some extent. H_2O_2 levels and POD activity were also markedly increased in oligochitosan-treated fruits ($P < 0.05$), aiding in the formation of an effective structural barrier.

Phenylalanine ammonialyase is the key enzyme in the phenylpropanoid metabolic pathway, which could regulate the metabolic flow from the primary metabolisms to phenylpropanoid metabolism. Other oxidative enzymes, such as POD and PPO, catalyze the formation of lignin and other oxidative phenols that contribute to the formation of defense barriers for reinforcing the cell structure [47, 51]. PAL and POD activities were positively related to OIPK in tobacco, whereas OIPK could upregulate three PR genes, enzymatic activity and gene expression of PAL, and expressions of CHI and GLU [4, 11, 21, 39]. In the present study, oligochitosan treatment decreased the peak value of PAL activity and delayed the peak time (about 1 week) in citrus fruit peels. Two activity peaks were found, indicating that a relatively higher PAL activity occurs later. Correspondingly, higher total phenol content was found during the later time of storage. In addition, the activity of GLU was sharply induced during the first 14 days by oligochitosan treatment, which agrees with results obtained from the previous studies mentioned above. These results indicate that increase in PAL and GLU activity by oligochitosan occurs at different time points.

PPO, which catalyzes O_2-dependent oxidation of phenolic compounds to form quinines, has been proposed as a component of plant defense mechanisms and is associated with induced defense responses in plants [52]. In the present study, oligochitosan was demonstrated to induce disease resistance, which is partly attributed to the enhancement in PPO activity, and the increase in the production of flavonoids, another important secondary metabolite of the phenylpropanoid metabolic pathway.

Conclusions

This study showed that oligochitosan treatment can effectively inhibit anthracnose caused by *C. gloeosporioides* Penz. in citrus fruit. Oligochitosan-induced strong disease resistance in citrus fruits during storage by increasing the contents of structurally related compounds and the activities of defense enzymes. In addition, a complex regulation of H_2O_2 and its related metabolism system can be helpful for the maintenance of a steady-state level of redox in fruits. However, different mechanisms take place at different storage times.

Collectively, the obtained results indicate that oligochitosan treatment can be an effective alternative to the conventional control methods of postharvest anthracnose in citrus fruits.

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Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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