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Quaternary chitosan oligomers enhance resistance and biocontrol efficacy of *Rhodosporidium paludigenum* to green mold in satsuma orange

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ABSTRACT

This study investigated the capacity of chitosan oligomers (COS), applied before harvest singly or in combination with antagonists, in controlling postharvest green mold caused by *Penicillium digitatum* in satsuma orange. Oranges treated with COS or *Rhodosporidium paludigenum* were observed having a delay in onset and progression of disease symptoms relative to wounded controls. Preharvest application of COS at different concentrations achieved similar biocontrol efficiency rates in green mold control after 4 days storage. However, the combination of pre-COS (1%, w/v) and *R. paludigenum* showed a more effective decay control than any other treatments. COS (1%, w/v) alone did not negatively affect *R. paludigenum* growth in wounds, but severely inhibited *P. digitatum* spore germination than lower dose treatments *in vitro*. The expression levels of the defense-related genes chitinase and phenylalanine ammonia lyase increased with decreased disease symptoms. Moreover, this phenomenon was more prominent in the integrated treatments than in the individual ones.

1. Introduction

Satsuma orange is a small seedless and easy-peeling cultivar of citrus well known worldwide because of its sweetness, hardness, and simple cultivation. Postharvest fungal disease caused by *Penicillium digitatum* (Pers.) Sacc., however, severely affects the storage and transportation of oranges, particularly during "ethylene degreening" (Kinay, Yildiz, Sen, Yildiz, & Karacali, 2005). At present, the primary method for controlling postharvest decay of fruits is still fungicides, but synthetic chemicals may cause increasing public concern over the potential impact of fungicide residues on human health and the environment. Therefore, it has a great interest to develop new and safer alternatives for the disease management. While field infections are the main source of *Penicillium* decay because fungal spores generally infect fruits through wounds produced by mechanical injuries during the growing season and harvest handling operations (Teixidó et al., 2010). Hence, the non-fungicide strategy of integrating pre- and postharvest decay control methods yielded more attention, which was expected to reduce the amount of both products without compromising decay control.

Among non-fungicide, environmentally friendly approaches, biological control strategy holds great promise of providing an alternative to present postharvest chemical fungicides for the control of postharvest diseases during the whole periods of fruit growth and storage (Sharma, Singh, & Singh, 2009; Wilson, Droby, Wisniewski, & Chalutz, 2011). The field application of biological control agents, including microbes, bioactive compounds and physico-chemical methods, prior to harvest also efficiently reduces latent, quiescent, and incipient infections; decreases pathogen inoculum in the environment; and increases innate resistance in fruit (Ippolito & Sanzani, 2011; Karabulut et al., 2004). Notably, increasing the fruit's natural defense capabilities through induction of resistance by pre- or postharvest application has made a significant contribution to non-fungicidal *Penicillium* decay control in citrus fruit (Lu et al., 2013a, 2013b; Youssef, Sanzani, Ligorio, Ippolito, & Terry, 2014).

Chitosan, the linear cationic (1-4)-2-amino-2-deoxy-<beta>-D-glucan produced from chitin by partial deacetylation, is known for its biochemical significance and biocompatibility (Muzzarelli, 2012; Muzzarelli et al., 2012). Chitosan oligomers (COS) are readily soluble in water and exert superior antimicrobial, antitumor and

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immunomodulatory activities (Muzzarelli et al., 1990; Muzzarelli, 2009). COS and its derivatives can form physical barriers around the penetration sites of pathogens, preventing conidia from spreading to healthy tissues, germ tube elongation, and mycelial growth (Bautista-Baños et al., 2006; El Hadrami, Adam, El Hadrami, & Daayf, 2010), such as *Botrytis cinerea* (Badawy & Rabea, 2009), *Penicillium expansum* (de Oliveira et al., 2014), *P. digitatum*, *Penicillium italicum* (Chien & Chou, 2006), and *Rhizopus stolonifer* (Hernández-Lauzardo et al., 2008). In addition to as physical barriers, COS can also reduce the host defense responses (Ma, Yang, Yan, Kennedy, & Meng, 2013). A recent study demonstrated that chitosan could be perceived as a pathogen-mimicking elicitor to powerfully activate a large number of biotic stress-related genes (Povero et al., 2011). Moreover, this polymer was proved to be just as effective in the field. Preharvest COS treatment significantly inhibits various postharvest diseases of table grape (Romanazzi, Gabler, & Smilanick, 2006), sweet cherry (Feliziani, Santini, Landi, & Romanazzi, 2013), and jujube fruit (Yan, Cao, Jiang, & Zhao, 2012).

Rhodosporidium paludigenum Fell & Tallman, a potential biocontrol yeast, was well investigated for its wide range mode of actions against fungal. It was mainly characterized by mechanisms of space and nutrient competition (Wang et al., 2008, 2011), and by resistance induction (Lu et al., 2013a,b). In addition, the biocontrol efficacy and population growth of *R. paludigenum* were enhanced by salt-adaptation (Wang et al., 2010), acid-adaptation (Wang, He, Xia, Yu, & Zheng, 2013), and chitin-adaptation (Lu et al., 2014). A single postharvest application of *R. paludigenum* can significantly reduce decay. However, the potential protection that can be elicited by additional COS application before harvest, particularly in the presence of *R. paludigenum* that can degrade chitin as a carbon source for growth, has yet to be determined.

The present study has the following objectives: (1) to investigate the biocontrol ability and synergistic effect of preharvest COS on inhibiting postharvest green mold in satsuma orange; and (2) to reveal the mechanisms by which COS inhibit postharvest disease development and improve biocontrol efficiency of antagonistic yeast at the physiological and molecular levels, including the detection of defense-related genes expression and microbes growth.

2. Materials and methods

2.1. Chemical and microorganisms

Crab-shell COS mixture (deacetylation of about 90%) with the molecular weight from 200 up to 500 kDa was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). For experimental use, the COS were dissolved in 0.1% (v/v) acetic acid, under continuous stirring. When dissolved, the pH of the COS solution was adjusted to pH 5.6 using 1 M NaOH, and then diluted with sterile distilled water as required.

The antagonistic yeast *R. paludigenum* (IMI 394084, CABI Bioscience Identification Services, U.K.) was originally isolated from the south East China Sea and cultured in 250 mL flasks containing nutrient yeast dextrose broth (Wang et al., 2008). After incubation on a gyratory shaker at 28 °C for 36 h, yeast cells were collected by centrifugation at 950 × *g* for 15 min, washed twice and resuspended with sterile distilled water.

The pathogen *P. digitatum* (Pers.:Fr.) Sacc. was isolated from decayed orange fruit and cultured on potato dextrose agar (PDA) medium in the dark at 25 °C. A conidial suspension was prepared by flooding 1-week-old plates with sterile distilled water containing 0.05% Tween-20. The number of yeast cells and fungal spore concentration were counted with a hemocytometer.

Table 1
Primers for defense-related genes β-1,3-glucanase (*GLU*), chitinase (*CHI*) and phenylalanine ammonia lyase (*PAL*), and *actin* in satsuma orange.

Accession number	Gene name	Primer	Sequence (5′–3′)
AY971953	*GLU*	-F	TGCTAATCGCTACCCTGGAC
		-R	ATTCTTCGGAGGAGGTCAA
AF090336	*CHI*	-F	CTGCCAAGGCTTATCCTCA
		-R	GCTCCGTAGTTTCCATTTCC
U43338	*PAL*	-F	GATTACGGATTCAAGGGTGC
		-R	TTGGTGACAGGATTGGCGAG
GU911361	*Actin*	-F	ATGAAGATCAAGGTCGTGGCT
		-R	CTGGAAGGTGCTGAGGGAT

2.2. Preharvest COS spraying treatments

The preharvest COS treatment was strictly applied as we described previously (Lu et al., 2013b). Satsuma orange (*Citrus unshiu* Marcow.) trees were grown in an experimental orchard, located in Chun'an city (Zhejiang Province, China). At one day before harvest in November, 2013, the fruit was sprayed with distilled water at pH 5.6 (as the control), 0.2%, 0.5%, or 1% (w/v) COS solution until all fruits were wet to run-off. Treated trees were separated by non-treated trees to prevent cross-contamination by spray drift. Commercial matured fruit was harvested and collected carefully, then transported to the storage room at 25 °C.

2.3. Improved biocontrol efficiency by preharvest spraying COS against green mold

After different intervals (0, 2 and 4 days) of storage at 25 °C, fruit from different treatments was gently wounded with a sterile borer around the blossom end to form two wounds of 2 mm depth and 5 mm diameter. Each wound was treated with 50 μL of one of (1) sterile distilled water as the control or (2) cell suspension of *R. paludigenum* at 1×10^8 cells mL^{-1}. After 2 h, 30 μL of a spore suspension of *P. digitatum* (0.5×10^5 spores mL^{-1}) was inoculated to fruit surface wounds. The fruit was then stored in enclosed plastic trays to maintain high (90–95%) relative humidity (RH) at 25 °C. The number of infected wounds and lesion diameters of wounds were recorded twice at 2 and 3 d (or 4 d for combination treatment) after inoculation. There were three replicates per treatment and each replicate contained 12 randomly selected fruits. The percentages of infected wounds and average lesion diameters of twelve fruits in each treatment were computed as one observation value for statistical analysis, respectively.

2.4. Effect of COS on population growth of R. paludigenum on the surface wound

To determine the effect of COS on the colonizing ability of the yeast *R. paludigenum*, the population dynamics of this yeast on the satsuma orange surface wounds was investigated. Fruit samples were taken at different time intervals (0, 24, 48, 72 and 96 h) after inoculation by removing the peel tissue with a cork borer. The resulting cylinders of excised tissue (2 mm deep × 1 cm wide) from three fruits were placed in a mortar with 15 mL of sterile distilled water and ground with a pestle. Then, serial ten-fold dilutions were made and sprayed on the nutrient yeast dextrose agar (NYDA, containing 20 g agar in 1 L of NYDB) glass plates. After 2 days of inoculation at 25 °C, the amount of the yeast colonies was counted. Each treatment contained three replicates and the entire experiment was repeated twice.

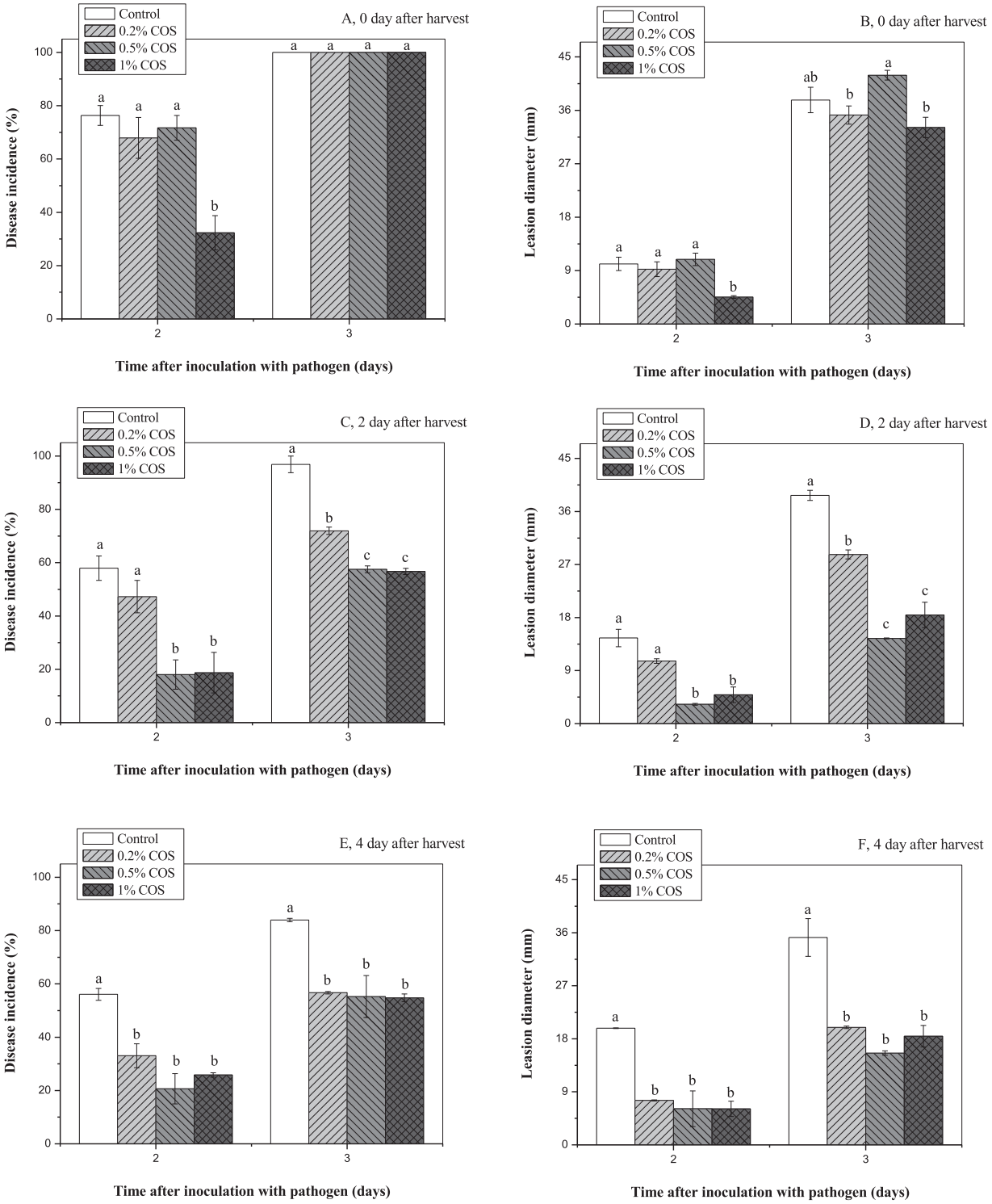

Fig. 1. Inhibition of green mold rot in satsuma orange wounds by preharvest COS at various concentrations and incubation at 25 °C for 0, 2, and 4 d postharvest. Lesion diameter (A – 0 d, C – 2 d and E – 4 d) and disease incidence (B – 0 d, D – 2 d and F – 4 d) in orange fruit were measured twice at 2 and 3 d after pathogen inoculation at 25 °C and 90–95% relative humidity. Bars represent standard errors of three replications. Different letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range tests.

2.5. Effect of COS on spore germination of *P. digitatum* in vitro

The effect of COS on spore growth of *P. digitatum* was assessed by spraying conidial suspension with COS solutions at 0, 0.2%, 0.5% or 1% (w/v) on PDA medium. Spore growth was evaluated following 48 h incubation at 25 °C in the dark. Each treatment was replicated three times and 150–200 spores per replicate were observed manually. The experiment was conducted twice.

2.6. Quantification of mRNA accumulation by quantitative reverse transcription

Total RNA was extracted, using a modified CTAB method as described by Ballester, Lafuente, and González-Candelas (2006).

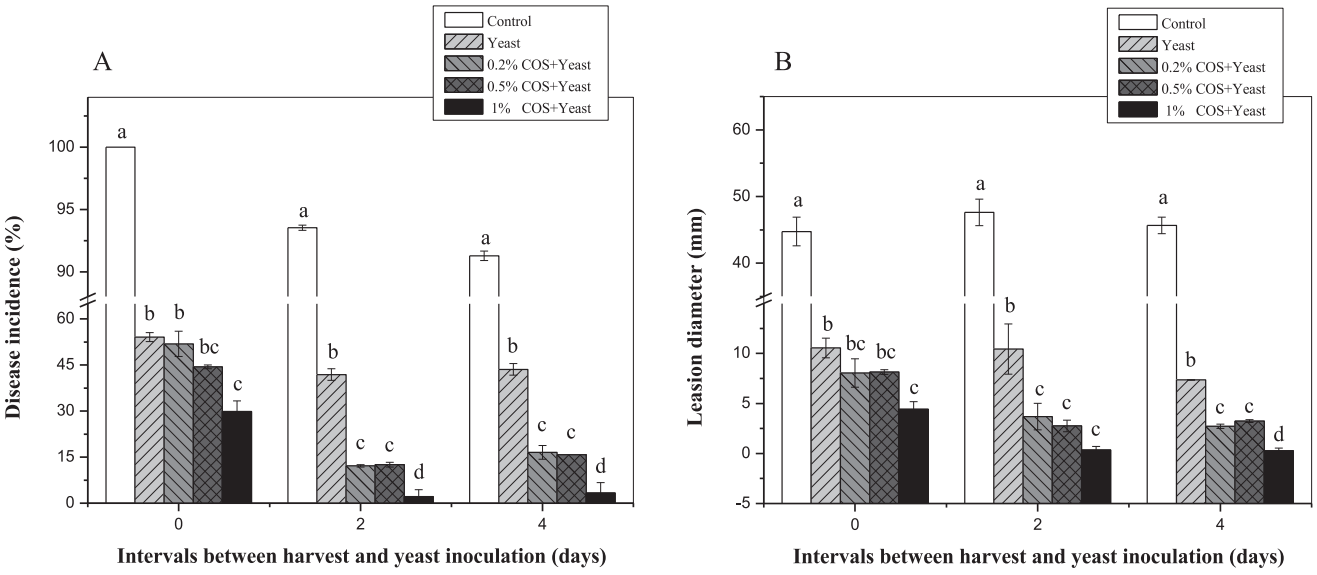

Fig. 2. Inhibition of green mold rot in satsuma orange wounds by preharvest COS at various concentrations and inoculation with *Rhodosporidium paludigenum* at 0, 2, and 4 d postharvest at 25 °C. Disease incidence (A) and lesion diameter (B) in orange fruit were measured 4 d after pathogen inoculation at 25 °C and 90–95% relative humidity. Bars represent standard errors of three replications. Different letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range tests.

First-strand cDNA was synthesized with a PrimeScript® RT reagent kit with gDNA eraser (RR047A, TaKaRa) from 1 μg of DNA-free RNA according to the manufacturer's instructions. The cDNA was diluted 10-fold and 2 μl of the diluted cDNA was used as the template for the real-time quantitative PCR (RT-qPCR) analysis.

For quantification of the mRNAs accumulated in the surface wound, RT-qPCR analysis was performed with iTaq™ SYBR® Green Supermix with ROX (172-5851, Bio-Rad, USA) using a StepOne real-time PCR instrument (v. 2.2.2, Applied Biosystems). The gene-specific primers were designed with the Primer Premier 5.0 program (PREMIER Biosoft International, Palo Alto, CA, USA) and are listed in Table 1. Primers were used at a concentration of 0.2 μM each with the equivalent of 50 ng reverse-transcribed RNA template per reaction. The thermal cycling conditions were 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s. The melt curve conditions were 95 °C for 15 s, 60 °C up to 95 °C by 0.3 °C steps and 95 °C for 15 s. Fluorescent signals were collected during the annealing and extension cycles. Cycle threshold (*Ct*) values were determined using autothreshold. For each sample, the relative expression (ΔCt) value for the gene of interest was obtained by normalizing to *Actin* transcript frequency. To enable comparisons to be made between treatment samples, all values were adjusted so that the controls at all-time points were set to one. The $\Delta\Delta Ct$ was calculated by subtracting the ΔCt of the controls from the ΔCt values of all other measurements at that time point. The relative mRNA expression was derived by the formula: $2\,[\exp(-\Delta\Delta Ct)]$.

2.7. Fruit quality assay

The effect of preharvest spraying of COS on postharvest quality of satsuma orange was assayed as described by our previous study (Lu et al., 2013b). Fruit hardness was measured at three points in the equatorial region by using Texture analyzer (GY-1; Top instrument Co., Ltd., China) and the maximum force was recorded as fruit's hardness (N). Total soluble solids (TSS) content was determined by measuring the refractive index of mandarin orange juice with a hand-held refractometer (WZ-103; Top instrument Co., Ltd., China) and the results were expressed as percentages. The titratable acidity (TA) content was measured by titration with 0.1 M NaOH to pH 8.1 and calculating the result as percentages of citric acid. Ascorbic acid (AA) content was determined using the 2,6-dichloro-indophenol method and the results were expressed as milligrams of ascorbic acid per 100 g sample.

2.8. Statistical analyses

All statistical analyses were performed using the statistics program SPSS/PC ver. II.x (SPSS Inc. Chicago, IL, USA) with a one-way analysis of variance and Duncan's multiple range tests. The values were the means of three replicated samples ± standard deviation (SD). Mean differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Effect of different COS pre-applications on inhibiting green mold in satsuma orange

The concentration and time courses of preharvest COS application for inhibiting green mold in satsuma orange were investigated after different storage intervals at 25 °C (Fig. 1). Compared with the water control, the disease incidence and severity were significantly reduced by 1.0% (w/v) COS treatment at 0 d postharvest (Fig. 1A and B). However, the decay increased with decreased concentration of COS application. At 2 d postharvest, low levels of COS (0.2% and 0.5%, w/v) could also effectively reduce green mold incidence and severity in satsuma orange (Fig. 1C and D). Particularly, 0.2% (w/v) or higher COS treatment showed clear inhibitory effects at 4 d postharvest relative to water control. The effect of 0.2% (w/v) COS treatment was 4 d or more delayed than that of 1.0% (w/v) COS treatment when applied at the same time (Fig. 1E and F). After 4 d of storage, different COS pre-applications achieved similar and effective biocontrol efficiency rates in green mold control (Fig. 1E and F).

3.2. Enhancement of biocontrol efficiency to yeast R. paludigenum by preharvest COS applications

Compared with the water-treated control, *R. paludigenum* markedly reduced the disease incidence (Fig. 2A) and disease severity (Fig. 2B) in satsuma orange. The percentages of infection and average lesion diameters in the *R. paludigenum* treatment group were reduced by 51.0% and 78.4% (the average value in three

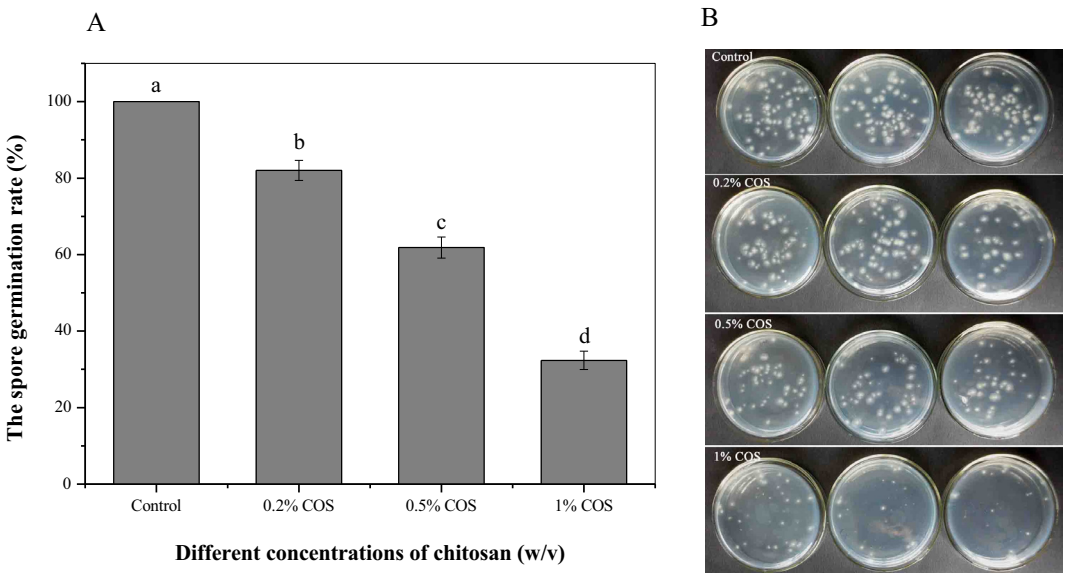

Fig. 3. Effects of COS at various concentrations on the spore germination of *Penicillium digitatum* on PDA medium. Each plate was sprayed with 100 μL of conidial suspension and COS solutions at 0, 0.2%, 0.5%, or 1% (w/v), respectively. Bars represent standard errors of three replications. Different letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range tests.

intervals) than in the control group, respectively. The combination of different preharvest COS applications and postharvest yeast *R. paludigenum* significantly reduced green mold compared with the control or yeast *R. paludigenum* treatment alone. The biocontrol efficiency of the different COS applications and the yeast also increased with prolonged storage time and increased application concentration. The largest enhancement occurred when *R. paludigenum* was applied at 4 d postharvest. Particularly, 1% COS pre-application reduced the disease incidence and average lesion diameters by 96.3% and 99.4% compared with the water control, and by at least 61.7% and 51.8% relative to 1% COS alone, respectively (Fig. 1E and F).

3.3. Spore germination of *P. digitatum* in vitro

The spore germination rate of *P. digitatum* on PDA medium was investigated to determine the inhibitory effect of different COS concentrations on this pathogen. As shown in Fig. 3, the selected highest concentrations of 1% (w/v) COS significantly inhibited the spore germination of *P. digitatum* on PDA medium. The spore germination rate was 32.4%, a value lower by 67.6% than that in the control group. However, the adverse effect of COS at ≤0.5% (w/v) concentration on *P. digitatum* was significantly reduced. 0.2% (w/v) COS showed a less toxic to the spore germination of *P. digitatum*, by an 18.0% reduction than that of the water control.

3.4. Population growth of *R. paludigenum* in vivo

In surface wounds, *R. paludigenum* grew rapidly, especially during the first 0 to 12 h after inoculation (Fig. 4). COS applied on fruit surface significantly promoted the population growth of *R. paludigenum* during 24–48 h *in vivo*. The yeast populations in the 0.2%, 0.5%, and 1% (w/v) COS-pretreated satsuma oranges were 2.9×10^7, 3.2×10^7, and 3.5×10^7 cells per wounds. These values were higher by 38.1%, 52.4%, and 66.7%, respectively, than those in the control groups at 48 h, the time point at which the maximum yeast population was achieved.

3.5. Expression of defense-related genes in satsuma orange peel tissue

Preharvest COS treatment alone clearly increased fruit chitinase (*CHI*) mRNA level (Fig. 5A). The highest *CHI* mRNA level was observed in the 1% (w/v) COS treatment group. Low levels of COS (0.2% and 0.5%, w/v) also showed a minimal promoting effect during 4 d of storage. Treatment of satsuma orange with preharvest COS (1%, w/v) and postharvest *R. paludigenum* induced *CHI* transcription, as shown by an approximately 2.2-fold increase within 2 d and was 3.0- or 5.8-fold higher than that measured in the treatment with 1% (w/v) COS alone or the water control, respectively (Fig. 5B). Meanwhile, the low levels of COS (0.2% and 0.5%, w/v) also promoted *CHI*

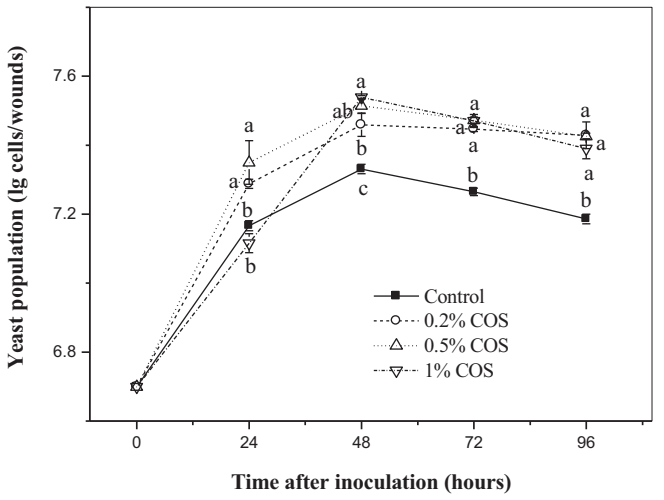

Fig. 4. Effect of COS at various concentrations on the population growth of *Rhodosporidium paludigenum* in satsuma orange. Each wound on preharvest COS treatment orange was treated with 50 μL of a cell suspension of *Rhodosporidium paludigenum* at 1×10^8 cells mL^{-1}. Bars represent standard errors of three replications. Different letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range tests.

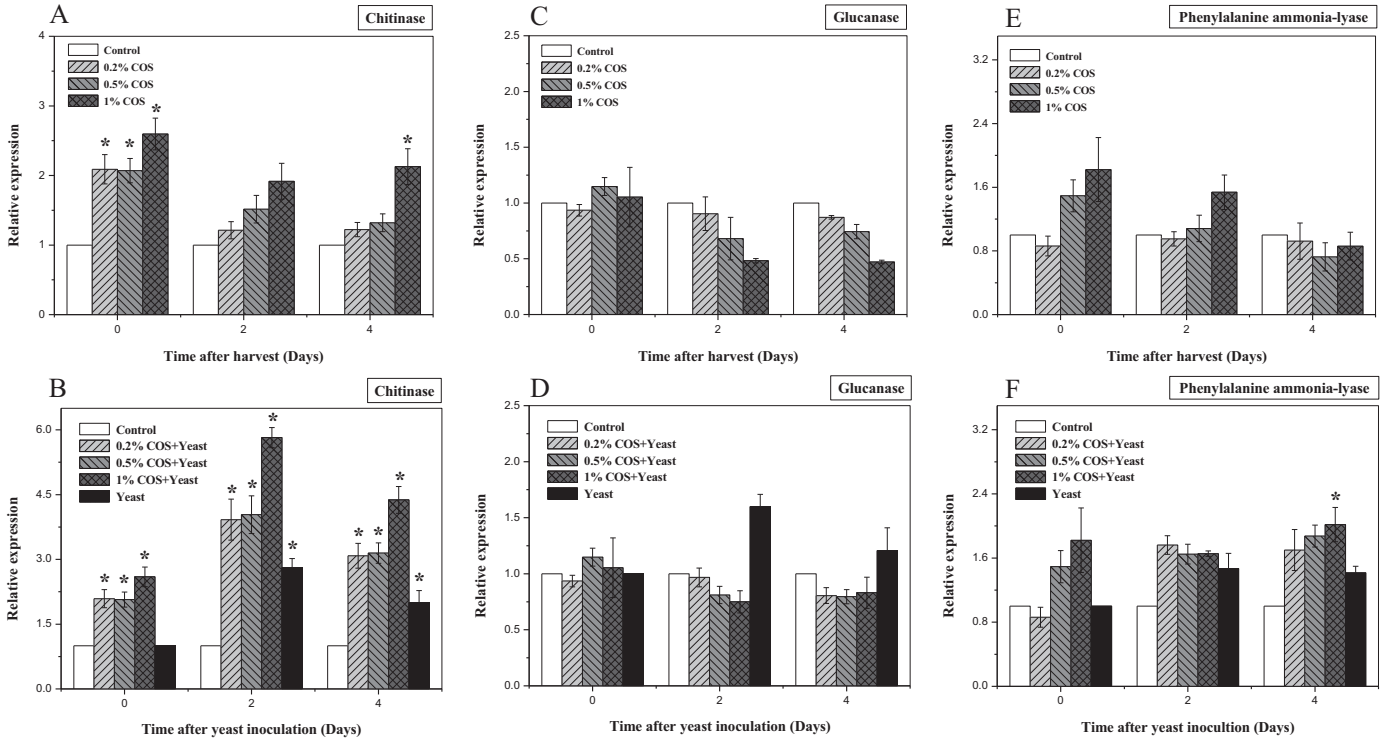

Fig. 5. RT-qPCR analysis of mRNA accumulation of three defense-related genes encoding chitinase (A, B), β-1,3-glucanase (C, D), and phenylalanine ammonia lyase (E, F) in satsuma orange peel tissue during resistance induction. Fruit were pre-sprayed with COS at various concentrations (0, 0.2%, 0.5%, and 1%, w/v) or inoculated with a cell suspension of *Rhodosporidium paludigenum* at 1×10^8 cells mL^{-1}. * Indicates up-regulated expression (fold change ≥ 2) for each time point. Bars represent standard errors of three replications.

transcription induced by *R. paludigenum* in orange surface wounds at 2 or 4 d after inoculation.

The expression pattern of β-1,3-glucanase (*GLU*) in response to the different concentrations of COS was different from that of *CHI*. The *GLU* transcripts in the COS-pretreated oranges decreased at 2 d postharvest and maintained thereafter (Fig. 5C). By contrast, *R. paludigenum* increased *GLU* mRNA accumulation after 2 d of incubation, and the COS-induced reduction was relieved when the biocontrol yeast was applied after pre-COS spraying (Fig. 5D).

Similar to the effect on gene *CHI*, preharvest application of different COS (0.5% and 1%, w/v) in satsuma orange clearly increased phenylalanine ammonia lyase (*PAL*) at the initial after harvest. However, these increases in *PAL* expression disappeared by the prolonged incubation time (Fig. 5E). Interestingly, the treatment of satsuma orange with postharvest *R. paludigenum* maintained and extended the increases in *PAL* mRNA transcripts by different concentrations of preharvest COS application (Fig. 5F). In the pre-COS (0.2%, 0.5%, and 1%, w/v) and post-yeast combination groups, the expression of *PAL* was 1.7-, 1.9-, and 2.0-fold higher that in the water control at 4 d after inoculation, respectively.

3.6. Effect of preharvest COS spraying on satsuma orange quality

Quality parameters, such as fruit firmness, TSS content, TA, and AA content, were determined at 0, 5, 10, and 15 d postharvest to investigate the effect of preharvest COS spraying on satsuma orange quality. As shown in Table 2, the preharvest spray treatment of satsuma orange using different COS concentrations had no significant effect on these fruit quality parameters during the 15 d of storage period at 10–15 °C. However, these fruit quality parameters decreased with prolonged storage time.

4. Discussion

COS and its derivatives, which are soluble in acidic aqueous media, have fruit protective, moisture conservation, and antifungal properties (Romanazzi, Feliziani, Santini, & Landi, 2013). The current result demonstrated that the preharvest application of COS alone was effective in controlling green mold decay caused by *P. digitatum* in satsuma orange (Fig. 1). This systemic protection gradually developed in a concentration- and time-dependent manner. When applied at a high concentration (1%, w/v), COS can effectively reduce disease incidence and severity after spraying; it can also decrease the spore germination of *P. digitatum* on PDA medium (Fig. 3A). The low level (0.2%, w/v) of COS showed a minimal toxic effect on the spore germination of *P. digitatum*. However, the low concentration of COS showed an equivalent effect with those at high dose in controlling green mold at 4 d postharvest when the incubation time was prolonged enough (Fig. 1E and F). Similarly, several studies reported that COS at very low concentrations can trigger defensive responses in plants and fruit against pathogenic attacks (Bautista-Baños et al., 2006; Ma et al., 2013). In the present study, antifungal activity and host resistance induction were proven to be the dual dominant mechanisms of COS to control green mold decay. The period of exposure to COS was firstly found to be more decisive for the increase of disease resistance in satsuma orange than COS concentration. The advantage of disease control by increasing disease resistance emphasized in this study is the long-lasting systemic resistance to a broad spectrum of pathogens and pests, and its applicability in whole fruit grown stage. What's more, it would be more effective to enhance the fruit's natural protection before or near harvest than postharvest when it could not acquire additional energy supplies for the tree.

However, the disease control ability of COS alone was inefficient. For instance, even at the highest concentration (1%, w/v),

Table 2
Effect of preharvest applications of COS at various concentrations on postharvest quality of satsuma orange.

Storage time (days)	Treatments	Quality parameters			
		Fruit firmness (N)	Total soluble solids (%)	Titratable acidity (%)	Ascorbic acid contents (mg/100 g)
0 d	Control	2.52 ± 0.06 a	16.60 ± 0.12 a	1.00 ± 0.01 a	37.22 ± 0.39 a
	0.2% COS	2.65 ± 0.04 a	16.07 ± 0.18 a	1.03 ± 0.02 a	36.61 ± 0.32 a
	0.5% COS	2.60 ± 0.09 a	16.30 ± 0.59 a	1.04 ± 0.01 a	36.91 ± 0.60 a
	1% COS	2.52 ± 0.08 a	15.93 ± 0.24 a	1.02 ± 0.01 a	36.48 ± 0.18 a
5 d	Control	2.18 ± 0.09 a	15.65 ± 0.25 a	1.06 ± 0.03 ab	35.95 ± 0.39 a
	0.2% COS	2.44 ± 0.07 a	16.23 ± 0.32 a	1.07 ± 0.01 a	34.77 ± 0.80 a
	0.5% COS	2.17 ± 0.04 a	16.37 ± 0.13 a	1.07 ± 0.02 a	35.65 ± 1.37 a
	1% COS	2.30 ± 0.15 a	15.58 ± 0.26 a	1.04 ± 0.01 b	33.64 ± 0.66 a
10 d	Control	1.87 ± 0.04 a	16.00 ± 0.34 a	0.96 ± 0.01 a	32.06 ± 0.95 a
	0.2% COS	1.99 ± 0.05 a	16.37 ± 0.15 a	0.98 ± 0.01 a	31.54 ± 0.64 a
	0.5% COS	1.98 ± 0.05 a	15.98 ± 0.21 a	0.97 ± 0.03 a	31.16 ± 0.48 a
	1% COS	1.98 ± 0.05 a	15.20 ± 0.27 b	0.97 ± 0.01 a	31.02 ± 0.18 a
15 d	Control	1.91 ± 0.04 a	15.57 ± 0.15 a	0.98 ± 0.03 a	31.78 ± 0.56 a
	0.2% COS	1.94 ± 0.06 a	15.73 ± 0.12 a	0.96 ± 0.01 a	31.45 ± 0.63 a
	0.5% COS	1.88 ± 0.06 a	15.59 ± 0.08 a	0.97 ± 0.02 a	31.01 ± 0.33 a
	1% COS	1.88 ± 0.08 a	15.44 ± 0.29 a	0.95 ± 0.01 a	31.05 ± 0.70 a

Fruit hardness, total soluble solids, the titratable acidity and ascorbic acid content were determined after storage for 0, 5, 10 and 15 days under normal condition. Different letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range tests.

the disease incidence and severity in the COS-treated oranges were just nearly half reduced. A similar level of control of decay by COS alone was also observed in apple and pear fruits against *P. expansum* (Yu et al., 2012) and in table grapes against *B. cinerea* (Meng & Tian, 2009; Romanazzi et al., 2006). Controlling postharvest diseases with a single alternative means, such as COS, is not often as consistent as control with synthetic fungicides. Therefore, integration with one or more additional biocontrol agents is necessary to improve the biocontrol efficiency of COS to satisfactory levels of decay control.

Biocontrol yeast is an important element in the integration of pre- and postharvest strategy with biocontrol products (Teixidó et al., 2010). Antagonistic microbes, such as the biocontrol yeast used in this study, often work in concert to control disease development. Their mechanisms may involve the microecology of the pathogen and antagonist, antibiosis, and host resistance responses (de Capdeville, Wilson, Beer, & Aist, 2002). The performance of yeast antagonists, including *R. paludigenum*, could be further improved by adding biocontrol agents or natural compounds (Wang et al., 2011; Yu et al., 2012). In the present study, the combination of COS and *R. paludigenum* can exploit an additive or synergistic effect in green mold reduction or elicit defense-related responses. The largest reductions in green mold incidence and severity were observed in preharvest COS and postharvest *R. paludigenum* combination treatment groups at 4 d postharvest in satsuma oranges. Specifically, disease development was almost completely inhibited in the oranges treated with high COS concentrations and *R. paludigenum* when most of the wounds in the control were rotten (Fig. 2). A similar synergistic effect of control of decay by COS was also observed in table grapes against *B. cinerea*, in which gray mold incidence was reduced by 89.5% when combined COS and UV-C irradiation treatments were applied (Romanazzi et al., 2006). Based on these results, we proposed that preharvest COS can markedly improve the biocontrol efficiency of antagonists to postharvest pathogens in store fruits and provide an efficient pre- and postharvest combination strategy or products in controlling postharvest decays.

Fruits have developed an intricate defense system against pathogen attack, including the accumulation of various types of stress proteins, and the production of secondary metabolites with antimicrobial activity. *PR-2* and *PR-3* genes coding for GLU and CHI can potentially degrade the fungal cell wall, which is mainly composed of β-1,3-glucan and chitin (van Loon, Geraats, & Linthorst, 2006). In several plant–pathogen interactions, the induction and accumulation of PR proteins, including CHI and GLU, often correlate with the onset of induced resistance (El Ghaouth, Wilson, & Wisniewski, 2003). In the present study, the mRNA expression of *CHI* was clearly induced in response to preharvest COS treatment, whereas the gene encoding for GLU was not induced (Fig. 5A and C). Meanwhile, *PAL*, the gene encoding for the first enzyme in the phenylpropanoid metabolism pathway, showed a similar behavior to the former one. The treatments that increased disease resistance induced the accumulation of large transcripts, but had no effect at the end of this test (Fig. 5E). The changes in *CHI* and *PAL* expression in response to the treatment that elicited fruit resistance were similar to those in the activity of their respective enzymes in other fruits (Meng & Tian, 2009; Trotel-Aziz, Couderchet, Vernet, & Aziz, 2006). Therefore, *CHI* and *PAL* might be involved in delaying fungal growth and decreasing the incidence of infection caused by *P. digitatum* in satsuma orange.

Aside from inducing disease resistance in fresh oranges by COS, *R. paludigenum* also caused a rapid accumulation of *CHI*, *GLU*, and *PAL* mRNA locally in the treated wound site and systemically in tissues distant from the initial wound. This result suggests the involvement of fruit resistance induction in the biocontrol mechanisms of antagonistic yeast. Moreover, the expression of *CHI* and *PAL* increased in response to combined COS–yeast treatments compared to their treatments alone; as a result, the resistance to *P. digitatum* markedly increased (Fig. 5B and F). COS at 1% (w/v) observably enhanced the biocontrol efficiency of *R. paludigenum* against *P. digitatum* and closely paralleled the increase in *CHI* mRNA accumulation in surface wounds. These phenomena may well explain the reasons causing the synergistic effect of an integrated strategy in satsuma orange.

In conclusion, combining preharvest COS with postharvest yeast can effectively integrate multiple biological activities from COS and yeast to suppress the fungal decay of orange fruit, suggesting it may be a promising management strategy to partially substitute the utilization of synthetic fungicides for postharvest diseases. However, whether combined treatment could be effective in controlling infections using as an alternative to synthetic in the field remain unknown. Further investigation of its ability to control the other

postharvest diseases is warranted before to use this novel strategy as a practical and effective biocontrol product.

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