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Biocontrol of gray mold in grapes with the yeast *Hanseniaspora uvarum* alone and in combination with salicylic acid or sodium bicarbonate

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ABSTRACT

The yeast *Hanseniaspora uvarum* alone or in combination with salicylic acid or sodium bicarbonate was investigated for control of gray mold, postharvest quality and antioxidant enzymes of grapes. The results indicated that the treatment of *H. uvarum* suspension of 1×10^8 CFU mL^{-1} combined with either 2 mmol L^{-1} SA or 2% SBC resulted in a remarkably improved control of *Botrytis cinerea* infections on grapes. Disease incidence and lesion diameter in treated fruit by *H. uvarum* alone or combined with SA or SBC were significantly lower than those of the control fruit ($P < 0.05$) at 25 °C, RH 90–95%. Integration of *H. uvarum* with SA and with SBC both significantly reduced the browning index, the decay incidence, weight loss while maintained the fruit appearance, firmness, total soluble solids (TSS) and titratable acidity (TA) of the grapes ($P < 0.05$) at 2 ± 1 °C, RH 90–95%. Furthermore, the combination of yeast and aforementioned chemicals led to increases of peroxidase (POD), superoxide dismutase (SOD), catalase (CAT), phenylalanine ammonia lyase (PAL), ascorbate peroxidase (APX) and polyphenoloxidase (PPO) activities. Enhanced control by SA or SBC could be due to either induced resistance or direct effects of these chemicals on *Botrytis*. The proper combination of *H. uvarum* and chemical reagent can thus provide an effective strategy to reduce postharvest decay of grape berries.

1. Introduction

Postharvest diseases limit the storage period and marketing life of grapes. Gray mold caused by *Botrytis cinerea* is one of the most destructive postharvest diseases of grapes (Masih et al., 2001). Although the use of synthetic fungicide is still the primary means for controlling postharvest diseases, the indiscriminate use of synthetic fungicides has caused increasingly worldwide concerns over their possible side effects on human health and the environment (Droby et al., 2009). Biological control utilizing antagonistic microorganisms has shown great potential as an alternative measure to synthetic fungicides for disease control (Kinay and Yildiz, 2008; Manso and Nunes, 2011; Liu et al., 2013). However, application of antagonistic microorganisms alone does not provide commercially acceptable control of postharvest diseases (Spadaro et al., 2004). Therefore, more environmentally friendly and harmless compounds should be developed as alternative methods for postharvest diseases (Ippolito et al., 2005; Janisiewicz et al., 2008; De Curtis and De Cicco, 2012; Ebrahimi et al., 2013).

Hanseniaspora uvarum, in inhibiting spore germination and lesion growth of *B. cinerea*, has an antagonistic property. Previous studies have demonstrated that competition for living space may be one of the mechanisms of action (Liu et al., 2010a). Liu et al. (2010b) found that *H. uvarum* could affect POD, SOD and CAT activity, which were considered to be the main enzymatic systems for protecting cells against oxidative damage and significantly increased the activities of PPO, PAL and β-1,3-glucanase, which were considered as key enzymes related to defense reaction against pathogen infections (Wallace and Fry, 1999).

Salicylic acid (SA), a natural phenolic compound, has been involved in local and systemic resistance to pathogens (Meena et al., 2001). Anthracnose disease caused by *Colletotrichum gloeosporioides* in mango (Joyce et al., 2001), occurance of *P. expansum* in sweet cherry fruit (Qin et al., 2003) and gray mold decay in peach (Zhang et al., 2008) can all be significantly controlled by SA treatment. Sodium bicarbonate (SBC), a commonly used food additive

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and listed as generally regarded as safe by the United States Food and Drug Administration, has shown ability in postharvest decay control of fruit (Larrigaudiere et al., 2002). The biocontrol efficacies of *Metschnikowia pulcherrima* against blue mold of apples (Spadaro et al., 2004) and *Aureobasidium pullulans* against rots caused by *B. cinerea* on sweet cherries (Ippolito et al., 2005) have been effectively improved by the use of sodium bicarbonate (SBC). The integration of an antagonist microbe with other treatments has been proposed and combined treatments with SA or SBC and different antagonist microbes have been tested and reported (Wan et al., 2003; Janisiewicz et al., 2008; Cao et al., 2013).

To our knowledge, no study has been conducted to determine the ability of SA or SBC to improve the biocontrol activity of *H. uvarum*. The objectives of this study were, first to optimize the concentrations of SA and SBC, combined with *H. uvarum* on inhibiting postharvest disease of grapes at 25 °C. Second, the effects of the treatments of *H. uvarum* alone, combined with SA and combined with SBC on postharvest quality and enzyme activities of grapes (2 ± 1 °C, RH 90–95%), including quality (browning index, decay incidence, weight loss, firmness, TSS, TA), and the defensive enzymes (POD, PPO, SOD, CAT, PAL, APX) were investigated.

2. Materials and methods

2.1. Pathogen inoculum

B. cinerea was isolated from a grape berry showing typical gray mold and stored on potato dextrose agar (PDA, 200 mL extract of boiled potatoes, 20 g dextrose and 20 g agar in 1000 mL distilled water) at 4 °C. Before use, *B. cinerea* was freshly cultured on PDA plates at 23 °C Spore suspensions were prepared by removing the spores from a 7-day old culture with a sterile inoculator and then suspending in sterile distilled water to the required concentration of 1×10^5 spores mL^{-1}, which was estimated using a hemacytometer (XB-K-25; Shanghai, China).

2.2. Antagonist

The antagonist yeast was isolated from the surface of strawberries and identified as *H. uvarum* based on the similarity analysis of its morphologies, physiological–biochemical characteristics and 26S rDNA D1/D2 domain sequence (GenBank accession number: JX125041). The yeast was cultured in 250 mL Erlenmeyer flasks with 100 mL potato dextrose (PDB, 200 mL extract of boiled potatoes, 20 g dextrose in 1000 mL distilled water) on a gyratory shaker at 180 r min^{-1}, 28 °C for 24 h. The yeast cells were acquired by centrifuging at 6000 × *g* for 15 min (at 4 °C) and then re-suspended in sterile distilled water. The yeast concentrations were adjusted as needed for different experiments using a hemacytometer.

2.3. Fruit

Grape berries (*Vitis vinifera* L. cv. Kyoho) were harvested early in the morning from a vineyard in Jiangxin Zhou, Nanjing City, China, and selected depending on the size and the absence of physical injuries or infection. Grapes were surface-disinfected with 2% sodium hypochlorite for 2 min, rinsed with tap water and dried in air before use.

2.4. Efficacy of H. uvarum combined with different concentrations of SA (or SBC) on control of gray mold in grape fruit

Grapes berries were divided into 5 groups. Three groups of berries were immersed in SA solutions with concentration of 1, 2 and 4 mmol L^{-1} for 2 min, respectively. The fourth group was not treated. The last group that was immersed in sterile distilled water was used as the control. After air-drying, the treated fruit were stored at 25 °C, RH 90–95% condition for 1 day. A uniform wound of 4 mm deep and 3 mm wide was made at the equator of each fruit using the tip of sterile dissecting needle. Aliquots (10 μL) of 1×10^8 CFU mL^{-1} yeast suspensions were pipetted into the wound site, except for the control group which was treated with sterile water. After 2 h, 10 μL of the 1×10^5 spores mL^{-1} suspension of *B. cinerea* was inoculated into each wound. All these fruit were air-dried again and then stored at 25 °C, RH 90–95% for 3 days. The lesion diameters and disease incidence were determined after incubation. Thirty fruit constituted a single replicate. The experiment was repeated twice with three replicates per treatment.

The effect of *H. uvarum* combined with different concentrations of SBC on grape lesion diameter and disease incidence of *B. cinerea* infection was studied using the similar procedure as for SA. The SBC solutions of 1, 2 and 4% concentrations were used.

2.5. Effects of H. uvarum in combination with SA or SBC on postharvest quality and enzyme activities of intact fruit during cold storage

The intact fruit were sprayed with: Control – sterile distilled water as the control, H – the cell suspension of *H. uvarum* (1×10^8 CFU mL^{-1}), H + SA – SA (2 mmol L^{-1}) after 2 h first and then in combination with *H. uvarum* suspension (1×10^8 CFU mL^{-1}), H + SBC – SBC (2%) after 2 h first and then in combination with *H. uvarum* suspension (1×10^8 CFU mL^{-1}). The fruit were air-dried and sealed in polyethylene-lined plastic boxes at 2 ± 1 °C, RH 90–95% to retain high humidity. Postharvest quality and enzyme activities of the fruit were evaluated at intervals of 10 days. 100 fruit constituted a single replicate. The experiment was repeated twice with three replicates per treatment.

2.6. Quality analysis of grapes

2.6.1. Browning index

Browning index (BI) of spike-stalk and pedicel was assessed according to the different browning of scales as follows: 0, no browning; 1, browning of scales less than a quarter of browning area; 2, browning of scales less than 1/2 of browning area; 3, browning of scales less than three-quarters of browning area; 4, more than 3/4 of browning area. The browning index was calculated by the formula, $BI = \sum(df)/ND$, where d is the browning of scales on the grape and f is its respective quantity; N is the total number of grapes examined and D is the highest browning of scales.

2.6.2. Decay incidence and weight loss

The number of decayed fruit (A) against the number of all fruit (B) was calculated as the decay incidence (A/B). Results were shown as percentage of fruit with decay. The mass of the grapes was measured by an MP2000-2 balance (±0.001 g) before treatment (a) and after storage (b). The mass loss was calculated as $(a - b)/a$.

2.6.3. Firmness

The firmness value of each individual grape was measured at the point of the equatorial region using the TA-XT2i Texture Analyser (Stable Micro Systems Ltd, UK) equipped with a 6 mm diameter flat probe. The probe descended toward the sample at the speed of 1 mm s^{-1} and the maximum force (N) was defined (Castillo et al., 2010).

2.6.4. Total soluble solids

The total soluble solids (TSS) were determined by measuring the refractive index of the fruit with a hand refractometer (WYT-4, Top instrument Co., Ltd.), China at room temperature and the result was

expressed as a percentage (g per 100 g fruit weight) (Larrigaudiere et al., 2002).

2.6.5. Titratable acidity

The titratable acidity of the grape was measured by titration with 0.1 mM NaOH to pH 8.3; 10 g of grape diluted with 100 mL of distilled water was evaluated for each replicated. The results were expressed as tartaric acid (%) (Porat et al., 2000).

2.7. Defensive enzyme assays

2.7.1. POD activity

For the determination of POD activity, fresh grapes (1.5 g) were first mixed with 8 mL of ice-cold Phosphate Buffered Saline solution (50 mM, pH 7.8) and then mashed thoroughly. The homogenate was centrifuged at 10,000 r min^{-1} for 15 min at 4 °C, and the supernatant was used for the POD activity assay. Guaiacol and H_2O_2 were used as the substrates for POD activity determination (Lurie et al., 1997). Enzyme activity was defined as the increase in absorbance at 460 nm with a spectrophotometer (UV Bluestar A, Beijing, China) at 25 °C within 2 min. The specific activity was expressed as units per gram of fresh weight. Each sample consisted of fruit tissues that were collected from five grapes. There were four samples per treatment.

2.7.2. SOD activity

For the determination of SOD activity, fresh grapes (1.5 g) were mixed with 8 mL of ice-cold sodium phosphate buffer (50 mM, pH 7.8), ground thoroughly and centrifuged as described above. SOD activity was evaluated following the method of (Wang et al., 2004). The reaction mixture was illuminated with a fluorescent lamp at 400 lx for 10 min and the absorbance was measured at 560 nm. The same solutions held in the dark were used as the blank. One unit of SOD activity was defined as the amount of enzyme that caused a 50% decrease of the SOD-inhibitable NBT reduction. The specific activity was expressed as units per gram of fresh weight. Each sample consisted of fruit tissues that were collected from five grapes. There were four samples per treatment.

2.7.3. CAT activity

For the determination of CAT activity, fresh grapes (1.5 g) were mixed with 8 mL of ice-cold sodium phosphate buffer (100 mM, pH 7.0) and ground thoroughly. The homogenate was centrifuged as above. CAT activity determination was performed following the method of (Wang et al., 2004). The reaction mixture contained 2 mL of sodium phosphate buffer, 0.4 mL of H_2O_2 and 1 mL of crude extract. The decomposition of H_2O_2 was measured at 240 nm. One unit was defined as the change in 0.001 absorbance units per minute and the specific activity was expressed as units per gram of fresh weight. Each sample consisted of fruit tissues that were collected from five grapes. There were four samples per treatment.

2.7.4. APX activity

For the determination of APX activity, fresh grapes (1.5 g) were first mixed with 8 mL of ice-cold phosphate buffered saline solution (50 mM, pH 7.8) and then mashed thoroughly. The homogenate was centrifuged at 10,000 r min^{-1} for 15 min at 4 °C. APX activity was evaluated according to the method described by Nakano and Asada (1981). One unit was defined as the change in 0.001 absorbance units per minute at 290 nm. The specific activity was expressed as units per gram of fresh weight. Each sample consisted of fruit tissues that were collected from five grapes. There were four samples per treatment.

2.7.5. PAL activity

For the determination of PAL activity, fresh grapes (1.5 g) were mixed with 8 mL of ice-cold boracic acid buffer (100 mM, pH 8.7), ground thoroughly and centrifuged as described above. PAL activity was analyzed using the method of Assis et al. (2001). One unit was defined as the change in 0.001 absorbance units per minute at 290 nm. The specific activity was expressed as units per gram of fresh weight. Each sample consisted of fruit tissues that were collected from five grapes. There were four samples per treatment.

2.7.6. PPO activity

For the determination of PPO activity, fresh grapes (1.5 g) were homogenized with 8 mL of ice-cold citric acid buffer (0.2 M, pH 6.8) and centrifuged at 10,000 r min^{-1} for 15 min at 4 °C. PPO activity was evaluated according to the method described by Tian et al. (2002). The assay was performed using 2 mL of citric acid buffer (pH 6.8), 1 mL of 100 mM 4-methylcatechol and 2 mL of the supernatant. The increase in absorbance at 398 nm at 25 °C within 2 min was recorded. The specific activity was expressed as units per gram of fresh weight. Each sample consisted of fruit tissues that were collected from five grapes. There were four samples per treatment.

2.8. Statistical analysis

All statistical analyses were performed using SAS Software (Version8.2; SAS Institute, Cary, NC, USA). The data were analyzed by one-way analysis of variance (ANOVA). Comparison of means was performed by Duncan's multiple range tests. Statistical significance was assessed at the level of $P = 0.05$ or $P = 0.01$.

3. Results

3.1. Efficacy of H. uvarum combined with different concentrations of SA (or SBC) on control of gray mold in grape fruit

Disease incidence and lesion diameter in all treated fruit were significantly lower than those of the control fruit ($P < 0.05$) (Fig. 1). *H. uvarum*, as a stand-alone treatment, reduced the disease incidence from 93.1% (control) to 51.8%. The combination of the chemical reagents and yeast decreased the disease incidence to a lower level, 38.5% for *H. uvarum* combined with 2 mmol L^{-1} SA and 34.3% for *H. uvarum* combined with 2% SBC, respectively.

At the same concentration of *H. uvarum*, the lesion diameter of fruit treated by 2 mmol L^{-1} SA was significantly ($P < 0.05$) lower than in those treated by 1 mmol L^{-1} SA and 4 mmol L^{-1} SA (Fig. 1) after 3 days of incubation. The lesion diameter in fruit treated

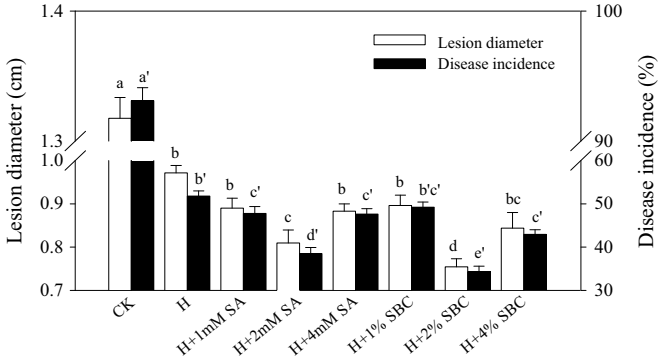

Fig. 1. Effect of *H. uvarum* combined with SA (SBC) on grape lesion diameter and disease incidence of *B. cinerea* infection. Vertical bars represent the standard errors of the means. Values with different letters at the same time within the same figure are significantly different according to Duncan's multiple range test at $P = 0.05$ level. CK: control; H: *H. uvarum*; SA: salicylic acid; SBC: sodium bicarbonate.

Table 1
Effect of *H. uvarum* combined with SA (or SBC) on spike-stalk and pedicel browning index of grape after 90 days cold storage.

Treatment	Fruit appearance	Spike-stalk appearance	Pedicel appearance	Spike-stalk browning index (%)	Pedicel browning index (%)
CK	Purplish red	Fulvouos	Fulvouos	43.65a	23.33a
H	Purplish red	Olivine	Olivine	22.22b	8.33b
H + SA	Purplish red/bright	Light green	Bright green	11.11c	5.00b
H + SBC	Purplish red/bright	Light green	Bright green	11.90c	3.33b

Values with different letters at the same time within the same figure are significantly different according to Duncan's multiple range test at $P = 0.05$ level. CK: control; H: *H. uvarum*; SA: salicylic acid; SBC: sodium bicarbonate.

by 2 mmol L^{-1} SA together with 1×10^8 CFU mL^{-1} *H. uvarum* was 0.81 cm, while the corresponding diameter in fruit treated by yeast alone was 0.97 cm. The inhibitory effects increased by 16.5% compared to the treatment of the yeast alone. As can be seen, SA was able to increase the inhibition of *H. uvarum* against *B. cinerea.*

The lesion diameter in fruit treated with 2% SBC was significantly ($P < 0.05$) lower than in those treated with 1% SBC or 4% SBC after 3 days of incubation, when the concentration of *H. uvarum* was identical (Fig. 1). The lesion diameter in fruit treated with 2% SBC in combination with 1×10^8 CFU mL^{-1} *H. uvarum* was 0.75 cm, which was 22.6% lower than that in fruit treated by *H. uvarum* alone. Therefore, the combined treatment of *H. uvarum* with 2% SBC gave a remarkably improved control of *B. cinerea* infections in grapes.

3.2. Effect of H. uvarum combined with SA (or SBC) on quality appearance of grape

As shown in Table 1, the spike-stalk browning indexes of *H. uvarum* combined with SA and with SBC were 11.11% and 11.90%, which were significantly lower than those of fruit treated by 22.22% for *H. uvarum* alone and 43.65% for the control respectively. There was no significant difference ($P \geq 0.05$) in spike-stalk browning index between grapes treated by *H. uvarum* combined with SA and with SBC. The pedicel browning indexes of *H. uvarum* combined with SA and with SBC were 5.00% and 3.33%, which were significantly lower than the 23.33% in the control. There was no significant difference ($P \geq 0.05$) of pedicel browning index among treated grapes. The results showed that *H. uvarum* had a significant role. However, in terms of fruit appearance, the effect of *H. uvarum* combined with SA and with SBC was the best treatment, which was purplish red and bright. SA and SBC contribute to promoting the quality appearance of grape fruit.

Our experiments evaluated the efficacy of yeast antagonist and SA (or SBC), as stand-alone treatments or in combination, in reducing the natural infections after cold storage. Decayed grape fruit first showed that grape stems were covered with mold, and then the typical symptom of gray rot developed gradually (Fig. 2). The appearance of treated groups was significantly better than that of control.

3.3. Effects of H. uvarum in combination with SA or SBC on quality of intact fruit at 2 °C

The results presented in Table 2 indicate that the decay incidence of grapes treated by the yeast alone and in combination with SA and SBC were all significantly ($P < 0.05$) lower than that of the control treatment. The application of *H. uvarum* together with SBC resulted in an average decay incidence of 4.09% at 90 days, and the application of *H. uvarum* combined with SA, which gave the result of average decay incidence of 5.94%. They were both significantly ($P < 0.05$) lower than the results of the treatment of yeast alone (10.22%) and the control (15.89%). The weight loss of grapes treated by *H. uvarum* combined with SA and with SBC was 8.87% and 6.96% respectively, which was significantly lower ($P < 0.01$) the treatment of the control and the yeast alone (Table 2).

Fig. 2. The appearance of different treatment on grape fruit during cold storage after 90days; (A) control; (B) *H. uvarum*; (C) *H. uvarum* in combination with 2 mmol L^{-1} SA; (D) *H. uvarum* in combination with 2% SBC.

Table 2
Effect of *H. uvarum* combined with SA (or SBC) on Decay incidence, Weight loss, Firmness, TSS and TA of grape during cold storage.

Storage condition	Treatments	Quality parameters				
		Decay incidence (%)	Weight loss (%)	Firmness (*N*)	Total soluble solids (%)	Titratable acidity (%)
2 °C for 90 days	CK	15.89 ± 1.49a	24.90 ± 0.78a	4.66 ± 0.12a	13.25 ± 0.14a	0.32 ± 0.00a
	H	10.22 ± 0.05b	16.71 ± 0.54b	4.97 ± 0.23b	13.68 ± 0.00b	0.35 ± 0.00b
	H + SA	5.94 ± 0.70c	8.87 ± 2.30c	5.40 ± 0.05c	13.95 ± 0.10c	0.39 ± 0.00d
	H + SBC	4.09 ± 0.03d	6.96 ± 0.95c	5.37 ± 0.16c	14.00 ± 0.01c	0.36 ± 0.00c

Values with different letters at the same time within the same figure are significantly different according to Duncan's multiple range test at $P = 0.05$ level. CK: control; H: *H. uvarum*; SA: salicylic acid; SBC: sodium bicarbonate.

During the first 10 days, no significant differences in fruit firmness, TSS and TA were observed between the treatments and control. As the storage period was prolonged, the treated berries were significantly ($P < 0.05$) firmer compared to those in the control. Moreover, the fruit treated with the yeast combined with SA or SBC were firmer than those treated with the yeast alone. The treatments of the yeast in combination with SA or SBC generally had higher TSS than the other treatments. After 20 days, the combined treatments caused higher TA in fruit compared with the control. They also resulted in higher TA values compared to the yeast alone throughout the whole storage period (Table 2). In this study, the chemical reagent integrated with *H. uvarum* resulted in significantly higher firmness, TA, TSS and lower decay incidence, and weight loss rate than the control.

3.4. Effects of H. uvarum in combination with SA or SBC on enzyme activities of intact fruit at 2 °C

The result of POD activity are shown in Fig. 3A. No significant changes were detected at the beginning of storage ($P \geq 0.05$). After 40 days, a more rapid increase in POD activity was observed in the treated fruit (*H. uvarum* alone, *H. uvarum* combined with SA and combined with SBC) compared to the control fruit. The POD activities of these treated grapes reached the highest value after 90 days of storage and the activities of the fruit treated together with SA or SBC were significantly higher than that of control samples after 40 days ($P < 0.05$). Moreover, the activities of the fruit treated by *H. uvarum* combined with SA or SBC were higher than those of yeast alone.

The results in Fig. 3B showed that SOD activity increased continuously in grapes treated with and without antagonistic yeast within 70 days of storage. After that, the SOD activity declined rapidly. The yeast alone and in combination with chemical reagents (SA, SBC) could induce the SOD activity at 2 °C, which reached its maximum value at day 70. The SOD activities of grapes treated by *H. uvarum* alone, as well as in combination with SA or SBC were higher than those of the control samples before day 80. However, no significant ($P \geq 0.05$) differences were detected among grapes treated in different ways.

As for the CAT activity of grapes in all treatments (Fig. 3C), its value increased slowly at the early stage of storage and then declined continuously after reaching the maximum value. On the other hand, the CAT activities of grapes treated by the antagonistic yeast with chemical reagents (SA, SBC) were significantly ($P < 0.05$) higher than those of control grapes and grapes treated by yeast alone after 30 days. Furthermore, after 40 days, significantly higher CAT activity in yeast alone treated fruit was observed compared with the control during the whole storage period except the 70th day ($P < 0.05$).

As can be seen in Fig. 3D, the peak values of APX activities in yeast with chemical reagents treated fruit and control fruit were induced at 30 and 40 days of incubation, respectively. This suggests that the chemical reagents could induce the appearance of peak APX activity 10 days earlier. Additionally, the trend for APX activities to change in treated fruit and the control were similar. Both increased, reaching a peak value, and then declined. Throughout the whole storage period, the APX activities of grapes treated by the antagonistic yeast with chemical reagents (SA, SBC) were significantly ($P < 0.05$) higher than those of control grapes.

The change in PAL activity is shown in Fig. 3E. Both yeast-treated fruit and the control demonstrated similar changes. PAL activity continuously increased until it reached its peak value at day 60 of incubation, and then declined rapidly. With prolonged storage time, the yeast-treated fruit demonstrated continuously higher activity than the control. Especially at 30 or 50 days of incubation, the PAL activities of grapes treated by the antagonistic yeast with chemical reagents (SA, SBC) were significantly ($P < 0.05$) higher than those of control grapes. This result indicated that the antagonistic yeast *H. uvarum* with SA or SBC could induce PAL activity markedly and rapidly, but this effect could not be maintained after 80 days.

Application of the yeast *H. uvarum* alone and combined with SA led to a marked increase of PPO activity (Fig. 3F) and the activity reached the maximum value at day 20 and day 60, respectively. The highest PPO activity of control samples was obtained at day 60. This result indicated that antagonistic yeast *H. uvarum* alone and combined with SA could result in the emergence of a peak value in PPO activity 40 days earlier. In addition, after reaching the peak value, PPO activity in control fruit was always lower than that in the treated fruit.

4. Discussion

The objective of the present study was to evaluate the feasibility of combined application of a microbial antagonist (*H. uvarum*) and chemical reagent to control postharvest rots of grape fruit. In our experiment, we found that *H. uvarum* alone or in combination with SA and with SBC could significantly reduce gray mold incidence and induce the accumulation of defense-related enzymes. These results suggest that the disease resistance of fruit was enhanced by treatments with *H. uvarum* alone or in combination with SA and with SBC.

Combinations with biocontrol agents have proved to be an effective method to improve control of postharvest pathogens while reducing chemical residues on the fruit in other studies (Arrebola et al., 2010). The potential of *H. uvarum* as a biological control agent has been previously reported (Liu et al., 2010a,b). This is, however, the first report where *H. uvarum* isolate was evaluated in combination with SA and with SBC to improve its efficacy. The results of our experiment showed that *H. uvarum* in combination with SA and with SBC resulted in better control of *B. cinerea* than the antagonist alone at 25 °C. It is also important that the colonizing ability of *H. uvarum* in wounds is not inhibited by SA or SBC. This probably indicates that a proper low dosage of SA or SBC solution showed no statistical effect on the growth of *H. uvarum* and could be compatible with the antagonist. SA at 2 mmol L^{-1} and SBC at 2% were found to enhance the biocontrol efficacy of *H. uvarum* in reducing the gray mold disease on grapes caused by *B. cinerea* significantly,

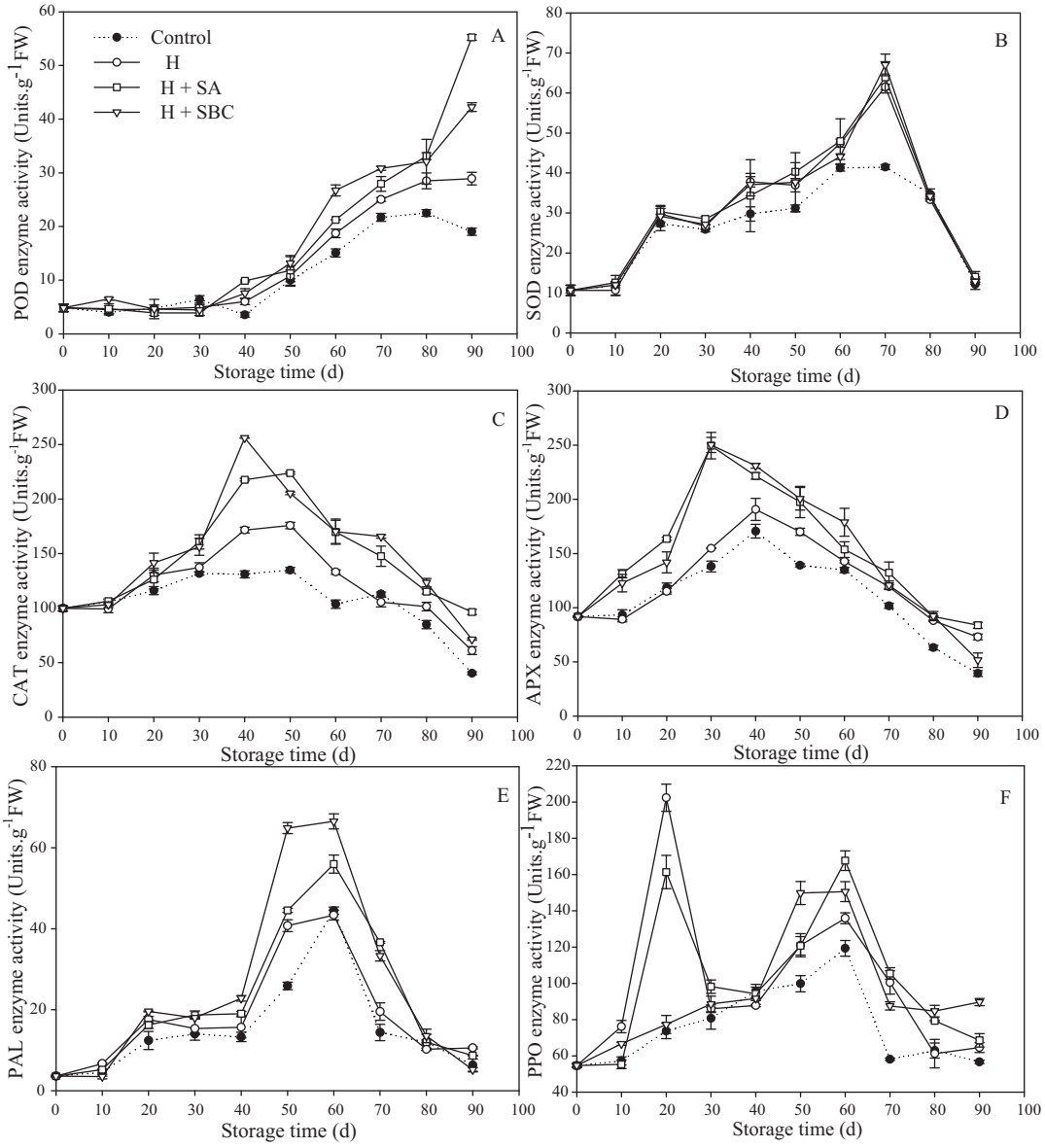

Fig. 3. Effects of the antagonistic yeast *H. uvarum* alone and in combination with SA, SBC on POD(A), SOD(B), CAT(C), APX(D), PAL(E), PPO(F) activities in grape fruits at 2 °C. Control: distilled water; H: *H. uvarum*; H + SA: *H. uvarum* combined with SA (2 mmol L^{-1}); H + SBC: *H. uvarum* combined with SBC (2%). Vertical bars represent standard deviations of the means.

similar to the findings in other studies (Wan et al., 2003; Yu et al., 2007).

The application of *H. uvarum* integrated with the chemical reagent delayed the postharvest ripening of grapes. This delay was characterized by reducing the browning index and weight loss and retention of fruit firmness. It is known that grape shelf-life is affected by weight loss, since as grapes lose weight they are more susceptible to fungal decay (Martínez-Romero et al., 2003). Weight loss can lead to wilting and shriveling which reduce both market value and consumer acceptability. Our results showed that all postharvest treatments prevented weight loss in comparison with the control, which are in agreement with Castillo et al. (2010) who demonstrated that weight loss was reduced significantly, while losses over 30% were detected in control grapes at the end of experiment. In addition, the application of *H. uvarum* in combination with SA and with SBC did not impair quality parameters including TSS and TA.

One of the possible action mechanisms of yeast combined with chemical regents is induction of antifungal-like substances that inhibit fungal development in fruit tissue and defense-related enzymes (Castoria and Wright, 2010). The reason for the observed increase in activity is not well understood. Our present study showed that *H. uvarum* in combination with SA or SBC had higher activities of POD, PPO, PAL SOD, CAT and APX than the application of *H. uvarum* treatment alone and untreated group. POD, PPO and PAL are commonly studied in the postharvest biocontrol area and known to be involved in plant disease resistance (Zhao et al., 2008). Thus, induction of enhanced activities of POD, PPO and PAL may be closely correlated with the mechanism by which *H. uvarum* and chemical regents induce resistance against *B. cinerea* in grape fruit.

POD controls the availability of H_2O_2 in the cell wall, which is a prerequisite for the cross-linking of phenolic groups in response to various external stress, such as wounds, pathogen interactions, and environmental constraints (Passardi et al., 2004). Ballester et al. (2010) found that soluble POD contributes to the beneficial effect of pathogen infection treatment in reducing disease incidence. Thus, the increase in POD is one of the markers of induced resistance. Besides POD, rapidly elevated levels of PPO are also important for

resistance genotypes following infection (Mayer, 2006). PPO can produce antimicrobial phenolic substances through oxidizing phenolic compounds (Mayer and Harel, 1979). This result suggests that PPO may potentially affect the progress of plant disease resistance. This finding is in agreement with the study of Zhao et al. (2009), who found that PPO activity in postharvest peach fruit was induced by antagonistic yeast, and this phenomenon was related to a decline in infection rate. PAL is responsible for the biosynthesis of *p*-coumaric acid derivatives, phytoalexin, and lignins that contribute to plant defense systems (Qin et al., 2003). PAL also participates in the biosynthesis of the defense hormone salicylic acid, which is required for both local and systemic acquired resistance in plants (Dixon and Paiva, 1995).

The accumulation of ROS has the potential to serve as barriers against invading pathogens and as signals for activating further plant defense reactions (Lamb and Dixon, 1997). Generally, the metabolism of ROS is controlled by an array of enzymes including SOD, CAT and APX. H_2O_2 is also destroyed predominantly by APX and CAT. It has been reported that a salicylic acid induced increase in H_2O_2 content is mediated by an inhibition of CAT and APX in several plants (Landberg and Greger, 2002). The present study showed CAT and APX were significantly increased and correspondingly, the capacity of the tissue to scavenge excess ROS was increased and the concentration of ROS was maintained at a relatively low level. The balance between POD, CAT and SOD activities in cells was crucial for determining the steady-state level of O_2^- and H_2O_2 (Chan and Tian, 2006). Thus, treatments with *H. uvarum* alone or in combination with SA or SBC activated protective enzyme, balanced the content of O_2^- and H_2O_2 and induced the disease resistance.

In general, the combination of *H. uvarum* and SA or SBC is more effective to control postharvest diseases of grape berries than the single treatments or the control. It may provide a reliable solution for control of postharvest gray mold of grape fruit. Moreover, the combination of SA (or SBC) with *H. uvarum* overcomes the limitations of each individual treatment. Thus, the increase in enzyme activity and the extension of storage time of grape berries by the yeast combined with chemical reagents demonstrated here suggests that the application of the *H. uvarum* combined with SA or SBC could be considered for use during commercial storage.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.postharvbio.2014.09.010.

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