Induction of H$_2$O$_2$-metabolizing enzymes and total protein synthesis by antagonistic yeast and salicylic acid in harvested sweet cherry fruit

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Abstract

The immersion of sweet cherry fruit in Pichia membranefaciens at a concentration of 5 × 10$^7$ cells ml$^{-1}$ or in salicylic acid (SA) at 0.5 mM for 10 min reduced the incidence of decay and lesion size caused by Penicillium expansum. Without pathogen inoculation, peroxidase (POD) activity was enhanced in yeast-treated fruit, but activities of catalase (CAT) and superoxide dismutase (SOD) showed a decrease in the same fruit. SA-treatment significantly inhibited CAT activity, but stimulated SOD and POD activities. After inoculation with P. expansum, CAT activity decreased and SOD activity increased in both yeast- and SA-treated fruit. No obvious difference was found in POD activity between treatments and water control. Treatments with yeast and SA changed the expression of POD isozymes. In addition, yeast and SA treatment increased total protein content of sweet cherry and up-regulated 33 and 47 kDa protein bands shown by SDS-PAGE. These results indicated that yeast- and SA-treatments induced synthesis of anti-oxidant enzymes and specific proteins, which may play a role in the resistance against postharvest blue mold.

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1. Introduction

Recently, considerable attention has been placed on postharvest application of antagonistic agents for the inhibition of plant disease (Cook et al., 1999; Castoria et al., 2001; Droby et al., 2001; El-Ghaouth et al., 2003). Treatment with antagonistic yeasts has suggested that intensification of defense mechanisms has potential in reducing postharvest decay (El-Ghaouth et al., 1998; Fan and Tian, 2000). El-Ghaouth et al. (2003) found that, along with the induction of a systemic protection in fresh apples, Candida sakecause a rapid accumulation of chitinase (EC 3.2.1.14) and β-1, 3-glucanase (EC 3.2.1.6) activities locally in the treated wound site and systemically in tissues distant from the initial wound.

In addition, as a hormone-like substance, salicylic acid (SA) has proved to be a major component in signal transduction pathways and plays an important role in the regulation of plant growth and development, including transpiration, stomatal closure, seed germination, fruit yield, glycolysis, flowering and heat production (Klessig and Malamy, 1994; Ananieva et al., 2004). It is believed that systemic acquired resistance (SAR) is dependent on SA-mediated signalling and is associated with the production of PR proteins (Lind, 2001). In the fields of disease control, SA has received particular attention because its accumulation is essential for expression of multiple modes of plant disease resistance. Exogenous application of SA at non-toxic concentrations to susceptible plants could enhance resistance to pathogens (Murphy et al., 2000; Gális et al., 2004).

In a previous study, we observed the induced resistance of Pichia membranefaciens and SA against postharvest diseases. Significant changes in polyphenoloxidase (PPO, EC 1.10.3.1), peroxidase (POD, EC 1.11.1.7), phenylalanine
ammonia-lyase (PAL, EC 4.3.1.5), and β-1, 3-glucanase activities were found to be involved in the action (Fan and Tian, 2000; Qin et al., 2002, 2003; Yao and Tian, 2005). To date, although the interaction between SA and active oxygen has been characterized (Lamb and Dixon, 1997), little information is available on whether the positive effect of antagonistic yeasts on postharvest diseases is mediated by an antioxidant system. The objective of this study was to determine the relationship between antioxidant enzymes and host resistance induced by \( \textit{P. membranefaciens} \) and SA. The time-course and magnitude of CAT (EC 1.11.1.6), POD and superoxide dismutase (SOD, EC 1.15.1.1) have been investigated after treatment with SA or antagonists in sweet cherry. Additionally, total protein metabolism was assayed.

2. Materials and methods

2.1. Fruit material

Sweet cherry (\textit{Prunus avium L. cv. Hongdeng}) fruit were harvested at commercial maturity from the experiment orchard of the Institute of Forest and Fruit, Beijing Academy of Agricultural Sciences in Beijing. Fruit were directly transported to our laboratory and sorted based on size and the absence of physical injuries or infections. Sweet cherries were disinfected with 2% (v/v) sodium hypochlorite solution for 2 min, washed with tap water, and air-dried prior to use.

2.2. Antagonistic yeast and pathogen

\( \textit{P. membranefaciens} \) was isolated from the wounds of peach fruit in our previous work (Fan and Tian, 2000) following the method of Wilson and Chalutz (1989) and identified by CABI Bioscience Identification Services (International Mycological Institute, UK). The yeast was cultured in 250 ml conical flasks containing 50 ml of nutrient yeast dextrose broth (NYDB: 1 g of beef extract, 10 g of glucose, 5 g of soya peptone, 5 g of NaCl, and 5 g of yeast extract in 1000 ml water) on a rotary shaker at 200 rpm for 48 h at 28°C. Yeast cells were centrifuged at 3365 C × g for 10 min, resuspended in sterile distilled water, and adjusted to a concentration of 5 × 10^7 cells ml^{-1} with a haemocytometer.

\textit{P. expansum} was obtained from infected apple fruit and cultured on Potato Dextrose Agar (PDA) for 14 days (PVPP) with a Kinematica tissue grinder (CrL-6010, Kriens-LU, Switzerland). The homogenate was centrifuged at 6730 C × g for 40 min at 4°C and the resulting supernatants were used directly for enzyme assays. There were three replicates in each treatment, and the experiment was conducted twice.

2.4. Evaluation of fruit decay

Fruit with pathogen inoculation from the third group were used for decay evaluation. Disease incidence and lesion diameters caused by \( \textit{P. expansum} \) were determined at 24, 48, and 72 h after treatment. Fruit with no infection were not counted for lesion size measurements. There were three replicates in each treatment, and the experiment was conducted twice.

2.5. CAT, POD and SOD enzyme assay

At various time intervals (0, 24, 48, and 72 h) after treatment, flesh samples from 10 fruit in the first and second groups were obtained for the enzyme assays and protein extraction. A flesh sample of 5 g from the middle part (the first group) or near the wound (the second group) was collected using a sampler (5 mm deep and 7 mm in diameter) in each treatment and homogenized in 25 ml of ice-cold sodium phosphate buffer (50 mM, pH 7.8) and 0.5 g polyvinyl pyrrolidone (PVP) with a Kinematica tissue grinder (CrL-6010, Kriens-LU, Switzerland). The homogenate was centrifuged at 6730 C × g for 40 min at 4°C and the resulting supernatants were used directly for enzyme assays. There were three replicates in each treatment for enzyme assays, and the experiment was conducted twice.

CAT activity was determined by adding 0.2 ml of enzyme preparation to 2.8 ml of 40 mM \( \text{H}_2\text{O}_2 \) (dissolved with 50 mM sodium phosphate buffer, pH 7.0) as a substrate (Wang et al., 2004). The decomposition of \( \text{H}_2\text{O}_2 \) was measured by the decline in absorbance at 240 nm with UV-160 spectrophotometer (Shimadzu, Japan). The specific activity was expressed in units per mg protein, where one unit of catalase converts 1 μmol of \( \text{H}_2\text{O}_2 \) per minute.

POD activity was analyzed using guaiacol as substrate (Yao and Tian, 2005). The reaction mixture consisting of 0.5 ml of crude extract, 2 ml of guaiacol substrate (100 mM sodium phosphate, pH 6.4 and 8 mM guaiacol) was incubated for 5 min at 30°C. The increase in absorbance at 460 nm was spectrophotometrically assayed after 1 ml \( \text{H}_2\text{O}_2 \) (24 mM) was added. Enzymatic activities were defined as the increase...
of absorbance, and one unit was defined as the increase in one absorbance unit per minute under the conditions of the assay.

For the SOD assay (Wang et al., 2004), the reaction mixture (3 ml) contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM nitroblue tetrazolium (NBT), 10 μM EDTA, 10 μM riboflavin, and 0.1 ml enzyme extract. The mixtures were illuminated by a fluorescent lamp (60 μmol m⁻² s⁻¹) for 10 min and then the absorbance was determined at 560 nm. Identical solutions held in the dark served as blanks. One unit of SOD was defined as the amount of enzyme that caused a 50% decrease of the SOD-inhibitable NBT reduction. The specific activity was expressed as U mg⁻¹ protein.

2.6. POD isozyme electrophoresis and gel staining

Isoenzymes of POD were separated on 7% nondenaturing polyacrylamide gels using Tris–HCl buffer 1.5 M, pH 8.8 at 4°C with Mini Protean electrophoresis unit (Bio-Rad, USA). Equal amounts of 50 μg protein per lane were loaded for the crude enzyme extracts. After running, gels were incubated in a 10 g l⁻¹ o-dianisidine:15 ml l⁻¹ H₂O₂:0.2 ml 0.05 M sodium phosphate buffer, pH 6.5, solution at 25°C for 15 min, for POD isoenzyme detection (Cano et al., 1998).

2.7. Preparation of total proteins and SDS-PAGE

Extraction of total proteins was performed according to the method of Sarry et al. (2004) with slight modifications. All procedures described below were carried out at 4°C. Fresh sweet cherries were ground in a small mortar and pestle in liquid nitrogen. The powdered sample was mixed with cold 12.5% TCA-acetone solution containing 28 mM mercaptoethanol in a ratio of 1/10 (w/v). The solution was allowed to precipitate at −20°C for 2 h and the proteins were precipitated by centrifugation at 14,000 × g for 15 min. After two washes with ice-cold 100% acetone, the pellet was air-dried and resuspended in SDS-PAGE sample buffer (Laemmli, 1970). Samples were heated at 100°C for 10 min to complete the protein denaturation and reduction of disulphide bonds (Laemmli, 1970). Finally, to remove any insoluble material, which might interfere with electrophoresis, the suspension was centrifuged (20,000 × g, 20°C, 10 min). The pellet was discarded and the supernatant was stored at −20°C for analysis.

SDS-PAGE was carried out on mini-gels (12% resolving gels, 4% stacking gel) according to Laemmli (1970). Samples were heated at 100°C for 10 min to complete the protein denaturation and reduction of disulphide bonds (Laemmli, 1970). Finally, to remove any insoluble material, which might interfere with electrophoresis, the suspension was centrifuged (20,000 × g, 20°C, 10 min). The pellet was discarded and the supernatant was stored at −20°C for analysis.

The protein concentration was determined according to Bradford’s method (1976) using bovine serum albumin as standard. Each treatment contained three replicates of 10 fruit and the entire experiment was repeated twice.

2.8. Statistics

All data were analyzed by analysis of variance with SPSS (SPSS Inc., Chicago, IL). When the treatment effects were statistically significantly (P < 0.05), the least significant difference test was used for means separation. Results presented were pooled across repeated experiments.

3. Results

3.1. Inhibition effect of antagonistic yeast and SA on disease severity

The inhibiting effect of antagonistic yeast and SA on disease severity was assessed at 24, 48, and 72 h after treatments. In comparison with the water control, fruit immersed with antagonistic yeast and SA showed significantly lower disease incidence at 24 h (P = 0.05). After this period, SA treatment still delayed the occurrence of blue mold, but no evident difference was observed between yeast-treated fruit and the control (Fig. 1).

Comparatively, antagonistic yeast and SA treatments were more effective in inhibiting disease development. Lesion

Fig. 1. Disease incidence and lesion diameters of P. expansum in sweet cherry fruit treated with 0.5 mM salicylic acid, 5 × 10⁷ cells ml⁻¹ antagonistic yeast P. membranefaciens and water control at 25°C during 72 h postharvest storage.
diameter caused by *P. expansum* was inhibited in both yeast- and SA-treated fruit (Fig. 1). In addition, the SA-treatment was more effective than the yeast treatment.

### 3.2. Effect of treatments on catalase, peroxidase, and superoxide dismutase activities

Without pathogen inoculation, SA-treated fruit showed significantly lower CAT activities compared with the control during the whole storage period (*P* = 0.05). Comparatively, the yeast treatment decreased CAT activities at 48 and 72h, but no difference was observed at 24h. After inoculation with *P. expansum*, CAT activity decreased in both yeast- and SA-treated fruit (Fig. 2).

POD activity in all fruit showed a sustained increase before 48h storage and then declined and remained at a relatively low level without pathogen inoculation. Both yeast and SA treatment enhanced POD activity, and the performance of SA was better than that of antagonistic yeast. After inoculation with *P. expansum*, no significant differences were found between yeast- or SA-treated fruit and the control (Fig. 2).

SOD activity in all treated fruit without pathogen inoculation declined at first, and then tended to be steady. Moreover, SOD in yeast-treated fruit showed continuously lower activity during the whole storage period compared to the control. By comparison, a lower decrease of SOD activity was observed in SA-treated fruit (Fig. 2). After pathogen inoculation, SOD activities increased in yeast and SA treatments during storage, and SA was more effective than antagonistic yeast.

### 3.3. Effect of treatments on isozyme of peroxidase

Three isoperoxidase bands were observed commonly in all treatments (Fig. 3). Without pathogen inoculation, a more marked band a was evident in both treatments at 24h. No difference was found for band b, which was faint at 24 and 72h, during storage time. Band c was intensified only in yeast-treated fruit. After inoculation with *P. expansum*, isozymes...
Fig. 3. Effect of salicylic acid and antagonistic P. membranefaciens on isoenzymes of peroxidase (POD) in sweet cherry at 25°C during postharvest storage. Without pathogen inoculations, both treatments intensified band a, whereas band b and c in treated fruit were more marked after inoculation with P. expansum (A: control; B: treatment with SA; C: treatment with P. membranefaciens; D: inoculation with P. expansum; E: treatment with SA and then inoculation with P. expansum; F: treatment with P. membranefaciens and then inoculation with P. expansum).

of POD in three treatments (D, E, and F) showed a gradual increase in the time-dependent process, and bands a and c became more marked after yeast and SA treatments, except at 72 h.

3.4. Effect of treatments on the yields of total protein

The total protein content increased at first in all the treatments, and then decreased with time. Without pathogen inoculation, the SA treatment enhanced total protein content, but no difference was found between yeast-treated fruit and the control. After inoculation with P. expansum, total protein contents in both SA- and yeast-treated fruit were higher than those in the control fruit during storage (Fig. 4).

3.5. SDS-PAGE

One hundred micrograms of protein were separated by one-dimensional polyacrylamide gel electrophoresis and

Fig. 4. Effect of salicylic acid and P. membranefaciens yeast on yields of total protein with or without postharvest pathogen in sweet cherry at 25°C during postharvest storage (A: control; B: treatment with SA; C: treatment with P. membranefaciens; D: inoculation with P. expansum; E: treatment with SA and then inoculation with P. expansum; F: treatment with P. membranefaciens and then inoculation with P. expansum).

Fig. 5. SDS-PAGE profiles of sweet cherry after SA and antagonistic yeast treatments with or without postharvest pathogen at 24 h. Arrowheads show different protein bands. Five new protein bands (33, 41, 47, 53, and 67 KDa) were differentially induced after SA- or yeast-treatment (A: control; B: treatment with SA; C: treatment with P. membranefaciens; D: inoculation with P. expansum; E: treatment with SA and then inoculation with P. expansum; F: treatment with P. membranefaciens and then inoculation with P. expansum; M: protein molecular weight marker).
stained with Coomassie blue R-250 to observe variations in the banding patterns (Fig. 5). SDS-PAGE analysis of the samples showed that there were differences in expression of major proteins between treatments and the control (Fig. 5).

For example, two large protein bands with a molecular weight of 33 and 47 kDa appeared to be more abundantly expressed in both yeast- and SA-treated fruit with or without pathogen inoculation. A protein band with 53 kDa was up-regulated in yeast-treated fruit without pathogen inoculation. The activity of a 41 kDa protein band was enhanced in yeast-treated fruit, and activity of another 67 kDa protein band was increased in both yeast- and SA-treated fruit, after inoculation with pathogen.

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4. Discussion

In this study, fruit treated with 5 × 10⁷ cells ml⁻¹ antagonistic yeast and 0.5 mM SA showed lower disease incidence (especially at 24 h) and smaller lesion diameters caused by P. expansum (Fig. 1). These results indicated that both treatments significantly reduced postharvest decay of cherries caused by P. expansum, which supported our previous study that antagonist treatment could induce fruit resistance and inhibit fruit decay caused by pathogenic fungi (Qin et al., 2003; Yao and Tian, 2005). Although the differences in disease incidence between the treatments and the water control at 48 and 72 h were not as evident as lesion diameter (Fig. 1), it might be due to the time limit of induced resistance, and indicates that induced resistance individually by biotic or abiotic agents was not powerful enough to control postharvest pathogens. The mechanisms of SA and antagonistic yeasts against postharvest pathogens are complicated. For example, SA at a higher concentration (2 mM) showed direct fungistaticity on postharvest Monilinia fructicola and inhibited mycelial growth and spor germination of the pathogen in vitro (Yao and Tian, 2005), which indicated that SA, like BABA, and JA (Droby et al., 1999; Porat et al., 2003), is most effective in inducing resistance when applied at an optimal concentration. Antagonistic yeasts could compete for space and nutrients with pathogens or directly attach to the hyphae of pathogens (Cook et al., 1999; Castoria et al., 2003; Chan and Tian, 2005).

In our experiments, treatment with exogenous SA affected H₂O₂-metabolizing enzymes in harvested sweet cherry. Compared with the water control without pathogen inoculation, SA treatment significantly increased activities of POD and inhibited CAT activity (Fig. 2). A lower decrease of SOD activity was also observed in SA treated fruit. In addition, SA changed the expression of POD isozymes (Fig. 3), which indicated that SA treatment could directly or indirectly activate antioxidant enzymes. POD catalyses the dismutation of O₂⁻ to H₂O₂, catalase (CAT) dismutates H₂O₂ to oxygen and water, and peroxidase (POD) decomposes H₂O₂ by oxidation of phenolic compounds. These enzymes are considered to be the main enzymatic systems for protecting cells against oxidative damage (Tommasi et al., 2001; Wang et al., 2005). The balance between SOD and POD or CAT activities in cells is crucial for determining the steady-state level of O₂⁻ and H₂O₂. Thus, SA interaction with the above mentioned enzymes leads to low levels of H₂O₂ accumulation in sweet cherry fruit, which activated protective enzymes and then induced fruit resistance against postharvest pathogens (Fig. 1).

It is worth noting that the antagonistic yeast treatment enhanced POD activity, but inhibited SOD and CAT activities in sweet cherry fruit without pathogen inoculation. Then after inoculation with P. expansum, yeast treatment also increased SOD activity but decreased CAT activity compared with the pathogen-inoculation control (Fig. 2). As mentioned above, POD, together with SOD and CAT, are antioxidant enzymes, which modulate the concentration of H₂O₂ (Barceló, 1997). De Gara et al. (2003) considered that H₂O₂ can hinder micro-organism penetration in plant tissues because it contributes to wall stiffening by facilitating peroxidase reactions catalysing intra- and inter-molecular cross-links between structural components of cell walls and lignin polymerisation. The consequent increase in mechanical barriers also slows down pathogen penetration allowing plant cells to arrange defences that require more time to be activated (Durner et al., 1997). However, active oxygen species (AOS), when produced at higher concentrations during pathogenesis, may initiate degradative reactions, causing lipid peroxidation, membrane deterioration, protein degradation and chlorophyll quenching (Bowler et al., 1992). Therefore, efficient antioxidant activity is essential in order to maintain the concentration of AOS at relatively low levels (Larrigaudière et al., 2004). Results in this experiment showed that antagonistic yeast treatment might affect H₂O₂ metabolism and then induce resistance in sweet cherry fruit. Castoria et al. (2003) have also found that the combined application of biocontrol yeasts and ROS-deactivating enzymes in apple wounds enhanced colonization and antagonistic activity of biocontrol yeasts against Botrytis cinerea and P. expansum. This indicates that biocontrol efficiency of antagonistic yeast may be related to ROS metabolism.

In this study, both exogenous SA and antagonistic yeast treatment influenced H₂O₂-metabolizing enzyme metabolism. In addition, a large body of evidence has shown that exogenous SA treatment can induce the accumulation of H₂O₂ levels in plant tissues. This increased H₂O₂ has been proposed to be a signal leading to the hypersensitive response and activates many tolerance-related genes against pathogen attack (Prasad et al., 1994; Lamb and Dixon, 1997). It is well known that SA is a signal and our results indicate that induced resistance by antagonistic yeast might share some pathways with the SA signal.

Total protein content also varied after exogenous elicitor treatment (Fig. 4). As described above, a general reaction of plants to stress or pathogen attack is the production of reactive oxygen species. To avoid damage of endogenous components, antioxidant defense proteins and metabolites are
essential to maintain cellular functions (Larrigaudière et al., 2004). The patterns of proteins using SDS-PAGE indicated that a number of protein bands were induced with the treatments of antagonistic yeast and SA. Two protein bands of 33 and 47 kDa were up-regulated by the treatments (Fig. 5), which further favors our hypothesis that the yeast treatment may elicit some pathways with SA treatments. Further studies using proteomics are being carried out in order to fully understand function of different proteins in fruit defense response and physiological changes.

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