ORIGINAL ARTICLE

Reducing oxidative stress in sweet cherry fruit by *Pichia membranaefaciens*: a possible mode of action against *Penicillium expansum*

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Abstract

Aims: To investigate the effect of antagonistic yeast *Pichia membranaefaciens* on alleviating oxidative stress caused by *Penicillium expansum* in sweet cherry fruit.

Methods and Results: At two maturity stages of sweet cherry fruit, *P. membranaefaciens* restrained blue mold rot caused by *Pe. expansum*. There was not any decay in yeast-treated fruit even at 5 days after inoculation. Carbonylated proteins accumulated to a lesser extent in yeast-treated fruit than in control fruit, particularly in non-full-matured stage fruit. Higher activities of catalase (CAT) and glutathione peroxidase (GPX) were observed in yeast-treated fruit, which consisted of the transcript expressions of *CAT* and *GPX* genes. In addition, yeast treatment also stimulated the transcript expression of *Gns1* and activity of β-1,3-glucanase.

Conclusion: Induction of antioxidant defence response may be an important mechanism of antagonistic yeast in mitigating pathogen-induced oxidative stress to postharvest fruit and controlling postharvest disease.

Significance and Impact of the Study: The results of this study showed a potential mode of action of antagonistic yeast in postharvest fruit disease control, which may be an important development in the understanding of antagonists in postharvest biocontrol and may provide important guidance for their application in the future.

Introduction

Biological control using microbial biocontrol agents against fungal pathogens is considered to be a potential alternative to chemical fungicides. Many research results indicated that antagonistic yeasts could effectively reduce postharvest diseases in different fruits (Fan and Tian 2000; Janisiewicz and Korsten 2002; El Ghaouth *et al.* 2003; Conway *et al.* 2004, 2007; Tian *et al.* 2004). Induction of host resistance is an important action of microbial biocontrol agents against fungal pathogens (Ippolito *et al.* 2000; Castoria *et al.* 2001). Regarding defence response induced by antagonists, numerous reports were focussed on investigating defence-related enzymes. Jijakli and

Lepoivre (1998) reported that antagonist *Pichia anomala* (Hansen) Kurtzman was able to produce an exo-β-1,3-glucanase and inhibit infection by *Botrytis cinerea* Pers. in apple fruit. Fan *et al.* (2002) indicated that *Pichia membranaefaciens* (Hansen) Hansen and *Candida guilliermondii* (Cast) Langeronet Guerra, two antagonistic yeasts, could produce chitinase and β-1,3-glucanase *in vitro* and induce an increase in β-1,3-glucanase and chitinase activities in the wounds of nectarine fruit, resulting in an effective decrease in decay caused by *Rhizopus stolonifer* (Ehrenb.:Fr.) Vuill. *Cryptococcus laurentii* (Kufferath) Skinner was also found to be effective in inducing the activity of β-1,3-glucanase and expression of *Glu-1* gene in jujube fruit, and suppressing *Alternaria* rot and blue

mold rot caused by *Alternaria alternata* (Fr.) Keissler and *Penicillium expansum* Link (Tian *et al.* 2006, 2007). In addition, the activities of polyphenol oxidase (PPO), peroxidase (POD), superoxide dismutase (SOD) and phenylalanine ammonia-lyase (PAL) in peaches increased significantly after inoculation with *C. laurentii* or *P. membranaefaciens*, and maintained at high levels throughout the experiment (Qin *et al.* 2002; Wang *et al.* 2004). However, to date, little evidence is demonstrated for antioxidant proteins induced by microbial biocontrol agents against pathogen-induced oxidative stress in postharvest disease control of fruit.

Fungal pathogen infection can cause oxidative stress by inducing the generation of reactive oxygen species (ROS) in plants (Campo *et al.* 2004), such as superoxide, hydrogen peroxide, and the hydroxyl radical. The oxidative stress can damage nucleic acids, lipids or carbohydrates, and induce irreversible introduction of aldehyde and ketone (carbonyl) groups to amino acid side chains, leading to protein inactivation, aggregation and degradation (Levine *et al.* 2000) and affecting the integrity of cell membranes and inactivating key cellular functions (Halliwell and Gutteridge 1999). Immunodetection of carbonylated proteins is a good indicator of protein damage owing to oxidative stress (Ghezzi and Bonetto 2003), and this approach has been widely used in mammalian and yeast systems (Conrad *et al.* 2000). In the biocontrol of postharvest disease, it may be a potential method for studying the effects of antagonists on pathogen-induced oxidative stress.

In order to fully understand the mechanism of microbial biocontrol agents against fungal pathogens in harvested fruit, we investigated the antioxidant defence response induced by *P. membranaefaciens* in sweet cherry fruit, and evaluated whether the antagonist was functional in alleviating pathogen-generated protein carbonylation in this study.

Materials and methods

Fruit material

Sweet cherry (*Prunus avium* L. cv. Hongdeng) fruit was harvested at two maturity stages from an experiment orchard of Institute of Botany, Chinese Academy of Sciences, Beijing, China. The maturity of fruit was defined according to skin colour and development days after full bloom. At maturity stage A, the fruit developed 43 days after full bloom with pale red skin, and at maturity stage B, the fruit developed 53 days after full bloom with full red skin. All fruits were selected without physical injuries or infections, and then were surface disinfected with 2% (v/v) sodium hypochlorite

for 2 min, washed with tap water and dried in air before used.

Antagonist and pathogen

Antagonist yeast, *P. membranaefaciens* was isolated in our previous experiment (Fan and Tian 2000) and identified by CABI Bioscience Identification Services (International Mycological Institute, UK). The yeast was cultured in nutrient yeast dextrose broth (NYDB) at 28°C for 48 h, and suspension concentration was adjusted to 1×10^8 cells per ml with a haemocytometer.

Fungal pathogen, *Pe. expansum* was isolated from decaying sweet cherry fruit, and cultured on potato dextrose agar (PDA) at 25°C for 2 weeks. Conidia of the fungi were obtained by flooding the cultures with sterile distilled water containing 0·05% (v/v) Tween 80. The conidial suspension was filtered through three layers of sterilized cheesecloth and adjusted to a concentration of 1×10^5 conidia per ml with a haemocytometer.

Biocontrol experiment

Sweet cherry fruit were wounded (3 mm deep and 3 mm wide) with a sterilizing nail, and 10 μl of cell suspension of *P. membranaefaciens* at 1×10^8 colony-forming units (CFU) per ml was put into each wound; after 12 h, 10 μl of conidial suspension of *Pe. expansum* at 1×10^4 conidia per ml was inoculated in the same wound. As control, the fruit were first wounded, and after 12 h, directly inoculated with 10 μl of conidial suspension of *Pe. expansum* at 1×10^4 conidia per ml. All fruits were put in plastic trays with plastic film (0·04 mm) to maintain a high relative humidity of about 95%, and then stored at 20°C. Decay rate was measured at 3, 4 and 5 days after inoculation. There were 15 fruits for each treatment with three replicates, and the experiment was performed twice.

Preparation of total proteins and immunodetection of carbonylated proteins

Extraction of total proteins of sweet cherry fruit was performed according to the method of Chan *et al.* (2007). Protein carbonylation was detected by using the OxyBlot™ Protein Oxidation Detection kit (Chemicon International, Temecula, CA, USA) (Qin *et al.* 2007). Briefly, protein sample (30 μg) was mixed with the same volume of 12% sodium dodecyl sulfate (SDS). The 2,4-dinitrophenylhydrazine (DNPH) at 10 mmol l^{-1}, dissolved in 10% trifluoroacetic acid (v/v), was added to the sample. The reaction mixture was incubated for 15 min at 25°C and then neutralized by the addition of 2 mol l^{-1}

Tris base containing 30% glycerol. The proteins were then separated by 15% SDS-PAGE and transferred to Immobilon-P polyvinylidene diflouride membrane (Millipore, Bedford, MA, USA) using an electroblotting apparatus (Bio-Rad, USA). The carbonylated proteins were detected with anti-DNP antibody and the chemiluminescence blotting substrate SuperECL plus (Applygen Technologies Inc., Beijing, China).

Gene cloning by RT-PCR and RACE

The total RNA was extracted from sweet cherry fruit after treatment for 24 h. The first-strand cDNA was synthesized with first-strand cDNA synthesis kit (Trans, Beijing, China) and used for PCR reaction. The primers for *catalase* (*CAT*) were designed based on the conserved amino acid sequences of peach and pumpkin: 5′-ATG GAT CC(A/T) TAC A(A/G)G (C/T)AC-3′ (forward), 5′-TCA AAT GCT (G/T)GG CCT CAC-3′ (reverse). Conditions for PCR reaction were: 94°C for 5 min, 33 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 100 s, and finally 72°C for 10 min. Primers for *glutathione peroxidase* (*GPX*) were designed according to the conserved amino acid sequences of peach and apple fruit: 5′-ATG GCT (A/G)GC CA(G/T) TC(C/T) G(A/G)G-3′ (forward), 5′-TCA GTT TCT T(A/C)(A/T) (C/T)A(C/T) AAG -3′ (reverse). The following thermal profile was used for PCR reaction: 94°C for 5 min, 33 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 45 s; and finally 72°C for 10 min. PCR products were cloned into pGEM-T easy vector (Tiangen, Beijing, China) and sent for sequencing. The conserved domain of *β-1,3-glucanase* gene was amplified by PCR performed with the synthesized cDNA, *Taq* DNA polymerase and a pair of degenerate primers as follows: 5′-TAC AT(C/T) GC(G/C/T) GT(A/T) GGA AA(C/T) GAA-3′ (forward primer) and 5′-A(A/T)G GCC AAC C(A/G)(G/C) TCT C(G/T)G ATA-3′ (reverse primer). The PCR cycling parameters were as follows: 94°C for 3 min; six cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s; 20 cycles of 94°C for 30 s, 60 to 50°C for 30 s, and 72°C for 30 s; eight cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 30 s; and, finally, 72°C for 10 min. PCR product was cloned into pGEM-T easy vector (Tiangen, Beijing, China) and sequenced. Full-length coding sequences for *β-1,3-glucanase* genes were obtained by 3′ and 5′ RACE. For 3′ RACE, RT was carried out as described earlier using an adaptor oligo (dT) 3′ primer (5′-GGC TCG AGT TTT TTT TTT-3′). PCR was then performed using a gene-specific 5′ primer (5′-AGC CCT CAG ACT CCT TGC ACA-3′) designed according to the sequence of the cloned conserved domain and the *adaptor* primer. For 5′ RACE, RT was per-

formed with a 3′ gene-specific primer (GSP1: 5′-TGG GGT CTT TCT ATT CTC ATC AA-3′) designed according to the sequence obtained from the 3′ RACE fragment; PCR was carried out with the second gene-specific primer (GSP2: 5′-TCT CAA GAG CAG CAT AAA CAC CA-3′) and the anchor primer-1 (5′-GGC CAC GCG TCG ACT AGT ACG$_{(18)}$-3′). In order to improve specificity, a further round of PCR was performed with a nested gene-specific primer (GSP3: 5′-CCA CTG TAA GCA AAA TAA GGG TA-3′) and an anchor primer-2 (5′-GGC CAC GCG TCG ACT AGT AC-3′). The resulting PCR product was cloned and sequenced. The nucleotide sequence of the full-length cDNA was analysed by using BLAST search of the GenBank Database at NCBI.

RNA isolation and northern blot

About 10 g of frozen flesh tissue (about 2 mm away from the infection or wounded site) from 15 fruits was ground in liquid nitrogen. Total RNA was extracted by the method hot-phenol isolation protocol (Chan *et al.* 2007) and stored at −80°C. Twenty micrograms of RNA was denatured in formamide and formaldehyde at 65°C and separated in a 1% agarose gel containing 6% formaldehyde. After electrophoresis, RNA was transferred overnight onto a Hybond-N$^+$ membrane (Amersham International Ltd) and hybridized with [^{32}P] dCTP-labelled cDNA probe. Prehybridization and hybridization were done at 65°C. After hybridization for 16 h, the membrane was washed twice for 15 min in $2 \times SSC$, 0.1% SDS, and once for 10 min in $0.1 \times SSC$, 0.1% SDS at 65°C. RNA bands were visualized by autoradiography. Equal loading of total RNA was identified by visualization of rRNA that had been stained with ethidium bromide. Autoradiographs were digitally scanned and band densities quantified using Scion Image Software (Scion Corporation, Frederick, MD, USA). Hundred per cent was assigned to the maximum optical density value achieved in each northern and the rest were normalized to the maximum value and expressed as percentage of relative accumulation (RA).

Enzyme activity assays

All extract procedures were carried out at 4°C, and enzyme activities were determined by a Shimadzu UV-160 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Activities of GPX and *β*-1,3-glucanase were measured at 460 and 500 nm, respectively, following the methods of Yao and Tian (2005). The activity of CAT was assayed at 240 nm according to the methods reported by Wang *et al.* (2004). Protein content was measured

according to the method of Bradford (1976), using BSA as the standard protein.

Statistical analysis

All data were analysed by one-way analysis of variance (ANOVA). Mean separations were performed by Duncan's multiple range test. Differences at $P \leq 0.05$ were considered as significant.

Results

Yeast completely inhibited blue mold rot

At both maturity stages, *P. membranaefaciens* with a concentration of 1×10^8 CFU ml^{-1} could completely inhibit *Pe. expansum* infection and there was no decay in yeast-treated fruit even at 5 days after inoculation (Fig. 1). The decay rate of control fruit at both maturity stages A and B increased rapidly with increased time, and reached 100% at 5 days after inoculation, respectively (Fig. 1).
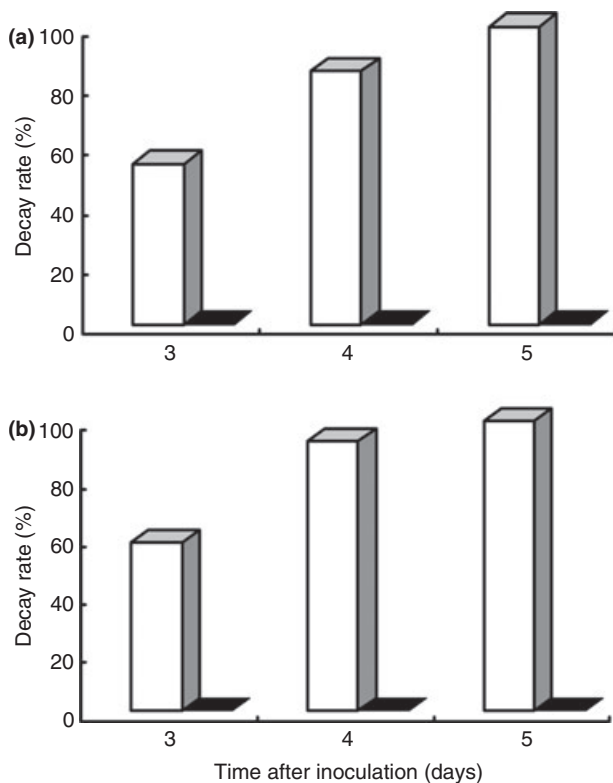

Figure 1 Inhibitory effect of *Pichia membranaefaciens* on decay caused by *Penicillium expansum* in sweet cherry fruit at A (a) and B (b) maturity stages at 20°C. (□) Control treatment; (■) *P. membranaefaciens* treatment. Data were the means from two experimental results.

Yeast alleviated protein carbonylation

Immunodetection of carbonylated proteins is a good indicator of protein damage owing to oxidative stress. The assessment of protein damage in sweet cherry fruit was undertaken by immunoassay with anti-DNP antibodies. It is clear that protein carbonylation occurred in all fruits (Fig. 2b,d). The levels of carbonylated proteins increased with prolonged storage time, ranging in size of apparent molecular masses at 29, 35 and 45 kDa. At maturity stage A, *P. membranaefaciens* significantly alleviated carbonylation of 35 and 45 kDa proteins at 1 and 2 days after inoculation with *Pe. expansum* compared with the control. At maturity stage B, although no significant differences were found between yeast-treated and control fruit 2 days after pathogen inoculation, the intensity of carbonylated proteins was weaker in yeast-treated fruit than that in the control fruit at day 1 (Fig. 2c,d).

Yeast sustained expression of relevant genes

Catalase, glutathione peroxidase and *β-1,3-glucanase* genes were cloned from sweet cherry fruit and designated as *CAT2*, *GPX* and *Gns1* (GenBank accession nos. EF165590, EF165591 and EF177487) by RT-PCR and RACE. The expressions of *CAT2*, *GPX* and *Gns1* genes were analysed by northern blotting. At maturity stage A, although expressions of *CAT2* and *GPX* genes showed a decreasing tendency with prolonged time, there were higher transcripts of these genes in yeast-treated fruit at days 2, 3 and 4 compared with the control (Fig. 3a). At maturity stage B, *GPX* expression was also found to be higher at days 2 and 3 in yeast-treated fruit, but *CAT2* expression was not significantly different between yeast-treated and control fruit at the same time (Fig. 3b). However, at both maturity stages, the expression of *Gns1* gene still kept a higher level in yeast-treated fruit in 2–4 days than that in the control (Fig. 3).

Yeast-affected activities of relevant enzymes

At maturity stage A, activities of CAT and GPX showed obviously higher levels in yeast-treated fruit than that in the control during all examined periods (Fig. 4a,c). However, at maturity stage B, except at day 2, there was no significant difference in the activities of two enzymes between yeast-treated and control fruit (Fig. 4b,d). Moreover, *P. membranaefaciens* significantly induced *β-1,3-glucanase activity in sweet cherry fruit at both maturity stages. In yeast-treated fruit, *β-1,3-glucanase* activity was observed to be significantly higher in 1–3 days after inoculation compared with that in the control (Fig. 4e,f).
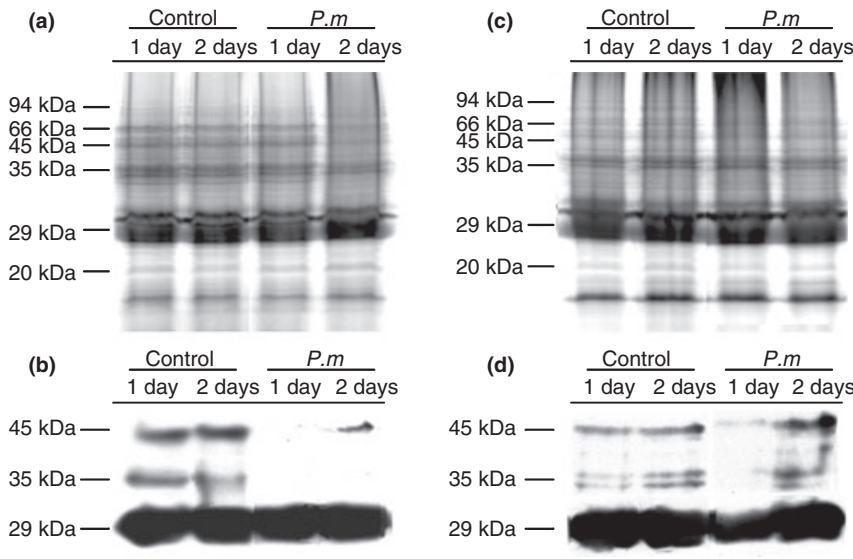
Figure 2 Effects of *Pichia membranaefaciens* on oxidative damage of protein in sweet cherry fruits. *P. m* = *P. membranaefaciens*. (a) Proteins of the fruit at maturity stage A, stained with Coomassie Brilliant Blue R-250; (b) immunodetection of carbonylated proteins of the fruit at maturity stage A; (c) proteins of the fruit at maturity stage B stained with Coomassie Brilliant Blue R-250; (d) immunodetection of carbonylated proteins of the fruit at maturity stage B.
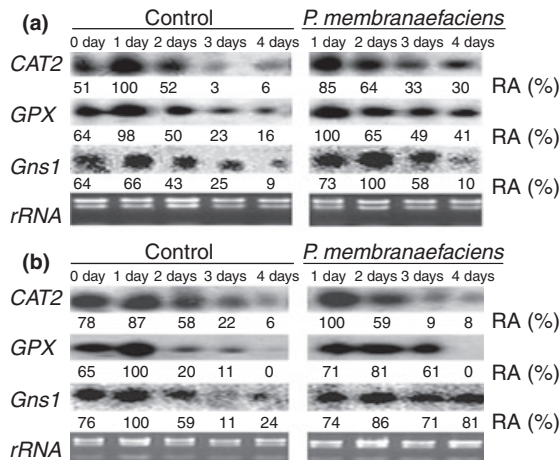

Figure 3 Effects of *Pichia membranaefaciens* on expressions of *CAT2*, *GPX* and *Gns1* genes in fruit at A (a) and B (b) maturity stages at 20°C. Twenty micrograms of total RNA from fruits were fractionated by gel electrophoresis, blotted and hybridized with probes. Equal loading of samples of total RNA was identified by visualization of rRNA that had been stained with ethidium bromide.

Discussion

Although the mechanisms by which microbial biocontrol agents against fungal pathogens are quite complicated, the mode of action of antagonist yeasts has been concluded as competition for nutrients and space (Janisiewicz and Korsten 2002; Chan and Tian 2005), direct parasitism (Wisniewski *et al.* 1991; Wan and Tian 2002), production of lytic enzymes (Jijakli and Lepoivre 1998; Fan *et al.* 2002; Masih and Paul 2002), induction of host resistance (E1 Ghaouth *et al.* 2003; Yao and Tian 2005; Tian *et al.* 2006), and resistance to oxidative stress (Castoria *et al.*

2003). In our recent study on the proteins induced by antagonistic yeast *P. membranaefaciens* in peach fruit based on proteomic approach, five antioxidant proteins and two pathogenesis-related (PR) proteins were found to be coordinately responded to *P. membranaefaciens*; the expression of *CAT* gene in peach fruit was also enhanced by the antagonist treatment, resulting in delaying decay and reduced lesion diameter of blue mold rot caused by *Pe. expansium* (Chan *et al.* 2007). Based on this, we hypothesized that antioxidant proteins induced by microbial biocontrol agent might be involved in the resistance of peach fruit against pathogen-induced oxidative stress. To further verify whether the antagonistic yeast *P. membranaefaciens* influenced pathogen-induced oxidative damage in postharvest fruit, carbonylated proteins of sweet cherry fruit were detected with anti-DNP antibody in this study. Results showed that the content of protein carbonylation in yeast-treated fruits significantly decreased during storage period, particularly in younger fruit (Fig. 2), demonstrating that the antagonistic yeast plays a significant role in alleviating pathogen-induced oxidative stress in sweet cherry fruit.

In general, an appropriate intracellular balance between ROS generation and scavenging exists in all cells. CAT and POD are two important detoxifying enzymes which work together with other enzymes of the ascorbate–glutathione cycle to promote the scavenging of ROS (Hernandez *et al.* 2001). CAT is present in the peroxisomes of nearly all aerobic cells (Dionisio-Sese and Tobita 1998) and protects the cell from H_2O_2 by catalysing its decomposition into O_2 and H_2O (Foyer and Noctor 2000). POD is widely distributed in all higher plants and protects cells against the destructive influence of H_2O_2 by catalysing its decomposition through the oxidation of
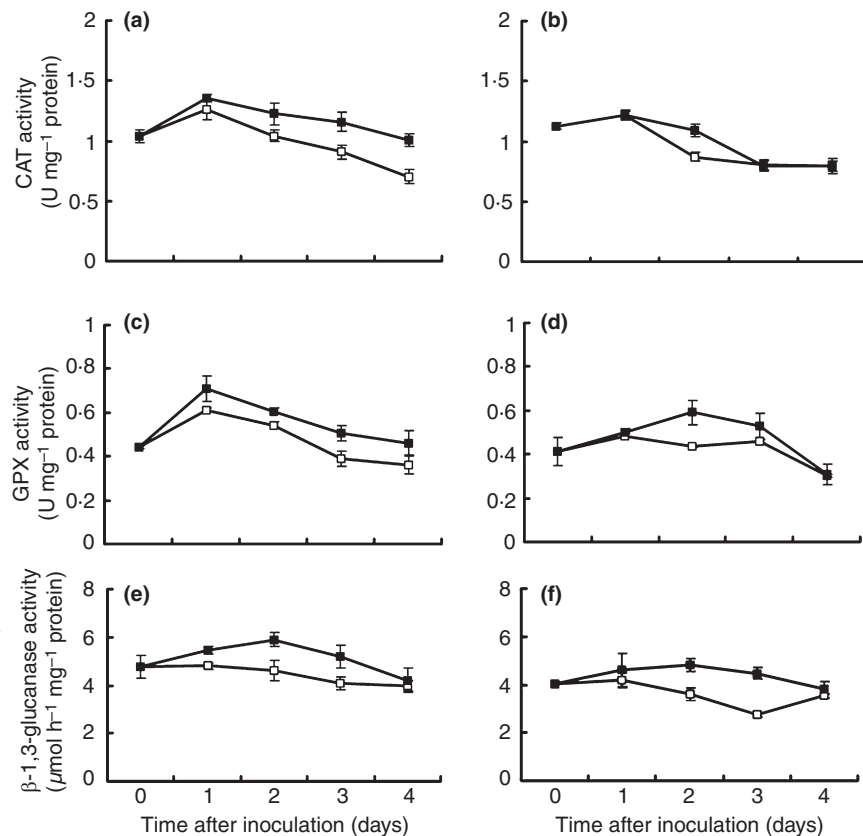
Figure 4 Effects of *Pichia membranaefaciens* on activities of catalase (CAT), glutathione peroxidase (GPX) and *β*-1,3-glucanase in sweet cherry fruit at A (a, c, e) and B (b, d, f) maturity stages at 20°C. (□) Control treatment; (■) *P. membranaefaciens* treatment. Vertical bars represent standard deviations of the means.

phenolic and enodiolic cosubstrates (Asada 1992; Borsani *et al.* 2001). In the present study, *P. membranaefaciens* induced the expressions of *CAT2* and *GPX* genes (Fig. 3) and significantly increased the activities of CAT and GPX in sweet cherry fruit (Fig. 4), particularly in younger fruit, suggesting that fruit maturity was an important factor affecting the induction of antioxidant defence response. The high levels of antioxidant enzymes elicited by microbial biocontrol agent might play important roles in reducing oxidative stress and alleviating protein carbonylation caused by pathogen-induced ROS, hence, restraining the infection of fungal pathogen in fruit. Previous study also showed that the application of exogenous ROS-detoxifying enzymes in apple wounds enhanced colonization and antagonistic activity of *C. laurentii* LS-28 and *Rhodotorula glutinis* LS-11, and inhibited decay caused by *B. cinerea* and *Pe. expansum* (Castoria *et al.* 2003). Therefore, besides directly restraining oxidative stress, the induction of antioxidant enzymes may play a role in enhancing colonization and antagonistic activity of *P. membranaefaciens* and indirectly restraining the oxidative stress in sweet cherry fruit.

Contrary to the antioxidant enzymes, the expression of *Gns1* gene and *β*-1,3-glucanase activity in yeast-treated fruit maintained higher levels than that in the control at both maturity stages (Figs 3 and 4). In the PR proteins, *β*-1,3-glucanase is one of the most fully characterized enzymes which is capable of hydrolysing polymers of fungal cell walls (Bartnicki-Garcia 1968); the accumulation of *β*-1,3-glucanase is important in retarding fungal growth and decreasing spoilage of fruits caused by fungal pathogens. Our previous study proved that an increase of *β*-1,3-glucanase activity in jujube fruit induced by *C. laurentii* was the result of the stimulating expression of *β*-1,3-glucanase gene, resulting in a significant decrease in disease incidence caused by *Pe. expansum* and *A. alternata* (Tian *et al.* 2007). In this study, the accumulation of *β*-1,3-glucanase induced by *P. membranaefaciens* may be also related to retarding fungal growth and decreasing spoilage of fruit, and hence, alleviating pathogen-induced oxidative stress in sweet cherry fruit.

Pathogen infection in nature starts soon after wounding, but resistance response of fruit induced by yeasts usually occurs after some hours (Qin *et al.* 2002; Tian *et al.* 2007). In this study, we preinoculated yeast in fruit to activate metabolic changes against pathogen infection. Our previous study also reported that *P. membranaefaciens* could stimulate the activities of other defence

enzymes, such as PPO and PAL, in peaches after treatment for 24 h, and inhibit decay caused by *R. stolonifer* (Qin *et al.* 2002). When the concentration reached 1×10^8 CFU ml^{-1}, it could rapidly propagate and inhibit *Pe. expansum* growth in the wound of sweet cherry fruit (Chan and Tian 2005). These integrative actions of *P. membranaefaciens* may also be a reason for retarding fungal growth in sweet cherry fruit.

In summary, the integrative actions of *P. membranaefaciens* resulted in completely decay control in sweet cherry fruit. *Pichia membranaefaciens* elevated the activities of antioxidant enzymes and the expressions of relevant genes and mitigated protein carbonylation, particularly in younger fruit, suggesting that induction of antioxidant defence response against pathogen-induced oxidative stress may be an important mechanism of antagonistic yeast in postharvest disease control.

Acknowledgements

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