ELSEVIER

Available online at www.sciencedirect.com
SCIENCE DIRECT®

Postharvest Biology and Technology 36 (2005) 215–223

Postharvest Biology and Technology

www.elsevier.com/locate/postharvbio

Interaction of antagonistic yeasts against postharvest pathogens of apple fruit and possible mode of action

Zhulong Chan[a, b], Shiping Tian[a, *]

[a] *Key Laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Nanxingcun 20, Xiangshan, Haidian, Beijing 100093, China*
[b] *Graduate School of Chinese Academy of Sciences, Beijing 100039, China*

Received 22 July 2004; accepted 13 January 2005

Abstract

The interactions between two antagonistic yeasts (*Pichia membranefaciens* and *Cryptococcus albidus*) and three fungal pathogens (*Monilinia fructicola, Penicillium expansum* and *Rhizopus stolonifer*) were examined both on apple juice agar plates and in apple wounds. Light microscopy and scanning electron microscopy (SEM) observations indicated that *P. membranefaciens* had a stronger capability of attaching to the fungal hyphae than *C. albidus* did. The attachment was blocked thoroughly by SDS and β-mercaptoethanol. Addition of nutrients had no visible effect on the interaction between yeasts and pathogens. Culture extract of *P. membranefaciens* had higher β-1,3-glucanase and exo-chitinase but less endo-chitinase activity than that of *C. albidus* in Lilly–Barnett medium supplied with cell wall preparation (CWP) of pathogens as the sole carbon source. This indicated that tenacious attachment, along with the secretion of extracellular lytic enzymes, may play a role in the biocontrol activity of yeast antagonists, and the interaction between yeasts and pathogens was hampered by a protein denaturant at low concentrations.
© 2005 Elsevier B.V. All rights reserved.

Keywords: Antagonistic yeast; Cell wall preparation (CWP); Glucanase; Chitinase; Scanning electron microscopy (SEM)

1. Introduction

Fruit and vegetables are highly perishable products, especially during the postharvest phase, when considerable losses due to microbiological diseases, disorders, transpiration and senescence can occur. Traditionally, postharvest disease is often controlled by the application of synthetic fungicides (Eckert and Ogawa, 1988). However, due to problems related to fungicide toxicity, development of fungicide resistance by pathogens, and potential harmful effects on the environment and human health, alternatives to synthetic chemicals have been proposed (Eckert et al., 1994).

In recent years, considerable attention has been placed on postharvest application of antagonists for the inhibition of plant disease (Cook et al., 1999; Tian et al., 2002a; El-Ghaouth et al., 2003). Utilization of antagonistic yeasts as an alternative appears to be a promising

* Corresponding author. Tel.: +86 10 6283 6559; fax: +86 10 8259 4675.
E-mail address: tsp@ibcas.ac.cn (S. Tian).

0925-5214/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.postharvbio.2005.01.001

technology (Wilson and Chalutz, 1989; Droby et al., 1991; Elad et al., 1994; Ippolito et al., 2000; Fan et al., 2002). Some antagonist-based products are commercially available and others are currently under varying degrees of development (Castoria et al., 2001). Several mechanisms have been reported to play a significant role in the biocontrol activity of antagonistic yeasts. Among them, interaction between yeast and postharvest pathogens is involved. It has been suggested that attachment of the yeast to fungal hyphae and extensive production of an extracellular matrix by yeasts may play a key role by either enhancing nutrient competition or by some other undetermined mechanisms (Wisniewski et al., 1991; Jijakli and Lepoivre, 1998; Wan and Tian, 2002).

Our previous study showed that *Pichia membranefaciens* was effective in controlling *Rhizopus stolonifer* on nectarine fruit (Fan and Tian, 2000), whereas *Cryptococcus albidus* provided a good control of *Penicillium expansum* on apples and pears (Tian et al., 2002b). The principal objective of the present study was to compare the direct mode of action of the yeasts as biocontrol agents against *Monilinia fructicola*, *P. expansum* and *R. stolonifer*, which cause decay in apple fruit. In addition, possible mechanisms involved in the attachment of the antagonistic yeasts to the hyphae of postharvest pathogens were demonstrated.

2. Materials and methods

2.1. Fruit material

Apples (*Malus domestica Borkh* cv. Fuji) were harvested at commercial maturity and kept at 0 °C until use. Fruit were disinfected with 2% sodium hypochlorite for 2 min, washed with tap water and dried in air prior to wounding.

2.2. Microorganisms and growth conditions

P. membranefaciens and *C. albidus* were isolated in our previous research experiments (Fan and Tian, 2000, 2001). Yeast cells were obtained from a culture grown in 250 ml flasks with 50 ml 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 24 h at 28 °C. Cells were harvested by centrifugation at 3365 × *g* for 10 min, resuspended in sterile distilled water and adjusted to the desired concentration with a hemocytometer. *M. fructicola*, *P. expansum* and *R. stolonifer* were obtained from infected apple fruit and cultured in 200 ml of potato dextrose broth (PDB) for 4 days at 25 °C. The mycelium was collected on Whatman No.1 filter paper for the preparation of cell walls (Wisniewski et al., 1991; Tian et al., 2002a).

2.3. Preparation of cell wall and colloidal chitin

Cell wall preparations (CWP) of each pathogen were prepared as reported by Saligkarias et al. (2002) with some modifications. Briefly, the mycelium collected by Whatman No.1 filter paper was washed four times with deionized water, homogenized for 2 min and centrifuged 2 min at 480 × *g*. After removing the supernatant, the fungal material was sonicated with a probe type sonicator (Kinematica CH-6010 Kriens-Lu, Switzerland) for 10 min and centrifuged for 5 min at 480 × *g*. The supernatant was discarded and the pellet was resuspended in water. The samples were subjected to sonication and centrifugation as above for a total of six times. Then the crushed mycelium was resuspended in an equal volume of Tris/HCl buffer (50 mmol l^{-1} and pH 7.2), centrifuged for 10 min at 1920 × *g*, and the supernatant was discarded. The pellet was subject to three successive cycles of centrifugation and resuspension. The final pellet was frozen in liquid N_2, lyophilized and stored at −20 °C thereafter.

Colloidal chitin was prepared according to the method provided by Roberts and Selitrennikoff (1988) from shrimp shell chitin: 5 g of chitin powder was added slowly into 100 ml of concentrated HCl and left at 4 °C overnight with vigorous stirring. The mixture was added to 2 l of ice-cold 95% ethanol with rapid stirring and kept overnight at 25 °C. The precipitant was collected by centrifugation at 3000 × *g* for 20 min at 4 °C and was washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0). Colloidal chitin solution (5 mg ml^{-1}) was prepared and stored at 4 °C for further detection.

2.4. Yeast–pathogen direct interaction in vitro

The possible interaction of yeasts with pathogen hyphae was assessed in Petri dishes (60 mm in diameter)

each containing 8 ml apple juice agar. Plugs (5 mm in diameter) of agar containing 3–4-day old mycelium were placed on the agar surface. After 72 h at 25 °C, 50 μl of each yeast cell suspension (1×10^8 cells ml^{-1}) were placed at the margin of the fungal mycelium. The dual cultures were washed under tap water for about 2 min after 48 h co-culture, and the plates were directly observed with a light microscope (Axioskop 40, Germany). Experiments were repeated twice.

2.5. Effectiveness of nutrients concentration and chemicals on yeast–pathogen interaction in vitro

To characterize the attachment mechanism, several compounds were applied to the yeast and pathogen to test if a disruption of attachment would occur. Suspensions of yeasts (1×10^8 cells ml^{-1}) with 0.1% sodium dodecyl sulfate (SDS) or 15 mmol l^{-1} β-mercaptoethanol were applied to the pathogens cultured on 1% apple juice agar. At the same time, apple juice agars with the concentrations of 1, 10 and 20% were used to examine the effect of nutrients on the attachment of yeast cells to fungal hyphae. The inoculation concentrations of yeasts were 1×10^8 cells ml^{-1} with distilled water. The interactions of yeast–pathogen were observed under a light microscope after washing with tap water for about 2 min as described above.

2.6. Scanning electron microscopy (SEM) in vivo

A uniform 4 mm deep × 3 mm wide wound was made at the equator of fruit using a sterile nail. Then, 30 μl aliquots of *P. membranefaciens* or *C. albidus* at 1×10^5 cells ml^{-1} were pipetted into each wound site. After 2 h, 30 μl suspensions of *M. fructicola*, *P. expansum* and *R. stolonifer* at 1×10^5 spores ml^{-1} were inoculated into each wound separately. After air-drying, fruit were put into a 400 mm × 300 mm × 100 mm plastic tray wrapped with a high density polyethylene sleeve in order to retain high humidity (about 95%). Wounded tissue (1–2 mm^2) was excised from five treated fruit 24 h after the treatment and immediately immersed into FAA fixing solution (89 ml 50% ethanol, 6 ml acetic acid and 5 ml formaldehyde) for at least 24 h. After that, samples were dehydrated in a graded ethanol series, critical-point dried with CO_2 and coated with gold–palladium for cell interaction assays. The tissues were then viewed using a Hitachi S-800 SEM (Japan).

2.7. Enzyme production and activity assay

Yeast strains were cultured in modified Lilly–Barnett minimal salt medium (Lilly and Bernett, 1951), which contains 2 mg ml^{-1} CWP as the sole carbon sources. A 250 ml Erlenmeyer flask containing 100 ml culture media was incubated on a rotary shaker at 200 rpm at 25 °C for 0, 24, 48, 72 and 96 h. Culture filtrates from each single culture were harvested by centrifuging at 7680 × *g* for 5 min, and the supernatant was used for enzyme assays.

β-1,3-Glucanase activity assays were performed by measuring the amount of reducing sugars released from laminarin, using glucose as a standard (Masih and Paul, 2002). A reaction mixture was prepared by adding 250 μl of 0.05 M potassium acetate buffer (pH 5.0) containing 2.5 mg of laminarin per ml to 250 μl of culture filtrate. The enzyme–substrate mixture was incubated for 2 h at 40 °C. Then 0.5 ml dinitrosalicylic acid reagent was added, boiling at 100 °C for 5 min. After cooling, 2 ml of deionized water was added directly and measured spectrophotometrically at 595 nm. Background levels of reducing sugars were determined with a time 0 supernatant substrate just prior to boiling at 100 °C for 5 min. The protein content of the enzyme solution was determined according to Bradford (1976) using bovine serum albumin as a standard. The specific activity was expressed as micromoles of glucose per milligram protein per hour. Each experiment contained three replicates. These experiments were repeated twice.

For the exo-chitinase assay (Abeles et al., 1970), a reaction mixture was prepared by adding 0.5 ml of 5 mg ml^{-1} colloidal chitin containing 1.2 μmol l^{-1} sodium azide and 56 μmol l^{-1} sodium acetate to 0.5 ml enzyme supernatant. For endo-chitinase assay, 0.5 ml of 5 mg ml^{-1} colloidal chitin, 0.1 ml of 3% (w/v) desalted snail gut enzyme (cytohelicase) (Sigma) and 0.1 ml of 1 mol l^{-1} potassium phosphate buffer (pH 7.1) was added to 0.5 ml of enzyme supernatant (Cabib and Bowers, 1971). The enzyme–substrate mixture was incubated for 2 h at 37 °C with constant shaking. After centrifuging at 7680 × *g* for 5 min, the supernatant was harvested. Then 0.5 ml dinitrosalicylic acid reagent was added, boiling at 100 °C for 5 min. After cooling, 1.5 ml

deionized water was added directly and measured spectrophotometrically at 550 nm. Background levels of reducing sugars and the protein content of the enzyme solution were determined as described above. The specific activity was expressed as micromoles of *N*-acetyl-D-glucosamine per milligram protein per hour according to Reissig et al. (1955). There were three replicates in each experiment. The experiment was done twice.

2.8. Statistical analysis

Data from all experiments conducted in this work were analyzed using analysis of variance (ANOVA) and the SPSS version 9.0 (SPSS, 1989–1999). The treatment means were separated at the 5% significance level using Duncan's multiple range tests.

3. Results

3.1. Yeast–pathogen direct interaction in vitro

After 48 h incubation on 1% apple juice agar at 25 °C, both antagonists were shown to adhere to the hyphae of most pathogens. *P. membranefaciens* attached to the hyphae of *M. fructicola* so firmly that the hyphae were almost invisible under the light microscope. Other observations showed that *P. membranefaciens* was in contact with the hyphae of *P. expansum* loosely and *R. stolonifer* sparsely. By contrast, *C. albidus* adhered to the hyphae of *M. fructicola* and *P. expansum* loosely, but no attachment was observed between *C. albidus* and *R. stolonifer* (Fig. 1).

3.2. Effectiveness of nutrients concentration and chemicals on yeast–pathogen interaction in vitro

The results indicated that apple juice culture concentration had no effect on the attachment modes between yeasts and pathogens in our experiment. However, the effects of chemicals on attachment of yeasts to hyphae of pathogens were significantly different from those of nutrients. The results of co-culture experiments in the presence of the test compounds indicated that 0.1% SDS and 15 mmol l^{-1} β-mercaptoethanol almost totally blocked attachment of yeast cells to fungal hyphae (Table 1).

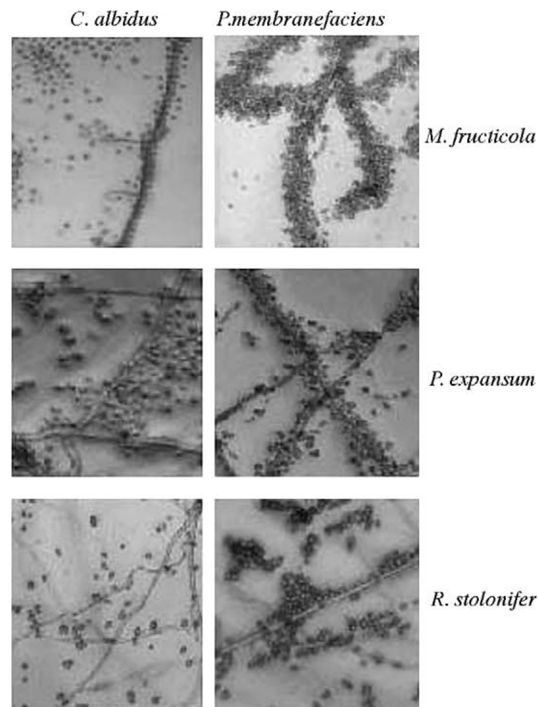

Fig. 1. Attachment of antagonistic yeast cells to hyphae of pathogens on a 1% apple juice agar plate for 48 h at 25 °C in the dark, after which the plate was washed with a stream of tap water for at least 60 s (magnification: 100×).

3.3. Ultrastructure of the yeast–pathogen interaction in vivo

In the wounds of apple fruit inoculated with antagonists and pathogens, *P. membranefaciens* was tenaciously attached to the hyphae of *M. fructicola* and *R. stolonifer*, but loosely to that of *P. expansum*. Comparatively, cells of *C. albidus* were less firmly attached to the hyphae of pathogens than *P. membranefaciens* or they were at a distance from fungal hyphae (Fig. 2).

Under SEM observation, extracellular matrix was accumulated around the hyphae of pathogens and *M. fructicola* hyphae had undergone some swelling (Fig. 3A and B). Closer examination of attachment of *P. membranefaciens* to *M. fructicola* hyphae indicated that, in many instances, a pitting appearance in the hyphae cell wall was detected, resulting in a concave appearance of the hyphal surface under the attached yeast cell (Fig. 3C). In some areas, *M. fructicola* hyphae were totally surrounded by *P. membranefaciens* cells and in

Table 1
Effect of nutrients and chemicals on attachment of yeast cells to hyphae of pathogens, examined under a light microscope

Yeasts	Nutrients or chemicals	Plant pathogens		
		R. stolonifer	*P. expansum*	*M. fructicola*
C. albidus	1% apple juice agar	−	++	++
	10% apple juice agar	−	++	++
	20% apple juice agar	−	+	++
P. membranefaciens	1% apple juice agar	+	++	+++
	10% apple juice agar	+	++	++
	20% apple juice agar	+	++	++
C. albidus	SDS (0.1%)	−	−	−
	β-meracaptoethanol (15 mM)	−	−	−
P. membranefaciens	SDS (0.1%)	−	−	+
	β-meracaptoethanol (15 mM)	−	−	−

+++, close attachment (>1000 cells $unit^{-1}$); ++, loose attachment (100–1000 cells $unit^{-1}$); +, attachment but sparse (<100 cells $unit^{-1}$); −, no attachment. Note: 1 unit = 0.04 mm^2.

particular at the end of the terminal region of the hypha (Fig. 3D).

3.4. Secretion of hydrolytic enzymes

The extracellular β-1,3-glucanase, exo- and endo-chitinase activities produced in culture were detected, when yeasts grew in the presence of CWP as the sole carbon source. Both *P. membranefaciens* and *C. albidus* produced extracellular β-1,3-glucanase immediately after the inducing treatment, and reached the maximum level at 72 h for *C. albidus* and 48 h for *P. membranefaciens*, respectively. *P. membranefaciens* culture extracts had higher β-1,3-glucanase activity than *C. albidus* culture extracts during the incubation period. The highest β-1,3-glucanase activity of both the yeasts was detected when they were incubated with CWP of *M. fructicola* as the sole carbon source, while the lowest was with CWP of *R. stolonifer* (Fig. 4).

The maximum level of exo-chitinase activity of *C. albidus* and *P. membranefaciens* was detected after incubation for 48 and 72 h, respectively (Fig. 5). For endo-chitinase activity, both yeasts appeared at the maximum level at 72 h of incubation time (Fig. 6). Extracellular culture extracts of *P. membranefaciens* exhibited at least two- to four-fold greater exo-chitinase activity than *C. albidus* culture extracts, but endo-chitinase activity from *P. membranefaciens* was significantly lower than that from *C. albidus* ($p = 0.05$). With the increase in incubation time, the chitinase activity increased to the highest point, and then decreased to a lower level at 72 h. Furthermore, the yeasts presented the highest exo- and endo-chitinase activities in the medium supplemented with CWP of *M. fructicola* as the sole carbon source (Figs. 5 and 6). This indicated CWP of *M. fructicola* might be a good carbon source for *C. albidus* and *P. membranefaciens*.

4. Discussion

Antagonist–pathogen interaction has been shown to play a major role in their biological activities (Arras, 1996; Cook, 2002). El-Ghaouth et al. (2003) found that *Candida saitoana* attached to *Botrytis cinerea* hyphae and restricted the proliferation of *B. cinerea*. Earlier study also showed that the isolate of *Pichia guilliermondii* tenaciously attached to the hyphae of *B. cinerea* despite extensive rinsing of samples with distilled water or 1% Tween during sample preparation (Wisniewski et al., 1991). In this study, we found that *P. membranefaciens* and *C. albidus* showed different attachment capability to the hyphae of three fungal pathogens both in vitro and in vivo (Figs. 1 and 2). These results indicated that attachment might universally exist between the interactions of yeast cells to fungal hyphae, and play a role in their biocontrol activity.

In the previous study, our results indicated that *P. membranefaciens* was able to produce significantly higher levels of chitinase in vitro than *Candida guil-*

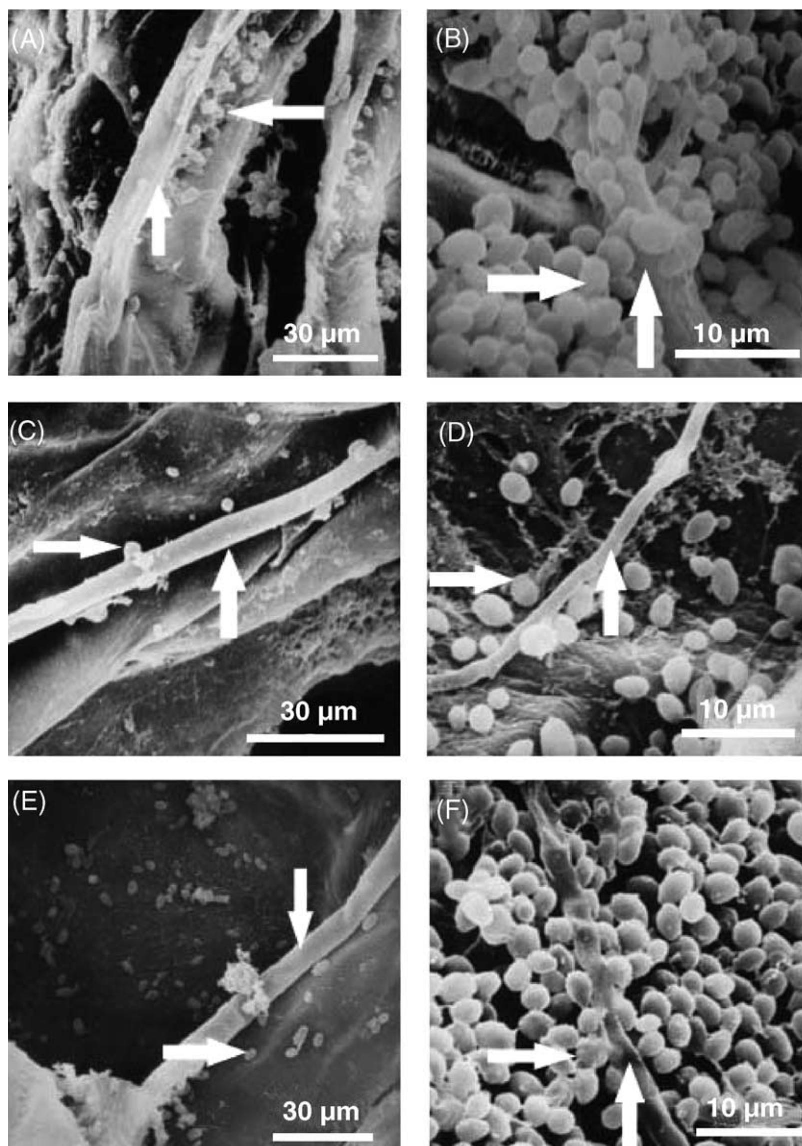

Fig. 2. Scanning electron micrographs of antagonistic yeast cells interacting with hyphae of pathogens in apple wounds 24 h after incubation at 25 °C. Treatments were (A) *C. albidus* + *M. fructicola*; (B) *P. membranefaciens* + *M. fructicola*; (C) *C. albidus* + *P. expansum*; (D) *P. membranefaciens* + *P. expansum*; (E) *C. albidus* + *R. stolonifer*; (F) *P. membranefaciens* + *R. stolonifer*. Note the interaction of yeasts (horizontal arrowhead) and fungal hyphae (vertical arrowhead).

liermondii grown in Czapeck minimal medium (Fan et al., 2002). From the data presented in Figs. 4–6, *P. membranefaciens* produced higher or at least equal amounts of β-1,3-glucanase, exo-chitinase than *C. albidus* during incubation times employed. This was correlated with the observation that *P. membranefaciens* had stronger attachment capability to hyphae of pathogens than *C. albidus* did in vitro and in vivo. Wisniewski et al. (1991) indicated that in the presence of the pathogen, the yeast cells produced lytic enzymes that could enhance the attaching ability of yeast to hyphae of pathogens. The degradation of fungal hyphae observed in our study would further support their findings. However, an interesting result that endo-chitinase

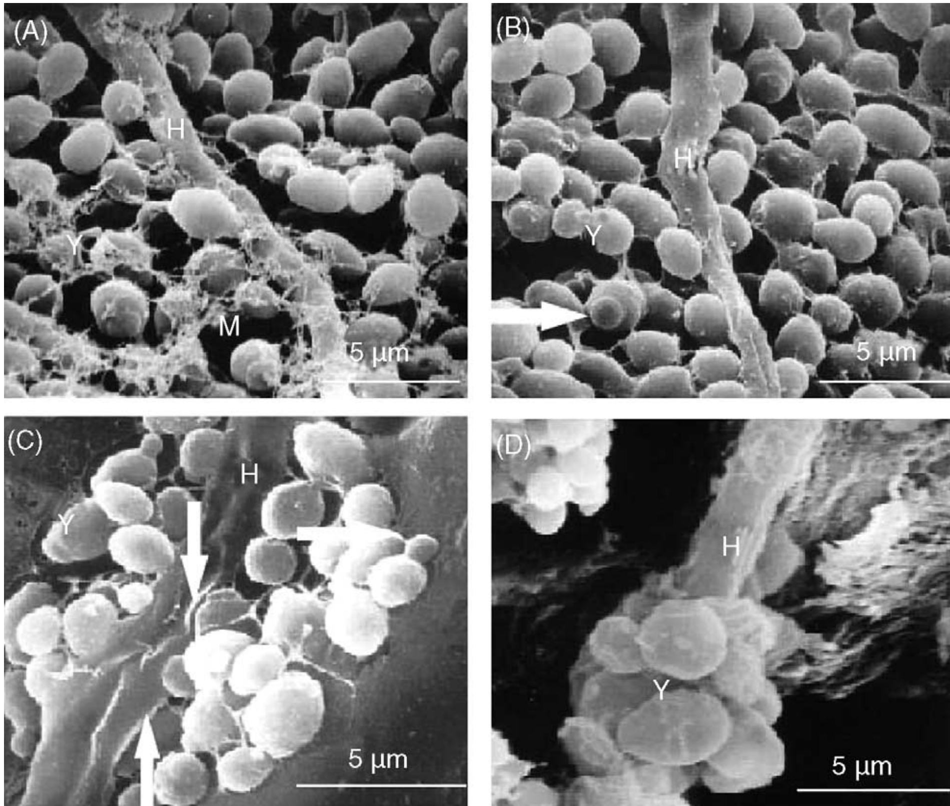

Fig. 3. Scanning electron micrographs of interactions between *P. membranefaciens* and postharvest pathogens in the apple wounds after 24 h inoculation. (A) Fungal hyphae (*M. fructicola*) encircled by yeast matrix; (B) the distortion of *M. fructicola* hyphae, note the budding of yeast cells (horizontal arrowhead); (C) the pitted appearance of *M. fructicola* hyphae (vertical arrowhead), note the budding of yeast cells (horizontal arrowhead); (D) *M. fructicola* hyphae totally surrounded by yeast cells and in particular at the end of the terminal region of the hypha. H = hypha, S = spores, M = yeast matrix and Y = yeast cells.

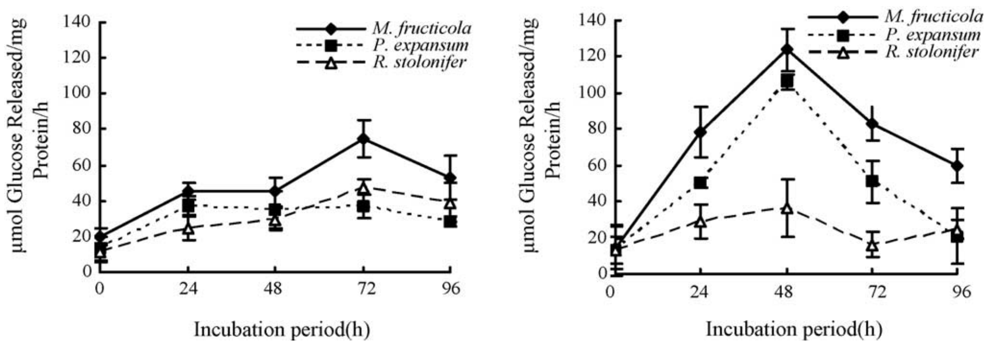

Fig. 4. β-1,3-glucanase activities of *C. albidus* (left) and *P. membranefaciens* (right) grown in Lilly–Barnett minimal salt medium supplemented with 2 mg ml^{-1} CWP for 96 h at 25 °C. Bars represented standard deviations of the means.

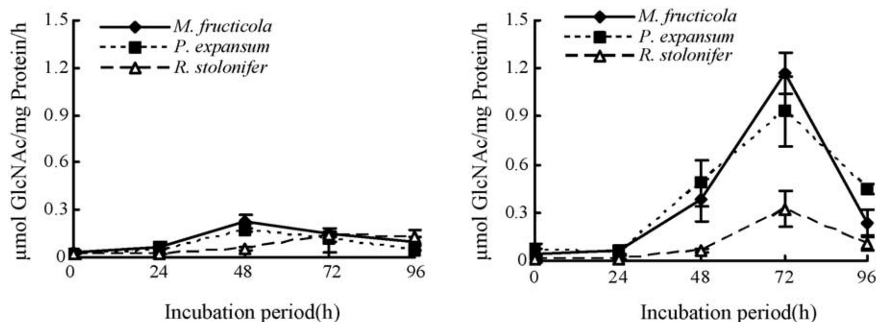

Fig. 5. Exo-chitinase activities of *C. albidus* (left) and *P. membranefaciens* (right) grown in Lilly–Barnett minimal salt medium supplemented with 2 mg ml^{-1} CWP for 96 h at 25 °C. Bars represented standard deviations of the means.

of *P. membranefaciens* was lower than that of *C. albidus* was found in this experiment. It indicated that endo-chitinase activity might play less significant role in the antagonism of yeast against pathogen.

On the other hand, the results present in Fig. 1 showed that yeast cells consistently adhered to fungal hyphae on nutrition limiting media (1% apple agar plate). In addition, Cook (2002) found that there was no hyphal adhesion by yeast when pathogens were grown on PDA. Therefore, it seems that the interaction between yeast and pathogen is dependent on nutrients. However, it was worth noting that yeast cells still tightly adhered to the hyphae of pathogens, when they were co-cultured on 10 and 20% apple juice agar plate. Additionally, when *C. albidus* and *P. membranefaciens* were cultured on 10 and 20% apple agar plate, the attachment of both yeasts to all pathogens was almost the same as on 1% apple agar (Table 1). This implied that the concentration of nutrients might not be the prerequisite for yeast–pathogen interaction. Further experiments indicated that cell membrane surface proteins appeared to play an important role in the attachment between yeasts and pathogens, because protein-denaturing agents, such as SDS and β-mercaptoethanol, blocked the attachment (Table 1). This was in accord with the result of Wisniewski et al. (1991), who found that SDS and β-mercaptoethanol were mildly effective in blocking attachment. Of course, physicochemical forces (such as microbial cell surface charges) and other compounds (including mannose residues) may influence adhesion, which have been shown to mediate adhesion of *Rhodosporidium toruloides* to surfaces of barley leaf (Buck and Andrews, 1999a,b). The results of this experiment suggest the possibility that yeast–pathogen interactions might be dependent on protein signal recognition between yeast cells and hyphae of pathogens. Additionally, the attachment efficacy of yeasts appears to be, at

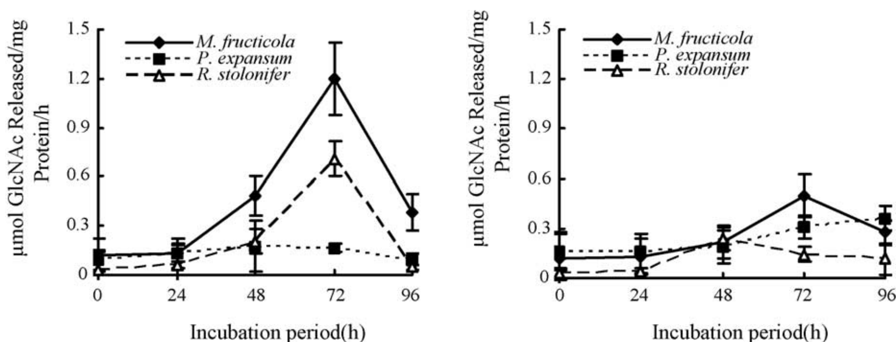

Fig. 6. Endo-chitinase activities of *C. albidus* (left) and *P. membranefaciens* (right) grown in Lilly–Barnett minimal salt medium supplemented with 2 mg ml^{-1} CWP for 96 h at 25 °C. Bars represented standard deviations of the means.

least partially, dependent on the secretion abilities of lytic enzymes, and this adhesion might be involved in the biocontrol efficacy of antagonistic yeasts. Further work will be needed to verify this hypothesis.

Acknowledgements

This study was supported by the National Science Fund for Distinguished Young Scholars of China (30225030) and the National Natural Science Foundation of China (30430480).

References

Abeles, F.B., Bosshart, R.P., Forrence, L.E., Habig, W.H., 1970. Preparation and purification of glucanase and chitinase from bean leaves. Plant Physiol. 47, 129–134.

Arras, G., 1996. Mode of action of an isolate of *Candida famata* in biological control of *Penicillium digitatum* in orange fruit. Postharvest Biol. Technol. 8, 191–198.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 1105–1112.

Buck, J.W., Andrews, J.H., 1999a. Attachment of the yeast *Rhodosporidium toruloides* is mediated by adhesives localized at sites of bud cell development. Appl. Environ. Microb. 65, 465–471.

Buck, J.W., Andrews, J.H., 1999b. Localized, positive charge mediates adhesion of *Rhodosporidium toruloides* to barley leaves and polystyrene. Appl. Environ. Microb. 65, 2179–2183.

Cabib, E., Bowers, B., 1971. Chitin and yeast budding. J. Biol. Chem. 246, 152–159.

Castoria, R., de Curtis, F., Lima, G., Caputo, L., Pacifico, S., de Cicco, V., 2001. *Aureobasidium pullulans*(LS-30) an antagonist of postharvest pathogens of fruit: study on its modes of action. Postharvest Biol. Technol. 2, 7–17.

Cook, D.W.M., 2002. A laboratory simulation for vectoring of *Trichosporon pullulans* by conidia of *Botrytis cinerea*. Phytopathology 92, 1293–1299.

Cook, D.W.M., Long, P.G., Ganesh, S., 1999. The combined effect of delayed application of yeast biocontrol agents and fruit curing for the inhibition of the postharvest pathogen *Botrytis cinerea* in kiwifruit. Postharvest Biol. Technol. 16, 233–243.

Droby, S., Chalutz, E., Wilson, C.L., 1991. Antagonistic microorganisms as biological control agents of postharvest diseases of fruit and vegetables. Postharvest News Inform. 2, 169–173.

Eckert, J.W., Ogawa, J.M., 1988. The chemical control of postharvest diseases: deciduous fruit, berries, vegetables and root tuber crops. Annu. Rev. Phytopathol. 26, 433–446.

Eckert, J.W., Sievert, J.R., Ratnayake, M., 1994. Reduction of imazalil effectiveness against citrus green mold in California packinghouse by resistant biotypes of *Penicillium digitatum*. Plant Dis. 78, 791–794.

Elad, Y., Köhl, J., Fokkema, N.J., 1994. Control of infection and sporulation of *Botrytis cinerea* on bean and tomato by saprophytic yeasts. Phytopathology 84, 1193–1200.

El-Ghaouth, A., Wilson, C.L., Wisniewski, M., 2003. Control of postharvest decay of apple fruit with *Candida saitoana* and induction of defense responses. Phytopathology 93, 344–348.

Fan, Q., Tian, S.P., 2000. Postharvest biological control of rhizopus rot on nectarine fruit by *Pichia membranefaciens* Hansen. Plant Dis. 84, 1212–1216.

Fan, Q., Tian, S.P., 2001. Postharvest biological control of grey mold and blue mold on apple by *Cryptococcus albidus*(Saito) Skinner. Postharvest Biol. Technol. 21, 341–350.

Fan, Q., Tian, S.P., Liu, H.B., Xu, Y., 2002. Production of β-1,3-glucanase and chitinase of two biocontrol agents and their possible modes of action. Chin. Sci. Bull. 47, 292–296.

Ippolito, A., El-Ghaouth, A., Wilson, C.L., Wisniewski, M., 2000. Control of postharvest decay of apple fruit by *Aureobasidium pullulans* and induction of defense responses. Postharvest Biol. Technol. 19, 265–272.

Jijakli, M.W., Lepoivre, P., 1998. Characterization of an exo-beta-1,3-glucanase produced by *Pichia anomala* Strain K, antagonist of *Botrytis cinerea* on apples. Phytopathology 88, 335–343.

Lilly, V.G., Bernett, H.L., 1951. Physiology of the Fungi. McGraw-Hill, New York.

Masih, E.I., Paul, B., 2002. Secretion of β-1,3-glucanase by the yeast *Pichia membranefaciens* and its possible role in the biocontrol of *Botrytis cinerea* causing grey mould of grapevine. Curr. Microbiol. 44, 391–395.

Reissig, J.L., Strominger, J.L., Leloir, L.F., 1955. A modified colorimetric method for the estimation of *N*-acetylamino sugars. J. Biol. Chem. 217, 959–966.

Roberts, W.K., Selitrennikoff, C.P., 1988. Plant and bacterial chitinases differ in antifungal activity. J. Gen. Microbiol. 134, 169–176.

Saligkarias, I.D., Gravanis, F.T., Epton, H.A.S., 2002. Biological control of *Botrytis cinerea* on tomato plants by the use of epiphytic yeasts *Candida guilliermondii* strains 101 and US 7 and *Candida oleophila* strain I-182. I. In vivo studies. Biol. Contr. 25, 143–150.

Tian, S.P., Fan, Q., Xu, Y., Jiang, A.L., 2002a. Effects of calcium on biocontrol activity of yeast antagonists against the postharvest fungal pathogen *Rhizopus stolonifer*. Plant Pathol. 51, 352–358.

Tian, S.P., Fan, Q., Xu, Y., Liu, H.B., 2002b. Biocontrol efficacy of antagonist yeasts to grey mold and blue mold on apples and pears in controlled atmospheres. Plant Dis. 86, 848–853.

Wan, Y.K., Tian, S.P., 2002. Antagonistical mode of *Pichia membranefaciens* to *Rhizopus stolonifer* in wounds of peach fruit by scanning electron microscope. Acta Bot. Sin. 44, 1384–1386.

Wilson, C.L., Chalutz, E., 1989. Postharvest biologicial control of *Penicillium*rots of citrus with antagonistic yeasts and bacteria. Sci. Hortic. 40, 105–112.

Wisniewski, M., Biles, C., Droby, S., McLaughlin, R., Wilson, C., Chalutz, E., 1991. Mode of action of the postharvest biocontrol yeast *Pichia guilliermondii*: characterization of attachment to *Botrytis cinerea*. Physiol. Mol. Plant Pathol. 39, 245–258.