Biocontrol Efficacy of Antagonist Yeasts to Gray Mold and Blue Mold on Apples and Pears in Controlled Atmospheres

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ABSTRACT

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Biocontrol capability of the yeasts *Trichosporon* sp. and *Cryptococcus albidus* against *Botrytis cinerea* and *Penicillium expansum* was evaluated in apple (cv. Golden Delicious) and pear (cv. Jingbai) fruits at 1°C in air and under controlled atmospheres (CA) with 3% O₂ + 3% CO₂ or 3% O₂ + 8% CO₂. *Trichosporon* sp. controlled gray mold and blue mold of apple fruits more effectively than *C. albidus* ($P < 0.05$). Apple fruits treated with *Trichosporon* sp. and *C. albidus* had a lower incidence of gray mold rot than blue mold rot in the same storage conditions. Biocontrol efficacy of the yeasts for controlling gray mold and blue mold was better in apples than in pears. Populations of the yeasts in drop-inoculated wounds in fruits increased rapidly after 20 days at 1°C both in air and in CA conditions. There was no significant difference in colony diameters of the two pathogens cultured in 0 to 15% CO₂ concentrations after 7 days at 20°C, but the colony diameter of both *B. cinerea* and *P. expansum* at 20% CO₂ was significantly less than in other treatments ($P < 0.05$). CA with 3% O₂ + 8% CO₂ inhibited the pathogenic fungi more than CA with 3% O₂ + 3% CO₂.

Additional keywords: *Malus*, postharvest disease, *Pyrus*

Postharvest diseases caused by pathogenic fungi result in great losses in fruits and vegetables (27). Application of synthetic fungicides is generally the main method to control postharvest diseases, but public concern over food safety and the development of fungicide resistance by pathogens has increased the search for alternative means potentially less harmful to human health and the environment (9,15,33). Biological control has been advanced as an alternative to synthetic fungicides, and considerable success has been achieved by utilizing antagonistic microorganisms (17,20,36). Some yeasts and bacteria have been reported to effectively reduce various postharvest diseases of fruits (3,12,18). However, previous research often focused on isolation and selection of antagonists (13,19,25) and comparing their biocontrol effectiveness for control of postharvest diseases (20,21). In addition, attempts were made to enhance efficacy by adding calcium, carbohydrates, amino acids, and other nitrogen compounds (7,8,14), or by combining antagonists with fungicides (4,5,10,28,34). There is little information about biocontrol efficacy of antagonists in low temperatures

and under controlled atmosphere (CA) storage, except that Chand-Goyal and Spotts (6) reported biological control of postharvest diseases of apple and pear under semi-commercial and commercial conditions using three saprophytic yeasts. Recently, we isolated *Trichosporon* sp. and *Cryptococcus albidus* from peach fruits (*Prunus persica*) and found them to be very effective for control of gray mold and blue mold of apple fruits at 20°C (11,34). Many postharvest pathogenic fungi grow at -4°C (1,31) and cause decay of fruits and vegetables below -2°C (2,30,32). CA storage is considered to be effective for reducing fruit decay (24,35). CA with CO₂ concentrations above 2.8% effectively reduced the development of lesions incited by *B. cinerea* and *P. expansum* in apple fruits (26). Therefore, it is necessary to study the effect of low temperature and CA conditions on the activity of antagonists and their biocontrol capacity to pathogenic fungi causing postharvest decay of fruits. Successful commercial use of biocontrol of postharvest decay of fruits and vegetables will depend on the adaptability of antagonists to commercial low-temperature and CA conditions.

The objectives of this study were: (i) to determine population dynamics of *Trichosporon* sp. and *C. albidus* in wounds of fruits kept at 25°C or in cold storage and CA at 1°C; (ii) to compare the biocontrol efficacy of the yeasts to gray mold and blue mold on apple and pear fruits in CA with 3% O₂ + 3% CO₂ or 3% O₂ + 8% CO₂ at 1°C; and (iii) to investigate growth of *B.*

cinerea and *P. expansum* in CA with different CO₂ concentrations at 20 and 1°C.

MATERIAL AND METHODS

Pathogen inoculum. *B. cinerea* and *P. expansum* were isolated from naturally infected apple fruit and cultured on oatmeal agar (OMA) (oatmeal 60 g, sucrose 30 g, NaNO₃ 10 g, agar 12 g/liter) and potato dextrose agar (PDA), respectively, at 25°C for 2 weeks. Conidial suspensions were prepared by flooding the cultures with sterile distilled water containing 0.05% (vol/vol) Tween 80. The conidial concentrations of the pathogens were determined with the aid of a hemacytometer and adjusted to 5×10^4 spores per ml.

Antagonist. The yeast antagonists were isolated from peach fruits according to the method of Wilson et al. (37) and identified as *Cryptococcus albidus* (Saito) Skinner and *Trichosporon* sp. by CABI Bioscience Identification Services (IMI 382212; 382211). The yeasts were cultured in 250-ml Erlenmeyer flasks with 50 ml of nutrient yeast dextrose broth (NYDB: 8 g of nutrient broth, 5 g of yeast extract, and 10 g of dextrose in 1 liter of water) on a rotary shaker at 200 rpm for 24 h at 28°C. The yeast cells were harvested by centrifugation at 6,000 rpm (about $2,500 \times g$) for 10 min, washed twice with 0.05 M phosphate buffer at pH 7.0, and resuspended in distilled water. The concentrations of cells of both yeasts in the suspensions were adjusted to 1×10^6 and 5×10^6 CFU/ml, respectively, with the aid of a hemacytometer.

Apple and pear fruits. 'Golden Delicious' apple (*Malus domestica* Borkh.) and 'Jingbai' pears (*Pyrus ussuriensis* Maxim.) were used in this study. The fruits were harvested from an orchard at the Institute of Forest and Fruits, Beijing Academy of Agricultural Sciences, and immediately transported to the laboratory after harvest. The firmness of the apples and pears was 32.3 and 49.9 N as determined by a penetrometer (FT-327, UC Fruit Firmness Tester, Milano, Italy), and total soluble solids were 12.9 and 13.8%, respectively. Apple and pear fruits were washed with 2% sodium hypochlorite for 2 min, rinsed with tap water, and air-dried before wounding.

Biocontrol efficacy of yeasts in different storage conditions. A uniform wound, 3 mm deep and 3 mm wide, was made at the equator of each fruit (put on its side) using the tip of sterile dissecting needle.

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Aliquots of 20 μ l of 1×10^6 CFU/ml suspension of each yeast and sterile distilled water (control) were pipetted into each wound site. After 4 h, 20 μ l of a suspension of *B. cinerea* or *P. expansum* (5×10^4 spores per ml) was placed in each wound, and fruits dried in air at room temperature for 4 h. Fruits were placed in a plastic tray (400 $\times$ 300 $\times$ 100 mm) and transferred to a controlled atmosphere cabinet (Fruit s.r.l. Control, type FC-701, Milano, Italy) with 3% O₂ + 3% CO₂ or 3% O₂ + 8% CO₂ at 1°C (RH > 95%). There were three replicate trials of 10 fruits per treatment with a complete randomization of trays in each test. The same trays wrapped with high-density polyethylene sleeves to keep high humidity (about 95%) were stored in air at 1°C as the control. The test was conducted twice. Disease incidence and lesion size in the fruits were measured every 20 days of storage. Two diameters were measured at right angles across each lesion on the surface of infected fruit. Data presented are the means of lesion diameters. The fruits both in air and under CA conditions were stored for 60 days.

Population dynamics of yeasts in different storage conditions. Three wounds per fruit were made equidistant around the stem, and then 20 μ l of each yeast suspension (5×10^6 CFU/ml) was applied to each wound site. The fruits were stored in different conditions: (i) in air at 25°C, and populations were determined daily for 8 days; and (ii) in air and CA 3% O₂ + 3% CO₂ or 3% O₂ + 8% CO₂ at 1°C, and fruit samples were taken to determine antago-

nist populations 1 h after inoculation and after 20, 40, and 60 days of storage. The yeasts were recovered as described by Janisiewicz (16) by removing the wound tissue with a cork borer. The resulting cylinder (1 cm diameter $\times$ 1 cm deep) was placed in a mortar with 1 ml of 0.05 M phosphate buffer at pH 7.0 and was ground with a pestle. Serial 10-fold dilutions were made in phosphate buffer, and 100 μ l of each dilution was plated on nutrient yeast dextrose agar (NYDA) medium (10 dishes). The colony counts were expressed as the log₁₀-transformed numbers of cells per wound. An analysis of variance of the log₁₀-transformed data was carried out for each sampling date. There were three replications of each treatment, and the experiment was conducted twice.

Pathogen growth in vitro under different storage conditions. A 50- μ l aliquot of suspensions of *B. cinerea* and *P. expansum* (5×10^4 spores per ml) was placed into a hole (6 mm) in the center of each dish (60 mm diameter) containing 10 ml of OMA or PDA, respectively. There were two tests in this experiment. In the first test, dishes were placed in cabinets and ventilated with a continuous flow of air or different CO₂ concentrations. The high CO₂ atmosphere was established with a flow-through system by mixing N₂ and CO₂. The concentrations were 0, 3, 8, 15, and 20% ($\pm 0.5\%$) for CO₂, and 21, 20.7, 19, 17, and 15% ($\pm 0.5\%$) for O₂, measured with a gas analyzer (Fruit s.r.l. Control, type FC-701, Milano, Italy) daily. The cabinets were kept at 20°C for 7 days. In

the second test, dishes were kept in ventilation storage and in CA cabinets with 3% O₂ + 3% CO₂ or 3% O₂ + 8% CO₂ at 1°C for 40 days. The colony diameters of the fungi were measured at the end of the storage period. There were 10 dishes in each treatment and the treatments were replicated three times. The test was conducted twice.

Statistical analysis. All statistical analyses were performed with SAS version 6.12 (SAS Institute, Cary, NC). The data were analyzed by one-way analysis of variance (ANOVA). Means were separated with the Student-Newman-Keuls test.

RESULTS

Efficacy of antagonists for controlling gray mold and blue mold rots in different storage conditions. The antagonists *Trichosporon* sp. and *C. albidus* were more effective for control of apple decay in CA with low O₂ and high CO₂ than in air, according to the results of two experiments (Figs. 1 and 2). *Trichosporon* sp. was more effective than *C. albidus* ($P < 0.01$) in the same storage conditions. Gray mold rot in apple fruits treated with *C. albidus* reached 40% and 10% incidence after 40 days in air storage and CA, respectively, but no disease was found in apples treated with *Trichosporon* sp. in the same storage conditions. However, blue mold rot in apples treated with the yeasts showed relatively higher decay incidence compared with gray mold rot in the same storage conditions. The similar results obtained in repeated experiments demonstrated that bio-control efficacy of *Trichosporon* sp. and *C.*

Fig. 1. Incidence of gray mold and blue mold decay in apple fruits treated with *Trichosporon* sp. and *Cryptococcus albidus* in air and controlled atmosphere (CA) with 3% O₂ + 3% CO₂ at 1°C. Concentration of the yeasts was 1×10^6 CFU/ml. There were 10 fruits with three replicates in each treatment and the test was conducted twice. The data were pooled from two experiments. Bars represent standard errors of the mean. CK = nontreated control; B = *Botrytis cinerea*; P = *Penicillium expansum*.

albidus for control of gray mold and blue mold in apples (Figs. 1 and 2) was better than that in pears (Figs. 3 and 4). There was no significant CA effect on incidence of gray mold and blue mold rots in control fruits (of both apple and pear) stored for 40 and 60 days. Lesions enlarged as storage

time increased. Again, the two antagonists provided better control of gray mold than of blue mold in apple fruits (Figs. 1 and 2).

Effects of storage conditions on population dynamics of the yeasts. *Trichosporon* sp. and *C. albidus* multiplied rapidly in fruit wounds after 1 day at 25°C

(Fig. 5). At the beginning of the experiment, population of the yeasts was 1.2×10^4 to 1.5×10^4 CFU per wound. Within 24 h, the population size of *Trichosporon* sp. and *C. albidus* increased more than 30-fold in apples and 20-fold in pears at 25°C, then stabilized at the same density ($4.58 \times$

Fig. 2. Diameters of gray mold and blue mold lesions in apple fruits treated with *Trichosporon* sp. and *Cryptococcus albidus* in air and in controlled atmosphere (CA) with 3% O₂ + 3% CO₂ at 1°C. Concentration of the yeasts was 1×10^6 CFU/ml. There were 10 fruits with three replicates in each treatment and the test was conducted twice. The data were pooled from two experiments. Bars represent standard errors of the mean. CK = nontreated control; B = *Botrytis cinerea*; P = *Penicillium expansum*.

Fig. 3. Incidence of gray mold and blue mold decay in pear fruits treated with *Trichosporon* sp. and *Cryptococcus albidus* in air and in controlled atmosphere (CA) with 3% O₂ + 3% CO₂ at 1°C. Concentration of the yeasts was 1×10^6 CFU/ml. There were 10 fruits with three replicates in each treatment and the test was conducted twice. The data were pooled from two experiments. Bars represent standard errors of the mean. CK = nontreated control; B = *Botrytis cinerea*; P = *Penicillium expansum*.

10^5 to 1.50×10^6 CFU per wound), except *Trichosporon* sp. in pear, which fluctuated and reached a maximum value of 5.1×10^6 CFU per wound after 5 days (Fig. 5).

Based on results from two experiments, low temperature and CA conditions did not significantly affect populations of the yeasts in wounds (Fig. 6). Populations of *Trichosporon* sp. and *C. albidus* in fruit wounds increased rapidly in the first 20 days of storage at 1°C both in air and in CA at 3% O₂ + 3% CO₂ and 3% O₂ + 8% CO₂. Even after 60 days, the populations of *Trichosporon* sp. and *C. albidus* in fruit wounds remained 6.1×10^4 to 5.45×10^5 CFU per wound in air and 4.33×10^4 to 2.52×10^5 CFU per wound in CA conditions. The highest populations of *Trichosporon* sp. were 5.45×10^5 , 2.60×10^5 , and 2.89×10^5 CFU per wound in pear fruits after 40 days in air, 3% O₂ + 3% CO₂, and 3% O₂ + 8% CO₂, respectively, which were 43.6, 20.8, and 23.1 times the initial populations (Fig. 6). *C. albidus* reached the highest populations of 8.28×10^5 , 3.3×10^5 , and 5.72×10^5 CFU per wound after 60 days in apples kept in air, 40 days in apples stored in 3% O₂ + 3% CO₂, and 20 days in pears stored in 3% O₂ + 8% CO₂, respectively. These populations increased 66.2, 26.4, and 45.8 times the initial populations.

Effects of storage conditions on pathogen growth in vitro. The concentration of CO₂ affected growth of *B. cinerea* and *P. expansum* in vitro (Fig. 7). There was no difference in colony diameters of the pathogens cultured in 0 to 15% CO₂ after 7 days at 20°C, but the colony diame-

ter both of *B. cinerea* and of *P. expansum* at 20% CO₂ was significantly smaller than in other treatments ($P < 0.01$). Colony diameters of *B. cinerea* and *P. expansum* kept at 20% CO₂ were 40 and 16 mm,

respectively, which were only 66.7 and 57% of the control treatment (21% O₂ + 0% CO₂). In addition, *B. cinerea* grew more rapidly than *P. expansum* in the same treatments ($P < 0.01$). There was no sig-

Fig. 5. Population dynamics of *Cryptococcus albidus* and *Trichosporon* sp. in wounds of apple and pear fruits incubated at 25°C in air. Fruits were wounded and 20 µl of suspension at 5×10^6 CFU/ml was applied to each wound. The antagonists were recovered by removing the wounded tissue, grinding it in a mortar with 0.05 M phosphate buffer, and plating 0.1 ml of a 10-fold dilution on nutrient yeast dextrose agar (NYDA). There were three replicates of one fruit each per treatment and the data were pooled from two experiments. Bars represent standard errors of the mean.

Fig. 4. Diameters of gray mold and blue mold lesions in pear fruits treated with *Trichosporon* sp. and *Cryptococcus albidus* in air and in controlled atmosphere (CA) with 3% O₂ + 3% CO₂ at 1°C. Concentration of the yeasts was 1×10^6 CFU/ml. There were 10 fruits with three replicates in each treatment and the test was conducted twice. The data were pooled from two experiments. Bars represent standard errors of the mean. CK = nontreated control; B = *Botrytis cinerea*; P = *Penicillium expansum*.

nificant difference in the data obtained in repeated experiments.

Low temperature and CA conditions also influenced pathogen growth (Fig. 7). However, effects of CA on growth of *B. cinerea* and *P. expansum* were greater than low temperature alone ($P < 0.01$). CA with the higher CO₂ concentration (3% O₂ + 8% CO₂) inhibited growth of both fungi more than CA with lower CO₂ concentration (3% O₂ + 3% CO₂).

DISCUSSION

Controlled atmosphere with high CO₂ and low O₂ concentrations has been widely

used in commercial storage for reducing decay, delaying senescence, and maintaining quality of fruits and vegetables. In general, pathogenic fungi are suppressed by low temperature and CA with high CO₂ and low O₂ (28,30), but many studies show that *Botrytis* spp. and *Penicillium* spp. can grow and cause decay in fruits and vegetables even at -2 and -4°C (1,2,29,31). Therefore, the ability of antagonists to grow at low temperature and CA conditions will affect their control of postharvest diseases. The results of two experiments demonstrated that the application of *C. albidus* and *Trichosporon* sp. resulted in

better control of gray mold and blue mold rots in apples compared with pears both in air and under CA conditions at 1°C (Figs. 1 and 3), which indicated there were differences in biocontrol efficacy of yeast to the same disease in different hosts.

Many yeasts require CO₂ as a carbon source for growth, and about 10% CO₂ enhances sporulation in yeast fungi (28). Miller and Sugar (22) reported that the combination of *C. infirmo-miniatus* and modified atmosphere packaging (MAP) with 2 to 6% O₂ and 1% CO₂ reduced blue mold better than MAP without yeast or treatment with *C. infirmo-miniatus* fol-

Fig. 6. Population dynamics of *Cryptococcus albidus* and *Trichosporon* sp. in wounds of apple and pear fruits incubated at 1°C in air and controlled atmospheres. Fruits were wounded and 20 µl of suspension at 5×10^6 CFU/ml was applied to each wound. The antagonists were recovered by removing the wounded tissue, grinding it in a mortar with 0.05 M phosphate buffer, and plating 0.1 ml of a 10-fold dilution on nutrient yeast dextrose agar (NYDA). There were three replicates per treatment and the test was conducted twice. The data were pooled from two experiments. Bars represent standard errors of the mean. CA-I: 3% O₂ + 3% CO₂; CA-II: 3% O₂ + 8% CO₂.

Fig. 7. Growth of *Botrytis cinerea* and *Penicillium expansum* in vitro under different storage conditions. Five replications per treatment were used and the experiment was conducted twice. The data were pooled from two experiments. Bars represent standard errors of the mean. CA-I: 3% O₂ + 3% CO₂; CA-II: 3% O₂ + 8% CO₂.

lowed by storage in air. Spotts et al. (28) reported that treatment of sweet cherry with *C. infirmo-minutus* and storage in MAP (5.1% O₂ and 11.4% CO₂) reduced brown rot incidence by 98.2%. Our results that *C. albidus* and *Trichosporon* sp. under CA storage conditions effectively controlled gray mold and blue mold in apples agree with these previous studies. The CA effect may be attributed to a combination of suppression of *B. cinerea* and *P. expansum* and stimulation of the yeasts by the high CO₂. Although *C. albidus* and *Trichosporon* sp. controlled gray mold and blue mold of apple fruits, neither yeast controlled decay of pear fruits, even in CA. This may be related in part to 'Golden Delicious' apple having a better storability both in cold air storage and under the CA conditions than 'Jingbai' pears (data not shown).

Rapid colonization of wounds by a microbe is important if it is to be an effective antagonist (23,36,37), and there is a direct relationship between the population of antagonist and the biocontrol efficacy (10,13,16). We found that populations of *Trichosporon* sp. and *C. albidus* increased rapidly at 25°C and then stabilized for the remaining period (Fig. 5), while in cold storage and CA conditions at 1°C, an initial increase often was found after 20 days (Fig. 6). This result indicated that *Trichosporon* sp. and *C. albidus* were well adapted to the wound environment in fruit and have considerable potential as biocontrol agents. The ability of yeasts to multiply rapidly in wounds, and perhaps preemptively utilize available nutrients, may facilitate biocontrol by nutrient competition (36).

In addition, an effective antagonist must be compatible with routine postharvest treatments (37). Our previous study showed that *C. albidus* and *Trichosporon* sp. are compatible with several postharvest practices, including fungicide and Ca²⁺ treatment, making feasible an integrated control strategy under commercial conditions (11,34). This approach not only makes it possible to reduce fungicide concentrations while maintaining adequate decay control, but also ensures a reduction of the chemical residue on the fruit. However, further research is needed to determine the best concentrations of O₂ and CO₂, both for the yeasts and the fruits, and the mechanisms of biocontrol by *C. albidus* and *Trichosporon* sp.

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