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Efficacy of *Pichia caribbica* in controlling blue mold rot and patulin degradation in apples

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ABSTRACT

The efficacy of *Pichia caribbica* in controlling postharvest blue mold and natural decay development of apples and degrading the patulin produced by *Penicillium expansum* was investigated. The decay incidence of the blue mold of apples treated by *P. caribbica* was significantly reduced compared with the control samples, and the higher the concentration of *P. caribbica*, the better the efficacy of the biocontrol. *P. caribbica* significantly controlled the natural decay development of apples following storage at 20 °C for 35 days or 4 °C for 45 days followed by 20 °C for 15 days. Germination of spores and growth of *P. expansum* were markedly inhibited by *P. caribbica* in *in vitro* testing. Rapid colonization of apple wounds by the yeast was observed in fruit stored at 20 °C or 4 °C. After incubation with *P. caribbica* at 20 °C for 15 days, patulin production by *P. expansum* in apples was significantly reduced compared with the control. *In vitro* testing indicated that *P. caribbica* can degrade patulin directly.

1. Introduction

Apple fruit (*Malus domestica* Borkh) is one of the most important fruits produced in China. To provide fruit throughout the year, fresh apples are stored after harvest (Li et al., 2011). However, postharvest diseases, especially those originating from wound infection at harvest or during postharvest handling and packing, can be a serious limiting factor for such storage (Bondoux, 1992). Blue mold decay caused by *Penicillium expansum* Link is one of the important postharvest diseases of apples (Saravanakumar et al., 2008; Tolaini et al., 2010). *P. expansum* is a psychotropic mold and the most common pathogen of pome fruit, causing blue mold rot during storage (Speijers et al., 1988; Sanderson and Spotts, 1995; Vero et al., 2002). Growth of *P. expansum* is greater in pears than in apples, however the latter tend to accumulate more patulin (Northold et al., 1978). Patulin (4-hydroxy-4H-furo [3, 2c] pyran, 2[6H]-one) is an unsaturated heterocyclic lactone produced by certain species of *Penicillium*, *Aspergillus* and *Byssochlamys* growing on fruit (Ritieni, 2003). Almost all isolates of *P. expansum* are patulin producers (Andersen et al., 2004). Patulin is toxic for animals, causing intestinal injuries, including epithelial cell degeneration, inflammation, ulceration, and hemorrhages; it has also been shown to be mutagenic, carcinogenic, immunotoxic, neurotoxic, genotoxic and teratogenic (Mahfoud et al., 2002; Iha and Sabino, 2006).

Traditionally, control of postharvest diseases of fruits relies mainly on the use of synthetic fungicides. However, the development of fungicide resistance in pathogens has prompted an urgent need for alternative control with good efficacy, low residues, and little or no toxicity to non-target organisms (Janisiewicz and Korsten, 2002). Biological control with antagonistic yeasts has emerged as a promising way to reduce synthetic fungicide usage (Droby et al., 2009). Various microbial antagonists have been reported to control several different pathogens on various fruits and vegetables (Fravel, 2005). Many yeast antagonists have been shown to have a significant effect on blue mold decay caused by *P. expansum* in apples, including *Cryptococcus albidus* (Fan and Tian, 2001), *Candida sake*, *Pantoea agglomerans* (Morales et al., 2008a) and *Rhodotorula mucilaginosa* (Li et al., 2011). In addition, many studies have shown that some yeast antagonists can directly inhibit the production of patulin, while also inhibiting *P. expansum*. Castoria et al. (2005) reported that *Rhodotorula glutinis* LS11 cells surviving in decaying apples could metabolize patulin and/or negatively affect its accumulation or synthesis. Coelho et al. (2007) reported that the initial patulin concentration of 223 μg in the presence of *Pichia ohmeri* 158 cells was decreased over 83% when incubated at 25 °C/2 days and >99% after 5 days of incubation, with an undetectable patulin level after 15 days. Although most published reports have demonstrated the efficacy of antagonistic yeasts in inhibiting patulin accumulation in apples, there is limited knowledge of its mechanism(s).

In this paper, a yeast antagonist *Pichia caribbica* is presented. It was isolated by our research team from the soil from an unsprayed peach orchard. The objectives of this study were: (1) to evaluate the efficacy of *P. caribbica* in inhibiting blue mold decay of apples; (2) to evaluate the control efficacy of *P. caribbica* on natural infections of apples and its effect on quality parameters of fruits; (3) to follow the population dynamics of *P. caribbica* in apple wounds; (4) to evaluate the effects of *P. caribbica* on survival of spores of *P. expansum in vitro*; (5) to

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evaluate the efficacy of *P. caribbica* in controlling patulin in apple wounds; and (6) to study the degradation of patulin by *P. caribbica in vitro*.

2. Materials and methods

2.1. Antagonist and growth conditions

The yeast antagonist *P. caribbica* was isolated from soil from a peach orchard (the central shoal of Yangtze River, Zhenjiang, Jiangsu Province). Classical methods based on colony and cell morphologies were used for a preliminary characterization of the yeast (Kurtzman and Fell, 1998). Subsequently, sequence analysis of the 5.8S internal transcribed spacer (ITS) ribosomal DNA (rDNA) region was used to identify the yeast (Li et al., 2010). *P. caribbica* has been shown to be safe in animal testing, including physiology experiments, acute toxicity studies, and the Ames test (unpublished data). *P. caribbica* isolates were maintained at 4 °C on nutrient yeast dextrose agar medium (NYDA—0.8% nutrient broth, 0.5% yeast extract, 1% glucose and 2% agar). Liquid cultures of the yeast were grown in 250 ml Erlenmeyer flasks containing 50 ml of nutrient yeast dextrose broth (NYDB) which had been inoculated with a loop of the culture. Flasks were incubated on a rotary shaker at 190 rpm at 28 °C for 20 h. Following incubation, cells were centrifuged at 6000×*g* for 10 min and washed twice with sterile distilled water in order to remove the growth medium. Cell pellets were re-suspended in sterile distilled water and adjusted to an initial concentration before being adjusted to the concentration required for the each experiment.

2.2. Fruits

Apples (*M. domestica* Borkh, cv. Fuji) were harvested at commercial maturity from an orchard in Yantai in Shandong province, and selected for uniformity of size, ripeness, and absence of apparent injury or infection.

2.3. Fungal pathogen

The pathogen *P. expansum* was isolated from infected apple. This culture was maintained on potato dextrose agar (PDA, extract of boiled potatoes, 200 ml; dextrose, 20 g; agar, 20 g and distilled water, 800 ml) at 4 °C, and fresh cultures were grown on PDA plates before use. Spore suspensions were prepared by removing the spores from the sporulating edges of a 7 day old culture with a bacteriological loop, and suspending them in sterile distilled water. Spore concentrations were determined with a hemocytometer, and adjusted as required with sterile distilled water.

2.4. Efficacy of P. caribbica in inhibiting blue mold decay of apples

Fruits were selected randomly and disinfected with 0.1% sodium hypochlorite for 1 min, washed with tap water, and allowed to air dry at room temperature. Three uniform wounds (5 mm diameter and approximately 3 mm deep) were made at the equator of each apple fruit using the tip of a sterile cork borer. Thirty μl of cell suspensions of *P. caribbica* at 1×10^6, 1×10^7, 1×10^8, 5×10^8, or 1×10^9 cells/ml respectively were pipetted into each wound site, and sterile distilled water was used as the control. Thirty μl of suspension of *P. expansum* (5×10^4 spores/ml) was inoculated into each wound after 2 h. After air drying, the apples were stored in enclosed plastic trays to maintain a high relative humidity (about 95%) at 20 °C. The percentage of infected fruit was recorded after 10 days of incubation. There were three replicates of ten fruits for each treatment. All treatments were arranged in a randomized complete block design, and the experiment was conducted twice.

2.5. Efficacy of P. caribbica for reducing natural decay development and its effect on quality parameters of apples

Intact fruits were inoculated by spraying them with a suspension of *P. caribbica* (1×10^8 cells/ml), using sterile distilled water as the control. After air drying, treated fruits were stored enclosed in plastic wrap to maintain high humidity (about 95%) and incubated at 20 °C for 35 days or at 4 °C for 45 days followed by 20 °C for 15 days in order to determine disease development under normal storage conditions, after which infection rate was measured. There were three replicate trials of 10 fruits per treatment with complete randomization. The experiment was repeated twice.

Quality parameters were measured after storage. Quality measurements were made on three replicates of ten fruits each, and performed at ambient temperature (about 20 °C). The experiment was repeated twice. The testing methods are described below.

Weight loss(%) The mass was measured by an MP2000-2 balance (±0.001 g) (Shanghai Balance Instruments, China) before treatment (A) and after storage (B), respectively, and the mass loss was calculated as (A − B)/A.

Fruit firmness Firmness values of each individual apple were measured at three points of the equatorial region by using the TA-XT2i Texture Analyser (Microstable Instruments, UK) with a 5 mm diameter flat probe. The probe descended toward the sample at 10.0 mm s^{-1} and the maximum force (N) was defined as firmness. The firmness of each apple was measured three times on different sides.

Total soluble solids Total soluble solids (TSS) were determined by measuring the refractive index of the same juice with a WYT hand refractometer (0–80%) and the results expressed as percentages (g per 100 g fruit weight) (Larrigaudière et al., 2002).

Titratable acidity Acidity was measured by titration with 0.1 N NaOH to pH 8.1; 4 g of juice diluted with 20 ml of distilled water was evaluated for each replicate. Titratable acidity was calculated as percent malic acid (Wright and Kader, 1997).

Browning The method was described by Lee et al. (2002) with some modifications. 20 g of fresh pressed fruit juice mixed with 20 ml of distilled water, was centrifuged at 10,000×*g* for 10 min at 4 °C. Then the supernatants were centrifuged at 10,000×*g* for 10 min at 4 °C with 15 ml 95% ethanol. The absorbance of supernatants were recorded at A_{420}.

Ascorbic acid The 2, 6-dichloroindophenol titrimetric method was used to determine the ascorbic acid content of pressed fruit juice. Results were expressed as milligrams of ascorbic acid per 100 g sample (Özden and Bayindirli, 2002).

2.6. Population studies of P. caribbica in apple wounds

Freshly harvested fruits were treated as described above to evaluate the efficacy of *P. caribbica* in inhibiting blue mold decay of apples. Each wound on fruit was inoculated with 30 μl of 1×10^8 cells/ml *P. caribbica*. Fruits were incubated at 20 °C or at 4 °C. *P. caribbica* was recovered from the wounds after incubation at 20 °C for 0 (1 h after treatment), 1, 2, 3, 4, 5, 6, 7 and 8 days, and at 4 °C for 0 (1 h after treatment), 3, 6, 9, 12, 15, 18 and 23 days, respectively. The wounded tissue was removed with a sterile cork borer (9 mm diameter) to a depth of 10 mm and macerated in 50 ml of sterile 0.85% sodium chloride solution in an Erlenmeyer flask using a glass rod. 10-fold serial dilutions were made and 0.1 ml of each dilution was spread by coated rod on the NYDA plates. These plates were incubated at 28 °C for 2 days and the colonies were counted. Population densities of yeast were expressed as $\log_{10}$ CFU per wound (CFU: colony forming units).