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Combination of *Pichia membranifaciens* and ammonium molybdate for controlling blue mould caused by *Penicillium expansum* in peach fruit

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ABSTRACT

The potential enhancement of *Pichia membranifaciens* by ammonium molybdate (NH_4Mo) to control blue mould caused by *Penicillium expansum* on peach fruit was investigated. Combining *P. membranifaciens* at 1×10^8 cell/ml with 1 mM NH_4Mo provided a more effective control of blue mould rot than applying the yeast or NH_4Mo alone. Addition of 1 mM NH_4Mo significantly increased the growth of *P. membranifaciens* in peach wounds, but did not affect the population in nutrient yeast dextrose broth medium. The *in vitro* experiment showed that the combined treatment inhibited spore germination and germ tube elongation of *P. expansum* in comparison with the treatment of *P. membranifaciens* or NH_4Mo alone. Moreover, *P. membranifaciens*, NH_4Mo, and the combination of them did not impair the quality parameters including fruit firmness and content of total soluble solids, titratable acidity and vitamin C of peach fruit after 6 days of storage at 20 °C. These results suggested that the use of NH_4Mo is a useful approach to improve the efficacy of *P. membranifaciens* for postharvest disease control in peach fruit.

1. Introduction

Blue mould caused by *Penicillium expansum*, is the major postharvest disease of peaches in China (Fan and Tian, 2000; Zhang et al., 2007). Currently, chemical fungicides are commonly used to prevent peach fruit from fungal infection and extend shelf life (Fernandez-Trujillo et al., 1998; Karabulut et al., 2002). However, problems related to development of pathogen resistance to many site specific fungicides and potentially harmful effects on the human safety and environment protection have stimulated research to look for alternative methods for disease control (Ragsdale and Sisler, 1994).

Biological control with microbial antagonists has emerged as a promising alternative, either alone or as part of integrated pest management to reduce synthetic fungicide usage (Wisniewski and Wilson, 1992). At present, a new yeast antagonist, *Pichia membranifaciens* Hansen, has been evaluated as a potential biological control agent for suppressing *Rhizopus* rot of peach and nectarine fruit (Fan and Tian, 2000; Tian et al., 2002), and mould decay in sweet cherry fruit (Qin et al., 2004; Chan and Tian, 2006), as well as anthracnose rot in loquat fruit (Cao et al., 2008a). However, when used alone, the biocontrol efficacy of *P. membranifaciens* is not as great as that of fungicides (Tian et al., 2002). Therefore, from a practical view, the effectiveness of the yeast antagonist must be enhanced.

Great interest has recently been focused on combining antagonists with some food additives. Among them, ammonium molybdate (NH_4Mo) shows broad-spectrum antifungal activity (Nunes et al., 2002a,b). The potential of NH_4Mo for the enhancement of biocontrol ability of antagonists has been investigated in apple, pear and jujube fruit (Nunes et al., 2002a,b; Wan et al., 2003; Wan and Tian, 2005). However, there is no information concerning the effect of NH_4Mo with the yeast *P. membranifaciens* on control of the postharvest diseases in fruit.

The objectives of this study were to evaluate (a) the effect of NH_4Mo at various concentrations and the antagonistic yeast *P. membranifaciens* used separately or in combination, on controlling postharvest blue mould decay of peach fruit caused by *P. expansum*; (b) the effect of NH_4Mo, used alone or in combination with *P. membranifaciens* on spore germination of *P. expansum in vitro*; (c) the effect of NH_4Mo on population dynamics of *P. membranifaciens in vivo* and *in vitro*; and (d) the efficacy of NH_4Mo and *P. membranifaciens*, separately or in combination, on quality of peach after storage, including firmness and total soluble solids (TSS) content, titratable acidity (TA) and vitamin C.

2. Materials and methods

2.1. Microorganisms, fruit and treatments

P. expansum was isolated from infected peach fruit. The culture was maintained on potato-dextrose agar medium (PDA: extract of

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boiled potatoes, 200 ml; dextrose, 20 g; agar, 20 g and distilled water, 800 ml). Spores of *P. expansum* were obtained from 2-week-old cultures incubated at 25 °C by flooding the cultures with sterile-distilled water containing 0.05% (v/v) Tween 80, and filtered through four layers of sterilised cheesecloth. The concentration of spores was adjusted to 1×10^5 spores/ml with a haemocytometer.

A strain of the yeast *P. membranifaciens* was obtained from the Institute of Microbiology, Chinese Academy of Science (Beijing, P.R. China). The yeast cultures were maintained at 4 °C on Nutrient Yeast Dextrose Agar (NYDA) medium containing 8 g nutrient broth, 5 g yeast extract, 10 g glucose and 20 g agar, in 1 l of distilled water. The yeast was transferred from NYDA with a sterile bacteriological loop to NYD Broth (NYDB: NYDA without agar), and then cultured in 250 ml conical flasks containing 50 ml of NYDB in a rotary shaker at 28 °C for 48 h. Following incubation, cells were centrifuged at $6000\times g$ for 10 min and washed twice with sterile-distilled water in order to remove the growth medium. The yeast was resuspended in sterile-distilled water and adjusted to a concentration of 1×10^8 cell/ml with a haemocytometer.

Peach fruit (*Prunus persica* Batsch cv Baifeng) were hand-harvested at firm-mature stage from a commercial orchard in Nanjing, China, and selected for uniform size, colour and absence of defects. Fruits were disinfected with 2% (v/v) sodium hypochlorite for 2 min, washed with tap water, and air dried prior to wounding. Disinfected fruits were wounded at two sites with a dissecting needle (2 mm diameter × 4 mm deep). An aliquot of 20 µl of each treatment was applied to the wounds, followed by inoculation with 15 µl of 1×10^5 spores/ml suspension of *P. expansum*. Treatments consisted of (i) sterile-distilled water; (ii) *P. membranifaciens* at 1×10^8 cell/ml; (iii) aqueous solutions of NH_4Mo at 1, 5 or 15 mM alone or in combination with *P. membranifaciens* at 1×10^8 cell/ml. The fruits were sealed in polyethylene bags to retain about 95% relative humidity and incubated at 20 °C for 6 days. The percentage of infected wound and lesion diameter were measured 3 and 6 days after inoculation. There were three replicates of 15 fruit each per treatment, and the experiment was conducted three times.

2.2. In vitro effect of NH_4Mo on growth of *P. membranifaciens*

Following the method of Wan and Tian (2005), aliquots of 50 ml of NYDB, with or without NH_4Mo at 1, 5 or 15 mM, in 250 ml conical flasks were autoclaved (121 °C, 15 min) and 100 µl of the suspension of *P. membranifaciens* (1×10^8 cell/ml) was added in the above solutions. The number of colony-forming unit (CFU) of the yeast was determined by dilution-plating at 0, 1, 2, and 3 days after incubation on a rotary shaker at 160 rpm at 28 °C and expressed as Log_{10} CFU/ml. Each treatment was replicated three times and the experiment was repeated twice.

2.3. Effect of NH_4Mo on growth of *P. membranifaciens* in peach wounds

Two wounds were made on each fruit, then 20 µl of *P. membranifaciens* (1×10^8 cell/ml), alone or in combination with NH_4Mo at 1, 5 or 15 mM was injected into each wound. Fruits were incubated at 20 °C (90% relative humidity). *P. membranifaciens* was recovered from the wounds 1 h after inoculation at 20 °C (time 0) and after 2, 4 and 6 days. Wounded tissue was removed with an ethanol-flamed, 5 mm (internal diameter) cork borer and ground in an autoclaved mortar with 5 ml of sterile 0.05 M phosphate buffer (pH 7.0), then plated 0.1 ml of a 10-fold dilution on NYDA. The plates were incubated at 28 °C for 2 days and the colonies were counted. Population densities of *P. membranifaciens* were expressed as Log_{10} CFU/wound. There were three single fruit replicates per treatment, and the experiments were repeated three times.

2.4. In vitro effect of NH_4Mo and *P. membranifaciens* on spore germination of *P. expansum*

The effect of NH_4Mo and *P. membranifaciens* on spore germination of pathogen was tested in potato-dextrose broth (PDB). Aliquots of 100 µl of spore suspensions of *P. expansum* (1×10^5 spores/ml) were added into 10 ml glass tube containing 5 ml of PDB. The PDB contained different concentrations of NH_4Mo (0, 1, 5 and 15 mM) with or without 100 µl of *P. membranifaciens* (1×10^8 cell/ml). All treated tubes were placed on a rotary shaker (100 rpm) at 26 °C. After 12 h incubation, at least 100 spores per replicate were observed microscopically to determine germination rate and germ tube length. Spores were considered germinated when germ tube length was equal to or greater than spore length. Each treatment was replicated three times and the experiment was repeated twice.

2.5. Effect of NH_4Mo and *P. membranifaciens* on quality parameters of peach fruit

Quality parameters of peach fruit treated with NH_4Mo, *P. membranifaciens*, or in combination were measured after storage. The testing methods used were described below.

The firmness of five fruits from each replicate was measured at three points of the equatorial region by using a FT327 firmness tester (Facchini FG, Alfonsine, Italy) fitted with a 5 mm diameter probe. The same five fruits from each replicate were then wrapped in cheesecloth and squeezed using a hand press. The resulting juice was analysed for its TSS and TA. TSS was determined at 20 °C using a portable refractometer (WYT-4; Quanzhou Zhongyou Optical Instrument Co., Ltd., Fujian, China). TA was determined by titrating 20 ml juice to pH 8.2 using 0.1 M NaOH. The vitamin C content of each sample of juice was measured using 2,6-dichloro-indophenol titration, as described by Jones and Hughes (1983). The results were expressed in mg/100 g fresh weight (FW).

2.6. Statistical analysis

Experiments were performed using a completely randomised design. Experimental data were the mean ± SE. All statistical analyses were performed with SPSS (SPSS Inc., Chicago, IL, USA) for this experiment. The data were analysed by one-way analysis of variance (ANOVA) to test the difference of the treatments. Mean separations were performed by Duncan's multiple range tests. Differences at $P<0.05$ were considered as significant.

3. Results

3.1. Effect of NH_4Mo and *P. membranifaciens* on the control of blue mould rot

As shown in Fig. 1, treatment with *P. membranifaciens* at 1×10^8 cell/ml or 1 mM NH_4Mo alone both resulted in significantly ($P<0.05$) smaller lesion diameter and lower disease incidence of blue mould rot on peach fruit wounds inoculated with *P. expansum* compared with the controls during 6 days of incubation at 20 °C. No significant difference of lesion diameter and disease incidence was observed between the two treatments. Treatments with NH_4Mo at 5 or 15 mM alone were not effective in reducing blue mould rot incidence and severity in fruit wounds. However, the combined treatment of *P. membranifaciens* with 1 mM NH_4Mo markedly ($P<0.05$) reduced the lesion diameter and disease incidence in comparison with the treatment of *P. membranifaciens* or NH_4Mo alone. In this combined treatment, the percentage of infected wounds and lesion diameter were reduced significantly ($P<0.05$) from 38.5 to 10.6% and 8.3 to 2.2 mm, respectively, compared with *P. membranifaciens* alone. With increase of the concentration of