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Improvement of *Hanseniaspora uvarum* biocontrol activity against gray mold by the addition of ammonium molybdate and the possible mechanisms involved

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ABSTRACT

The efficacy of *Hanseniaspora uvarum* against gray mold by adding ammonium molybdate (NH_4–Mo) and the mode of actions were evaluated. The results showed that *H. uvarum* at 1×10^6 CFU ml^{-1} plus 1 mmol l^{-1} NH_4–Mo greatly reduced gray mold in grape fruits. NH_4–Mo at concentrations of 1, 5, 10 and 15 mmol l^{-1} significantly inhibited spore germination and mycelium growth of *Botrytis cinerea*. Population growth of *H. uvarum* was markedly inhibited by NH_4–Mo at 5 mmol l^{-1} *in vitro* and not affected by addition of NH_4–Mo at 1 and 5 mmol l^{-1} in wounds combination of NH_4–Mo and *H. uvarum* induced higher activities of peroxidase (POD), polyphenoloxidase (PPO), phenylalanine ammonialyase (PAL), superoxide dismutase (SOD), catalase (CAT) and β-1,3-Glucanase than individual application of *H. uvarum* or NH_4–Mo. The enhancement of disease control may be directly because of the inhibitory effects of NH_4–Mo on spore germination and mycelial growth of *B. cinerea in vitro*, and indirectly because of the induced defense reactions by NH_4–Mo in grape berries.

1. Introduction

Gray mold caused by *Botrytis cinerea* is one of the most destructive postharvest diseases of grapes (Cappellini et al., 1986; Latorre, 2007). Currently, the disease is primarily controlled by sulphur dioxide fumigation (Smilanick et al., 1990) and SO_2 generator pads (Guzev et al., 2008). However, due to the public concern about environmental contamination and human health risks, biological control using microbial antagonists has shown potential as an alternative measure to synthetic fungicides for disease control (Ippolito et al., 2000; Smilanick, 2004). Nevertheless, application of antagonistic microorganisms alone does not provide commercially acceptable control of postharvest diseases (Droby et al., 1998; Spadaro and Gullino, 2004). In order to substitute synthetic fungicides, more environmentally friendly and harmless compounds should be developed as alternative methods for postharvest diseases (Janisiewicz and Korsten, 2002; Droby et al., 2003).

Ammonium molybdate has been reported to play an important role in controlling postharvest decay. Nunes et al. (2002a) observed that the performance of NH_4–Mo combined with *Candida sake* exhibited better control of blue and gray mold on pears than that of individual treatments. Palou et al. (2002) reported that satisfactory control of green and blue molds was obtained by dipping oranges for 150 s in solution of NH_4–Mo at 48 or 53 °C. Biological activity of *Rhodotorula glutinis* and *Cryptococcus laurentii* was enhanced by addition of NH_4–Mo (Wan and Tian, 2005). Qin et al. (2006) reported that biocontrol efficacy of *Pichia membranaefaciens* and *C. laurentii* against brown rot in sweet cherry was improved by the addition of NH_4–Mo when stored in air at 20 °C and 0 °C, and in controlled atmosphere storage with 10% O_2 + 10% CO_2 at 0 °C. Although NH_4–Mo was demonstrated to improve the biological activity of fruit decay, little information was given about the effects of induced host resistance when NH_4–Mo and antagonists were used together.

The objectives of the present work were: (i) to determine the potential of NH_4–Mo to enhance the efficacy of P-2 against *B. cinerea in vivo*; (ii) to assess the effect of NH_4–Mo on the spore germination and mycelial growth of *B. cinerea*; (iii) to evaluate the effect of NH_4–Mo on the growth of *Hanseniaspora uvarum in vitro* and *in vivo*; (iv) to examine the defense-related enzymes in grape treated with *H. uvarum* and NH_4–Mo.

2. Materials and methods

2.1. Strains, chemicals and fruits

B. cinerea, purchased from China Center for Type Culture Collection (CCTCC) in Wuhan University, was maintained on potato dextrose agar (PDA) at 25 °C. Spore suspensions were prepared by

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flooding 7-day-old PDA cultures with sterile distilled water. Spore concentrations were determined with a hemacytometer and adjusted to the required concentrations.

The antagonist *H. uvarum* was isolated from the surface of grape fruits using method of Wilson and Chalutz (1989) and identified by morphological, physiological experiments and ITS analysis (GQ480362). The yeast was cultured in 250-ml Erlenmeyer flasks with 50 ml of yeast peptone dextrose (YPD) on a gyratory shaker at 200 r/min for 48 h at 25 °C. Then the cells were harvested by centrifuging at 6000 g for 10 min and resuspended in sterile distilled water. The cell suspensions were adjusted to concentrations of 1×10^6 or 1×10^8 CFU ml^{-1}, respectively, with a hemocytometer.

Ammonium molybdate [$(NH_4)_6\ Mo_7O_{24}.4H_2O$] (analytical pure), purchased from Beijing Chemical Reagent Company (China), was dissolved at 50 mmol l^{-1} in sterile distilled water. Then, the original solution was diluted to a series of concentrations including 1, 5, 10 and 15 mmol l^{-1}, and sterilized by filtration.

Grape berries (*Vitis vinifera* L. Kyoho) were harvested from the vineyard in Huazhong Agricultural University, and selected on size and the absence of physical injuries or infections. Prior to use, fruits were surface disinfected with 2% (v/v) sodium hypochlorite for 5 min, rinsed with tap water and dried in air.

2.2. Efficacy of NH_4–Mo and *H. uvarum* strain P-2 in controlling gray mold in vivo

The inhibition activity of NH_4–Mo and *H. uvarum* strain P-2 was evaluated by means of two ways:

(a) The fruits were wounded (about 3 mm-deep and 3 mm-diameters) on the equator by sterile dissecting needles. Each wound was added with 20 μl of the treatment suspensions including aqueous solution of NH_4–Mo at 1, 5, 10 and 15 mmol l^{-1}, alone or in combination with P-2 at 1×10^6 CFU ml^{-1}, respectively, P-2 at 1×10^6 and 1×10^8 CFU ml^{-1} and distilled sterilized water control. After 2 h, 20 μl of spore suspensions of *B. cinerea* at 1×10^6 conidia ml^{-1} was added into each wound. All treated fruits were placed in an incubator to maintain a relative humidity of about 95% and keep at 25 °C. Disease incidence was determined by counting the number of infected wounds after 4 d. Each treatment contained three replications with 50 single berries per replication and the entire experiment was repeated twice.

Sample was taken from the fruit treated by NH_4–Mo (1 mmol l^{-1}), P-2 (1×10^6 CFU ml^{-1}) alone or in combination with 1 mmol l^{-1} NH_4–Mo at 0, 24, 48, 72 and 96 h after inoculation for enzyme assays and measurements of protein. Wounds treated with the same amount of distilled sterilized water served as control. Each treatment contained three replicates and the experiment was repeated twice.

(b) Intact fruit were dipped in the solutions of NH_4–Mo (1 mmol l^{-1}), *H. uvarum* (1×10^6 CFU ml^{-1}) alone or in combination for about 5 min, and air dried for 1 h. The fruits treated with water served as control. The treated fruits were placed in ventilated polyethylene bags to retain high humidity at 25 °C. Disease incidence was measured after 10 d. There were three replicates of 200 berries per treatment. The entire experiment was repeated twice.

2.3. Measurement of enzyme activities

All enzyme extract procedures were conducted at 4 °C. Two-gram of flesh samples from five fruits with 0.3 g polyvinyl polypyrrolidone (PVPP) (Biosharp, USA) were ground with 10 ml of 0.1 mol l^{-1} sodium phosphate buffer (pH 6.4) for POD and PPO, 8 ml of 0.005 mol l^{-1} mercaptoethanol–boric acid buffer (pH 8.8) for PAL, 10 ml sodium phosphate buffer (0.05 mol l^{-1}, pH 7.8) for SOD and CAT, and 10 ml citric acid–Na_2HPO_4 buffer (0.1 mol l^{-1}, pH 4.8) for β-1,3-Glucanase. The samples were homogenized and centrifuged at 12,000 rpm for 30 min. The supernatants were used as the crude enzyme source to assay enzyme activities.

POD activity was carried out as described by Zhu et al. (1990), with some modification. The reaction mixture consisted 1 ml crude extract and 3 ml reaction solution (50 ml of 100 mmol l^{-1} sodium phosphate (pH 6.4), 0.028 μl of guaiacol, and 0.019 μl of 3% H_2O_2). The activity was determined by measuring the increase in absorbance at 460 nm.

PPO activity was determined by adding 0.1 ml of enzyme preparation to 5.0 ml of catechol substrate (0.1 mol l^{-1}, in 100 mmol l^{-1} sodium phosphate, pH 6.4) and the increase in absorbance at 398 nm was measured immediately. The enzyme activity of POD and PPO were expressed in units (U) per microgram protein, where 1 unit was defined as ΔA of 0.01 per minute.

PAL activity was assayed referring to the method of Zhu et al. (1990), with some modifications. 0.5 ml of crude extract was incubated with 0.5 ml of L-phenylalanine borate buffer (20 mmol l^{-1}, pH 8.8) and 3.0 ml of distilled water for 30 min at 37 °C. PAL activity was determined by the production of cinnamate, which was measured at 290 nm absorbance. The L-phenylalanine borate buffer was insteaded of distilled water in the control. Specific enzyme activity was defined as nanomoles cinnamic acid per hour per milligram weight.

For the SOD assay (Li, 2000), the reaction mixture (6 ml) contained 50 mmol l^{-1} sodium phosphate buffer (pH 7.8), 130 mmol l^{-1} methionine, 750 μmol l^{-1} nitroblue tetrazolium (NBT), 100 μmol l^{-1} EDTA, 20 μmol l^{-1} riboflavin and 0.2 ml enzyme extract. The mixtures were illuminated by a fluorescent lamp (4000 lx) for 20 min and then the absorbance was determined at 560 nm. Identical solutions held in the dark served as blanks. One unit of SOD was defined as the amount of enzyme that caused a 50% decrease of the SOD-inhibitable NBT reduction.

CAT activity determination was performed according to the method of Beers and Sizer (1952) with slight modifications. The reaction mixture contained 2 ml of sodium phosphate buffer (0.05 mol l^{-1}, pH 7.0), 0.5 ml H_2O_2 and 0.5 ml crude extract. The decomposition of H_2O_2 was measured at 240 nm absorbance. One unit was of 0.01 absorbance change per minute.

β-1,3-Glucanase activity was carried out as described by El-Ghaouth et al. (2003). 1.0 ml of enzyme preparation was incubated with 0.5 ml of 1% laminarin (w/v) for 1 h at 30 °C. Then 50 μl reactive mixture was removed. The reaction was stopped by adding 0.5 ml anthranone–ethyl acetate and 5.0 ml concentrated sulphuric acid and boiling for 1 min on a water bath. The amount of reducing sugars was measured spectrophotometrically at 620 nm. One unit was defined as 1 μg glucose per hour.

Protein concentrations were measured according to the method of Bradford (1976), using bovine serum albumin (BSA) as a standard.

2.4. Effect of NH_4–Mo on spore germination and mycelium growth of *B. cinerea* in vitro

To assess the effect of NH_4–Mo on spore germination of *B. cinerea*, 100 μl of spore suspensions of *B. cinerea* (1×10^6 spores ml^{-1}) was transferred to glass tubes containing 5 ml of potato dextrose broth (PDB) with different concentrations (0, 1, 5, 10 and 15 mmol l^{-1}) of NH_4–Mo. All flasks were put on a gyratory shaker (200 r/min) at 28 °C and incubated for 12 h. Approximately 100 spores per replicate were measured for germination and at least 5 microscope fields were observed. Conidia were considered