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Biocontrol of *Botrytis cinerea* in apple fruit by *Cryptococcus laurentii* and indole-3-acetic acid

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Abstract

This study evaluated the effect of a yeast antagonist *Cryptococcus laurentii* and a plant regulator indole-3-acetic acid (IAA) on inhibition of *Botrytis cinerea* infection in harvested apple fruit. The results showed that the combined treatment with *C. laurentii* and IAA at 20 μg/ml was a more effective approach to reduce the gray mold rot in apple wounds than the *C. laurentii* alone. After 4 days of incubation, gray mold incidence in the combined treatment with *C. laurentii* and IAA was about 18%, which was a 50% reduction in incidence compared to the treatment with *C. laurentii* alone. Although IAA had no direct antifungal activity against *B. cinerea* infection when the time interval between IAA treatment and pathogen inoculation was within 2 h, application of IAA strongly reduced gray mold infection when IAA was applied 24 h prior to inoculation with *B. cinerea* in apple fruit wounds. Moreover, combination of IAA and *C. laurentii* stimulated the activities of superoxide dismutase, catalase and peroxidase with above 1.5-fold higher than that treatment with *C. laurentii* alone at 48 h. Therefore, combination of *C. laurentii* with IAA, which integrated the dual biological activity from the antagonistic yeast and plant regulator, might be developed to be a useful approach to control gray mold in harvested apple fruit.

Keywords: Apple; Biocontrol; *Cryptococcus laurentii*; Gray mold; Indole-3-acetic acid; Postharvest

1. Introduction

Gray mold, caused by *Botrytis cinerea*, is one of the major diseases of apple fruit (Sholberg and Conway, 2004). In recent years, the need for exploration of alternatives to synthetic fungicides has attracted attention because intensive use of synthetic chemical fungicides may give rise to a number of toxicological problems regarding human safety and environment protection (Droby, 2006).

Biological control with antagonistic yeasts is a promising strategy for postharvest diseases control (Janisiewicz and Korsten, 2002; Fravel, 2005) including gray mold in harvested apple fruit (Sansone et al., 2005; Lima et al., 2006). However, at present, most of the potential biocontrol agents alone cannot exert sufficient effectiveness compared with chemical fungicides (Janisiewicz and Korsten, 2002). Indications are that biological control agents must be combined with other disease control strategies to acquire adequate disease control (Sholberg and Conway, 2004).

Auxin, a well-studied plant hormone, regulates many plant growth- and development-associated processes including delay of ripening and senescence in harvested produce (Baldwin, 2003; Ludford, 2003) and induction of resistance in plants (Mayda et al., 2000a,b). Indole-3-acetic acid (IAA) is the earliest identified and the most important auxin in higher plants. In addition to its physiological regulation activity, recent studies have revealed an antifungal activity of IAA against *Saccharomyces cerevisiae* and *Ustilago maydis* (Prusty et al., 2004), *Phytophthora infestans* (Noël et al., 2001) and *Gibberella pulicaris* (Slininger et al., 2004) *in vitro* or *in vivo*. In addition, it was found that application of a

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synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), as a part of a combination treatment, effectively controlled the side rots caused by *Alternaria alternate* and stem end rots caused by *A. alternata*, *Phomopsis* spp. or *Lasiodiplodia* spp. in mango fruit (Kobiler et al., 2001).

However, little information is available evaluating the effect of IAA on fungal diseases of harvested fruit. Though it has been shown that IAA can enhance the control of blue and gray molds on the basis of antagonistic activity of the biocontrol yeast *Cryptococcus laurentii* in pear fruit, the mechanism for the improved fungal diseases inhibition by *C. laurentii* and IAA was still unclear (Yu and Zheng, 2007). The objective of this study was to investigate the potential of *C. laurentii* in combination with IAA for control of gray mold in apple fruit and the possible mechanisms involved.

2. Materials and methods

2.1. Material and microorganisms

Fuji apple fruit (*Malus domestica* Borkh.) were harvested at commercial maturity, selected for uniformity of size and any apparent injury or infection was removed. After being superficially disinfected in 0.1% (vol/vol) sodium hypochlorite for 1 min, fruit samples were rinsed with tap water and allowed to air dry at room temperature (20 °C).

The antagonist *C. laurentii* (Kufferath) Skinner was originally isolated from the surface of pear fruit and identified using VITEK 32 Automicrobic System (bioMerieux Company, Marcy l'Etoile, France) and maintained on nutrient yeast dextrose agar (NYDA, containing 8 g nutrient broth, 5 g yeast extract, 10 g glucose and 20 g agar in 1liter of distilled water) at 4 °C before used. The yeast was grown in 50 ml of nutrient yeast dextrose broth (NYDB) in 250-ml flask for 24 h at 28 °C on a gyratory shaker at 200 rpm and harvested by centrifuging at 3000 rpm for 10 min, then washed twice with sterile distilled water to remove the growth medium. The cell concentration was counted using a hemocytometer and diluted with sterile distilled water as required.

The pathogen *B. cinerea* was originally isolated from an apple fruit and cultured on potato dextrose agar (PDA, containing the extract from 200 g boiled potato, 20 g glucose and 20 g agar in 1liter of distilled water) at 28 °C in the dark. Spore suspension was prepared by flooding 7-day-old sporulating cultures of *B. cinerea* with sterile distilled water. The amount of spores was counted with a hemacytometer and diluted with sterile distilled water as required.

IAA was purchased from a local producer (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). IAA was dissolved with ethanol and the pH was adjusted to 6 with 1 mol/l HCl, then the solution was filter sterilized (0.45 μm) and used immediately.

2.2. Control of B. cinerea by C. laurentii with IAA

The apples were wounded uniformly (approximately 5 mm diameter and 3 mm deep) with a sterile cork borer and treated with 30 μl of one of the following: (1) *C. laurentii* suspension (1×10^8 cells/ml), (2) IAA solution (20 μg/ml), (3) a combination of both above and (4) sterile distilled water as the control. Two hours later, 30 μl of *B. cinerea* spore suspension (1×10^6 spores/ml) were inoculated onto each wound. After air-drying, samples were stored in enclosed plastic trays to maintain a high relative humidity (about 95%) at 20 °C. The number of the infected fruit and their lesion diameters were examined daily. There were four replicates consisting of 24 apples per replicate and the experiment was conducted twice with similar results.

2.3. Control of gray mold by IAA

The fruit were wounded as above and pre-treated with 30 μl of an IAA solution (20 μg/ml) or sterile distilled water as the control. After 2 h or 24 h, each wound was inoculated with 30 μl of *B. cinerea* spore suspension (1×10^6 spores/ml). After air-drying, samples were stored in enclosed plastic trays to maintain a high relative humidity (about 95%) at 20 °C. The number of infected fruit was examined daily. There were four replicates of 24 apples per replicate and the experiment was conducted twice with similar results.

2.4. Effect of IAA on inhibition of B. cinerea in vitro

2.4.1. Conidial germination in potato-dextrose broth (PDB)

Conidia of *B. cinerea* were cultured in PDB with IAA added in a final concentration at 0, 1, 10, 100 or 1000 μg/ml and incubated on a rotary shaker (200 rpm) at 28 °C for 12 h. The number of germinated spores was counted using a hemacytometer and expressed as percent germination. There were three replicates per treatment and three flasks per replicate with at least 150–200 spores per replication for calculation of percent germination.

2.4.2. Conidial survival on PDA

Conidia of *B. cinerea* were incubated in a solution of IAA in a final concentration at 0, 1, 10, 100 or 1000 μg/ml for 60 s, centrifuged and re-suspended in water to remove the IAA solution prior to plating on the agar medium, then 100 μl of each suspension were plated on NYDA. After 72 h of incubation at 28 °C, the number of colonies per plate was counted and the results were expressed as the mean number of colony forming units (CFU) per plate. There were three replicates per treatment and four plates per replicate.

2.5. Host enzyme activities

Fruit samples were wounded as described above. Then, each wound was treated with 30 μl of one of the following: