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Boron improves the biocontrol activity of *Cryptococcus laurentii* against *Penicillium expansum* in jujube fruit

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ABSTRACT

Boron in the form of potassium tetraborate was effective for control of blue mold rot caused by *Penicillium expansum* in jujube fruit. The control activity was positively correlated with the concentration of boron solution. Boron at 0.5% enhanced the biocontrol efficacy of the antagonistic yeast *Cryptococcus laurentii* against *P. expansum*. Analysis of population dynamics demonstrated that growth of *C. laurentii* was not significantly influenced by boron in the fruit wounds. *C. laurentii* multiplied quickly, regardless of whether the yeast was used alone or combined with boron. An *in vitro* study showed that boron at 0.25% even stimulated the growth of *C. laurentii* at the end of incubation period. By comparison, mycelial spread of *P. expansum* in the culture medium was completely inhibited by boron at 0.25%. Using the fluorescent probe rhodamine 123, we found that the mitochondrial membrane potential collapsed significantly after boron treatment. This indicated that boron inhibited the growth of *P. expansum* by targeting the mitochondria of the fungal pathogen. Taken together, our data suggest that the enhancement in biocontrol efficacy of *C. laurentii* may be related to the differential influence of boron on the antagonistic yeast and the fungal pathogen.

1. Introduction

Postharvest diseases caused by pathogens result in significant losses in fruit and have been controlled mainly by synthetic fungicides. However, the use of fungicides is becoming increasingly restricted because of concerns for environment and health, as well as the development of fungicide resistance by pathogens (Usall et al., 2000). A search for new alternatives to synthetic fungicides to reduce postharvest decay is becoming increasingly important (Conway et al., 2004). A number of alternatives, including near-harvest spraying of biological control agents (Karabulut et al., 2003), prestorage heat treatments (Conway et al., 2004), and postharvest application of calcium chloride (Conway et al., 1991), bicarbonates (Mlikota Gabler and Smilanick, 2001), chitosan (Xu et al., 2007), or ethanol (Lichter et al., 2002), have been shown to protect fruit against postharvest pathogens.

Biological control using microbial antagonists has emerged as one of the most promising alternatives to fungicides (Spadaro and Gullino, 2004; Janisiewicz et al., 2010; Li et al., 2011). The mechanisms by which antagonistic yeasts exhibit biological control ability are suggested to be competition for space and nutrients (Janisiewicz et al., 2000), production of cell wall lytic enzymes (Bar-Shimon et al., 2004), and induction of host resistance (El Ghaouth et al., 2003). Several biological control agents have been widely investigated for controlling diseases on different fruit crops. However, biological control alone often cannot provide adequate control of fruit decay and must be combined with diluted fungicides or other methods of control (Janisiewicz and Korsten, 2002). For example, Droby et al. (2003) have shown that biocontrol activity of *Candida oleophila* against *Penicillium expansum* and *Botrytis cinerea* in apples and *Monilinia fructicola* and *Rhizopus stolonifer* in peaches could be enhanced by the addition of sodium bicarbonate. Liu et al. (2011) observed that the performance of antagonistic yeast *Cystofilobasidium infirmominiatum* combined with glycine betaine exhibited better control of blue mold caused by *P. expansum* on apple fruit than that of yeast treatment alone. It has also been shown that the biocontrol activity of a well-known yeast antagonist *Cryptococcus laurentii* can be enhanced by combining it with other methods such as sodium bicarbonate (Wan et al., 2003), heat treatment (Conway et al., 2005), salicylic acid (Yu et al., 2007a), indole-3-acetic acid (Yu et al., 2009), and chitosan (Yu et al., 2007b) in various fruit crops.

Boron is an essential microelement that is required for the normal growth of seed plants (Ishii et al., 2002). Large amounts of boron can also be toxic to plants (Cervilla et al., 2007). Its toxicity to animals is generally slight and natural sources of boron often have

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a much higher boron content than that achieved from deliberate boron applications (Hunt et al., 1991; Anderson et al., 1994; Gentz and Grace, 2006). Boron and its salts were proposed to be exempted from residue tolerances in fresh products (USEPA, 1993), and so when used properly, boron is environmentally safe. More than 80 years ago, borax-boric acid solutions were used to control postharvest decay of citrus fruit (Winston, 1935). It has been reported that boron could be used for the control of disease in grapevines caused by the fungus *Eutypa lata* (Rolshausen and Gubler, 2005), and our previous work has demonstrated that boron was effective in inhibiting *B. cinerea* on table grapes (Qin et al., 2010). The mode of action of boron against *B. cinerea* may be directly related to the disruptive effects of boron on cell membranes of the fungal pathogen. However, little is known about the efficacy of boron on the activity of biological control agents.

Jujube fruit are susceptible to postharvest diseases caused by various pathogenic fungi (Zhu et al., 2010). Blue mold rot, caused by the wound-invading necrotrophic fungus *P. expansum*, is one of the most destructive postharvest diseases of jujube fruit (Qin and Tian, 2004). The objective of this study was to evaluate the synergistic effects of combining boron with the antagonistic yeast *C. laurentii* against blue mold rot caused by *P. expansum* in jujube fruit. The possible mechanisms by which boron enhanced the efficacy of biological control agents were also investigated.

2. Materials and methods

2.1. Fruit

Jujube (*Zizyphus jujuba* cv. Dongzao) fruit were harvested at commercial maturity. Fruit firmness was 45.6 N as determined by a penetrometer (FT-327, UC Fruit Firmness Tester, Milano, Italy), and total soluble solids were 15.4%. Fruit without physical injuries were surface sterilized with 2% (v/v) sodium hypochlorite for 2 min, washed with tap water, and air-dried prior to use.

2.2. Yeast and pathogen

The antagonistic yeast *C. laurentii* (Kufferath) Skinner was isolated from the surface of apple fruit following the method of Wilson and Chalutz (1989) and identified by CABI Bioscience Identification Services (International Mycological Institute, UK). The yeast was cultured in nutrient yeast dextrose broth (NYDB; 8 g of nutrient broth, 5 g of yeast extract, and 10 g of dextrose in 1000 mL water) for 48 h at 25 °C. Yeast cells were collected by centrifugation at 8000 × *g* for 10 min. After washing twice with sterile distilled water, the yeast cells were suspended and adjusted to a concentration of 1×10^7 or 1×10^8 cells/mL with a haemocytometer.

P. expansum was isolated from infected jujube fruit and maintained on potato dextrose agar (PDA). Spores were obtained from 2-week-old PDA cultures incubated at 23 °C. The spores were scraped from the surface of the cultures and suspended in 5 mL of sterile distilled water containing 0.05% (v/v) Tween 80. Spore suspensions were filtered through four layers of sterile cheesecloth to remove any adhering mycelia. Spore concentration was determined with a hemacytometer and adjusted to 5×10^4 spores/mL with sterile distilled water.

2.3. Control of fruit decay with boron at different concentrations

Jujube fruit of uniform size were wounded (3 mm deep × 3 mm wide) at the equator with a sterile nail. Wounded fruit were inoculated with 15 μL of conidial suspension of *P. expansum* at 5×10^4 spores/mL. After air-drying, 20 μL of boron solution in the form of potassium tetraborate (Sigma–Aldrich) was added to the same wound site. Boron solution at different concentrations (0, 0.25, 0.5, 0.75, and 1%, w/v) was applied. Fruit treated with water were used as the control. Fruit were put in 200 mm × 130 mm × 50 mm plastic boxes with sterile water to maintain high humidity (about 95%) and stored at room temperature (25 °C). Fruit decay was measured daily for the occurrence of blue mold. Each treatment contained three replicates (20 fruit per replicate), and the entire experiment was repeated twice.

2.4. Integrated control of blue mold by boron in combination with biological control yeast

Fruit were wounded with a sterile nail at the equator and each wound was added with 20 μL of the treatment suspensions as follows: potassium tetraborate (0.5%, w/v), *C. laurentii* (1×10^7 cells/mL), *C. laurentii* (1×10^8 cells/mL), and *C. laurentii* (1×10^7 cells/mL) + potassium tetraborate (0.5%, w/v). Fruit treated with sterile distilled water served as the control. After the wounds were air-dried, fruit were challenge-inoculated with 15 μL of a conidial suspension of *P. expansum* at 5×10^4 spores/mL. In another experiment, fruit were challenge-inoculated with a *P. expansum* conidial suspension (15 μL) at 5×10^4 spores/mL before the treatment suspensions as described above were added. Treated fruit were stored at 25 °C with high humidity (RH about 95%) and blue mold rot was measured daily after treatment. There were three replicates of each treatment with 20 fruit per replicate, and the experiment was repeated twice.

2.5. Effect of boron on populations of C. laurentii in fruit wounds

Fruit were wounded and 20 μL of a cell suspension of *C. laurentii* at 1×10^7 cells/mL, alone or containing 0.5% (w/v) potassium tetraborate, was added to each wound. Treated fruit were stored at 25 °C with high humidity. Fruit samples were taken with a cork borer at 0, 24, 48, 72, and 96 h after treatment following the method of Janisiewicz et al. (1992). The resulting cylinders of excised tissue (10 mm deep × 10 mm wide) were ground with a pestle in a mortar containing 10 mL of sterile distilled water. Then, 100 μL of the serial tenfold dilution was plated on nutrient yeast dextrose agar (NYDA) medium. After incubation at 25 °C for 72 h, colonies were counted and expressed as $\log_{10}$ colony forming units (CFU)/wound. There were three replicates of each treatment with 5 fruit per replicate and the experiment was conducted three times.

2.6. Effect of boron on growth of C. laurentii in vitro

The effects of boron on the growth of *C. laurentii in vitro* were carried out according to our previous method (Qin et al., 2003), with some modification. Briefly, *C. laurentii* was cultured in NYDB overnight. Then, 50 μL of *C. laurentii* culture was added to 5 mL NYDB containing different concentrations of potassium tetraborate. After culture at 25 °C on a rotary shaker at 200 rpm for indicated time (0, 12, 24, 48, 72, 96, and 120 h), the OD_{600} of the yeasts was measured with a spectrophotometer (UV-160, Shimadzu Corp., Kyoto, Japan). Each treatment was replicated three times and the experiment was repeated twice.

2.7. Effect of boron on mycelial growth of P. expansum

Mycelial growth of *P. expansum* was measured on PDA as we had previously reported (Qin et al., 2010). In brief, a 5 mm diameter plug of mycelial agar obtained from the growing edge of 7-day-old cultures of *P. expansum* was placed in the center of a 9 cm diameter Petri dish containing PDA medium with potassium tetraborate at different concentrations (0, 0.01, 0.05, 0.1, and 0.25%, w/v). Potassium tetraborate solutions were filtered through a Millipore filter