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Integrated control of green mold to reduce chemical treatment in post-harvest citrus fruits

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ABSTRACT

This study shows that *Saccharomyces cerevisiae* (ACB-K1 and ACB-CR1) and *Bacillus subtilis* (ACB-69 and ACB-84) isolates perform differently on the control of green mold (*Penicillium digitatum*), depending on the citrus variety. In 'Murcott' tangor, the yeast ACB-CR1 resulted in 47% of healthy fruits, which increased to 67% when combined with imazalil 0.5 mL L^{-1}. In the 'Hamlin' orange, ACB-CR1 (*S. cerevisiae*) provided 87% control when applied alone. However, when combined with 0.5 and 1.0 mL L^{-1} of fungicide (the lowest doses), the efficiency of ACB-CR1 was decreased, yielding 76 and 78% healthy fruits, respectively. Both yeasts controlled green mold in the 'Tahiti' acid lime by 40% when used as a curative treatment; however, the ACB-K1 isolate that was applied as a preventive measure was the best antagonist, yielding 73% healthy fruits. This yeast increased disease control, with healthy fruit percentages ranging from 84 to 89% when the microorganism was combined with the lowest doses of imazalil. In general, *B. subtilis* isolates provided only slight disease control when tested in the three citrus fruit varieties during this study. However, the results of preventive treatments with bacteria on 'Tahiti' acid lime fruits revealed an improvement in the degree of biocontrol. This study demonstrated the possibility of reducing the imazalil dose during the post-harvest citrus fruit treatment using a biocontrol agent without losing green mold control efficiency under storage conditions (27 °C and 70% RH [relative humidity]). The preventive application provided the best protection to 'Tahiti' acid lime fruits, suggesting that the mode of action of these biocontrol agents is through competition, or even resistance induction, considering the specificity of the antagonist–host relationship within the context of pathogen control. The yeast isolates decreased their antagonistic activity against *P. digitatum* under refrigeration (10 °C and 95% RH). However, the ACB-K1 isolate provided 100% disease control in 'Tahiti' acid lime fruits under these storage conditions when combined with a quarter dose of imazalil, despite its low activity.

1. Introduction

Green mold, which is caused by *Penicillium digitatum* (Pers.:Fr) Sacc., is one of the most significant diseases of post-harvest in citrus fruits causing extensive losses during harvest, transport, and storage processes (Eckert and Eaks, 1989). Synthetic fungicides, including imazalil (IMZ) and thiabendazole (TBZ), have traditionally been used to control green mold (Ismail and Zhang, 2004; Lahlali et al., 2005; Smilanick et al., 2006). However, the efficacy of chemical treatments has often been limited in the face of pathogen resistance to these compounds, as well as concerns about environment contamination and public health. Accordingly, a search for alternative control strategies is needed (Pérez et al., 2011).

The use of antagonistic microorganisms has emerged as a potential alternative to synthetic fungicides for controlling diseases. Previous research showed that the protective activity of the two selected antagonist yeasts, *Rhodosporidium kratochvilovae* (LS11) and *Cryptococcus laurentii* (LS28), is enhanced by combining them with a low dosage of fungicides and/or natural adjuvants, and that such strategies can control both resistant and sensitive strains of fungal pathogens (Lima et al., 2006, 2011). Therefore, selected biocontrol yeasts are very interesting candidates for their utilization in integrated control strategies aimed at reducing the use of fungicides.

Among the biological control agents, *Saccharomyces cerevisiae* and *Bacillus subtilis* have been used for the biocontrol of pathogens that occur during the post-harvest period (Coelho et al., 2003; Leelasuphakul et al., 2008; Sharma et al., 2009; Kupper et al., 2013). The main mechanisms of action of yeasts during post-harvest disease biocontrol include competition for space and nutrients, production of volatile metabolites, enzymes that degrade the plant pathogen cell wall (including β-1,3-glucanase and chitinase), host resistance induction, mycoparasitism, and the 'killer' factor (a toxic peptide) (Coelho et al., 2003; E.I-Tarabily and Sivasithamparam, 2006). The *Bacillus* species have been promising in the control of a variety of fungi that cause plant diseases, and their antagonistic action against pathogens occurs through the production of antibiotics (iturin, surfactin, and fengycin), by enzymes (chitinase, β-1,3-glucanase) that degrade structural polymers of the fungal and by production of volatile antifungal agents (Leelasuphakul et al., 2006; Pinchuk et al., 2002). In certain situations, volatile

Abbreviations: IMZ, imazalil; TBZ, thiabendazole; BCA, biological control agent; PDA, potato dextrose agar; NYDA, nutrient yeast dextrose agar; ANOVA, analysis of variance; CFU, colony forming unit.

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organic compounds that were secreted by *B. subtilis* cells were linked to plant growth and plant systemic resistance induction (Compant et al., 2005). The efficiency of a biological control agent can be enhanced with the addition of chemical control. Errampalli and Brubacher (2006) demonstrated the control of *P. expansum* in apples by integrating the use of *Pseudomonas syringae* with cyprodinil, which provided the best results.

Research on post-harvest disease has focused on application of biocontrol agents after harvest. However, application after harvest may be too late for the biocontrol agents to effectively compete with the decay pathogens already established on or in fruit tissues in the field. Antagonistic microorganisms that have curative action, controlling pre-existing infections, that prevents subsequent infections and retards fungal sporulation are desirable. On the other hand, the ability of the treatments to protect the fruit from future infections (preventive activity) should be evaluated. In commercial situations the reinfection of the same fruit or on healthy fruits may occur during handling and processing within the packing house, where the surface wounds can be infected by the pathogen for several days. The application of products based on curative or preventive forms of antagonistic microorganisms and an understanding of their modes of action against plant pathogens may benefit the field of biocontrol. Usall et al. (2008) reported that the combination of sodium carbonate with *Pantoea agglomerans* (CPA-2) bacteria was more efficient against *P. digitatum.*

Considering all these factors, this study was aimed at evaluating the use of *S. cerevisiae* and *B. subtilis* to control *P. digitatum*, the causal agent of citrus green mold, with or without a chemical product (imazalil).

2. Material and methods

2.1. Cultivars

The cultivars in this study were 'Murcott' tangor, a hybrid between tangerine (*Citrus reticulata* Blanco) and sweet orange (*Citrus sinensis* [L.] Osb.). The fruits were harvested from the Agricultural Experimental Station at the Sylvio Moreira APTA (São Paulo Agribusiness Technology Agency) Citrus Production Center/IAC (Agronomic Institute of Campinas) (Estação Experimental, Centro APTA Citros Sylvio Moreira/IAC), Cordeirópolis, São Paulo, Brazil. A 'Hamlin' orange tree (*C. sinensis* (L.) Osb.) Cv. and 'Tahiti' acid lime (*Citrus latifolia* Tanaka) were purchased from commercial orchards in the state of São Paulo (Brazil). The fruits in these experiments were not subjected to regular commercial treatment, which is usually applied post-harvest, and they were used on the same day or stored up to 2 weeks (5 ± 1 °C and 95% RH [relative humidity]) prior to their use in various assays.

2.2. Pathogen

A highly virulent strain of *P. digitatum* (PF-1) was obtained from decayed oranges and used to artificially inoculate the fruit. Conidial suspensions for fruit inoculation were obtained as follows: the pathogen was grown on potato dextrose agar (PDA) for 7 days at 27 °C. Ten microliters of sterile distilled water with 0.01% Tween 80 was dispensed into Petri dishes. Conidia were scraped from the agar using a sterile loop. The suspension was subsequently transferred to a test tube and was sonicated for 5 min to facilitate conidial suspension, and the concentration was adjusted with the aid of a hemocytometer.

2.3. Antagonists

B. subtilis strains ACB-84 and ACB-69 were obtained from the APTA Center Citros 'Sylvio Moreira' IAC, Cordeirópolis, São Paulo, Brazil. The *S. cerevisiae* strains (ACB-CR1 and ACB-K1) were obtained from the Laboratory for Biochemistry and Plant Pathology at the University of São Paulo (ESALQ), Piracicaba, São Paulo, Brazil. These strains were selected by assay *in vitro* and *in vivo* of antagonistic action of the BCAs against *P. digitatum* (Kupper et al., 2013).

The activated culture was maintained on NYDA (nutrient yeast dextrose agar) medium at 27 °C for 48 h and transferred, and the cell suspension was used as inoculum for mass production in a fermentation system at 27 °C in the dark.

2.4. Preparation and fermentation of biocontrol agents

B. subtilis was grown in the presence of foliar fertilizer under glutamic fermentation of molasses to 0.5% with agitation for 72 h (Bettiol et al., 2005). The residue, which is known as Ajifol®, was used to grow the bacteria because it contains carbon sources, nitrogen, and salts in addition to being low-cost and in common use by citrus orchards. A liquid medium containing potato dextrose was used to produce *S. cerevisiae* with stirring for 72 h.

2.5. In vitro sensitivity of biocontrol agents to imazalil

The aim of this experiment was to evaluate *B. subtilis* (ACB-69 and ACB-84) and *S. cerevisiae* (ACB-CR1 and ACB-K1) for their sensitivity to the imazalil (500 g L^{-1}, active ingredient) fungicide that is used to control green mold with the intention of developing integrated control methods.

B. subtilis and *S. cerevisiae* isolates were grown on NYDA (nutrient yeast dextrose agar) with stirring at 250 rpm at 30 °C for 48 h. A 200 μL aliquot of the microorganism suspension (10^8 CFU mL^{-1}) was subsequently plated on Petri dishes with NYDA medium using a Drigalski handle. The cells from each biological control agent (BCA) were subsequently scattered evenly over the culture medium. In Petri dishes containing the BCAs, two sterilized discs of filter paper (12.7 mm diameter) were placed and were soaked in the fungicide imazalil (IMZ) at concentrations of 0.5; 1.0; 2.0 (as recommended by the manufacturer); 4.0 and 8.0 mL L^{-1}, and five plates were made by treatment.

The cultures were incubated at 27 °C for 72 h. Five replicates were used per treatment. An evaluation was performed on the basis of the presence or absence of an inhibition halo between the filter paper discs and the bacterial or yeast growth.

2.6. Use of biocontrol agents and imazalil in the integrated control of citrus fruit green mold

Citrus fruits from the 'Murcott' tangor, 'Hamlin' orange, and 'Tahiti' acid lime cultivars with no postharvest treatment were washed using a soft sponge, neutral detergent, and water and were surface-disinfected with 0.7% (v/v) sodium hypochlorite for 3 min. The fruits were wounded at two equidistant points on the equatorial region with a sterilized stylus at a depth of 3 mm and inoculated with 20 μL of *P. digitatum* conidial suspension (1×10^5 spores mL^{-1}) 24 h prior to starting the different treatments. A preventive biological control effect was only done for 'Tahiti' acid lime fruits under ambient conditions; therefore, those fruits were treated 24 h prior to inoculation with the plant pathogen.

The treatments were as follows: (i) each BCA was applied separately, with cell suspensions corresponding to 1×10^8 CFU mL^{-1} for 2 min; (ii) imazalil 0.5 mL L^{-1} + fermented broth from each BCA (1×10^8 CFU mL^{-1}) for 2 min; (iii) imazalil 1.0 mL L^{-1} + fermented broth from each BCA (1×10^8 CFU mL^{-1}) for 2 min; (iv) imazalil 2.0 mL L^{-1} (as recommended by the manufacturer); (v) the control, in which fruits were inoculated with *P. digitatum* (1×10^5 spores