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Effects of *Cryptococcus laurentii* (Kufferath) Skinner in combination with sodium bicarbonate on biocontrol of postharvest green mold decay of citrus fruit

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Abstract. The potential of using *Cryptococcus laurentii* alone or in combination with sodium bicarbonate solution for control of *Penicillium digitatum* (green mold) on oranges was investigated. Agar disks of *C. laurentii* NYDA cultures placed on PDA plates seeded with pathogen did not inhibit the growth of *P. digitatum*. Spore germination of *P. digitatum* in PDB was significantly controlled in the presence of living *C. laurentii* cell suspensions. *Cryptococcus laurentii* significantly controlled green mold on oranges after challenge with 5×10^4 spores/ml of *P. digitatum*. The higher the concentrations of the antagonist, the lower the disease incidence regardless of whether the fruit was stored at 20°C for 5 days or 2°C for 50 days. The efficacy of *C. laurentii* for control of green mold was improved when combined with sodium bicarbonate. The combination of sodium bicarbonate and *C. laurentii* could be an alternative to fungicides for control of postharvest green mold disease on citrus fruits.

Keywords: Biocontrol; Citrus fruits; *Cryptococcus laurentii*; Green mold; Sodium bicarbonate.

Introduction

Green mold of citrus, caused by *Penicillium digitatum* (Pers.:Fr.) Sacc., is one of the most economically important postharvest diseases of citrus worldwide (Smilanick et al., 1997; Caccioni et al., 1998; Holmes and Eckert, 1999; Palou et al., 2002). Currently, the disease is primarily controlled by applying synthetic fungicides such as imazalil and thiabendazole (Holmes and Eckert, 1999; Palou et al., 2001). However, alternative methods are needed because of concerns about environmental contamination and human health risks associated with fungicide residues (Wisniewski and Wilson, 1992) and because the widespread use of these chemicals in commercial packinghouses has led to the proliferation of resistant strains of the pathogens (Palou et al., 2002).

Microbial biocontrol agents have shown great potential as an alternative to synthetic fungicides for control of the postharvest decay of fruits and vegetables (Chalutz and Wilson, 1990; Wilson et al., 1991; Janisiewicz and Marchi, 1992; EI-Ghaouth et al., 2000). Strains of the yeast *Cryptococcus laurentii* (Kufferath) Skinner have been studied for the postharvest biological control of gray and blue mold rots of apples (Roberts, 1990a) and other fruits such as strawberries, kiwifruits, and table grapes (Lima et al., 1998), as well as for *Mucor* rot of pears (Roberts, 1990b).

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Sodium bicarbonate (SBC) is another promising alternative to synthetic fungicides (Larrigaudière et al., 2002). SBC holds a generally recognized as safe (GRAS) classification by the United States Food and Drug Administration and is proposed to be exempt from residue tolerances on all agricultural commodities by the United States Environmental Protection Agency (Palou et al., 2001). It was previously reported that SBC salts reduce the development of blue and green mold decay in lemon, orange, and mandarin fruits (Smilanick et al., 1999; Palou et al., 2001, 2002).

Although both of the above-mentioned approaches have been shown to reduce postharvest rots of citrus, each exhibits limitations that can affect its commercial applicability. When used as independent treatments, none of these control methods has been clearly shown to offer a consistently economical level of disease control that would warrant its acceptance as a viable alternative to synthetic fungicides (Porat et al., 2002).

In the near future, the preferred alternative to synthetic fungicide treatments will probably be a combination of different methods (Teixidó et al., 2001). The objective of the present study is to evaluate the effects of combining SBC with the yeast biocontrol agent *C. laurentii*, in controlling green mold decay of citrus fruit.

Materials and Methods

Fruits

Oranges (*Citrus sinensis* [L.] Osbeck) cultivar 'Bendizao' fruits were harvested at commercial maturity

from Huangyan in Zhejiang Province. Fruits were used immediately after harvest or held at 2°C with 92-94% relative humidity for no longer than 2 days before use (Brown et al., 2000). Before each experiment, fruits were washed with tap water and then air dried (He et al., 2003).

Pathogen Inoculum

Penicillium digitatum was isolated from an infected orange and cultured on potato-dextrose agar medium (PDA: extract of boiled potatoes, 200 ml; dextrose, 20 g; agar, 20 g and deionized water, 800 ml). Spore suspensions were prepared by removing the spores from the sporulating edges of a 2-3-week-old culture with a bacteriological loop and suspending them in sterile distilled water. Spore concentration was determined with a hemocytometer, and adjusted as required (Zheng et al., 2004).

Antagonist

The yeast cultures of *C. laurentii* 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. Liquid cultures of the yeast were grown in 250-ml Ehrlenmeyer flasks containing 50 ml of NYD Broth (NYDB: NYDA minus agar) and inoculated with a loop of the culture. Flasks were incubated on a rotary shaker at 28°C for 20 h. Following incubation, cells were centrifuged at 6000 rpm for 10 min and washed twice in order to remove the growth medium. Cell pellets were re-suspended in distilled sterilized water and brought to the initial concentration of 2-5 × 10^9 CFU/ml (CFU: colony-forming units). Cell concentration was then adjusted as needed for different experiments (Wisniewski et al., 1995). Culture filtrates were prepared by filtering centrifuged culture of the antagonist through a 0.2 µm polycarbonate membrane filter. Autoclaved culture was prepared by autoclaving a sample containing yeast in culture broth for 20 min at 120°C. The unwashed cells were grown in the 20-h culture filtrate and adjusted to 10^8 CFU/ml with additional culture filtrate. The washed cell suspension was prepared as described above (Zhang et al., 2003).

Biocontrol Activity of C. laurentii in vitro

To evaluate the interactions between the antagonist and the pathogen in culture, 15-mm-diameter disks from 5-day-old NYDA cultures of *C. laurentii* were cut and then placed on PDA plates seeded with 0.5 ml of a conidial suspension of *P. digitatum*. The effect of the yeast antagonist on the growth of the pathogens was compared with that of *B. subtilis*, a known bacterial antagonist (Chalutz and Wilson, 1990).

The effect of *C. laurentii* on spore germination and germ tube elongation of the pathogen was tested in potato dextrose broth (PDB). A 100-µl quantity of 1×10^8, 1×10^6, or 1×10^4 CFU/ml living yeast cell suspensions, culture filtrate, autoclaved 1×10^8 CFU/ml cell suspension, or sterile distilled water (used as a control) was added to 10 ml glass tubes containing 5 ml PDB. At the same time, aliquot (100 µl) of spore suspensions (1×10^7 spores/ml) of *P. digitatum* was added into each tube. After 20 h incubation at 25°C on a rotary shaker (50 rpm), at least 100 spores per replicate were observed microscopically to determine germination rate and germ tube length. All treatments consisted of three replicates, and the experiments were performed three times (Droby et al., 1997).

Biocontrol of Green Mold Rot with C. laurentii Under Ambient and Cold Storage Conditions

Oranges were wounded with a sterile puncher, making one uniform 7 mm diameter by 3 mm depth wound in the equatorial region of each one. Aliquots of 30 µl of 1×10^6, 1×10^7, 1×10^8 and 1×10^9 CFU/ml washed cell suspension of the antagonist were pipetted to each wound. Wounds treated with sterile distilled water served as a control. Three hours later, 15 µl of 5×10^4 spores/ml of *P. digitatum* was inoculated into each wound. The fruits were placed in polyethylene-lined plastic boxes to retain high humidity. Treated fruits were stored at 20°C for 5 days or 2°C for 50 days, then data were recorded as the percentage of decayed fruits. There were three replicates of 10 fruits per treatment with complete randomization, the experiment was performed three times.

Efficacy of Combining C. laurentii and SBC for Reducing Green Mold Development on Artificially Inoculated Oranges

Suspensions of *C. laurentii* were prepared with washed cells adjusted to 1×10^7, 1×10^8, and 0 CFU/ml either in distilled water or in 2% SBC. Fruits were treated with these suspensions and challenged with pathogen as described above. Fruits were placed in polyethylene-lined plastic boxes to retain high humidity, incubated at 20°C, and observed for percent infection at 5 days after challenge inoculation. There were three replicates of 10 fruits per treatment with complete randomization, and the experiment was performed three times.

Efficacy of Combining C. laurentii and SBC for Reducing Natural Decay Development

Suspensions of *C. laurentii* were prepared with washed cells and adjusted to 1×10^7, 1×10^8, and 0 CFU/ml either in distilled water or in 2% SBC. Fruits were wounded, inoculated by dipping them in different suspensions as described above for 30 sec (without challenge with pathogen), and air dried. The treated fruits were placed in polyethylene-lined plastic boxes to retain high humidity. Fruits were incubated at 20°C and the numbers of infected fruit were determined after 10 days. There were three replicates of 10 fruits per treatment with complete randomization, and the experiment was performed three times.

Statistical Analysis of Data

All statistical analyses were performed using SAS (SAS Institute, Version 6.08, Cary, NC). To test for the effect of

the treatments, the data were analyzed by one-way analysis of variance (ANOVA). Data for percentages of decayed fruits (disease incidence) and percentages of germinated spores (spore germination) were transformed into the arcsine square root values to normalize distribution before analysis of variance (Sukhvibul et al., 1999). Mean separations were performed using the protected least significant difference (LSD) tests (P=0.05). The percentages shown are untransformed data.

Results

Biocontrol Activity of C. laurentii in vitro

Agar disks of *C. laurentii* NYDA cultures placed on PDA plates seeded with pathogens did not inhibit the growth of *P. digitatum*. However, under similar conditions, *B. subtilis* inhibited the growth of *P. digitatum* by forming an inhibition zone (3-to12-mm wide) around the disks.

Spore germination and germ tube elongation of *P. digitatum* in PDB was inhibited in the presence of living *C. laurentii* cell suspensions (Table 1). Culture filtrate did not inhibit spore germination or germ tube elongation, nor did autoclaved culture. The concentrations of living *C. laurentii* cells significantly influenced inhibition of spore germination of *P. digitatum*. The higher the concentration of the antagonist, the lower the spore germination rate and the smaller the germ tube length. When the concentration of the cell suspension of *C. laurentii* reached 1×10^8 CFU/ml, spore germination of *P. digitatum* was completely inhibited.

Biocontrol of Green Mold Rot with C. laurentii Under Ambient and Cold Storage Conditions

Disease incidence on fruits stored at 2°C and on those kept at 20°C was significantly lower than that on the untreated control (Table 2). The higher the concentration of the antagonist, the lower the disease incidence, regardless of whether the fruit was stored at 2°C for 50 d or at 20°C for 5 d. When antagonist concentrations were increased from 1×10^6 CFU/ml to 1×10^9 CFU/ml, the efficacy against the pathogen increased, achieving reductions in disease incidence from 34.5% to 89.7%, and from 20% to 76.7% in fruit stored at 20°C for 5d, and at 2°C for 50 d, respectively.

Enhancement of Biocontrol by C. laurentii with Sodium Bicarbonate on Green Mold Decay Development in Artificially Wounded and Inoculated with Pathogen Fruits

Disease incidence was significantly reduced by combining *C. laurentii* with 2% sodium bicarbonate, as compared to yeast treatments alone (Figure 1). SBC and 1×10^8 CFU/ml *C. laurentii*, as stand-alone treatments, reduced the percentage of decayed fruits from 96.7% in control fruit to 50% and 20%, respectively. In fruit treated with a combination of SBC and 1×10^8 CFU/ml *C. laurentii*, the percentage of decayed fruits fell to 6.7%. Greater control was achieved with 1×10^8 CFU/ml *C. laurentii* than with 1×10^7, with or without SBC.

Table 1. Effect of different concentration of washed cell suspensions, autoclaved cell, and cell-free culture filtrates of *C. laurentii* on spore germination and germ tube elongation of *P. digitatum*.

Treatments[1]	Spore germination (%)	Germ tube length (um)
1×10^4 cfu/ml	64.1b[2]	53.4b
1×10^6 cfu/ml	44.3c	39.1c
1×10^8 cfu/ml	0d	0d
Autoclaved culture	80.9a	67.9a
Culture filtrate	77.8a	67.9a
Control	88.1a	70.1a

[1]Germination rate and germ tube length were measured microscopically after 20 h incubation at 25°C in PDB.
[2]Each value is a mean of three replicates; means followed by different letters in a column are significantly different (P<0.05) according to the protected LSD test.

Table 2. Effects of cell concentration of *C. laurentii* on development of green mold (*P. digitatum*) after inoculation and storage under two temperature regimes.

Yeast concentration (CFU ml^{-1})	Disease incidence (%)[1]	
	2°C	20°C
0	100 a	96.7a
10^6	80 b	63.3b
10^7	73.3b	50 b
10^8	60c	20c
10^9	23.3d	10d

[1]Disease incidence was recorded after 50 days storage at 2°C or after 5 d storage at 20°C after inoculation with *P. digitatum*. Means are the averaged values of three trials. Values followed by different letters in a column are significantly different (P< 0.05) according to the protected LSD test.

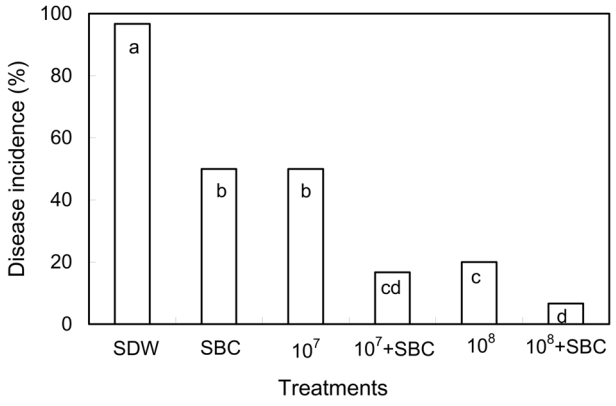

Figure 1. Enhancement of biocontrol of green mold by *C. laurentii* combined with sodium bicarbonate in artificially inoculated fruit. Data were measured 5 days after inoculation with the pathogen at 20°C. Means are the averaged values of three trials. Columns marked with different letters are significantly different at P=0.05 according to the protected LSD test.

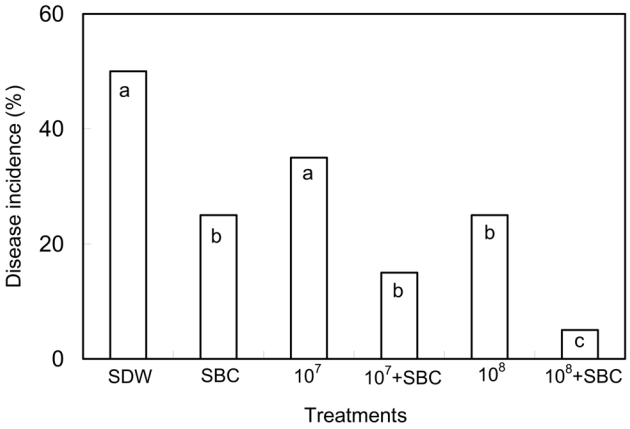

Figure 2. Efficacy of *C. laurentii* and sodium bicarbonate for reducing natural decay development. Data were measured after 10 days of storage at 20°C. Means are the averaged values of three trials. Columns marked with different letters are significantly different at P=0.05 according to the protected LSD test.

Efficacy of C. laurentii and SBC for Reducing Natural Decay Development

After application of 1×10^7 CFU/ml *C. laurentii*, 1×10^8 CFU/ml *C. laurentii* or SBC, alone, on fruit not inoculated with pathogen, the percentages of decayed fruits were 35, 25, and 25%, respectively, significantly lower than those of control fruit (50%) (Figure 2). Combining *C. laurentii* with 2% SBC appeared to have been more effective in inhibiting decay development than either stand-alone. When 1×10^8 CFU/ml *C. laurentii* was combined with 2% SBC, the percentage of decayed fruits was 5%, a 90% reduction compared with control.

Discussion

This study demonstrates that *C. laurentii* can significantly reduce green mold in oranges, caused by *P. digitatum*. The antagonistic activity of *C. laurentii* was dependent on its initial concentration. The higher the concentration of *C. laurentii*, the greater the biocontrol activity. Culture filtrate and autoclaved cell culture co-cultured with pathogen spores did not affect spore germination or germ tube elongation. In associated studies (unpublished data), autoclaved cell culture of *C. laurentii* and its culture filtrate had no antagonist activity against artificially inoculated pathogens on citrus fruits. On PDA plates, *C. laurentii* had no effect on the growth of *P. digitatum*. This suggests that *C. laurentii* does not produce antibiotics in the manner of *B. subtilis* (Chalutz and Wilson, 1990). Therefore, competition for space and nutrients may play a major role in the biocontrol capability of *C. laurenti* against pathogens, as previously demonstrated for other antagonistic yeasts (McLaughlin et al., 1992; Filonow, 1998; Fan and Tian, 2001).

An antagonist is very promising if it can be combined with routine postharvest treatments. Cold storage treatment is a routine practice in orchards to prolong the storage period of fruits. The demonstrated biocontrol effect of *C. laurentii* at low temperature (2°C) indicates that *C. laurentii* can be combined with cold storage to enhance control efficacy.

Laboratory tests revealed that the effectiveness of *C. laurentii* combined with SBC in controlling artificially inoculated *P. digitatum* as well as natural infections on wounded fruit was high. The results of this study indicate the beneficial effect of sodium bicarbonate on the biocontrol activity of *C. laurentii* for control of green mold rot of oranges. Biocontrol agents are poor eradicants of pathogens on citrus and are usually incapable of controlling green mold when fruits are inoculated at least 24 h before treatment (Smilanick and Denis-Arrue, 1992). Control of green and blue molds after inoculation is important because most infections occur through wounds inflicted during or just after harvest (Teixidó et al., 2001). SBC can control these infections (Porat et al., 2002). On the other hand, SBC does not provide persistent protection of the fruit from re-infection after treatment, while biological control antagonists persist for long periods after treatment and protect fruits from re-infection (Smilanick et al., 1999). In short, combination of SBC with the biological control agent overcomes significant limitations of either of these treatments alone. A combination of *C. laurentii* and SBC could be a reliable solution to control green mold rot on oranges.

Overall, this study offers a viable strategy by which combinations of different alternative control methods, whose modes of action complement one another, can effectively control postharvest decay of citrus fruit. Our results do not fully assess the control efficacy of *C. laurentii* under commercial practices. Further study should focus on testing the efficacy of combined application of *C. laurentii* with SBC under commercial practices as well as combined application of *C. laurentii* with other control strategies, such as controlled-atmosphere storage and heat treatment, so as to obtain even better control.

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羅倫隱球酵母與碳酸氫鈉結合使用對柑桔採後綠黴病的生物防治效果

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本文測定了羅倫隱球酵母單獨使用或與碳酸氫鈉結合使用對柑桔綠黴病的防治效果。在馬鈴薯葡萄糖瓊脂固體培養基 (PDA) 體外抑菌實驗中，把生長有羅倫隱球酵母的營養酵母葡萄糖固體培養基 (NYDA) 切塊放置在塗布有指狀青黴孢子的馬鈴薯葡萄糖瓊脂固體培養基上一起培養，羅倫隱球酵母不能抑制黴菌的生長；而在馬鈴薯葡萄糖液體培養基 (PDB) 體外抑菌實驗中，羅倫隱球酵母活細胞有效的抑制了黴菌孢子的萌發。在接種 5×10^4 個/毫升的指狀青黴孢子後，羅倫隱球酵母顯著地抑制了綠黴病的發展。無論柑桔在 20℃下貯存 5天或在 2℃下貯存 50天，拮抗酵母的濃度越高，綠黴病的發病率越低。碳酸氫鈉可以加強羅倫隱球酵母的抑菌效果。將碳酸氫鈉和羅倫隱球酵母結合使用可以取代殺菌劑用於柑桔採後綠黴病的防治。

關鍵詞：羅倫隱球酵母；碳酸氫鈉；綠黴病；柑桔；生物防治。