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# Combination of *Kluyveromyces marxianus* and sodium bicarbonate for controlling green mold of citrus fruit

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ABSTRACT

Biocontrol efficacy of an antagonistic yeast *Kluyveromyces marxianus* was evaluated individually or in combination with sodium bicarbonate (SBC) against green mold of citrus fruit caused by *Penicillium digitatum*. Their effects on postharvest quality of citrus fruit were also investigated. The results indicated that the antagonistic activity of *K. marxianus* at $1\times10^8$ CFU/mL on green mold of citrus fruit was enhanced by 2% SBC treatment. In artificial inoculation trials, disease control after 3 and 6 days, respectively, with the mixture of *K. marxianus* and 2% SBC (18.33%, 58.33%) was significantly improved over that obtained with *K. marxianus* (41.67%, 70.00%) or SBC (43.33%, 81.67%) alone. The combination of *K. marxianus* with SBC was as effective as the imazalil treatment in natural infection trials, which gave about 90% control of green mold. Addition of 2% SBC significantly stimulated the growth of *K. marxianus* in citrus fruit wounds after 72 h. Moreover, *K. marxianus*, SBC and their combination did not impair quality parameters including weight loss, fruit firmness, total soluble solids, titratable acidity and ascorbic acid at 4 °C for 30 days followed by 20 °C for 15 days. These results suggested that the use of SBC is a useful approach to improve the efficacy of *K. marxianus* for the postharvest green mold of citrus fruit.



## 1. Introduction

Green mold, caused by *Penicillium digitatum*, is one of the most important postharvest diseases of citrus fruit that often causes extensive losses during storage and transportation (Eckert and Eaks, 1989). Synthetic fungicides such as imazalil (IMZ), thiabendazole (TBZ) and sodium o-phenylphenol (SOPP) are traditionally used to control green mold, and have played an important role for the management of *P. digitatum* of citrus fruit (Lahlali et al., 2004, 2005; Smilanick et al., 2005, 2006; Ismail and Zhang, 2004). However, chemical treatment efficacy is frequently decreased by development of postharvest pathogen resistance (Holmes and Eckert, 1999). There is also increasing concern about the environmental contamination and human health problems caused by synthetic fungicides due to possible toxicological risks. Therefore, the need for alternative strategy to control postharvest disease of citrus fruits is urgent.

Antagonistic yeasts isolated from the surface of fruits and vegetables have emerged as alternative methods with great potential to control postharvest diseases (Wisniewski and Wilson, 1992). Microorganisms from these locations have been consumed by humans for a long period of time and have not shown negative effects on the human body. Antagonistic yeasts grow rapidly, colonize fruit surfaces and limit nutrient availability to pathogens that would cause damage to fruits and vegetables (Richard and Prusky, 2002). These advantages should aid registration of antagonistic yeasts. One such yeast, *Debaryomyces hansenii* has been reported to control green and blue mold of citrus fruit (Singh, 2002; Chalutz and Wilson, 1990), and reduce postharvest decays on many other fruits, such as Rhizopus rot caused by *Rhizopus stolonifer* of peach fruit. The efficiency of a biocontrol agent can be enhanced by physical treatment such as hot water, or in combination with other substances generally recognized as safe (GRAS), such as calcium salts (Mandal et al., 2007; Singh, 2004). *Pichia anomala* and *Pichia guilliermondii* are two other yeasts commonly used for controlling postharvest green mold of citrus fruit (Lahlali et al., 2004; 2011a, 2011b; Wilson and Chalutz, 1989). Some antagonistic yeasts such as *Candida oleophila*, *Cryptococcus albidus*, *Metschnikowia fructicola* and *Candida sake* are available on the market (Janisiewicz and Korsten, 2002; Fravel, 2005; Lahlali et al., 2011a, 2011b). The main mechanisms of antagonistic yeasts in the control of postharvest diseases include the competition for nutrients and space, production of antifungal diffusible and volatile metabolites, production of cell wall-degrading enzymes such as β-1, 3-glucanase and chitinase, induction of host resistance, and mycoparasitism (El-Tarabily and Sivasithamparam, 2006).

In our previous studies, one antagonistic yeast was isolated from surface of papaya fruit, and identified as *Kluyveromyces marxianus* (Geng et al., 2011). Unfortunately, like other biocontrol yeasts reported, when used alone it did not reduce postharvest decays as effectively as synthetic fungicides (Tian et al., 2002; Janisiewicz and Korsten, 2002; Spadaro and Gullino, 2004). Therefore, the effectiveness of antagonistic yeast needs to be enhanced before application.

The objectives of the present study were to evaluate: (1) the use of *K. marxianus*, as a stand-alone treatment and in combination with the

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application of SBC for the control of green mold decay of citrus fruit; (2) the effect of SBC on the growth of *K. marxianus in vitro* and *in vivo*; (3) the control efficiency of SBC and *K. marxianus*, separately or in combination, on quality of citrus after storage, including weight loss, firmness, soluble solids, titratable acidity and ascorbic acids.

## 2. Materials and methods

### 2.1. Chemicals and fruit

Sodium bicarbonate was purchased from Guangzhou Chemical Reagent Company (Guangzhou, China), and it was used at a concentration of 2% (weight/volume, *w*/*v*).

Imazalil (95% *w*/*v* technical grade, Gengreen Ltd., China) was used at 300 μg/mL.

Mandarin (*Citrus reticulata* Blanco cv. Wuzishatangju) fruit used were harvested from an orchard of Sihui city, China. Fruit were classified according to uniformity of size and maturity without wounds or rot. All fruits were surface-disinfected by immersion for 2 min in 2% sodium hypochlorite, rinsed with tap water, and allowed to air-dry at room temperature (25 °C).

### 2.2. Pathogen

The pathogen *Penicillium digitatum* was originally isolated from a citrus fruit showing the typical green mold. Cultures were maintained on potato-dextrose agar medium (PDA) (extract of boiled potatoes, 200 mL; dextrose, 20 g; agar, 20 g; and distilled water, 800 mL at 4 °C). Fresh cultures were grown on PDA plates before use. Spore suspensions were prepared by removing the spores from the sporulating edges of a 7-day old culture with a bacteriological loop, and suspending them in sterile distilled water. Spore concentrations were determined with a haemocytometer, and adjusted to $1\times10^5$ spores/mL as required with sterile distilled water.

### 2.3. Antagonistic yeast

The strain of *K.marxianus* used in this study was originally isolated from surface of papaya fruit in the horticulture orchard of South China Agricultural University. It was identified as *K. marxianus* based on 18S rDNA sequence homology (Genbank accession number: HQ414234). The yeast *K. marxianus* was cultivated in 250 mL Erlenmeyer flasks with 50 mL of nutrient yeast dextrose broth (NYDB) (8 g nutrient broth, 5 g yeast extract, and 10 g dextrose in 1 L distilled water) on a rotary shaker at 200 rpm for 2 days at 28 °C. The media were centrifuged at 6000 rpm for 10 min. The yeast cells were resuspended in sterile distilled water and adjusted to a concentration of $1\times10^8$ CFU /mL using a haemocytometer.

### 2.4. Effect of K. marxianus and sodium bicarbonate in controlling green mold in vivo

The biocontrol efficiency of *K. marxianus* and SBC was carried out in two ways:

(a) Artificial inoculation trials: In this experiment, all fruits were wounded (about 3 mm-deep and 3 mm-diameter) on the equatorial zone using the tip of a sterile dissecting needle. Each wound was inoculated with 20 μL of the treatment suspensions as follows: CK (sterile distilled water), *K. marxianus* ($1\times10^8$ CFU/mL), *K. marxianus* ($1\times10^8$ CFU/mL) + 2% SBC, 2% SBC and Imazalil (300 μg/mL). Three hours later, 15 μL of spore suspension of *P. digitatum* at $1\times10^5$ spores/mL was inoculated to each wound, respectively. After application as described above, all treated fruits were placed into trays and stored at 25 °C for 6 days (Yao et al., 2004). Disease incidence and lesion diameter were recorded after 3 days and 6 days, respectively. Each treatment included 30 fruits and the entire trials were repeated twice.

(b) Natural infection trials: Intact fruits were drenched in treatment solutions as described above for about 3 min, and air dried at room temperature (25 °C) for 2 h. All fruits were sealed in polyethylene-lined plastic boxes to retain high humidity (90%). Then the treatments were allowed to simulate disease development under normal shelf-life conditions, stored for 30 days at 4 °C followed by 20 °C for 15 days. The numbers of decayed fruits were recorded afterwards. There were three replicates of 100 citrus fruit per treatment. This experiment was repeated twice.

### 2.5. Effect of K. marxianus and SBC on spore germination of P. digitatum in vitro

The effect of *K. marxianus* and SBC on germination of spores of the pathogen was evaluated in potato-dextrose broth (PDB). Aliquots of 100 μL of spore suspensions of *P. digitatum* ($1\times10^5$ spores/mL) were placed into glass tubes containing 5 mL of PDB. The PDB contained treatment solutions as follows: *K. marxianus* ($1\times10^8$ CFU/mL), *K. marxianus* ($1\times10^8$ CFU/mL) + 2% SBC, 2% SBC and CK (sterile distilled water). All treated tubes were put on a rotary shaker (80 rpm) at 25 °C for 24 h. About 120 spores per replicate were observed microscopically to determine germination. Spores were considered to be germinated when germ tubes exceeded the spore diameter. Each treatment included three replicates and the experiment was repeated twice.

### 2.6. Effect of SBC on growth of K. marxianus in vitro and in vivo

The effect of SBC on growth of *K. marxianus in vitro* was carried out according to Liu et al. (2010), with some modifications. Flasks (250 mL) containing 50 mL of NYDB were autoclaved (120 °C, 15 min) prior to adding 0 and 2% SBC to each flask, respectively. 1 mL of *K. marxianus* suspensions was placed into the above solutions to reach an initial concentration of $1\times10^5$ CFU/mL, and incubated on a rotary shaker at 160 rpm at 28 °C for 120 h. The number of yeasts was determined by dilution-plating technique every 24 h and expressed as $Log_{10}$CFU/mL. Each treatment included three replicates and the experiment was repeated twice.

To determine the effect of SBC on population of *K. marxianus in vivo*, two wounds were made on each fruit as described above, each wound was inoculated with 20 μL of *K. marxianus* at $1\times10^6$ CFU/mL, alone or in combination with 2% SBC, Treated fruit were placed at 28 °C and 90% relative humidity. Fruit samples were taken as described by Janisiewicz et al. (1992), with some modifications. Wounded tissue was removed from five single fruits with a sterile cork border (5 mm deep × 5 mm wide) every 24 h and homogenized in 5 mL sterile distilled water. Then 100 μL of serial 10-fold dilutions was plated on Nutrient Yeast Dextrose Agar (NYDA) (8 g nutrient broth, 5 g yeast extract, 10 g dextrose and 20 g agar in 1 L distilled water). The plates were inoculated at 28 °C for 48 h and the colonies were counted. Colony counts were expressed as $Log_{10}$CFU/mL. Each treatment included three replicates and the experiment was repeated two times.

### 2.7. Effect of K. marxianus in combination with SBC on postharvest quality parameters of citrus fruit

Measurement of quality parameters of citrus fruit treated with *K. marxianus*, SBC, or in combination were made on fruit from the natural infection trials, but stored for 14 days at 25 °C and 95% RH. The experiment was repeated three times.