

## INTEGRATED CONTROL OF *PENICILLIUM DIGITATUM* BY THE PREDACIOUS YEAST *SACCHAROMYCOPSIS CRATAEGENSIS* AND SODIUM BICARBONATE ON ORANGES

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### ABSTRACT

Our investigation of integrated biological control (IBC) started with an assay testing activity of the predacious yeast *Saccharomyces crataegensis* UFMG-DC19.2 against *Penicillium digitatum* LCP 4354, a very aggressive fungus that causes postharvest decay in oranges. Under unfavourable environmental conditions, the yeast showed a high potential for control (39.9% disease severity reduction) of this fungus. This result was decisive for the next step, in which *S. crataegensis* was tested in association with sodium bicarbonate salt, a generally regarded as safe (GRAS) substance. The yeast was able to survive at different concentrations of the salt (1%, 2% and 5%), and continued to grow for a week at the wound site, remaining viable at high population for 14 days on the fruit surface. The yeast alone reduced the severity of decay by 41.7% and sodium bicarbonate alone reduced severity of decay by 19.8%, whereas the application of both led to a delay in the development of symptoms from 2 to 10 days. Ingredients of the formulations were not aggressive to fruits since no lesions were produced in control experiments.

**Key words:** biological control, *Saccharomyces*, *Penicillium*, postharvest disease, orange.

### INTRODUCTION

Biological control of postharvest diseases has been used as an alternative for synthetic fungicides mainly against fruit decays (15, 18). The mechanism of this biological control is based on ecological interactions, such as competition for space and nutrients, mycoparasitism, antibiosis, predation or induction of plant defenses (15, 19, 20, 23). Traditionally, yeasts are the organism used most frequently as biocontrol agents due to their fast colonization of plant surfaces and their production of extracellular polysaccharides that enhance their survival and to restrain both colonization sites and the flow of

germination cues to pathogen propagules (13). They use available nutrients to rapidly proliferate and to stay viable under different environmental conditions, seldom produce chemical antagonistic substances and are less affected by pesticides than filamentous (4, 9, 10, 14, 16, 23). However, for the most effective control of decays, biological control can be integrated with other alternative methods. This approach includes the utilization of a biological control agent in combination with substances Generally Regarded as Safe (GRAS) or other methods. Sodium carbonate, sodium bicarbonate, calcium chloride, and ethyl alcohol are GRAS substances frequently utilized in IBC programs (15). Other

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strategies utilize physical methods, such as UV irradiation, hot or cold water treatments, and modified atmosphere packaging (3, 18, 29). Some types of pathogens are satisfactorily controlled only when the additive or synergistic effect of yeast and GRAS substances take place. Primary pathogens, like *Penicillium*, *Aspergillus* and *Rhizopus* species, are non-specific and very aggressive, and because of this they are difficult to control. *Penicillium digitatum* grows saprophytically in soil and it is commonly isolated from plant material. It causes green mold of oranges, and is one of the most important disease of citrus fruit (25). The objectives of the present work was to study control of green mold of citrus after harvest using *Saccharomycopsis* species, determining population dynamics of *S. crataegensis* in fruit wounds, and evaluating efficacy of combination of the yeasts with sodium bicarbonate in controlling green mold caused by *P. digitatum* on oranges at room temperature.

## MATERIALS AND METHODS

### Pathogen

*P. digitatum* (LCP 004354) was obtained as a gracious gift from the Laboratoire Cryptogamie – Paris and maintained on Potato Dextrose Agar (PDA) slants at 4°C under mineral oil. To prepare conidial suspension the culture was grown on PDA for 7 -14 days and washed with 10 mL of GY broth (glucose 1% and yeast extract 0.01%) added with 20 µL of Tween 20 to harvest conidia and concentration of the conidia was adjusted with haemocytometer resulting in five ml of the suspension containing 10<sup>4</sup> conidia/ml. This conidia suspension was used to inoculate orange fruits.

### Antagonistic yeast

The antagonistic yeast *S. crataegensis* UFMG-DC19.2 was isolated from a tropical fruit (*Acrocomia aculeata*) collected in the Ecological Reserve of the Universidade Federal de Minas Gerais, Belo Horizonte, Brazil. The cultures were purified on YM agar (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose and 2% agar) and maintained on GYMP broth (2% glucose, 0.5 yeast extract, 1% malt Extract

and 0.2% Na<sub>2</sub>PO<sub>4</sub>) supplemented with 20% glycerol frozen at -80°C. The yeasts were grown at 25°C for 48 h on YM agar, collected with a bacteriological loop and suspended in 10 ml of GY broth supplemented with 500 µl of Tween 20. Cell concentration was determined with a haemocytometer and adjusted to 10<sup>8</sup> cells/ml (23).

### Fruits

Ripe oranges [*Citrus sinensis* (L.) Osbeck variety Pêra Rio] of approximately 20 cm in circumference were acquired from a local commercial orchard and selected for lack of lesions or injuries. The fruits were superficially disinfected by immersion in 1% sodium hypochlorite for 3 min, rinsed with sterile water and dried in a sterile chamber. When dry, a wound was made around the entire equatorial region of each orange with a sterile blade. The wound was approximately 3 mm wide and 1 mm deep. The pathogen and/or the antagonistic yeast were inoculated into these wounds.

### Biological control assay

Aliquots of 200 µl of pathogen suspension were deposited with a sterile pipette on the orange wound immediately after wounding and allowed to dry under aseptic conditions. After pathogen inoculation, 200 µl of a suspension of biocontrol agent was also deposited on the wound. The inoculated fruits were incubated in a 100% RH humidity chamber at 25°C for 10 days to simulate the best conditions for the growth of the pathogen. Oranges inoculated with the pathogen alone served as a positive control. Negative controls were performed by inoculation of GY without yeast or pathogenic fungus, and the yeast alone. Four fruits arranged in a randomized block design were used per treatment, the treatment being the combined inoculation of pathogen and the yeast strain. All assays were designed in random blocks, with three repetitions. Tukey's test with a 5% confidence interval was used to assess the reduction in disease severity.

### Maximum temperature of growth

The yeast was allowed to grow in glass test tubes with YM broth and was incubated at 25, 35, 36 and 37°C. The growth

was verified by turbidity of medium after 48 h of incubation. The experiment was repeated two times.

#### Compatibility of biocontrol agent with sodium bicarbonate solution

In this experiment one ml of a 10X concentrated sodium bicarbonate solution (SBC) was transferred to glass test tubes with 9 ml of *S. crataegensis* at a concentration of  $10^6$  cells/ml. The final concentrations of SBC were 1, 2 and 5%. The tubes were incubated at 25°C for 0, 1, 2, 5 and 7 days on a rotary shaker at 50 rpm, after which 100 µl of the culture was plated on YM agar. After incubation for 48 h, the colonies were counted and the results were expressed in colony forming units (cfu)/ml. The experiment was repeated three times.

#### Integrated Biological Control

The biocontrol tests on fruit were performed by inoculating the pathogen alone or in combination with the yeast, or both. Aliquots of 200 µl of pathogen suspension were deposited with a sterile pipette onto the orange wound immediately after wounding, allowed to dry, and 200 µl of a suspension of *S. crataegensis* and/or sodium bicarbonate at 5% was also deposited onto the wound. The inoculated fruits were incubated in a 100% humidity chamber at 25°C for 10 days. The controls included inoculation with pathogen alone, GY without the yeast or pathogenic fungus, the yeast alone, sodium bicarbonate at 5%, and the yeast and sodium bicarbonate at 5% together. There were three replicates of four fruits arranged in a randomized block design per treatment.

#### Assessment of lesion

The percentage of disease severity reduction (DSR%) was calculated by the equation:

$$DSR\% = \frac{DSc - DSt}{DSc} \times 100$$

Where DSc = lesion area on the positive control (pathogen alone) and DSt = lesion area on the treated fruit (1). Only

lesion originating from the mechanically wounding were used in the disease assessment.

#### Antagonist recovery

To assess the ability of *S. crataegensis* to survive on the surface of oranges, wounds of fruits wounded as described above were treated with 200 µl of GY broth supplemented with 5% sodium bicarbonate and  $2 \times 10^8$  cells/ml of the yeast. The survival of the yeast was determined immediately after application and after 4, 7 and 14 days of incubation at 25°C in a 100% RH. To recover the yeast from fruits, portions of wounds from the treated oranges were removed with a 1.0 cm<sup>2</sup> diameter cork borer, transferred to tubes containing GY broth, agitated in a vortex and serially diluted to  $10^{-2}$ ,  $10^{-4}$  and  $10^{-6}$ , followed by plating 100 µl of the suspension in triplicate on YM agar supplemented with 200 mg/l of chloramphenicol. The colonies were counted after 48h incubation at 25°C.

## RESULTS AND DISCUSSION

The Pera Rio orange variety has been shown to be sensitive to infection caused by *P. digitatum* (8). Our results showed that the yeast significantly reduce the disease severity (DSR%). The biocontrol efficacy of *S. crataegensis* was 60.1% (Fig. 1). The positive control containing only *P. digitatum* showed 100% of disease incidence. These results can be considered satisfactory, since incubation conditions (25°C in high humidity) and the time of inoculation (inoculation of *P. digitatum* before the yeast) strongly favored the pathogen. Because some of the post-harvest pathogens that affect fruits initiate their disease cycle even before harvest this antagonist may have potential for controlling these type infections. The experiments were designed to simulate the post-harvest environmental situation and showed that *S. crataegensis* has potential for use in IBC under commercial conditions, since very few studies with yeast antagonists were conducted under such adverse conditions. Usually, the combination of bicarbonate with biological control agents has been observed to be synergistic (11). However, some studies have reported an inhibitory effect of sodium

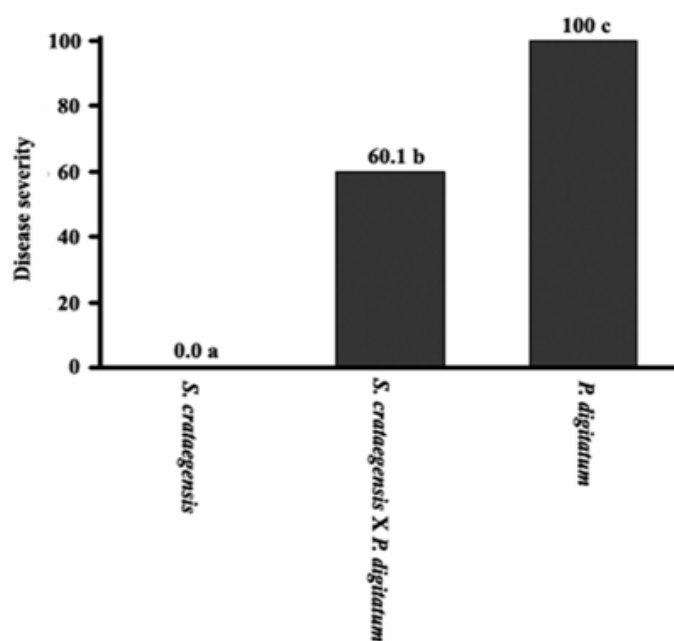
bicarbonate not only on pathogens but also on biocontrol agents (11, 17, 27). Most of the experiments about the combination of sodium bicarbonate and yeast utilize concentrations under 2% of the salt. The survival of *S. crataegensis* in formulations with 1, 2, and 5% sodium bicarbonate can be considered very satisfactory, since the yeast remained viable at all these concentrations (Fig. 2). After one week of incubation, the populations of the yeast were very similar to those at the beginning of the experiment. Strong resistance to SBC is an important characteristic of this yeast, because it allows for the use of SBC at higher concentrations, which may have stronger effect on reduction of fruit decay.

The ability of antagonists to survive at sufficient populations on the fruit surface after their application is very important for efficacy and persistence of control (2). The ability of *S. crataegensis* to rapidly colonize and to stay viable in fruit wounds (the major points of entry for pathogens) at room temperature (25°C) is a very important characteristic. Another important characteristic of *S. crataegensis* was the absence of growth at 37°C, which eliminates this yeast as a potential human pathogen (6, 12). The wounds, which inevitably occur during harvest, transport, and handling, not only damage the fruit (22), but also provide pathways for pathogen invasion, especially for the primary pathogens (15). The yeast *S. crataegensis* was re-isolated from treated oranges throughout the 2 week experiment (Fig. 3). However, similar to other yeasts utilized in biological control of fruit decays that only work at high cell concentrations (21, 28), in this study the antagonistic yeast only produced an efficient control of molds when inoculated at a concentration of  $10^8$  cells/ml (data not shown). Despite high density, application of this yeast alone to oranges did not result in any necrosis, chlorosis or fruit decay which is another important characteristic of a biocontrol agent.

Results from the IBC experiment are very promising, especially under unfavorable environmental conditions such as room temperatures and high humidity. The pathogen alone infected all of the oranges within 4 days. The 5% sodium bicarbonate treatment alone reduced the disease severity by 19.8%, and the yeast alone reduced the disease severity in

41.7% (Fig. 4). When these treatments were combined initially, total control of the decay was achieved and the first symptoms started to appear only on the tenth day after inoculation. The development of a biological control strategy for citrus decay caused by wound invading *P. digitatum* is very difficult, mainly because *Penicillium* spp. are aggressive pathogens and are good competitors. Pilot test in citrus packinghouses indicated that antagonist alone cannot provide adequate control and must be combined with diluted fungicides or other methods of control (7).

In the biocontrol tests the yeast significantly reduced decay severity and incidence on mature oranges. Decays originating from natural infection were also observed and were quite variable but they diminished after treatment with *S. crataegensis* (data not shown).



**Figure 1.** Disease severity (%) on oranges artificially inoculated with *Penicillium digitatum* LCP 4354 conidia at  $10^4$  /ml alone or in combination with *S. crataegensis* at  $10^8$  cells/ml. The treatments were statistically different according to Tukey's test ( $P=0.05$ )

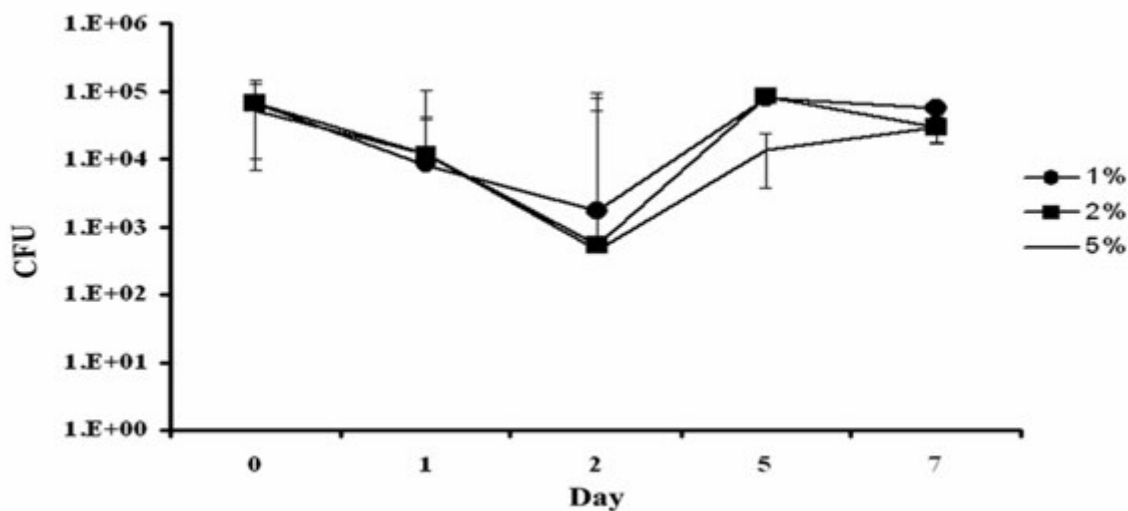


Figure 2. Effect of different concentrations (%) of sodium bicarbonate on survival of *S. crataegensis* *in vitro*

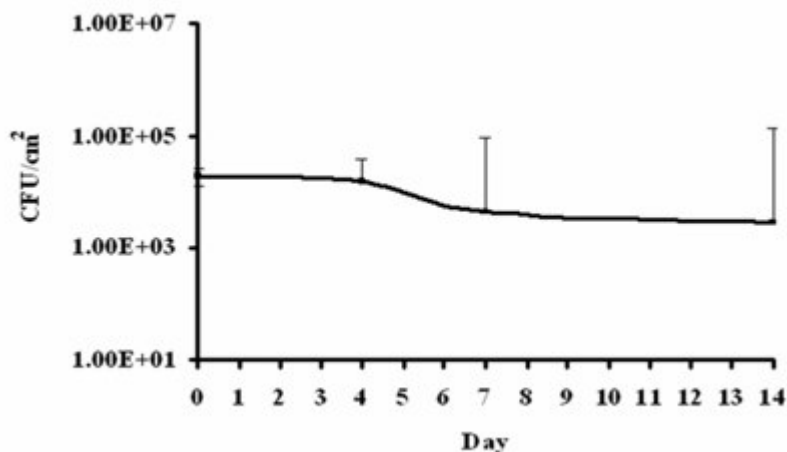


Figure 3. Recovery of *S. crataegensis* from oranges wounds after inoculation with 10<sup>8</sup> cells/ml and incubation for up to 14 days at 25°C. The bars represent the standard deviation of the mean (n=3)

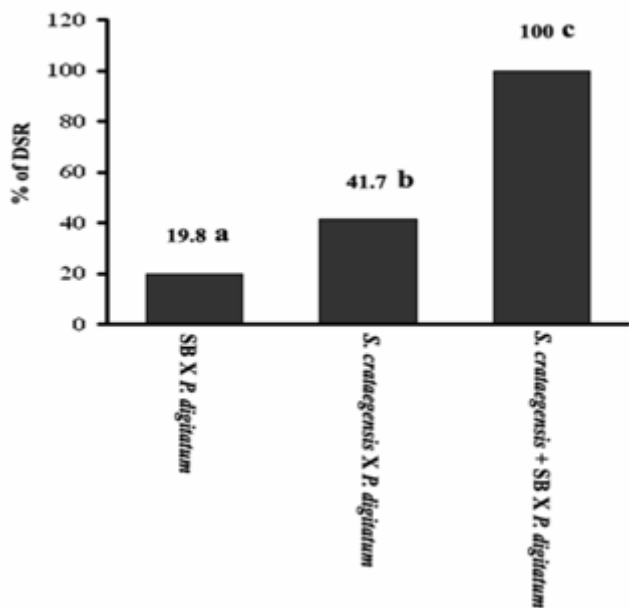


Figure 4. Effect of *S. crataegensis* alone or in combination with SB at 5% on control of green mold on oranges artificially inoculated with the pathogen and stored at 25°C in a humid chamber for 10 days. Data represent the means of three replicates. Bars with different letters are significantly different according to Tukey's test (P=0.05)

An antagonist yeast is very promising if it can be combined with routine postharvest treatments (29). The best time for the postharvest application of a water-based treatment to the fruit should be during the hydrocooling, drenching or washing processes (17). Citrus fruits are usually treated with chlorinated water after harvest to reduce decays, but the use of chlorine on fruits and vegetables is banned in some countries due to deleterious effect on human health resulting from its reaction with organic matter that lead to the formation of carcinogenic compounds (5). Accumulation of these substances also causes environmental pollution (24, 26). The findings provide a powerful incentive for development of this yeast as an alternative to green mold control by *P. digitatum* in postharvest orange fruits.

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