Microbiological Research 175 (2015) 93–99

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Saccharomyces cerevisiae: A novel and efficient biological control agent for *Colletotrichum acutatum* during pre-harvest

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ARTICLE INFO

Article history:
Received 6 March 2015
Received in revised form 1 April 2015
Accepted 4 April 2015
Available online 17 April 2015

Keywords:
Detached citrus flowers
Antifungal compounds
Killer yeast
Hydrolytic enzymes
Postbloom fruit drop

ABSTRACT

In this study, we evaluated the efficiency of six isolates of *Saccharomyces cerevisiae* in controlling *Colletotrichum acutatum*, the causal agent of postbloom fruit drop that occur in pre-harvest citrus. We analyzed the mechanisms of action involved in biological control such as: production of antifungal compounds, nutrient competition, detection of *killer* activity, and production of hydrolytic enzymes of the isolates of *S. cerevisiae* on *C. acutatum* and their efficiency in controlling postbloom fruit drop on detached citrus flowers. Our results showed that all six *S. cerevisiae* isolates produced antifungal compounds, competed for nutrients, inhibited pathogen germination, and produced *killer* activity and hydrolytic enzymes when in contact with the fungus wall. The isolates were able to control the disease when detached flowers were artificially inoculated, both preventively and curatively. In this work we identified a novel potential biological control agent for *C. acutatum* during pre-harvest. This is the first report of yeast efficiency for the biocontrol of postbloom fruit drop, which represents an important contribution to the field of biocontrol of diseases affecting citrus populations worldwide.

1. Introduction

Postbloom Fruit Drop (PFD), caused by the fungus *Colletotrichum acutatum* Simmonds, is one the most devastating diseases affecting citrus. Only flower tissues of citrus are infected and develop postbloom fruit drop (Fig. 1A). Natural citrus fruit abscission usually occurs at the base of the peduncle at the attachment to the stem, but with postbloom fruit drop, abscission is at the base of the fruit leaving persistent calyces and peduncles (Fig. 1B) (Peres et al. 2005). When conditions are favorable during bloom (*i.e.*, rain), the fungus can disperse and cause greater damage to the crop (Silva-Junior et al. 2014). In general, this disease is controlled with protectant and systemic fungicide sprays (Goes et al. 2008). Due to the numerous bloom cycles in certain commercial citrus varieties, the fungicidal method requires numerous sprays, which increases production costs and causes significant environmental risks. Therefore, new and effective control methods are needed that satisfy consumer needs and are also safe for workers and the environment.

One alternative method studied under field conditions in recent years is the bacterium *Bacillus subtilis*, which acts on a curative control mechanism (Klein et al. 2013; Kupper et al. 2012). In the current work, we were interested in identifying an alternative method that could act preventively. Species of yeast, which act by competing for nutrients and space, have been used as biocontrol agents against various pathogens, but only those that occur in postharvest fruits (Droby et al. 2009; Kupper et al. 2013; Moretto et al. 2014; Talibi et al. 2014). Furthermore, *S. cerevisiae* has been shown to act as an important biocontrol agent against plant pathogens (Nally et al. 2012; Platania et al. 2012; Gouvea et al. 2009).

Yeast rely on antagonistic mechanisms of action to exert biological control. In general, many yeast species compete for nutrients and space, induce resistance and secrete lytic enzymes (Droby et al. 2009; Zhang et al. 2011). Johnson and Stockwell (2000) reported that the antagonistic efficiency of microorganisms in flowers can be attributed to their competition for nutrients, and in some cases, to the production of compounds with an inhibitory action against pathogens. Fialho et al. (2010) demonstrated that the production of volatile organic compounds by *Saccharomyces cerevisiae* plays an essential role in the antagonistic activity on *Guignardia citricarpa*.

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http://dx.doi.org/10.1016/j.micres.2015.04.003
0944-5013/

Fig. 1. Symptoms caused by *Colletotrichum acutatum* on citrus: (A) infected flowers of sweet orange; (B) persistent calyces remaining following fruit drop.

Some yeast exhibit *killer* activity, which is defined as the ability to secrete proteins or glycoprotein *killer* toxins that generally are lethal to other species of yeasts, pathogenic bacteria and also to cells of filamentous fungi through different mechanisms, including the hydrolysis of the major cell wall component β-1,3-glucans (Schmitt and Breinig, 2006; Coelho et al. 2007; Hashem and Alamri, 2009; Muccilli et al. 2013).

The objectives of this study were to evaluate six yeast isolates of *S. cerevisiae* for the possible biological control of PFD caused by *C. acutatum* in citrus, and to determine the mechanisms of action that are involved in this biocontrol. In the case of most citrus pathogens detected post-harvest, infection occurs pre-harvest. Therefore, a yeast-based product that acts during the pre-harvest period would be particularly important for diseases such as PFD, which occurs pre-harvest. Such a yeast would also reduce the production of initial inoculum of pathogens, which would also indirectly help control diseases that occur during the post-harvest period.

2. Material and methods

2.1. Microorganisms

Six industrial yeast strains of *S. cerevisiae* (ACB-CR1, ACB-KD1, ACB-CAT1, ACB-BG1, ACB-K1 and ACB-PE2) isolated from the fermentation process for ethanol production, characterized by electrophoretic karyotyping, and stored in the collection of the Laboratory of Biochemistry and Plant Pathology at the University if São Paulo (ESALQ), Piracicaba-São Paulo, Brazil (Fialho et al. 2010) and later deposited in the microorganisms collection of the Apta Center Citrus "Sylvio Moreira"/IAC, Cordeirópolis, São Paulo State, Brazil. The *C. acutatum* strain used in the present study was obtained from the same collection.

2.2. Evaluation of in vitro antagonism

C. acutatum was co-cultured on Petri dishes with each yeast isolate studied. Petri dishes with PDA medium were inoculated with a 7-mm diameter disk of an actively growing fungal mycelium near the disk edge. Another disk of yeast (cultured for 48 h), of the same size, was inoculated at the opposite edge. The control corresponded to the growth of the fungus without yeast.

The experiment was performed with a completely randomized design and five replicates, incubating the cultures at 27 °C for 18 days with a 12 h photoperiod. Mycelial diameter of *C. acutatum* was measured in two perpendicular directions. The data were statistically analyzed by analysis of variance (ANOVA) and Tukey's test at 5% significance. The experiment was repeated at least twice.

2.3. Production of antifungal compounds by yeast isolates

2.3.1. Production of volatile antifungal compounds

To assess the production of volatile compounds, each of the yeast isolates was simultaneously cultivated with *C. acutatum* on split plates, which prevented nonvolatile compounds produced by the yeast from reaching the fungus. A *C. acutatum* culture disk (7 mm in diameter) was placed on PDA medium on one side of the split plate. On the other side, a disk of same size of the yeast strain (cultured for 48 h) was placed in the medium, and the plates were hermetically sealed. After incubating the fungus and different yeast strains at 25 °C for 7 days, fungal growth was measured as the mycelial diameter in the presence of yeast relative to the mycelial diameter in the absence of yeast (control).

2.3.2. Production of cell-free antifungal compounds in yeast

For each yeast isolate tested, a loop of inoculum from a 48 h culture was transferred to a 250 mL Erlenmeyer flask containing 50 mL of potato dextrose broth (PDB), followed by incubation at 150 rpm for 72 h in the dark.

Using a protocol adapted from the technique described by Frighetto and Melo (1995), each yeast culture was filtered through Whatman N° 4 filter paper and a 0.45-μm Millipore® membrane after the incubation period to remove the yeast cells. For each yeast cell-free filtrate, a 10 mL aliquot was added to 90 mL of PDA medium and poured into Petri dishes. After solidification, a 7 mm *C. acutatum* culture disk was placed in the center of each Petri dish. For the control, *C. acutatum* was grown in PDA medium containing sterile water instead of the yeast filtrate. The cultures were incubated in a Biochemical Oxygen Demand (BOD) chamber at 25 °C for 9 days. Mycelial diameter of *C. acutatum* was measured in two perpendicular directions.

2.3.3. Production of thermostable antifungal compounds

For each yeast isolate tested, a yeast culture disk was transferred to a 250 mL Erlenmeyer flask containing 50 mL PDB, followed by incubation at 150 rpm for 72 h in the dark, as described before. Thereafter, 10 mL aliquots of each isolate were transferred to vials containing 90 mL PDA medium and sterilized by autoclaving at 121 °C for 20 min. Each sterilized medium was poured into a Petri dish, and, following solidification, a 7 mm *C. acutatum* culture disk was transferred to the center of each plate. For the control, *C. acutatum* was grown on PDA medium containing sterile water instead