Biological Control 53 (2010) 32–38

Contents lists available at ScienceDirect

Biological Control

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

Isolation and *in vivo* screening of yeast and *Bacillus* antagonists for the control of *Penicillium digitatum* of citrus fruit

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ARTICLE INFO

Article history:
Received 4 December 2008
Accepted 9 December 2009
Available online 14 December 2009

Keywords:
Yeast
Bacillus
Penicillium digitatum
Antagonism
Citrus fruit

ABSTRACT

A total of 60 yeast and 92 *Bacillus* isolates were isolated from the fruit surface of papaya and several varieties of citrus from various orchards in South Africa, and screened for antagonism to *Penicillium digitatum*. Ten yeast and 10 *Bacillus* isolates reduced the surface area of visible *P. digitatum* growth ⩾50%, when applied 3 h before inoculation with the pathogen. Two yeast isolates (B13 and Grape), when applied 48 h prior to inoculation with *P. digitatum*, prevented decay of navel oranges and lemons, and ⩽5% incidence on Valencia oranges, compared with an untreated control that had ⩾50% incidence of infection. The application of isolates to lemons and Valencia oranges did not produce a curative action against *P. digitatum* when applied 3 h postinfection. The yeast isolates B13 and Grape were superior to all the *Bacillus* isolates, and provided excellent control of *P. digitatum*, when applied to citrus fruit prior to artificial inoculation by *P. digitatum*.

1. Introduction

Green mold of citrus, caused by *Penicillium digitatum* (Pers.: Fr) Sacc. is a major cause of postharvest disease of citrus fruit worldwide (Bancroft et al., 1984). Control of the disease has depended upon the use of synthetic fungicides. However, the development of strains of *P. digitatum* and other fungi that are resistant to fungicides (El-Goorani et al., 1984; Eckert et al., 1994) and increasing public concern over food safety, and the environment (Holmes and Eckert, 1999) are driving a search for alternative postharvest disease control methods. Biological control has been proposed as a key alternative control method, and some effective antagonistic microorganisms have already been used in the global fruit industry (McLaughlin et al., 1992).

Successful control of infections caused by a number of postharvest pathogens using biological control agents have been reported on citrus fruit (Wilson and Chalutz, 1989; Chalutz and Wilson, 1990; Cheah and Tran, 1995; El-Ghaouth et al., 2000; Bouzerda et al., 2003). Numerous yeast antagonists have been reported to successfully control postharvest infection on a variety of fruit (Chalutz and Wilson, 1990). Yeasts are particularly suitable as antagonistic agents because they grow rapidly, colonizing fruit surfaces and limiting nutrient availability to pathogens through competition, and are tolerant of most agrochemicals (Richard and Prusky, 2002). Two yeast products, Aspire® (*Candida oleophila* Montrocher) and Yield-Plus® (*Cryptococcus albidus* (Saito) Skinner) are commercially available (Vero et al., 2002).

Bacillus spp. are also used as biological control agents (Leifert et al., 1995; Kim et al., 1997; Enebak and Carey, 2000). *Bacillus subtilis* Ehrenberg Cohn strains typically have the ability to survive on citrus fruit surfaces and some are antagonistic to pathogens (Gutter and Littauer, 1953).

The objectives of the study were: (1) to identify yeast and *Bacillus* isolates antagonistic to *P. digitatum*; and (2) to investigate their efficacy in controlling infection by *P. digitatum in vivo*.

2. Materials and methods

2.1. Fruit used for isolation of potential antagonists

Papaya (*Carica papaya* L.), granadilla (*Passiflora quadrangularis* L.) and a range of citrus [i.e., navel orange (*Citrus sinensis* [L.] Osbeck), Valencia orange (*C. sinensis* [L.] Osbeck), lemon (*Citrus limon* Burmann), grapefruit (*Citrus paradisi* Macf.), and mandarin (*Citrus reticulata* Blanco)] were harvested from commercial orchards and home gardens in KwaZulu-Natal and Mpumalanga, South Africa. Undamaged fruit were processed either immediately, after storage for 2–3 days at room temperature or after storage in a cold room at 8 ± 1 °C for 5–7 days.

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doi:10.1016/j.biocontrol.2009.12.009

2.2. Preliminary investigation of microorganisms on the fruit surface

Navel and Valencia oranges that had not been sprayed with fungicides were collected from citrus orchards located at Ukulinga Research Farm, University of KwaZulu-Natal, Pietermartizburg (29.36S 30.24E), South Africa, and stored at 25 °C for 2 weeks. Whole oranges were rinsed with sterile water in order to remove any potential antagonists. The rinsing water was then serially diluted (10-fold dilution series was made up to 10^{-4}) and plated onto potato dextrose agar (PDA) (Merck Laboratory, South Africa). The plates were incubated at 25 °C for 4 days. Yeasts or/and *Bacillus* colonies were selected and identified visually by their typical colony morphologies.

2.3. Isolation of antagonistic yeasts and Bacillus

Bacillus and yeast isolates were recovered from the peel of 3–5 mature fruit from a range of the fruit samples. The fruit peel was cut into 10–15 small pieces, weighing 50 g and placed in separate 250 ml Erlenmeyer flasks containing 100 ml sterile distilled water plus quarter strength Ringer's Solution and shaken in a water bath (G.F.L. 1083, Labortechnik, Germany) at 120 rotations per minute (rpm) for 1 h at 30 °C. Fruit peel pieces were removed and the liquid suspension was used to make a serial dilution of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}. An aliquot of 0.2 ml of each dilution was plated onto PDA amended with 0.15 g l^{-1} of Rose Bengal (BDN Laboratory, England) for recovery of yeast isolates and incubated at 25 °C for 3 days. Pure cultures of yeast were made by sub-culturing from discrete colonies on the plates. For isolation of *Bacillus*, the same serial dilution prepared for the yeast isolates was used, after heat treatment at 80 °C for 15 min in a water bath. Each aliquot of 0.2 ml was poured onto tryptone soy agar (TSA) (Merck Laboratory, South Africa) plate. Plates were incubated for 3 days at 28 °C, after which representative colonies were arbitrarily selected and streaked onto fresh TSA plates to obtain single colonies.

2.4. Preliminary screening of yeasts and Bacillus isolates to P. digitatum on navel oranges

A total of 60 yeast and 92 *Bacillus* isolates were tested on navel oranges. Each fruit was surface disinfected with 70% alcohol for 1 min, dried, and then wounded (2 mm in width and 1 mm in depth) with a disinfected needle 10 times at both ends of the fruit. The wound was dipped into a suspension of yeast cells or *Bacillus* (1×10^8 cells ml^{-1}) for 1 min. Three hours after the wound site had dried, each wounded fruit was dipped into a suspension of conidia of *P. digitatum* at 1×10^4 conidia ml^{-1}, isolated from infected navel oranges, collected at Gateway Packhouse (29.53S 30.17E), Thornville, Pietermaritzburg, South Africa. The conidial suspension was quantified using a hemocytometer and then adjusted to the final concentrations by dilution. Control fruit were treated with sterile distilled water. Fruit were kept at room temperature (24 ± 1 °C) for 10 days. One box with three fruit was used per treatment. Treatments were placed on a bench. Fruit were examined for percentage fruit surface area covered by *P. digitatum* using visual estimations for the initial screening. The criterion used to select the antagonists was the ability to reduce growth or development of *P. digitatum* to $\leqslant$50%.

2.5. Further screening of selected yeasts and Bacillus isolates antagonistic to P. digitatum on Valencia oranges

Further tests were conducted on the most promising yeast (10) and *Bacillus* (10) isolates following the procedures described in Section 2.4. However, in this instance Valencia oranges were used for the evaluation, and fruit was wounded (3 mm in length × 3 mm in depth) at one site on the fruit equator with a dissecting needle. The wound was then treated with a 100 μl cell suspension of the test organism (yeast or *Bacillus* at 1×10^8 cells ml^{-1}). After the wound site had dried for 3 h, 100 μl of the conidial suspension of *P. digitatum* (1×10^4 conidia ml^{-1}) was inoculated into the wound. Wounds inoculated with the same amount of *P. digitatum* isolate, but no biocontrol pretreatment, served as the control. Fruit were kept at room temperature (24 ± 1 °C). Two boxes, with five fruit per box, were used per treatment and placed on a bench in a complete randomized block design (CRBD). The criterion used to select the antagonist was the reduction of lesion diameters (mm) caused by *P. digitatum* 10 days after inoculation. Lesion diameter was measured by taking the mean of the horizontal and vertical diameters of each lesion.

2.6. Preventative action of selected yeast and Bacillus isolates antagonistic to P. digitatum on navel and Valencia oranges, and lemons

Ten yeasts and 10 *Bacillus* isolates were tested for their preventative action against *P. digitatum*. This was achieved by treating fruit with an antagonist 48 h before inoculation with the pathogen. The procedures described in Section 2.5 were followed for the preparation of the antagonist, *P. digitatum*, and for the treatment application. Navel and Valencia oranges, and lemons were used in this trial. The fruit wound was extended to 25 mm in length × 3 mm in depth. The wound was treated with 100 μl of cell suspension of the test organism (yeast or *Bacillus* at 1×10^8 cells ml^{-1}). After the wound site had dried for 48 h, each wound was inoculated with 100 μl of the suspension of conidia of the *P. digitatum* isolate (1×10^4 conidia ml^{-1}). Wounds inoculated with the same amount of *P. digitatum* isolate served as the control. Fruit were kept at room temperature (24 ± 1 °C). Two boxes, with five fruit per box, were used per treatment and placed on a bench in a CRBD. Lesion diameter (mm) of each infected wound was determined 10 days after inoculation. Lesion diameter was measured by taking the mean of the horizontal and vertical diameters of each lesion.

2.7. Curative action of selected yeast and Bacillus isolates antagonistic to P. digitatum on Valencia oranges, and lemons

Similar procedures as described in Section 2.6 were followed, with the difference that navel oranges were not included because they were out of season. Valencia oranges or lemons were wounded, and then inoculated with 100 μl of the suspension of *P. digitatum* conidia (1×10^4 conidia ml^{-1}). After the wound site had dried for 3 h, the wound was treated with a 100 μl of the test organism (yeast or *Bacillus* at 1×10^8 cells ml^{-1}). Wounds inoculated with *P. digitatum* conidial suspension served as a control. Fruit were kept at room temperature (24 ± 1 °C). Two boxes, with five fruit per box, were used per treatment and placed on a bench in a CRBD. Lesion diameter was measured by taking the mean of the horizontal and vertical diameters of each lesion.

2.8. Dose effect of two yeast isolates, B13 and Grape, applied preventatively on lemons for the control of P. digitatum

The effect of various concentrations of two yeast isolates, namely, B13 and Grape (identified as strains of *Candida fermentati* (Saito) Bai. by Botes (2005), were studied for their preventative action on lemons against *P. digitatum*. Lemons were surface disinfected with 70% alcohol for 1 min, dried, and then wounded. Fruit were wounded (25 mm in length × 3 mm in depth) at one site on the equator with a dissecting needle as described in Section 2.6. Cell suspensions (100 μl) of both yeasts of 1×10^5, 1×10^6, 2.5×10^6, 1×10^7, and 1×10^8 cells ml^{-1} were inoculated into