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ScienceDirect
Scientia Horticulturae 111 (2007) 179–185
SCIENTIA HORTICULTURAE
www.elsevier.com/locate/scihorti

Short communication

Selection and testing of epiphytic yeasts to control anthacnose in post-harvest of papaya fruit

Guy de Capdeville [a,*], Manoel Teixeira Souza Jr.[a], Jansen Rodrigo Pereira Santos [a], Simone de Paula Miranda [a], Alexandre Rodrigues Caetano [a], Fernando Araripe Gonçalves Torres [b]

[a] *Embrapa Genetic Resources and Biotechnology, Final av. W/5 Norte, Brasília, DF 70770-900, Brazil*
[b] *Universidade de Brasília, Departamento de Biologia Molecular, Brasília, DF 70910-900, Brazil*

Received 9 March 2006; received in revised form 5 October 2006; accepted 12 October 2006

Abstract

Anthracnose, caused by *Colletotrichum gloeosporioides*, is a major post-harvest disease in papaya fruit. The major objectives of the present work were to isolate, select and test the *in vitro* and *in vivo* ability of epiphytic microorganisms, isolated from papaya fruit and leaf surfaces, in controlling anthracnose onset after harvest. A total of 75 bacteria, 67 yeasts and 22 mycelial fungi were isolated. Thirty yeast isolates were able to inhibit the mycelial growth of *C. gloeosporioide in vitro* and seven of those were used in *in vivo* assays, resulting in the identification of two very effective isolates. Isolate CEN63, identified molecularly as *Cryptococcus magnus*, was the most effective in controlling the disease and therefore was studied in more detail. The results of the assays with *C. magnus* provided evidence that when fruit were treated with the antagonists at concentrations of 10^7 to 10^8 cells/ml, as early as 24 h, preferentially 48 h, before inoculation with the pathogen, the development of disease was significantly reduced. *C. magnus* is a potential antagonist for the development of a commercial product. Additional studies on the modes of action of this yeast isolate, as on its ability to interact with fungicides are being conducted to generate solid basis for the development of an environmentally friendly control agent.

Keywords: *Cryptococcus magnus*; Papaya; Post-harvest; *Colletotrichum gloeosporioides*; Yeasts; *Carica papaya*

1. Introduction

Anthracnose is a major post-harvest disease caused by *Colletotrichum gloeosporioides* (Penz.) Sacc., which affects many tropical and subtropical fruit (Prusky, 1996; Prusky and Plumbley, 1992), including papaya (*Carica papaya* L., Snowdon, 1990). In general, the fungus initiates infection as soon as flowering starts and stays latent until the post-harvest environment conditions favor colonization of fruit tissue. Although infection occurs before harvest, symptoms usually become apparent only after harvest, when fungal development is resumed due to favorable storage conditions. Nonetheless, post-harvest infections also occur due to availability of inoculum in the processing environment and due to a considerable amount of wounds imposed to the fruit after harvest.

The control of Anthracnose has been accomplished almost exclusively by fungicide dip or drench during the packing process (Robs, 1996; Sholberg and Conway, 2001). The pursuit of alternative control methods has become increasingly more important due to rising concerns with potential harmful consequences of fungicide toxicity to human health and the environment. In addition, loss of effectiveness as a consequence of pathogens developing resistance to widely used fungicides is also a significant problem (Capdeville et al., 2002; Wilson and Wisniewiski, 1995). Among the current alternative methods are the use of UV irradiation and/or hot water (Alvarez and Nishijima, 1987), and the use of antagonistic microorganisms (Gamagae et al., 2004). The later have been very promising in controlling post-harvest diseases in papaya and other fruit due to controllable post-harvest conditions (Wilson and Wisniewiski, 1995).

A considerable number of antagonistic microorganisms have been tested for controlling different pathogens in different fruit-pathogen interaction systems (Capdeville et al., 2003;

* Corresponding author. Tel.: +11 55 61 4484707; fax: +11 55 61 3403658.
E-mail address: guy@cenargen.embrapa.br (G. de Capdeville).

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doi:10.1016/j.scienta.2006.10.003

Chand-Goyal and Spotts, 1996, 1997; Chalutz and Wilson, 1990; Janisiewicz and Bors, 1995). However, very few studies have been published on the control of papaya post-harvest diseases relying on antagonistic microorganisms, combined or not with other alternative methods (Gamagae et al., 2004). The main objective of the present work was to test the ability of epiphytic microorganisms isolated from papaya fruits and leaves in controlling the post-harvest development of anthracnose in papaya fruits.

2. Materials and methods

The research work was conducted at the plant–pathogen interaction lab of Embrapa Genetic Resources and Biotechnology, Brazil, during the years of 2002–2004. The papaya fruit (cv. Solo) used in the experiments were harvested at the commercial ripening stage (two thirds of fruit surface showing yellow color) and were provided by two papaya growers from southern Bahia state.

2.1. Isolation and storage of epiphytic microorganisms

Epiphytic microorganisms were isolated from leaves and fruit surfaces of randomly selected papaya plants from the cultivars 'Solo' and 'Formosa' held in an orchard at the experimental station of the University of Brasília, Brazil. Samples of leaves and fruit peel were collected, cut in pieces of 3 cm × 3 cm, and three to four pieces of each sample were immersed in 20 ml of sterile water in a test tube. The tubes were then agitated in a shaker for about 5 min and the mixture was filtered through a single layer of cheesecloth. One milliliter aliquots of the remaining solution were used to prepare serial dilutions up to 1:1000, and from each of those dilutions 20 μL drops were placed on Petri dishes containing PDA media amended or not with 0.1% streptomycin to separate fungi from bacteria. The dishes were incubated in a growth chamber at 25 °C and 80% RH. After one to two days of incubation, isolated colonies of micelial fungi, yeasts and bacteria were removed from the plates, and re-isolated on fresh PDA medium. The pure isolated cultures obtained were used to prepare cell suspensions for long-term storage of the microorganism under three methods: storage in mineral oil (fungi), on silica gel (all) and on filter paper (all) as described by Dhingra and Sinclair (1985).

2.2. Inoculum preparation

An isolate (Cg-32) of *C. gloeosporioides*, kept in the Plant Pathology laboratory of the University of Brasília (Brasília, DF), was used as the source of inoculum for the experiments. The pathogenicity of the isolate was checked by inoculating papaya fruit. From lesions formed in those inoculated fruit, pieces of tissue were removed from the edge of the lesions, immersed in 70% ethanol for one minute, transferred to a solution of 1% sodium hypochlorite for 1 min, washed three times for two minutes each in sterile distilled water, blotted on sterile filter paper, and plated on potato dextrose agar (PDA) medium amended with 0.1% streptomycin. The plates were placed in a growth chamber at 24 °C and, after colonies were formed, disks of mycelium were removed from the edge of a colony and transferred to assay tubes containing PDA plus 0.1% streptomycin, allowed to grow for 5 days at 25 °C, and then stored in the refrigerator at 5 °C. These stock cultures were tested for pathogenicity every 2 months. The stocks were then used for generating new 10-day-old cultures on peptone glucose agar medium (PGA), from which spores were collected in sterile water. The final concentration of the spore suspension used was adjusted using a improved Neubauer chamber (Boeco, Germany).

2.3. 'In vitro' testing of the antagonistc activity of yeasts against C. gloeosporioides

A preliminary *in vitro* screening was conducted with all yeast isolates, collected from the epiphytic microflora of papaya leaves and fruit, when they were evaluated for their antagonistic capacity against *C. gloeosporioides*. To test the antagonistic effect of the microorganisms, the potential antagonists were co-cultivated with the pathogen in Petri dishes containing PDA medium. The dishes had their bottom divided in two halves with a marker pen and a 5 mm micelial disc, obtained from the edges of a 5 days old culture of the fungus, was placed in the center of one side of the dish. On the other side of the dish, a 10 μl drop of a cell suspension at 10^8 cells/ml of the antagonist was applied and spread, with a Drigalski spreader, on a circular area with a diameter of 1.5 cm. The dishes were then incubated in a growth chamber at 25 °C and 80% RH, and the appearance of an inhibition zone (IZ) between the fungus and the antagonist was observed for about 7 days. Whenever an IZ was formed the antagonist was selected to be used in the *in vivo* assays.

2.4. In vivo testing of the antagonistic activity of yeast isolates against C. gloeosporioides

To test the efficacy of selected antagonists in controlling *C. gloeosporioides in vivo*, papaya fruit were harvested at the commercial harvest stage, washed with tap water, immersed in ethanol 70% for 1 min, followed by immersion in Sodium Hypocloride 2% for 3 min, washed in sterile water, and allowed to air dry. Following, a 3 mm × 3 mm wound was made on each fruit, and the wounds received the treatments as described bellow. The antagonistic yeasts tested were cultivated on yeast extract agar medium (YMA) for 48 h at 26 °C, before being used to prepare cell suspensions at the concentration of 0, 10^5, 10^6, 10^7 and 10^8 cells/ml. A 20 μl drop from each cell suspension was applied in the wounds (two different fruits were used for each cell suspension) at different times (0, 24, and 48 h) after inoculation of the wound with the antagonist (a 20 μl drop of a spore suspension at 10^4 spores/ml of *C. gloeosporioides* was applied to each wound). The spores of the fungus were obtained by cultivating the pathogen on PGA medium for 7–10 days at 25 °C. After applying the treatments, the fruit were placed in plastic trays and stored in a growth chamber at 25 °C and 80% RH. Development of disease was evaluated daily for 7 days by measuring the diameter of the

lesion formed. These measurements were used to calculate the area under the disease progress curve (AUDPC) using the equation proposed by Shaner and Finney (1977):

$$\text{AUDPC} = S\sum\left[\frac{y_i + y_{i+1}}{2x(t_{i+1} - t_i)}\right]$$

where y_i is the diameter of a lesion at time t_i, in days, and y_{i+1} is the diameter of the lesion at time t_{i+1}.

2.5. In vivo testing of the antagonistic activity of the most effective yeast against C. gloeosporioides

The most efficient isolate among all tested (CEN63) was further evaluated for its ability to control the disease according the following methodology: fruit at the harvest stage were picked, washed with tap water, immersed in ethanol 70% for 1 min followed by immersion in 2% sodium hypocloride for 3 min, washed in sterile water, and allowed to air dry. One 3 mm × 3 mm wound was made in each fruit, which were then sprayed with a CEN63 cell suspension (1.5 ml/fruit) at concentrations of 0, 10^6, 10^7, or 10^8 cell/ml. At different times after treatment with the antagonist (0, 24 and 48 h), the wounds in the fruit were inoculated with *C. gloeosporioides* by application of a 20 μl drop of a spore suspension at the concentration of 5×10^3 spores/ml (about 100 spores per wound). The fruit were placed in plastic trays and stored in a growth chamber at 25 °C and 80% RH. Development of disease was evaluated daily and AUDPC was calculated as described above. The experiment was performed three times in a period of two months.

2.6. Molecular characterization of the most efficient yeast isolate

Molecular identification of isolate CEN63 was based on the analysis of the intervening 5.8S ribosomal RNA (rRNA) and the adjacent ITS1 and ITS2 regions after PCR amplification with primers ITS1 (5′-TCCGTAGGTGAACCTGCG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), which target the conserved regions of 18S, 5.8S, and 28S rRNAs. DNA extraction was performed by boiling a suspension of yeast cells in 50 μl of sterile water for 10 min. After brief centrifugation, 2 μl of the supernatant were used as template in 50 μl PCR reactions as previously described by White et al. (1990), using a PTC-100 MJ Research thermocycler. Amplicons were cloned into pGEMT vector (Promega) and sequencing was carried out using the MegaBACE Dye Terminator procedure and analyzed in a MegaBACE 1000 automatic sequencer (Amersham Biosciences, USA), according to manufacturer recomendations. Sequence analysis was performed using the Blast program (Altschul et al., 1997).

2.7. Statistical analysis

The experiments using different antagonists, which were repeated three times, were factorials conducted in a randomized complete block design, with four replications. The values of AUDPC were used to perform analysis of variance using PROC GLM of SAS (SAS Institute, Cary, NC) and, whenever significant effects or interactions were identified, Statistical analysis was performed with a mixed model ANOVA approach using PROC MIXED from SAS (1996). The model fitted was

$$Y_{ijkl} = \mu + C_i + T_j + (\text{CT})_{ij} + R_k + (\text{BR})_{kl} + \varepsilon_{ijkl}$$

where Y_{ijkl} is the AUDPC mean measurement obtained from four antagonist ou CEN63 concentrations i ($i = 1, \ldots, 4$), time of treatment j ($j = 1, \ldots, 3$), block k ($k = 1, \ldots, 4$) and replicate l ($l = 1, \ldots, 3$), μ represents an overall mean value, C the main effect for antagonist concentration, T the main effect for time of treatment, CT the interaction effect of concentration and time of treatment, R the main effect of replicate, BR the interaction effect of block and treatment and ε is the stochastic error. C, T and CT were fitted as fixed effects and R and BR were fitted as random effects.

3. Results

3.1. Isolation and storage of epiphytic microorganisms

A total of 164 distinct microorganisms were isolated from the epiphytic microflora of papaya leaves and fruit. These were later classified as bacteria (75), yeasts (67) and micelial fungi (22).

3.2. Molecular identification of the most effective yeast isolate

BLASTN comparisons of the ribosomal RNA sequences from the most effective yeast isolate (CEN63) against the nr database showed a significant hit with *Cryptococcus magnus*. The 485bp amplified sequence was compared with the 5.8 S ribosomal RNA gene from *C. magnus* strain MZKI K-479 (GeneBank accession no. DQ631895.1), showing 100% similarity (Fig. 1).

3.3. In vitro testing of the antagonistc activity of yeasts against C. gloeosporioides

The results of the *in vitro* assay allowed for the selection of 30 yeast isolates, based on their ability to produce an inhibition zone when co-cultivated with the fungus, with inhibitory effect on the micelial growth of *C. gloeosporioides*. From those, 10 were very effective in inhibiting the growth of the fungus and, because of this they were selected for the *in vivo* assays (data not shown).

3.4. 'In vivo' testing of the antagonistc activity of yeasts against C. gloeosporioides

The results of the *in vivo* assay revealed a total of seven yeast isolates with considerable ability to reduce the progress of anthracnose. Although all seven isolates tested were effective in controlling the disease, the isolates CEN35 and CEN63 provided the best results. The graphs in Fig. 2 show that there was a clear effect of the concentration and time of application of antagonist

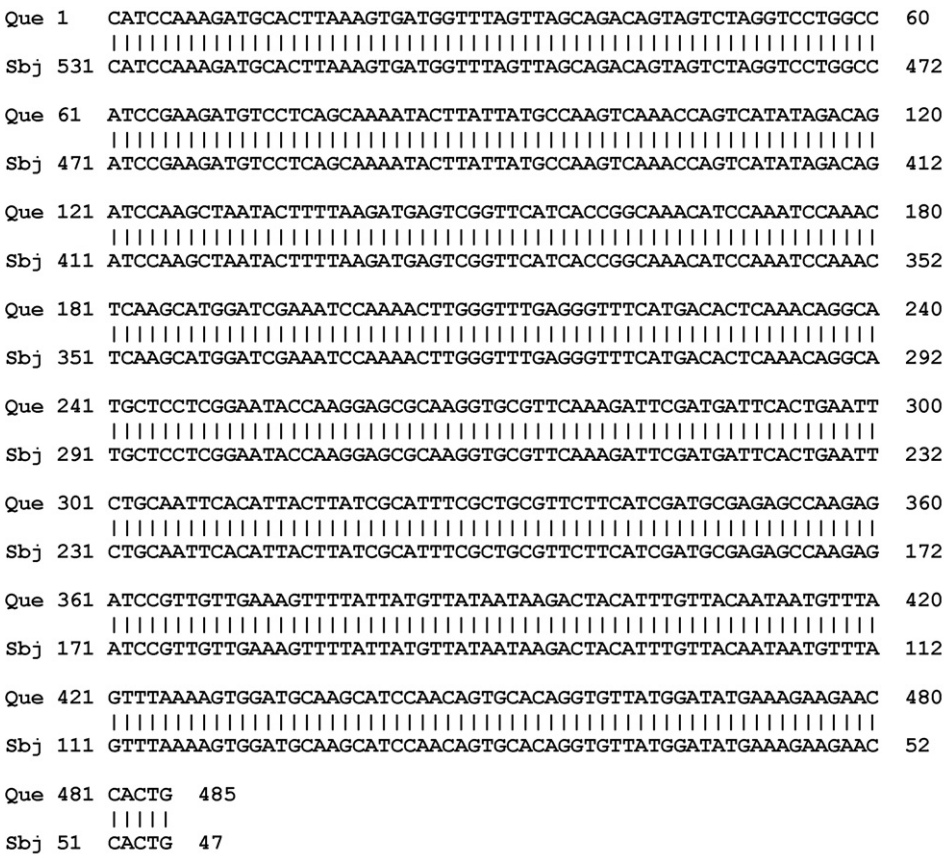

Fig. 1. Alignment of the 485 bp amplified sequence from CEN 63 showing 100% similarity with the 5.8 S ribosomal RNA gene from *Cryptococcus magnus* strain MZKI K-479 (GeneBank accession no. DQ631895.1).

on AUDPC. It was observed that the greater the concentration and time of application of the antagonist were, the better was the control of the disease. Best results were achieved with antagonist concentrations of 10^7 and 10^8 cells/ml and treatment time of 48 h. In the assay where the isolate of *C. magnus* (CEN63) was studied in more detail by spraying the whole fruit surface with the antagonist before inoculation with the pathogen, the results confirmed its effectiveness in controlling the disease. As it can be seen in Fig. 2, the effectiveness of the antagonist was significant even when it was applied to fruit simultaneously with the fungus (time 0) at the higher concentrations (10^7 and 10^8) of the antagonist. No significant differences were found with inoculation times of 24 and 48 h.

Analyzing the results of the *in vitro* and *in vivo* assays we found that some of the yeasts were effective in controlling the fungus *in vitro* but not the disease *in vivo*, some were effective in controlling the disease *in vivo* but did not generated a strong inhibition zone against the fungus *in vitro*, while others did control the fungus and the disease *in vitro* and *in vivo*, this last was the case of isolate CEN63 (data not shown).

3.5. 'In vivo' testing of the effectiveness of C. magnus against C. gloeosporioides

When *C. magnus* was evaluated on its effectiveness to control *C. gloeosporioides* by spraying the whole fruit surface prior to inoculation with the pathogen, the results clearly showed that there was a significant effect of concentration of antagonist applied and time of inoculation with the pathogen. In all cases, better results were achieved when fruit were inoculated 24 to 48 h after treatment with *C. magnus* at the concentration of 10^7 and 10^8 cells/ ml (Fig. 3).

4. Discussion

We were able to select and test a number of yeasts, which proved to be effective against the most important post-harvest pathogen of papaya fruit. One of those yeasts, *C. magnus*, was identified as a promising control agent for controlling anthracnose in papaya. The results of the *in vitro* and *in vivo* assays showed that when *C. magnus* was applied at the concentrations of 10^7 to 10^8 cells/ml, as early as 24 h before inoculation with *C. gloeosporioides*, there was a significant reduction in the progress of the disease as measured by the values of AUDPC.

Selection and testing of epiphytic microorganisms with ability to control post harvest diseases is a difficult task, mostly because some of the post-harvest pathogens that affect papaya and other fruit initiate their disease cycle even before harvest takes place (Prusky, 1996). However, many of the infections by post-harvest pathogens, including *C. gloeosporioides*, occur during and after harvest due to the amount of inoculum

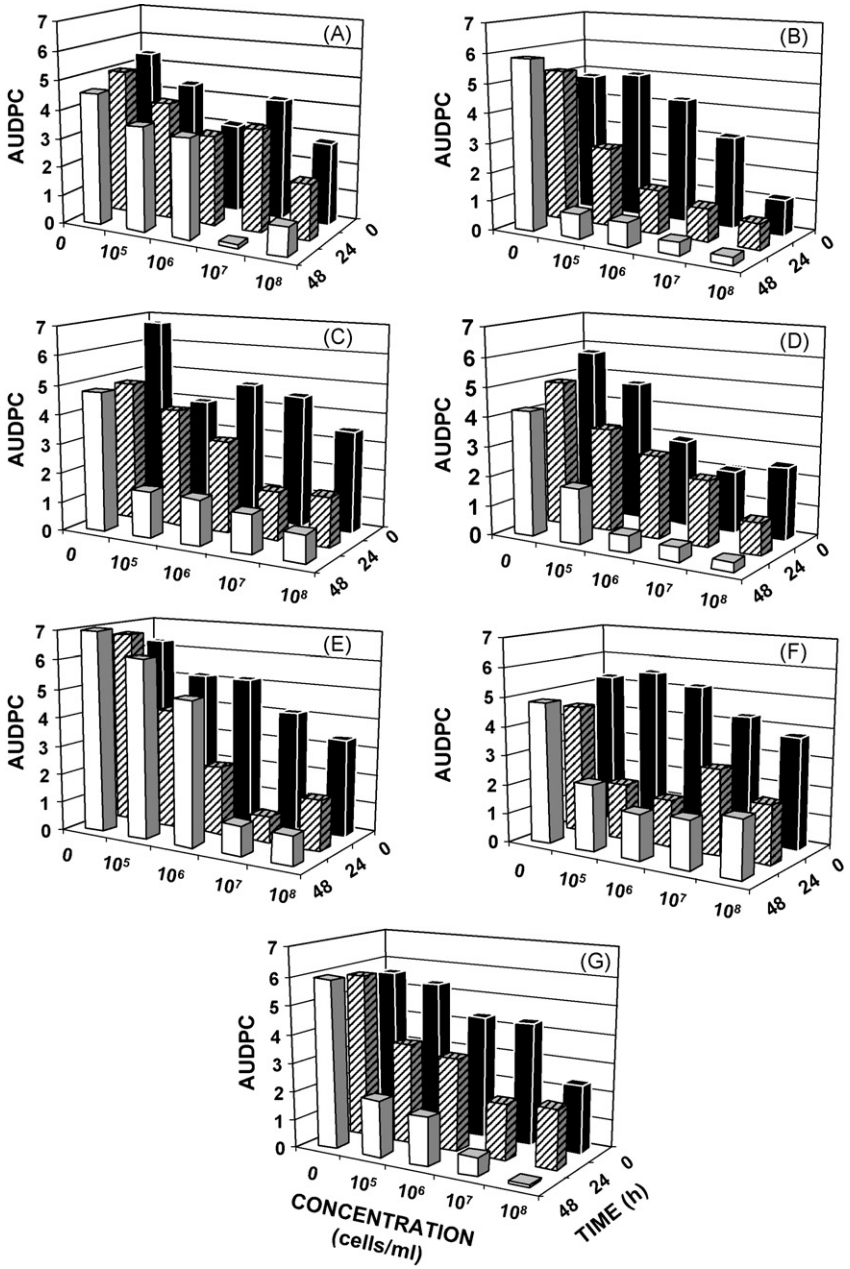

Fig. 2. Effect of seven yeasts isolates on the area under the disease progress curve (AUDPC) of anthracnose of papaya fruit inoculated at five different concentrations, and at three different times before inoculation with *C. gloesporioiedes*. (A) Isolate 20, $P = 0.0115$; (B) isolate 63, $P = 0.0217$; (C) isolate 34, $P = 0.0322$; (D) isolate 35, $P = 0.0023$; (E) isolate 44, $P = 0.0427$; (F) isolate 67, $P = 0.0187$; (G) isolate 45, $P = 0.0132$.

available and the large number of injuries and wounds brought about during the picking and processing stages. Therefore, in order for an antagonistic microorganism to be effective as a control agent it must protect the fruit against new incoming, as well as already established, inoculum. Therefore, an efficient antagonist must operate on different control mechanisms, which may include mycoparasitism, antibiosis, competition for space and nutrients, and ability to induce resistance in their hosts (Wilson and Wisniewiski, 1995). Because we were interested in evaluating the efficacy of different antagonists in controlling the onset of anthracnose initiated after harvest, as opposed to the quiescent manifestation of the disease, the experiments were designed to simulate the post-harvest environment conditions. The initial *in vitro* screening of isolated microorganisms provided preliminary information on possible modes of action used by each isolate, which leads to the control of *C. gloeosporioides*. Although there is great concern regarding the use of antagonists that rely on antibiosis as a control strategy, one most have in mind that antibiosis is a major mode of action for bacteria and not yeasts, which in general rely on the production of cell wall degrading enzymes or in the ability to compete for nutrients and space rather than production of antibiotics. To evaluate the efficacy of the antagonists on quiescent infections, field experiments must be

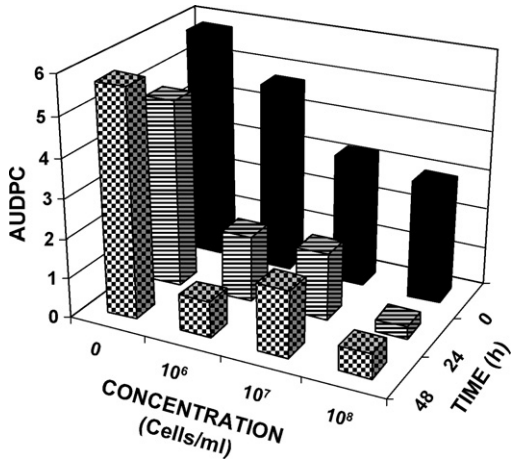

Fig. 3. Effect of *C. magnus* on the area under the disease progress curve of anthracnose of papaya fruit treated at five different concentrations and challenged with *C. gloeosporioides* at 0, 24 and 48 h after inoculation with the yeast antagonist. The graphic represents pooled data of three repetitions of the experiment ($P < 0.0001$).

performed, which was beyond the scope of the present work at the time.

A number of yeasts antagonists have been studied for their potential to control post-harvest diseases however, only a few have actually been registered for use on fruit crops. This is a consequence of the fact that many post-harvest fungi initiate their infection cycle while the fruit are still in the field, before harvest. Nonetheless, for those pathogens that come in contact with the fruit surface during harvest or processing, the prospect for effective control with biological antagonists increases considerably because the exact environmental conditions can be established and maintained during storage. The benefits from using such an approach on harvested commodities justify the extra costs involved (Sholberg and Conway, 2001; Wilson and Wisniewiski, 1995).

In order to be effective in controlling a post-harvest disease, it is very important that the antagonist be applied to the fruit surface as early as possible, otherwise the antagonist may encounter wounds colonized either by post-harvest pathogens or resident microbial flora (Ippolito and Nigro, 2000). Therefore, it is very important to get the control agent established on fruit surfaces before the pathogen arrives at the infection site.

Some studies have tested the effectiveness of *Cryptococcus* species in controlling post-harvest diseases of fruit (Benbow and Sugar, 1999; Chand-Goyal and Spotts, 1997; Roberts, 1991), but no studies were done with these agents with the objective of controlling anthracnose of papaya fruit. The studies in this research work allowed the selection of at least one significantly effective antagonistic microorganism, which proved to be effective against infections initiated after harvest. More studies are being done to determine the effectiveness of *C. magnus* in controlling anthracnose when applied before harvest of the fruit. In addition, studies are being conducted to understand the modes of action involved in the ability of *C. magnus* to control anthracnose of papaya fruit.

Acknowledgments

We thank Dr. Marisa Álvares da Silva V. Ferreira from the Plant Pathology Department of the Universidade de Brasília for providing the isolate of *C. gloeosporioides* used in the experiments. The research work was funded by the Empresa Brasileira de Pesquisa Agropecuária, Brazil.

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