



Biological control of *Rhizoctonia solani* in cowpea plants using yeast

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Abstract

Yeasts have been used to manage a large number of plant diseases, but little is known about the mechanisms used by these biocontrol agents. The objectives of the present study were to evaluate the antagonistic effect of yeasts against *Rhizoctonia solani* and possible mechanisms of action in cowpea plants. Seventy yeast isolates were obtained from leaf, root and stem tissues of cowpea and common bean plants. Screening experiments were conducted in a greenhouse at temperatures ranging from 15 to 26 °C in the first and from 22 to 31 °C in the second experiment. *Candida saopaulonensis* C6A, *Cryptococcus laurentii* FVC10 and *Bullera sinensis* FVF10 (R1) reduced disease severity by 57.4%, 48.5% and 66.3%, respectively. Cowpea plants treated with FVF10 (R1) showed the highest peroxidase and catalase activities. The mechanisms of action were based on competition and induction of enzymes such as peroxidase, catalase and ascorbate peroxidase in cowpea. *Candida saopaulonensis* C6A, *C. laurentii* FVC10 and *B. sinensis* FVF10 (R1) are potential biocontrol agents of damping-off and stem rot caused by *R. solani* on cowpea plants.

Keywords *Vigna unguiculata* · Antagonists · Biocontrol · Induction of plant resistance · Soil-borne pathogens

Introduction

Damping-off and stem rot caused by *Rhizoctonia solani* are important soil-borne diseases of cowpea plants (*Vigna unguiculata*) (González-García et al. 2006). These diseases are responsible for severe economic losses, mainly because they reduce seed germination and emergence (Sartorato et al. 2006; Sikora 2004). *Rhizoctonia solani*

is difficult to control because of its broad host range. This pathogen can persist in the soil due to its overwintering structures, its strong saprophytic capacity and high virulence (González et al. 2011). Therefore, taking actions to decrease inoculum density, promote rapid seedling development and prevent favorable environmental conditions for the pathogen to infect susceptible hosts is necessary (Sneh et al. 1996).

The adverse effects caused by fungicides have encouraged researchers to develop alternatives to synthetic chemicals such as biological control (Pal and Gardener 2006). Yeasts have been successfully used to suppress soil-borne plant pathogens and help plant growth (Botha 2011; El-Tarabily and Sivasithamparam 2006). El-Tarabily (2004) used *Candida valida*, *Trichosporon asahii* and *Rhodotorula glutinis* to control diseases caused by *Rhizoctonia solani* in sugar beet seedlings. The use of *Hansenula arabitolgenes*, *Candida incommunis* and *Candida steatolytica* was effective to inhibit *Rhizoctonia solani*, and these yeasts helped to reduce damping-off incidence in cotton plants (El-Mehalawy et al. 2006). El-Mehalawy et al. (2007) and Mohammed et al. (2008) showed the effectiveness of yeasts to reduce damping-off severity on potato plants and stem rot on cotton plants attacked by *Rhizoctonia solani*. According to El-

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Tarabily and Sivasithamparam (2006), yeasts promoted plant growth and induced resistance by increasing phenylalanine ammonia lyase, phytoalexins, peroxidase and ethylene levels in plant tissues.

There are many studies about yeasts controlling plant diseases, however, little is known about the mechanisms used by these biocontrol agents. The objectives of this study were (i) to isolate yeasts from the leaf, root and stem tissues of cowpea and common bean plants; (ii) to select the most effective yeast isolates against damping-off and stem rot caused by *Rhizoctonia solani* on cowpea plants; (iii) to investigate their possible mechanisms of action; and (iv) to assess antioxidative enzyme activity in cowpea plants.

Materials and methods

Pathogen and antagonistic yeasts

Rhizoctonia solani (CMM-3643) was obtained from the culture collection “Profa. Maria Menezes” (CMM) at the Federal Rural University of Pernambuco. It was isolated from the root of a bean plant and preserved by the Castellani method. This fungus was grown in potato-dextrose agar (PDA) medium and incubated for 3 days at 25 ± 2 °C. The *Rhizoctonia solani* inoculum was produced in Erlenmeyer flasks containing 150 g of sterile parboiled rice and 150 mL of water, which were colonized by the pathogen for 10 days at room temperature, according to the methodology described by Barbosa et al. (1995).

The yeasts were isolated from the leaf, root and stem tissue fragments of healthy cowpea and common bean plants. These fragments were separately immersed in test tubes (five pieces per tube) containing 10 mL of sterile water and chloramphenicol (50 mg/L). Subsequently, the tubes were stirred in an ultrasonic bath for 10 min, vortexed and the content was serially diluted. Aliquots of 0.1 mL of these dilutions were spread on Petri plates containing Sabouraud dextrose agar (SDA) composed of 40 g dextrose, 10 g neopeptone and 20 g agar per litre; the agar solution was supplemented with yeast extract (1.5 g/L). The plates were incubated for 72 h at 25 ± 2 °C and, thereafter, the yeast colonies were transferred to tubes containing SDA medium. The isolates were preserved in mineral oil at room temperature (25 ± 2 °C).

Screening the yeasts showing biocontrol potential

Seventy yeast isolates from cowpea and common bean plants were collected in production areas of Pernambuco state, Brazil.

The yeasts were cultivated on SDA medium supplemented with yeast extract and chloramphenicol for 48 h at 28 ± 2 °C under a 12 h photoperiod. The suspensions were adjusted to 10^7 CFU/mL using a Neubauer chamber. Cowpea seeds (cv. IPA-207) were immersed in yeast suspensions with 0.02% tween 80 for 10 min. Treated seeds were kept at 28 °C for 12 h before sowing. The experimental design was completely randomized with 71 treatments (70 yeast isolates plus the control) and five replications, using two seeds in each replication.

The *Rhizoctonia solani* inoculum prepared as described above was incorporated in the soil (50 mg of colonized rice per kg of soil) 48 h before sowing. The treated seeds were sown in expanded polystyrene trays containing humic dystrophic cohesive yellow latosol with a clayey texture. Two experiments with five replications containing two seeds each were conducted at different temperatures, 22–31 °C and 15–26 °C, under 80% humidity in a greenhouse. The control treatment was the soil infested with the pathogen and common bean seeds without treatment with yeasts.

After 15 days, damping-off and stem rot severity were assessed with a descriptive scale (0–4), where: 0 = no symptoms, 1 = hypocotyl with small injuries, 2 = hypocotyl with large lesions and no constrictions, 3 = completely constricted hypocotyl showing damping-off, and 4 = non-germinated seeds and/or non-emerged seedlings (Noronha et al. 1995). The disease index (DI) was calculated using data from the descriptive scale according to McKinney’s formula (McKinney 1923). The DI was subjected to variance analysis (ANOVA) and the means were compared with the Scott-Knott test at 5% probability.

Three isolates [C6A, FVC10 and FVF10 (R1)] were tested in a second experiment as they were among the ones that consistently reduced disease severity in the first two screening experiments. The same methodology was used in this experiment. The experiment was installed in a completely randomized design with four treatments (three yeast isolates plus the control) and five replications. Each replication contained nine plants. The control treatment consisted of soil infested with *Rhizoctonia solani* and cowpea seeds without yeasts. Two experiments were conducted at different temperatures (14–26 °C and 19–30 °C) under 80% humidity. The DI and statistical analyses were calculated as described above.

The *in vitro* antagonism of *Candida saopaulonensis* C6A, *Cryptococcus laurentii* FVC10 and *Bullera sinensis* FVF10 (R1) against *Rhizoctonia solani* was assessed through the method of dual cultures. The yeasts were cultivated in SDA agar as described above. Cell suspensions containing 10^7 CFU/mL were prepared in sterile saline solution. Sterilized glass funnels (7 cm diameter) were immersed in the yeast suspensions and used to stamp the surface of the PDA culture medium in each Petri dish (9 cm). A *Rhizoctonia solani* mycelial disk (5 mm) containing three-day-old colonies was placed at the center of each plate. The plates were kept at $25 \pm$

2 °C under a 12 h photoperiod. Control treatment contained *Rhizoctonia solani*, and the yeast suspension was replaced with sterile saline solution. Plates were kept in BOD for five days at 25 ± 2 °C under a 12 h photoperiod. Mycelial growth inhibition was calculated in relation to the control treatment without any yeast isolate.

Identification of the selected yeast isolates

Three yeast isolates in the biocontrol assays were identified according to classical (Kurtzman et al. 2011) and molecular taxonomies (Negri et al. 2014). The ITS and a fragment of the 26S rDNA genomic regions were amplified by PCR and sequenced using primers ITS1 and ITS4 (White et al. 1990). The DNA sequences generated from the isolates were compared to the sequences deposited in GenBank using Blastn (Altschul et al. 1997).

Induction of systemic resistance

The enzymatic activity was assessed in cowpea plants inoculated with the three selected yeast isolates. The inoculum of *R. solani* was prepared and incorporated in the soil as described above. Seeds were treated and seeded in plastic trays kept in a greenhouse. The design was completely randomized with four treatments (three yeasts plus the control) and five replications. Cowpea leaves were collected 15 days after the beginning of the experiment and processed as follows: 0.1 g of cowpea leaves were immersed in liquid N₂ added with 4 mL of 50 mM potassium phosphate buffer (pH 7.0) and 0.05 g of polyvinylpyrrolidone (Silva et al. 2016). This extract was placed in microtubes and centrifuged for 10 min at 10,000×g at 4 °C (Silva et al. 2017). The supernatant was kept in microtubes and stored at –20 °C. Catalase activity (CAT, EC 1.11.1.6) was determined according to Havir and Mchale (1987). Ascorbate peroxidase activity (APX, EC 1.11.1.11) was determined according to the method of Nakano and Asada (1981), which was modified by Koshiba (1993). Peroxidase activity (POX, EC 1.11.1) was determined according to Urbanek et al. (1991), using guaiacol and H₂O₂ as substrates. Polyphenol oxidase activity (PPO, EC 1.10.3.1) was determined through pyrogallol oxidation (Kar and Mishra 1976). All enzyme activities were expressed as U/min/mg of protein.

Results

Screening potential biocontrol yeast isolates

In the first assay (Fig. 1a), performed at 22–31 °C, 62 isolates showed disease indices significantly lower than

that of the control ($P < 0.05$) and eight isolates did not differ from the control ($P < 0.05$). In the second experiment (Fig. 1b), conducted at 15–26 °C, 36 isolates differed from the control, whereas 34 isolates showed disease indexes similar to the control. Isolates C6A, FVF10 (R1) and FVC10 were randomly chosen among the ones that significantly reduced disease severity in both assays for molecular identification and further experimentation. These isolates were able to decrease severity by 55–72% in the first and by 58–75% in the second experiment.

Yeast identification

The ITS and 26S rDNA sequences identified isolate C6A as *Candida saopaulonensis*, as its sequence was 99% identical to that of isolate JYC1092 (KM555179). The sequence of isolate FVC10 was identical to that of *Cryptococcus laurentii* isolate IMUFRJ 51996 (FN428921). Isolate FVF10 (R1) was identified as *Bullera sinensis* as its sequence was 97% identical to an isolate of this species recovered from a landfill site (DQ297411). The sequences varied from 644 bp to 837 bp and were deposited in GenBank under accession numbers KX781276, KX781277 and KX781278, respectively.

Biocontrol activity *in vitro* and *in vivo* of selected biocontrol isolates

A dual plate assay showed that isolate C6A of *Candida saopaulonensis* inhibited 52% of *Rhizoctonia solani* mycelial growth. This was the only isolate that significantly inhibited *Rhizoctonia solani* compared to the control (*data not shown*). Two experiments were performed to test the biocontrol activity of the three selected isolates against *Rhizoctonia solani*. All isolates significantly reduced disease severity in both experiments. This reduction varied from 49 to 66% in the first experiment and from 31 to 65% in the second experiment (Fig. 2).

Induction of systemic resistance

Cowpea plants exposed to *B. sinensis* FVF10 (R1) showed peroxidase and catalase activities significantly higher than other treatments, but a low activity of ascorbate peroxidase (Fig. 3). Plants treated with *Candida saopaulonensis* C6A showed low ascorbate peroxidase activity whereas the activities of the other enzymes were higher than the activities of their controls. The plants treated with *Cryptococcus laurentii* FVC10 showed peroxidase activity lower than that of the other treatments, but the activities of the other enzymes were higher than

Fig. 1 Screening of potential biocontrol yeast isolates in bioassays performed with cowpea. Treatments were 70 yeast isolates inoculated on cowpea seeds, and the control was non-inoculated with yeasts. Disease severity was determined 15 days after sowing. **a** Assay 1, temperature ranging from 22 to 31 °C. **b** Assay 2, temperature ranging from 15 to 26 °C. Means followed by the same letters are not significantly different according to Scott-Knott test ($P=0.05$). Disease index (DI) was calculated according to McKinney (1923)

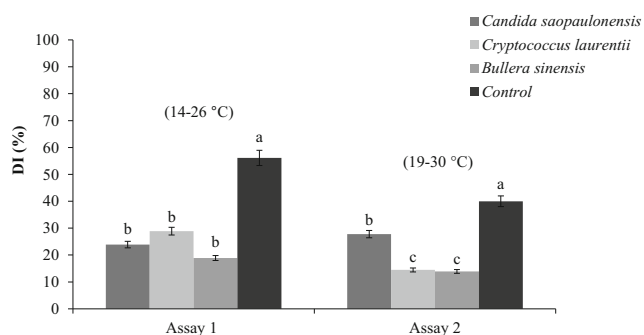
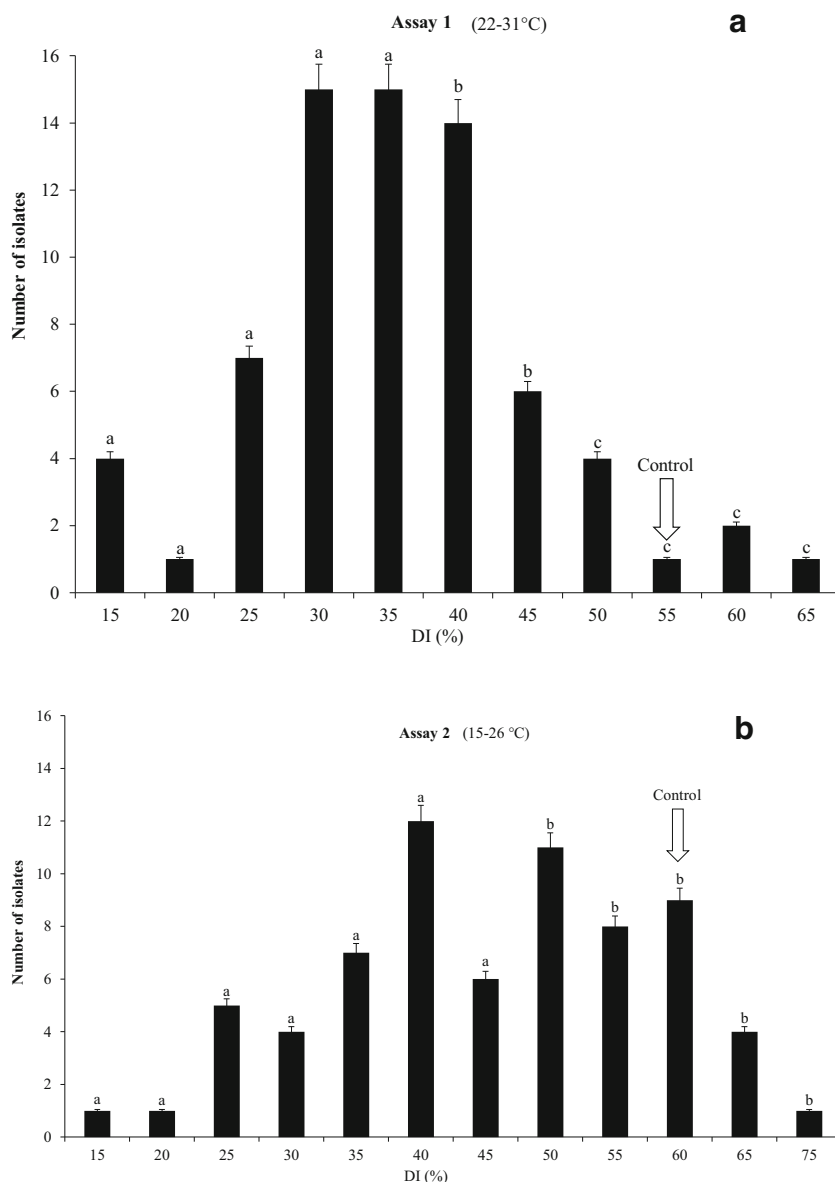


Fig. 2 Control of damping-off and stem rot caused by *Rhizoctonia solani* with three selected yeast isolates in assays with the temperature ranging from 14 to 26 °C (Assay 1) and from 19 to 30 °C (Assay 2). The data are representative of two independent experiments. Means with the same letters are not significantly different according to the Scott-Knott test ($P=0.05$). Disease index (DI) was calculated according to McKinney (1923)

that of their respective controls. The application of *Bullera sinensis* FVF10 (R1) led to maximum activity of the enzymes in the ROS group (peroxidase and catalase) in cowpea plants.

Discussion

Damping-off and stem rot diseases caused by *Rhizoctonia solani* are the most severe diseases of cowpea worldwide. There are no efficient control strategies against these diseases (González et al. 2011). In this study, 70 yeasts were isolated from cowpea and common bean and screened against *Rhizoctonia solani*. Three yeast isolates, *Candida saopaulonensis* C6A, *Cryptococcus laurentii* FVC10 and

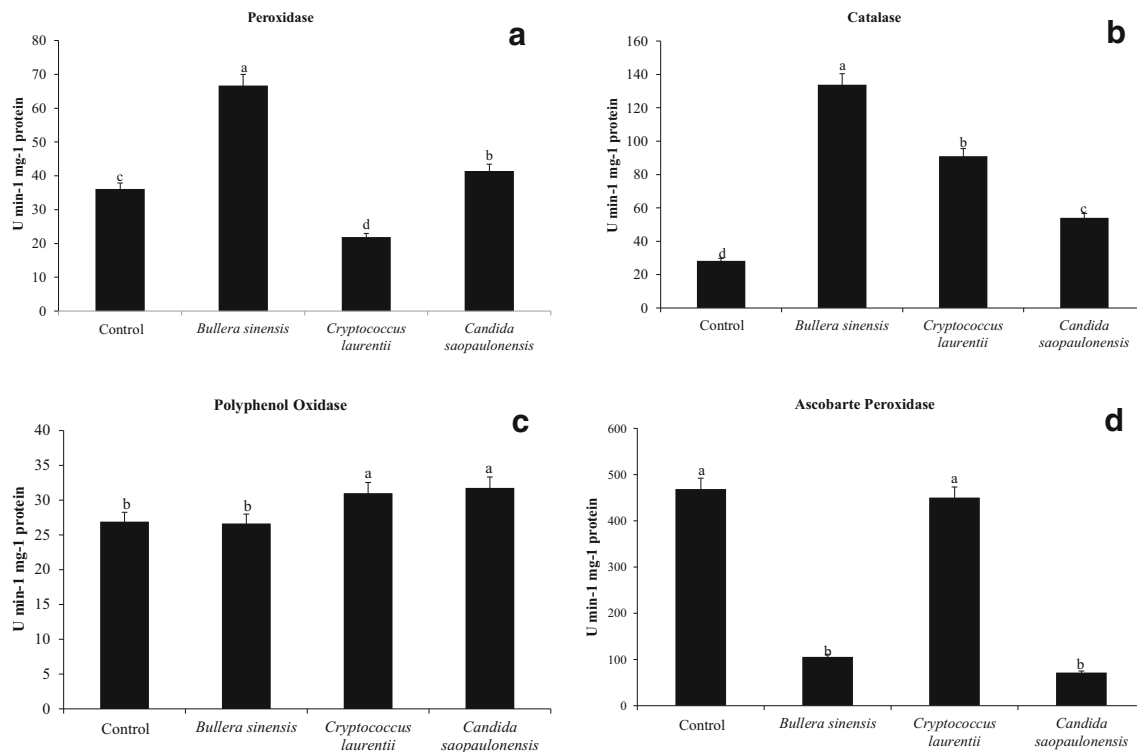


Fig. 3 Enzymatic activities in cowpea leaves inoculated with *Rhizoctonia solani* and treated with *Bullera sinensis*, *Cryptococcus laurentii* and *Candida saopaulonensis* suspensions at 10^7 CFU mL or sterile water

(control). **a** Peroxidase; **b** Catalase; **c** Polyphenol oxidase; **d** Ascobarte peroxidase. Means with the same letters are not significantly different based on the Scott-Knott test ($P = 0.05$)

Bullera sinensis FVF10 (R1), were selected as potential biocontrol agents of *Rhizoctonia solani*.

Candida saopaulonensis C6A, *Cryptococcus laurentii* FVC10 and *Bullera sinensis* (FVF10 (R1) decreased the severity of damping-off and stem rot caused by *Rhizoctonia solani* in cowpea plants. A higher reduction in disease severity was observed at higher temperatures (19–30 °C). Teixidó et al. (1998) demonstrated that yeasts were more efficient as biological control agents at approximately 30 °C. The temperature is one of the main environmental stresses that affect yeasts (Sui et al. 2015). When applied before harvesting in the field, antagonistic yeasts need to tolerate environmental stresses. Beneficial microorganisms need to overcome these challenges in order to effectively establish themselves and control plant diseases (Conway et al. 1999; Deacon 1991; Sui et al. 2015). In the present study, most yeasts significantly reduced the disease index (DI) at different temperatures. This relatively good response at different temperatures may be explained by the fact that the temperature could have influenced the virulence of *Rhizoctonia solani* (Goulart 2002; Santos et al. 2005; Tanaka 1994).

Candida species may produce antifungal compounds as observed in our study with *Candida saopaulonensis* C6A and by other authors (El-Tarabily and Sivasithamparam

2006). The activity of a biocontrol agent by antibiosis does not rule out other mechanisms (Tuzun and Kloepper 1995).

The significant increase in the concentrations of peroxidase and catalase in plants treated with yeast *Bullera sinensis* FVF10 (R1) and the absence of *in vitro* antibiosis are evidences that the mechanism of disease control in question is related to induced resistance. Khalid (2014) found that *Saccharomyces cerevisiae* reduced the incidence of *S. rolfisii* disease in common bean plants, resulting in increased peroxidase, polyphenoloxidase and chitinase activity.

Cryptococcus laurentii FVC10 and *Candida saopaulonensis* C6A increased polyphenol oxidase levels in plant tissues as observed for other yeast species (El-Tarabily and Sivasithamparam 2006; Zhao et al. 2008; Khalid 2014). Furthermore, there are reports that ascorbate peroxidase and catalase act in the modulation and removal of the excess of reactive oxygen species (ROS) that result from stresses. Thus, helping the plant's defense mechanism by preventing ROS accumulation and the consequent cell death (Mittler 2002; Tománková et al. 2006).

Candida saopaulonensis C6A, *Cryptococcus laurentii* FVC10 and *Bullera sinensis* FVF10 (R1) were effective against *Rhizoctonia solani* and are promising control agents against the pathogen in cowpea plants. The current study is the first to report the isolation of *Candida saopaulonensis* and *Bullera sinensis* from bean plants, as

well as their efficacy in biological control of *R. solani*. Understanding the role of yeasts in the control of soil pathogens will contribute to future sustainable agricultural practices.

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