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Yeasts from native Brazilian Cerrado plants: Occurrence, diversity and use in the biocontrol of citrus green mould

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ABSTRACT

Yeasts are some of the most important postharvest biocontrol agents. Postharvest oranges frequently deteriorate due to green mould (*Penicillium digitatum*), which causes significant losses. The aims of this study were to determine the composition and diversity of yeasts on plants of the Brazilian Cerrado and to explore their potential for inhibiting citrus green mould. Leaves and fruit of *Byrsonima crassifolia* and *Eugenia dysenterica* were collected from Cerrado conservation areas, and thirty-five yeasts were isolated and identified by sequencing the D1–D2 domain of the rDNA large subunit (26S). The isolates represented the *Aureobasidium*, *Meyerozyma*, *Candida*, and *Pichia* genera. Three isolates identified as *Aureobasidium pullulans* exhibited potential for the control of *P. digitatum* in both *in vitro* and *in vivo* tests; these isolates reduced the incidence of disease and increased the storage time of fruit. *Aureobasidium. pullulans* has immense potential for the biological control of filamentous fungi.

Introduction

The Cerrado, which occupies over 2.0 million square kilometres, is the second largest Brazilian biome (Brannstrom *et al.* 2008; Ribeiro *et al.* 2011) and is included among the world's 35 biodiversity hotspots due to its biological richness and the degree of anthropogenic pressure experienced by the region (Silva & Bates 2002; Wantzen *et al.* 2012). It is home to 15 000 plant species and is the most biodiverse savannah in the world (Eiten 1982). Because diverse nutrients are available, Cerrado plants provide an excellent habitat for bacteria, filamentous fungi, and yeasts (Kirk *et al.* 2008; Carvalho *et al.* 2012). It is estimated that at least 24 000 filamentous fungal species are associated with Cerrado plants (Furlaneto & Dianese 1997), but little is known about the yeast species in this region.

Yeasts have potential as biocontrol agents because they compete for nutrients and inhibit the growth of opportunistic microorganisms and plant pathogens. They also colonise possible sites of infection, such as injuries and natural openings,

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without producing toxins, which is a great advantage for consuming food *in natura*. Due to their adaptation to this wide variety of niches, they are potentially effective as biocontrol agents (Droby *et al.* 2009; Sharma *et al.* 2009; Liu *et al.* 2013).

Postharvest fungal diseases cause significant losses of fruits and vegetables; it is estimated that one fourth of all fruits and vegetables deteriorate due to pathogens after harvest (Droby 2006; Sharma *et al.* 2009; Liu *et al.* 2013). The citrus green mould caused by *Penicillium digitatum* is one of the important diseases that limits the commercial value of harvested fruits. It is also often associated with allergies due to the large number of spores that it produces and with toxicity due to its production of the mycotoxin patulin (Moss 2008; Souza Sant'Ana *et al.* 2008; Marcel-Houben *et al.* 2012). This pathogen is controlled primarily through the use of fungicides, but the efficacy of fungicides has become limited over time due to the emergence of fungicide-resistant strains (Sánchez-Torres & Tuset 2011). Additionally, growing concerns over public health and environmental pollution have been the primary driving force behind the search for alternative methods of disease control. In this context, biocontrol offers a possible approach to effective and ecologically sustainable disease control (Sharma *et al.* 2009; Liu *et al.* 2013).

Materials and methods

Isolation and identification of yeasts

Isolation of yeast samples

Two plant species native to the Cerrado, *Byrsonima crassifolia* (Malpighiaceae), and *Eugenia dysenterica* (Myrtaceae), were collected from the Olympic Centre of the University of Brazil (21/03/11; S 15°45′57″ and W 47°51′16″) and from the National Park of Brazil (07/10/11; S 15°47′ and W 47°56′), respectively. Mature healthy leaves and fruits were collected aseptically and processed within 48 h. The samples were cut into small pieces, ground and placed in Erlenmeyer flasks containing 0.8 % peptone aqueous solution (w/v) (Bio-Rad, Hercules, CA, USA) in a 9:1 ratio of peptone solution volume to sample volume. The samples were agitated on a rotary shaker at 150 rpm for 25 min. Later, each peptone solution were diluted by factors of 10^{-1} up to 10^{-3} in culture tubes containing 9 mL of peptone solution. Aliquots of 100 μL from these serial dilutions were pipetted onto and spread on Petri dishes; three Petri dishes were used per dilution. The yeast were isolated on Petri dishes with YM agar (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone, 1 % glucose, 2 % agar) containing 100 mg mL^{-1} chloramphenicol. The Petri dishes were incubated in a growth chamber at 28 °C for a period of 5–7 d, and after incubation, colonies were counted and expressed as colony-forming units (CFU). Representative colonies were selected according to their cultural characteristics (colony colour, size, surface, texture, border type and growth rate) and picked for purification 0, 5, and 7 d after processing the samples; these time points were chosen to allow for the growth of the yeast colonies and to avoid the growth of filamentous fungi on the plates. Each isolate was purified by subsequent streaking on the same medium with chloramphenicol (100 mg mL^{-1}) and microscope observation. Pure yeast cultures were catalogued and preserved at −80 °C in cryovials containing 700 μL of YM liquid culture medium and 300 μL of 50 % glycerol (v/v).

DNA extraction

DNA was extracted from yeast culture samples as described by Xin *et al.* (2009), with some modifications. The isolates were grown in 9 mL of YM liquid culture medium (pH 5.6) for 48 h at 28 °C without shaking, after which they were centrifuged for 5 min at 12 000 rpm. Cells were lysed by agitation at 2500 rpm with 0.1 g of glass beads in 200 μL of extraction buffer for 1.0 min. The culture was centrifuged for 10 min at 12 000 rpm after adding 200 μL of phenol:chloroform:isoamyl alcohol at 25:24:1 (v/v). DNA in the supernatant was precipitated with isopropyl alcohol for 1 h at −20 °C. After that, the DNA samples were washed and centrifuged twice with each of 70 % and 100 % ethanol. The precipitated DNA was then suspended in 30 μL of Milli-Q H_2O and stored at −20 °C until further use.

Amplification of rDNA, analysis of sequences and identification of yeasts

Fragments of the D1/D2 domain of the rDNA large subunit (LSU) were amplified as described by Kurtzman & Robnett (1999) using the primers NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL4 (5′-GGT CCG TGT TTC AAG ACG G-3′) (O'Donnell *et al.* 1993). The resulting PCR products were purified using a USB® ExoSAP-IT® Kit according to the manufacturer's instructions, and the purified PCR products were then sequenced at the Catholic University of Brazil using the Sanger method. The sequences that were obtained were compared with sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/) using BLAST (Basic Local Alignment Search Tool) software. The sequences were aligned and a phylogenetic tree was constructed using MEGA software version 5, which performs maximum likelihood and Bayesian inference analyses.

Biocontrol trials

Pathogen and yeast isolates

The plant pathogen *Penicillium digitatum*, which was isolated from oranges showing symptoms of citrus green mould, was obtained from the Department of Plant Pathology of the University of Brasilia. It was grown in Petri dishes containing potato-dextrose-agar (PDA). The fungus was grown on PDA culture medium for seven days at 25 °C, and the resulting spore suspension was prepared using a hemacytometer and adjusted with sterile distilled water to 10^5 spores mL^{-1}. The yeast isolates were added to Erlenmeyer flasks containing YM liquid culture medium, and the flasks were incubated on a rotary shaker at 150 rpm for 72 h. The resulting cell suspension was adjusted to 10^8 CFU mL^{-1} in sterile distilled water.

Screening antagonistic yeasts in vitro

The yeast isolates obtained from the leaves and fruit of *Byrsonima crassifolia* and *Eugenia dysenterica* and the plant pathogen samples were grown at 25 °C for 72 h in YM and PDA, respectively. The antagonistic activity of each yeast isolate was evaluated in Petri dishes containing PDA using the dual culture

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