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Yeasts isolated from figs (*Ficus carica* L.) as biocontrol agents of postharvest fruit diseases

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ABSTRACT

Fresh fruit is highly perishable during postharvest life, mainly due to fungal growth. Thus, fungal control is an important goal for the fruit industry. In this work, a selection of antagonistic yeasts isolated from fig and breba crops were screened *in vitro*. The isolated yeasts were challenged with three moulds isolated from decayed figs and breba crops, identified as *Penicillium expansum* M639 and *Cladosporium cladosporioides* M310 and M624, and pathogenic moulds *Botrytis cinerea* CECT20518 and *Monilia laxa* CA1 from culture collections. Two yeast isolates, *Hanseniaspora opuntiae* L479 and *Metschnikowia pulcherrima* L672, were selected for their ability to inhibit the growth of aforementioned moulds. These yeasts reduced the radial growth of moulds on PDA by between 45.23% and 66.09%. Antagonistic activity was associated with the interaction of live yeast cells with moulds. *M. pulcherrima* L672 apparently parasitised *C. cladosporioides* isolates. In addition, challenges were assayed using wounded apples and nectarines, with significant reductions in percent infection and lesion size for all moulds tested. To our knowledge, this is the first report identifying *H. opuntiae* as an antagonist against different pathogenic moulds.

1. Introduction

The control of postharvest fruit decay is an important challenge for the fruit industry. Moulds and other pests account for losses of 20–25% of fruit production (Sharma et al., 2009). Biological control is one of the most promising alternatives to unpopular synthetic fungicides, and research on postharvest biocontrol has increased in recent decades (Droby et al., 2009).

The main characteristics of an ideal biocontrol agent were defined by Wilson and Wisniewski (1989), and are related to biosafety, activity in a range of environments and against a variety of pathogens, and ease of management and use. Members of the genera *Bacillus* (Kumar et al., 2012; Ren et al., 2013), *Pseudomonas* (Zhou et al., 1999; Cirvilleri et al., 2005) and *Pantoea* (Nunes et al., 2002), among others, have been shown to be effective in the biological control of mould rots; the commercial product Biosave (*Pseudomonas syringae* Van Hall) is even available on the market in the USA (Stockwell and Stack, 2007). Bacteria control pathogens mainly by the production of antibiotics. However, the use of antibiotic-producing microorganisms in food products causes significant concern due to the possible development of resistance in pathogens. The focus, therefore, is to find antagonistic microorganisms that control postharvest pathogens by other mechanisms such as competition for space and/or nutrients, direct parasitism, cell-to-cell contact, production of antimicrobial compounds such as antifungal killer toxins or “mycocins” or induced resistance in the harvested fruits and vegetables (Ceugniez et al., 2015; Droby, 2006; Hatoum et al., 2012; Sharma et al., 2009; Spadaro and Gullino, 2004). Yeasts are promising antagonistic microorganisms because some species have the characteristics of an ideal biocontrol agent (Liu et al., 2013). For example, yeasts are genetically stable, do not need special nutrients to proliferate rapidly, are resistant to adverse environmental conditions, are effective against wide range of fruit pathogens, do not produce metabolites dangerous to human health and are not strongly affected by pesticides (Liu et al., 2013; Sharma et al., 2009). In fact, there are already several yeast-derived

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commercial products for the postharvest biocontrol of pathogens (Droby et al., 2009). Among the different yeast species, the most frequent antagonistic strains include *Aureobasidium pullulans*, *Candida oleophila*, *Cryptococcus laurentii*, *Metschnikowia pulcherrima* and *Pichia guilliermondii*.

In this study, yeasts isolated from different cultivars of *Ficus carica* L. stored in a modified atmosphere were selected on the basis of their antagonistic activities after challenge on agar plates with moulds isolated from decayed figs and two common pathogen fungal species, *Monilia laxa* and *Botrytis cinerea*. A preliminary characterisation of the mechanism of action of the selected yeasts was carried out. Finally, the antagonistic capacity was evaluated in a series of challenges of the antagonistic yeasts with pathogenic moulds on wounded apples and nectarines.

2. Materials and methods

2.1. Isolation of yeasts and moulds and preparation of inocula

The isolates of yeasts and moulds used in this work (Table 1) were obtained from different cultivars of breba and fig (*F. carica* L.) stored in equilibrium-modified atmosphere packaging (EMAP) (Villalobos et al., 2014), using potato dextrose agar (PDA; Oxoid, Unipath, Basingstoke, UK) acidified at pH 3.5 with a sterilised solution of tartaric acid (10%) and Rose Bengal chloramphenicol agar (RBCA; Oxoid). Yeasts were isolated from fruit before storage (day 0) or at day 7 of EMAP storage, while the moulds were isolated at day 14 or 21 of EMAP storage when fungal decay was clearly present. Two common pathogenic mould strains were also included in the study: *B. cinerea* CECT20518 from the Spanish Type Culture Collection, and *M. laxa* CA1, from the Department of Plant Health (Government of Extremadura).

The isolates of yeasts were grown in nutrient yeast dextrose broth (NYDB: 0.8% of nutrient broth, 0.5 g of yeast extract, and 1% of dextrose (w/vol); Scharlab, Barcelona, Spain) on an orbital shaker (Thermoshake THO 500, Gerhardt Analytical Systems, Germany) set up at 120 rpm for 24 h at 25 °C. Cells were collected by centrifugation for 10 min at 16,000 *g*, washed twice with 0.05 M phosphate buffer (pH 7), and then suspended in sterile distilled water. Finally, the cells were counted in a Neubauer chamber and adjusted to different concentrations with sterile distilled water for experimental treatments (5×10^8, 5×10^7, 5×10^6 cells/mL).

Spore suspensions were obtained by growing pathogen moulds (except *M. laxa* CA1) on PDA (Oxoid) at 25 °C for 7–10 days. The spores were collected in distilled water with 0.05% (v/v) Tween 80 (Scharlab). Finally, spores were counted in a Neubauer chamber and adjusted to the required concentrations for experimental treatments. In the case of *M. laxa* CA1, inoculations were performed using plugs of agar (6 mm) containing 5-day-old mycelia.

2.2. Identification of the isolates

2.2.1. Yeasts identification

Yeast isolates were identified by PCR-RFLP of the ITS1-5.8S rRNA-IT2 region and subsequent sequencing of this genomic region for isolates with different profile as described Gallardo et al. (2014).

To extract DNA, a ~1 mm yeast colony from the PDA plate was added to 50 μL of sterile deionised water, boiled at 95 °C for 10 min and centrifuged at 16000 *g* for 5 min. ITS region amplifications were performed using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), as described by White et al. (1990). PCR products were digested with the restriction enzymes *Cfo*I, *Hae*III, and *Hinf*I (Fermentas, Thermo Fisher Scientific Inc., MA, USA) in three independent reactions. The resulting fragments were separated by horizontal electrophoresis at a constant voltage (90 V) in 2% agarose gel and visualised by UV and PCR product sizes were estimated by comparison with a DNA ladder standard (Thermo Fisher Scientific Inc., MA, USA) using Image Analysis Software (Genetools, Syn-Gene, Cambridge, United Kingdom). The fragment profiles obtained were grouped on operational taxonomic units (OTUs). The ITS region of one member of each OTU was amplified as above. PCR products were purified using the GFX PCR DNA and Gel Band Purification kit (GE Healthcare, UK) and sequenced by the Facility of Applied Bioscience Techniques (STAB) at the University of Extremadura (Badajoz, Spain). Taxonomic identification was by comparing the genomic sequences with the GenBank database using the BLAST algorithm.

The identity of yeasts selected from *in vitro* antagonist assays (described below) was also confirmed by sequencing the D1/D2 domain of the 26S LSU of rRNA, as described Gallardo et al. (2014), using the primers NL1 (5′-GCA TAT CAA TAA GCG GAGGAA AAG-3′) and NL4 (5′-GGT CCG TGT TTC AAGACG G-3′) proposed by O'Donnell (1993).

2.2.2. Moulds identification

To extract the DNA of moulds, each isolate was grown on PDA plates at 25 °C for 4 days. Conidia and mycelia of each isolate were harvested by gently scraping the surface of the culture with a sterilised loop, and placing into a 1.5 mL microcentrifuge tube. Approximately 100 mg of fungal material was ground with a motor-driven pestle in 0.6 mL of extraction buffer (50 mM Tris–HCl, pH 8;

Table 1
Origin, day of isolation and code of the yeasts and mould isolates used in this study.

Cultivar	Days of storage	Isolates
Yeasts		
Banane	0	L379
	7	L411
Cuello de Dama Blanco	0	L240, L621, L638
	7	L341, L413, L437, L644
Cuello de Dama Negro	0	L412, L453, L498, L502, L519, L526, L561, L672, L688, L721, L755
	7	L414, L416, L475, L479, L538, L539, L541, L543, L545, L546, L553, L554, L558, L560, L563, L567, L571, L579, L580, L585, L588, L595, L596, L658, L788, L791, L793, L868, L873, L887, L912, L919, L921
San Antonio	0	L20, L120, L197
	7	L158, L196, L222
Moulds		
Cuello de Dama Blanco	14	M310
	21	M639
Cuello de Dama Negro	14	M624