International Journal of Food Microbiology 147 (2011) 211–216

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Short Communication

Yeast biocontrol of fungal spoilage of pears stored at low temperature

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ARTICLE INFO

Article history:
Received 29 November 2010
Received in revised form 18 March 2011
Accepted 6 April 2011
Available online 13 April 2011

Keywords:
Pyrus communis L.
Postharvest diseases
Penicillium expansum
Botrytis cinerea
Antagonists yeast
Biocontrol

ABSTRACT

To reduce the use of fungicides, biological control with yeasts has been proposed in postharvest pears. Most studies of antagonists selection have been carried out at room temperature. However, in regions like North Patagonia where fruits are stored at −1/0 °C during 5–7 months the selection of potential antagonist agents must be carried out at low temperature. In this study, 75 yeast cultures were isolated from healthy pears from two Patagonian cold-storage packinghouses. *Aureobasidium pullulans*, *Cryptococcus albidus, Cryptococcus difluens, Pichia membranifaciens, Pichia philogaea, Rhodotorula mucilaginosa* and *Saccharomyces cerevisiae* yeast species were identified. Additionally, 13 indigenous isolates of *Penicillium expansum* and 10 isolates of *Botrytis cinerea* were obtained from diseased pears, characterized by aggressiveness and tested for sensitivity to postharvest fungicides. The yeasts were pre-selected for their ability to grow at low temperature. In a first biocontrol assay using the most aggressive and the most sensitive isolate of each pathogen, two epiphytic isolates of *A. pullulans* and *R. mucilaginosa* were the most promising isolates to be used as biocontrol agents. They reduced the decay incidence by *P. expansum* to 33% and the lesion diameter in 88% after 60 days of incubation in cold. Foreign commercial yeast used as a reference in assays, only reduced 30% of lesion diameter in the same conditions. Yeasts were not able to reduce the incidence of *B. cinerea* decay. The control activity of the best two yeasts was compared with the control caused by the fungicides in a second bioassay, obtaining higher levels of protection against *P. expansum* by the yeasts. These two regional yeasts isolates could be promising tools for the future development of commercial products for biological control.

1. Introduction

Argentina is the largest pear producer country and the major exporter in the Southern Hemisphere. The main pear-growing area in Argentina is situated in the provinces of Rio Negro and Neuquén (North Patagonia). Both *Penicillium expansum* and *Botrytis cinerea*, are the most important spoilage fungi on pears and apples during refrigerated postharvest storage (−1/0 °C). Postharvest decay of apple and pear fruits can be reduced by avoiding injury to the fruit during harvest and subsequent handling, stringent sanitation practices, the use of fungicides and storage in cold or under modified atmosphere environment (Lennox and Spott, 2003; Zhang et al., 2005). However, these beneficial practices are usually not enough to completely protect harvested fruits from spoilage.

Due to different factors as: i) the increasing health and environmental concerns over pesticide disposal and residue levels on fresh commodities, ii) the development of fungicide-resistant isolates of postharvest spoilage fungi, and iii) the deregistration of some of the more effective fungicides, the need for new safer effective alternatives with no risks to human health and the environment has been proposed.

Several promising biological approaches that include the use of either antagonistic microorganisms or compounds of natural origin, have been proposed as potential alternatives to synthetic fungicides for postharvest diseases control (Droby et al., 2009; El-Ghaouth et al., 2002; Janisiewicz and Korsten, 2002; Usall et al., 2000; Wisniewski et al., 2007). Yeasts are interesting microorganisms to be used in Biological Control programmes because they are relatively easy to produce and maintain and they have several characteristics that can be manipulated in order to improve its use and efficiency (Pimenta et al., 2009). In particular, the high efficiency of yeasts applied as biocontrol agents (BCAs) is related to: i) their adaptation to both the immediate environment and the nutritional conditions prevailing at the wound site, ii) their capacity to grow at low temperatures and iii) their ability to colonize wounds (Janisiewicz et al., 2010; Sharma et al., 2009).

A number of antagonistic yeasts have been selected and evaluated for commercial use as postharvest biological treatment (Janisiewicz et al., 2010; Wisniewski et al., 2007). However, the financial costs involved in the registration of a foreign biological product inhibit its availability in several countries. On the other hand, BCAs isolated from the commercial environment and target fruits from specific

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doi:10.1016/j.ijfoodmicro.2011.04.007

geographic areas may be more adapted and, therefore, should be better antagonists than BCAs from other origins. (Pimenta et al., 2009).

The aim of the present work was to select regional yeast isolates to be used in the biocontrol of *P. expansum* and *B. cinerea* in pears stored at low temperature in commercial packinghouses in North Patagonia. A comparison of the antagonistic activity of these yeasts and a commercial culture of biocontrol yeast was also carried out.

2. Materials and methods

2.1. Source of spoilage fungi and biocontrol yeasts

Both spoilage fungi and epiphytic yeasts were isolated from pear fruits Packham's Triumph cultivar stored for seven months at −1/0 °C in two packinghouses from the Upper Valley region of Rio Negro and Neuquén provinces (Patagonia, Argentina) during 2007. Packinghouse A was characterized by the continuous use of postharvest synthesis fungicides (conventional) management. Packinghouse B has not used fungicides for the last two years (transition to certified organic management).

2.2. Spoilage fungi

The fruits that showed the typical symptoms of blue or grey mold were removed from storage, superficially sterilized with ethanol (70% v/v) and used for isolation of fungi. Each isolate of *Penicillium* sp. and *Botrytis* sp. was grown at 24 °C as a monoconidial culture on potato dextrose agar (PDA) and kept at 8 °C until use. Fungal cultures were maintained on PDA, and their virulence was assured by periodic transfers through pears. The *Botrytis* isolates were identified by phenotypic (cultural and morphological) features from cultures on PDA. The *Penicillium* isolates were identified by phenotypic features from cultures on Czapek yeast autolysate agar plates (CYA) and Czapek agar plates (Cz) according to Samson et al. (2000) and by ITS1-5.8S–ITS2 rDNA PCR-RFLP (Pianzzola et al., 2004). The PCR and restriction products were resolved by electrophoresis in 1.5 and 3% w/v agarose respectively.

2.3. Yeasts

Epiphytic yeasts were isolated from healthy pear surfaces. Two 2×2 cm blocks were removed from each fruit by using a sharp knife and immediately immersed in 9 ml of sterile distilled water with 0.05% w/v of Tween. Blocks were sonicated (5 times during 10 s), centrifuged (10 min at 3000 rpm) and resuspended in 100 µl of distilled water. Each resuspended pellet was seeded on GPY agar (w/v: 0.5% yeast extract, 0.5% peptone, 4% glucose, 1.5% agar-agar) supplemented with ampicillin (0.5 µg/ml). After 48 h of incubation at 26 °C, a representative number of yeast colonies were selected according to their frequencies and morphology and identified by ITS1-5.8S–ITS2 rDNA PCR-RFLP (Esteve-Zarzoso et al., 1999). Patterns obtained for each isolate after digestion with the restriction enzymes Cfo I, Hae III and Hinf I were compared with those of reference strains available in the yeast identification database (www.yeast-id.com). The identity of isolates representative of each different PCR-RFLP pattern was confirmed by sequencing the D1/D2 domains of the 26S rRNA gene (Kurtzmann and Fell, 1998). The sequences obtained for yeast isolates were compared with those published at GenBank.

The commercial yeast used in this study was isolated from a commercial preparation of *Cryptoccocus albidus* (YieldPlus state supplier) by culture on GPY.

2.4. Characterization and selection of fungal isolates

P. expansum and *B. cinerea* isolates were selected by their aggressiveness and sensitivity to fungicides thiabendazol (TBZ) and captan according to the following procedures.

2.4.1. Aggressiveness determination

The aggressiveness of each fungal isolate was determined by measuring the lesions diameter (mm) induced on pear fruits after wound inoculation with the respective fungal isolate. Pear fruits were surface-sterilized with 70% (v/v) ethanol, and air dried prior to wounding. One wound (3 mm deep and 3 mm wide) was made at the equatorial region of each fruit using a conk borer. Each wound was inoculated with 10 µl of an aqueous suspension (10^6 conidia/ml) of the respective fungal isolate. *B. cinerea* conidia preceded from 14-days-old cultures in light at 20 °C and *P. expansum* conidia from 7-days-old cultures grown in darkness at 20 °C. Treated fruits were placed in poly-ethylene bags and incubated at 20 °C and 95% relative humidity (RH) for seven days. Lesion diameters were measured and recorded. Each assay was conducted three times with three fruits per assay.

Minimal conidial concentration (MCC) was determined for *B. cinerea* and *P. expansum* on pears. Conidia suspensions were adjusted to 10^2 to 10^6 conidia/ml. Pear fruits were disinfected, wounded and inoculated as described above.

2.4.2. Fungicides sensitivity

Fungicide sensitivity was tested on PDA added with either thiabendazol (TBZ) or Captan. For this purpose, 10 µl of a conidial suspension (10^6 conidia/ml) was placed as a drop on PDA plates amended with 2000, 1000, 500, 250, 100, 50, 10, 5, 1 µg/ml of TBZ [2-(4-Thiazolyl)-1 H-benzimidazole as Tecto '50SC Syngenta Agro SA'] or 666, 333, 166, 88, 41, 20, 10, 5, 1 µg/ml of captan [N-(triclorometiltio) phtalimida as CAPTAN S. Ando and Cía.S.A.] (Pianzzola et al., 2004). After 72 h of incubation at 20 °C in darkness, fungal growth was visually determined (Table 1). Minimal inhibitory concentration (MIC) of TBZ and captan, defined as the lowest concentration that inhibited fungal

Table 1
Aggressiveness and sensitivity to fungicides of *P. expansum* and *B. cinerea* isolates.

Pathogens	Isolates	Lesion diameter * (mm)	MIC† (µg/mL)	
			Captan	TBZ
P. expansum	AP1	22.6 ± 2.0 abc	5	250
	AP2	22.3 ± 5.0 abc	5	>2000
	AP3	24.6 ± 4.0 abc	5	250
	AP4	27.0 ± 1.0 bc	5	250
	AP5	20.0 ± 5.0 ab	5	250
	AP6	22.3 ± 7.0 abc	5	1
	AP7	29.0 ± 2.0 c	5	250
	AP8	22.3 ± 3.0 abc	5	250
	AP9	20.0 ± 5.0 ab	5	250
	AP10	17.6 ± 3.0 a	5	250
	AP11	26.0 ± 6.0 bc	5	1
	AP12	20.3 ± 0.5 ab	5	5
	AP13	29.0 ± 6.0 c	5	>2000
B. cinerea	AB1	37.6 ± 8.0 ab	41	250
	AB2	45.3 ± 9.0 b	88	250
	AB3	24.0 ± 6.0a	41	250
	AB4	28.6 ± 10.0ab	88	250
	AB5	38.0 ± 1.0 ab	41	250
	AB6	34.0 ± 11.0 ab	41	250
	AB7	33.6 ± 6.0 ab	41	250
	AB8	34.3 ± 11.0 ab	41	250
	AB9	39.6 ± 12.0 ab	5	1
	AB10	39.3 ± 4.0 ab	41	250

*Results presented as mean ± standard deviations. Values within a same column and fungi followed by the same letter are not significantly different according to Fisher's test ($P>0.05$).
†MIC: Minimal Inhibitory Concentration. TBZ: thiabendazole.