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Short Communication

Yeast biocontrol of fungal spoilage of pears stored at low temperature

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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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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.

growth, was determined for *B. cinerea* and *P. expansum* (Pianzzola et al., 2004). Experiments were carried out in duplicates.

2.5. Yeasts growth at low temperatures

Native epiphytic yeasts were pre-selected by growth at storage temperature (−1/0 °C). Aqueous suspensions of the yeasts (10^6 cells/ml) were prepared for each isolate and 5 µl were seeded as a drop on GPY agar plates surface. Plates were incubated at −1/0 °C for seven days and daily evaluated for growth. Assays were carried out in triplicate.

2.6. In vivo biocontrol assays

Biocontrol assays were carried out on Packham's Triumph and Beurre D'Anjou cv pear fruits. Yeasts were prepared by growing cultures on GPY-agar for 24 h at 20 °C. A loop of the respective young yeast culture was suspended in sterile water at a concentration of 10^6 cell/ml. Pears selected by size uniformity and absence of injuries were surface-disinfected with 70% (v/v) ethanol. One wound (3 mm deep and 3 mm wide) was made at the equator of each fruit with a disinfected tool and 20 µl of the corresponding yeast suspension was placed into each wound. After 1 h, the treated wounds were inoculated with 20 µl of either *P. expansum* or *B. cinerea* conidia suspension at MCC. Wounds inoculated only with spoilage fungi were used as controls. After inoculation, pears were placed in polyethylene bags and stored in boxes under standard conditions (−1/0 °C and 95% RH) for four months. Each pear constituted a single replicate and each assay was replicated nine times. The wounds were examined for decay and lesion diameters (mm) every 15 days. Both incidence – calculated as number of decayed wounds over the total number of wounds – and decay reduction (DR) – calculated as (mean lesion diameter in control − mean lesion diameter in treatment) × 100/mean lesion diameter in control – were recorded during the assay storage time.

The most promising yeast strains were further tested in a second bioassay during the following postharvest period to confirm their biocontrol activity against *B. cinerea* and *P. expansum*. In these bioassays, treatments with TBZ and captan were also evaluated with comparative purposes. Fungicides were applied by dipping the wounded pears in fungicide solution at commercial concentration (530 µg/mL of TBZ and 660 µg/mL of captan) for 30 s. Each pear constituted a single replicate and a total of 15 replicates were carried out.

2.7. Statistical analysis

Data were subjected to analysis of variance (ANOVA) and means were separated according to the Fisher least-significant-difference test ($P = 0.05$) using STATISTICA data analysis software system, version 6 (Stat-Soft, 2001, France).

3. Results

3.1. Identification and characterization of spoilage fungi

Ten isolates recovered from pears with grey mold symptoms and 13 isolates obtained from pears exhibiting blue mold symptoms were identified by morphological methods as *B. cinerea* and *P. expansum* respectively. The taxonomic identity of all isolates *P. expansum* was confirmed by PCR-RFLP. A same PCR product of about 600 pb and the restriction pattern after digestion with Hinf I (300 + 180 + 120 pb) were obtained for all the isolates (data not shown). This pattern was coincident with that reported for *P. expansum* type strain (Pianzzola et al., 2004).

Results of the aggressiveness tests *in vivo* indicated that all isolates of *P. expansum* and *B. cinerea* produced different lesion diameters on pear fruits (Table 1). The isolates that showed the highest aggressiveness levels (the highest lesion diameters) were *P. expansum* AP7 and AP13 and *B. cinerea* AB2 (Table 1).

MIC was also a variable feature among the fungal isolates evaluated. Most isolates belonging to *P. expansum* (62%) showed a MIC for TBZ of 250 µg/mL (Table 1). Two isolates of *P. expansum* (AP2 and AP13) were resistant to the commercial doses of this fungicide (528 µg/mL) and three sensitive isolates (AP6, AP11 and AP12) only grew in plates containing as much as 10 µg/mL of TBZ. All *P. expansum* isolates evidenced a MIC for captan of 5 µg/mL (Table 1).

On the other hand, 70% of the *B. cinerea* isolates showed a MIC for captan of 41 µg/mL and 90% evidenced a MIC for TBZ of 10 µg/mL. The isolate AB9 evidenced the highest level of sensitivity to both evaluated fungicides; it was sensitive to 5 µg/mL captan and 1 µg/mL TBZ (Table 1). The highest levels of resistance to captan were observed for isolates AB2 and AB4 (88 µg/mL). All *B. cinerea* isolates were sensitive to the commercial doses of both fungicides.

For both spoilage fungi analyzed, no direct relationship between aggressiveness and MIC was detected. However, the isolates AP13 and AB2, belonging to *P. expansum* and *B. cinerea* respectively, showed both the highest aggressiveness values and high levels of resistance to the evaluated fungicides. They were selected to be used in *in vivo* biocontrol assays.

3.2. Identification and characterization of yeasts

A total of 75 epiphytic yeast isolates were obtained from the surfaces of healthy pear fruits stored for seven months at −1/0 °C in two different packinghouses (named A and B in this work). Sixty-four percent of the total isolates were isolated from the packinghouse B (Table 2).

Six different yeast species were detected in packinghouse B and four species in packinghouse A. In particular, *Saccharomyces cerevisiae* was only detected in packinghouse A and *Cryptococcus difluens, Pichia membranifaciens* and *Pichia philogaea* were only isolated from packinghouse B (Table 2). The yeast-like fungi *Aureobasidium pullulans* was the dominant species in both origins (Table 2). *Aureobasidium pullulans, Cryptococcus albidus* and *Rhodotorula mucilaginosa* were identified in both A and B packinghouses.

Taking into account that the most important postharvest pear spoilage fungi grow below −1/0 °C (storage temperature), the capacity of the potential antagonists to grow in cold as well as their biocontrol activity were evaluated at this temperature. The capacity of each yeast isolate to grow in cold was evaluated and used as a pre-selection criterion.

Sixty-seven percent of the total yeast isolates obtained from pear surfaces were capable to grow at −1/0 °C in GPY-agar medium (Table 2).

Table 2
Origin, identification and growth in cold (−1/0 °C) of epiphytic yeast.

Yeast species	Number of isolates[a] (%)			Growth at low temperature[b]		
	A	B	Total	+	w	−
Aureobasidium pullulans	10 (37)	24 (50)	34 (44)	28	4	2
Cryptococcus albidus	1 (4)	1 (2)	2 (4)	1	–	1
Cryptococcus difluens	nd	9 (19)	9 (12)	7	1	1
Pichia membranifaciens	nd	1 (2)	1 (2)	1	–	–
Pichia philogaea	nd	7 (15)	7 (9)	7	–	–
Rhodotorula mucilaginosa	5 (18)	6 (12)	11 (14)	7	2	2
Saccharomyces cerevisiae	11 (41)	nd	11 (14)	–	–	11
Total	27 (100)	48 (100)	75 (100)			

[a] A: conventional packinghouse that uses fungicides; B: transition packinghouse that does not use fungicides. nd: not detected.

[b] Number of yeast isolates that grew in GPY agar plates at −1/0 °C according to the following scale: +, growth; w, weak growth; –, no growth.

Table 3
Antagonistic efficacy of six epihytic yeast isolates and a commercial yeast culture against *P. expansum* and *B. cinerea* in pear wounds.

Antagonist source	Yeast isolate	*P. expansum* (60 days)			*B. cinerea* (30 days)		
		Incidence	Lesion diameter*	% DR[y]	Incidence	Lesion diameter*	% DR[y]
Pear surfaces	*A. pullulans* 4	33	3 ± 5 a	88	100	21 ± 4 cd	22
	C. albidus 43	100	22 ± 2 de	8	100	28 ± 1 fg	0
	C. difluens 9	100	9 ± 3 ab	66	100	27 ± 2 e–g	0
	P. membranifaciens 39	100	19 ± 1 c–e	20	100	22 ± 0.5 c–e	18
	P. philogaea 21	100	15 ± 4 b–d	38	100	24 ± .5 c–f	11
	R. mucilaginosa 34	33	3 ± 5 a	88	100	19 ± 4 c	30
Comercial	*C. albidus*	100	17 ± 0.2 b–e	30	100	24 ± 2 c–f	11
Control without yeasts		100	24 ± 0.6 de	–	100	27 ± 0.2 e–g	–

*Values presented as means ± standard deviation (mm). Values within a column followed by the same letter are not significantly different according to Fisher's test ($P>0.05$).
[y]Represent mean percentage of decay reduction (DR) with regard to control (calculated: see Materials and methods).

3.3. First biocontrol assay

One isolate representative of each identified yeast species was selected for *in vivo* biocontrol assays: *A. pullulans* 4, *C. difluens* 37, *C. albidus* 43, *P. membranifaciens* 39, *P. philogaea* 21 and *R. mucilaginosa* 34. All six isolates exhibited good growth capacity at −1/0 °C (Table 2). No *S. cerevisiae* isolate was included in the analysis because of its incapability to grow at low temperatures. A commercial strain *C. albidus* was also evaluated with comparative purpose (Table 3). Minimal conidial concentrations (MCC) of each spoilage fungi were employed in *in vivo* biocontrol assays. MCC values determined in the present work were 1×10^2 conidia/ml for *P. expansum* AP13 and 1×10^4 conidia/ml for *B. cinerea* AB2.

In particular, the pear epiphytic isolates *A. pullulans* 4 and *R. mucilaginosa* 34 caused the minimum percentage of disease incidence (33%) and the highest percentage of decay reduction (88%) after 60 days of incubation with *P. expansum*. The remaining pear epiphytic isolates and the commercial culture were not able to reduce the disease incidence caused by this spoilage fungus; however, they showed different percentages of decay reduction. *C. albidus* commercial strain was unable to control the incidence percentage and only achieved 30% of decay reduction (Table 3). No decay was observed in wounds treated only with the yeasts. The incidence percentage values of 33% in wounds inoculated with *P. expansum* and *A. pullulans* remained stable until the end of the experiment (105 days) while co-inoculation with *R. mucilaginosa* 34 gave incidence values of 65% at the same final time (data not shown).

On the other hand, no yeast was able to reduce the disease incidence caused by *B. cinerea* in assayed conditions (Table 3). Nevertheless, the isolates *A. pullulans* 4 and *R. mucilaginosa* 34 showed the highest levels of decay reduction (22% and 30%, respectively) against this spoilage fungus.

3.4. Second biocontrol assay

A. pullulans 4 and *R. mucilaginosa* 24 that stood out by their antagonistic activity in the first biocontrol assay were reevaluated in a second bioassay under the same conditions of inoculation and conservation (−1/0 °C). Additionally, TBZ and captan treatments at commercial doses were evaluated with comparative purposes.

The protection levels achieved in the second biocontrol assay with the selected epiphytic yeasts were higher than those obtained in the first assay for both spoilage fungi (Fig. 1). Both yeast isolates controlled completely the rot development caused by *P. expansum* after 75 days of incubation and revealed incidence values of 40% after 120 days. After 75 days, 100% decay incidence was observed with TBZ and 40% with captan (Fig. 1A). Inoculation with *A. pullulans* and *R. mucilaginosa* also produced the least lesion diameter in wounds co-inoculated with *B. cinerea* all along the process (Fig. 1B). After 75 and 100 days of assay, *A. pullulans* showed a better biocontrol activity than *R. mucilaginosa*. The fungicides completely controlled *B. cinerea* spoilage after 120 days of assay (Fig. 1D).

As it was observed in the first biocontrol assay, no decay was evidenced in the fruit wounds treated with yeasts and without spoilage fungi.

4. Discussion

A potential limitation in the use of biocontrol agents is their adaptability to conditions prevailing in each particular fruit and storage environment. In this sense, the selection and use of regional antagonistic microorganisms isolated from the same environment in which fruits are stored, became the most effective strategy to prevent postharvest diseases caused by regionally established spoilage fungi.

The characterization of regional spoilage fungi isolates, particularly those causing the major losses in pear production, is also necessary. Based on the discriminatory concentration of TBZ established by FRAC (Fungicide Resistance Action Committee) as the threshold to determine resistance to this fungicide (10 μg/mL) (Smith et al., 2006); 77% and 90% of our regional isolates of *P. expansum* and *B. cinerea* respectively, showed resistant phenotype. Additionally, 15% of the *P. expansum* isolates were resistant to the commercial concentration of this fungicide (530 μg/mL). Studies carried out on spoilage fungi isolated from different packinghouses reported resistance values between 65% (Pianzzola et al., 2004) and 82% (Baraldi et al., 2003) for *P. expansum* and 3% for *B. cinerea* (Lennox and Spotts, 2003). The high levels of resistance to TBZ found in the present study could be associated with the broad, long-term use of this fungicide in regional conventional packinghouses (Dobra et al., 2008).

A threshold concentration to determine resistance to captan was not established by FRAC. For this reason, the concentration employed in regional packinghouses (660 μg/mL) was used in this work as a reference parameter. All isolates belonging to both spoilage fungi were sensitive to the commercial concentration of captan. *P. expansum* showed the highest sensitivity level (MIC 5 μg/mL) in comparison to *B. cinerea* (maximum 88 μg/mL); this last value was similar to those found in other studies (Pepin and MacPherson, 1982; Barak and Edgington, 1984). The multi-site activity of this fungicide has been related to its overall effectiveness, despite their extensive and sometimes exclusive use over many years (Brent and Hollomon, 2007).

Resident fruit microbiota has been proposed as source of microbial agents for the control of fruit postharvest diseases. Some yeast species have been repeatedly isolated from mature pears immediately after harvesting in various geographical locations: *A. pullulans, C. albidus, Cryptococcus flavus, Cryptococcus infirmominiatus, Cryptococcus laurentii, Debaryomyces hansenii, Rhodotorula aurantiaca, Rhodotorula fujisanenis, Rhodotorula glutinis, Rhodotorula minuta* and *Sporobolomyces roseus* (Borges et al., 2004; Chand-Goyal and Spotts, 1996a; Janisiewicz and Bors, 1995; Roberts, 1990). However, only a small proportion of these species have been associated with an effective reduction of incidence and severity of spoilage caused by *P. expansum* and *B. cinerea* (Chand-Goyal and Spotts, 1996b; Sugar and Basile, 2008; Zhang et al., 2005, 2008). In our work, in which yeasts were recovered from fruits

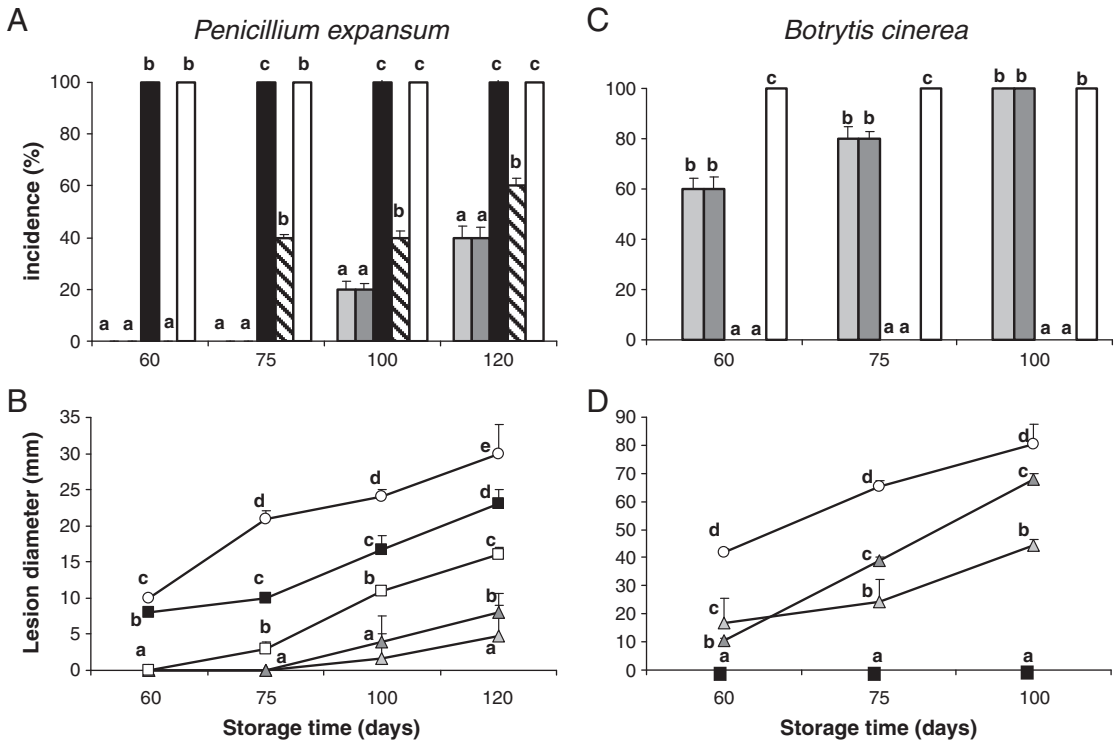

Fig. 1. Spoilage incidence and lesion diameter caused by *P. expansum* (A and B respectively) and *B. cinerea* (C and D respectively) on pear fruits inoculated with fungi alone (▭, △) or in combination with *A. pullulans 4* (▭, △), *R. mucilaginosa 34* (■, ■) , TBZ (▨, □) or captan (▭, ○) and stored at −1/0 °C. The bars represent standard deviations of the means. Values with the same letter at each particular time of assays are not significantly different at $P = 0.05$ (Fisher's test).

after seven months of storage at −1/0 °C in two differentially managed packinghouses, a mostly different biota composed by the species *A. pullulans, C. difluens, C. albidus, P. membranifaciens, P. philogaea, R. mucilaginosa* and *S. cerevisiae* was observed. Our results evidenced the existence of some common species to both analyzed packinghouses, as well as some other species that seem to be associated with only one origin. This behaviour could be due to different technological management: packinghouse A has made an uninterrupted use of postharvest synthesis fungicides (conventional management), while packinghouse B has not used chemical products for the last two years (transition to certified organic management). In this sense, Janisiewicz and Korsten (2002) suggested that fungicides may decrease the microbial diversity on fruits surfaces.

The epiphytic yeast isolates *A. pullulans* 4 and *R. mucilaginosa* 34 showed remarkable biocontrol activity against *P. expansum* under storage conditions (−1/0 °C) in two different bioassays carried out in two consecutive years. The protection levels reached by both *R. mucilaginosa* and *A. pullulans* against the two spoilage fungi during the second year were higher than those obtained in the first bioassay. The different effectiveness of the antagonists in two consecutive annual assays could be due to small physiological differences in the host, even when a same pear cultivar and a same packinghouse were used.

Furthermore, the selected epiphytic yeasts *A. pullulans* 4 and *R. mucilaginosa* 34 were more effective against *P. expansum* than the evaluated fungicides and the commercial yeast after 120 days of conservation in cold. These yeasts resulted to be particularly promising because of its high biocontrol activity against the fungicides resistant *P. expansum* strain tested in this work.

This is the first report about the selection of *R. mucilaginosa* as a microbial antagonist of postharvest spoilage fungi in fruits; however, other species belonging to the same genus, like *Rhodotorula glutinis* have been reported (Castoria et al., 2005; Spotts and Cervantes, 2002; Zhang et al., 2008). The yeast-like fungus *A. pullulans* is a wide-spread saprophyte present in the phyllosphere and it has been previously described as a biocontrol agent to prevent against postharvest fruit spoilage (Spotts and Cervantes, 2002; Vero et al., 2009; Castoria et al., 2001). It is important to remark that most of these studies on the selection of biocontrol agents have been performed at room temperature (Borges et al., 2004; Chand-Goyal and Spotts, 1996b; Sugar and Basile, 2008; Zheng et al., 2007).

Taking into account that: i) the temperature during postharvest storage is −1/0 °C, ii) different strains belonging to the same yeast species can show a differential capacity to grow in cold and iii) the potential antagonist must be evaluated against regional spoilage fungi isolate in real conditions; we emphasize that the probability to find good antagonistic strains for postharvest application became greater when the isolation is made from cold-stored fruits such as it was carried out in this work.

The results of this study indicate that the microbiota associated with pear fruits stored in cold exhibited a better biocontrol efficacy against postharvest spoilage fungi than commercial yeast. Two yeast strains belonging to the species *A. pullulans* and *R. mucilaginosa* were selected for their potential as biocontrol agents against *B. cinerea* and *P. expansum* on pear wounds.

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