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RESEARCH ARTICLE

A new improved strategy for the selection of cold-adapted antagonist yeasts to control postharvest pear diseases

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Postharvest diseases cause considerable losses of harvested fruits during transportation and storage. Many yeast species have been reported as good antagonists against postharvest pear pathogens. In this work, we used a novel selection strategy that involves the isolation of yeasts from washing fluids, showing biocontrol activity against a regional *Penicillium expansum* strain (primary screening), originally obtained from fruit wounds after long time storage at −1/0°C. About 26 isolates representative of the 11 yeast species identified in the 27 selected washing waters were chosen to be evaluated in a secondary screening against a regional *Botrytis cinerea* strain on pear wounds. Among yeasts tested, 38% showed complete control of *P. expansum*, but only 15% reduced the decay incidence of *B. cinerea* to 60–80% at −1/0°C. These results reveal that some of the yeasts found can be biological alternatives to fungicides in the control of *P. expansum* and *B. cinerea* infections. Based on the data obtained, our strategy seems to be much more effective than the previously reported methods in obtaining successful biocontrol agents.

Keywords: postharvest diseases; antagonists yeast; pear; biocontrol

Introduction

Decay of pear fruits during long-term storage, caused by any of several fungal pathogens, can result in significant economic losses in Patagonia (Argentina). Postharvest diseases limit the storage period and marketing life of pears. Blue mould and grey mould decays caused by *Penicillium expansum* Link and *Botrytis cinerea* Pers. ex Fr., respectively, are two of the most important postharvest pear diseases in the world (Janisiewicz and Marchi 1992; Lennox, Spotts, and Cervantes 2003; Zhang, Zheng, Fu, and Xi 2005; Spotts and Cervantes 2002; Sugar and Basile 2008). In Patagonia, *P. expansum* represents the principal pathogen of pear fruit in storage conditions (Dobra, Di Masi, Rossini, and Engler 1995) while *B. cinerea* is the second most relevant pathogen on this substrate (Dobra, Sosa, and Dussi 2008). The control of postharvest mould decays has been traditionally carried out with synthetic fungicides; however, during the last decades, their effectiveness decreased due to the appearance of resistant strains (Mari, Bertolini, and Pratella 2003). A public demand

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to reduce the use of synthetic fungicides, stimulated by a growing awareness in environmental and health issues, also limits the postharvest application of these and other chemicals to agricultural products. In the last decades, research efforts have focused on developing alternative control methods against postharvest diseases of fresh commodities (Baker and Cook 1974; Janisiewicz and Roitman 1988; Wilson, Wisniewski, Droby, and Chalutz, 1993; Sharma, Singh, and Singh 2009).

The use of biocontrol agents (BCAs) occurring naturally on fruit surface has become one of the most preferred ways of controlling postharvest diseases (Wisniewski et al. 2007). Among them, six BCAs based on different yeast species have been registered for pears postharvest application: Aspire, based on the active ingredient (a.i.) *Candida oleophila* (Ecogen Inc. Langorne, USA); Yield Plus a.i. *Cryptococcus albidus* (Lallemand, Montreal, Canada), Shemer a.i. *Metschnikowia fructicola* (AgroGreen, Asgdod, Israel); Candifruit a.i. *Candida sake* (Sipcam-Inaagri, SA Valencia, Spain); Nexy a.i. *C. oleophila* (Lesaffre-Bionext, France) and Boni-Protect a.i. *Aureobasidium pullulans* (Biofa, Münsingen, Germany) (Janisiewicz, Pimenta, and Jurick 2011; Jijalki 2011). The legal permits required for the transfer of beneficial organisms and the financial costs involved in its registration have inhibited the widespread availability of these products in several countries (Sundh and Melin 2011). These restrictions, as well as the fact that BCAs isolated from and adapted to specific geographic areas and target fruits are generally more effective than foreign BCAs, make it necessary the isolation and development of new BCAs from each particular region (Pimenta, Morais, Rosa, and Corrêa 2009).

Many different strategies, directed to select potential BCAs against postharvest pathogens in fruits, have been reported (Wilson et al. 1993; Chand-Goyal and Spotts 1996; Lima, De Curtis, Castoria, and de Cicco 1998; Viñas, Usall, Teixido, and Sanchis 1998). A typical selection strategy for BCAs is constituted by the following steps: (1) the isolation of potential BCAs in a general medium at room temperature from fruit collected in the orchard; (2) the *in vitro* screening of properties of interest in the potential candidates and (3) a final *in situ* assay using individual isolates against the pathogens to be controlled in fruit wounds (Janisiewicz and Roitman 1988; Droby et al. 1999; Bleve, Grieco, Cozzi, Logrieco, and Visconti 2006; Zhang, Spadaro, Garibaldi, and Gullino 2010). However, one of the most relevant problems in the majority of these studies is the fact that the BCA isolation and bioassays are generally performed at room temperature (Viñas et al. 1998; Droby et al. 1999; Zhang et al. 2005; Zhang et al. 2010). In a previous work, we proposed a BCA selection protocol in which both yeast isolation and *in situ* assays of each yeast isolate against the pathogens were performed at the same cold storage conditions ($-1/0^{\circ}$C) (Robiglio, Sosa, Lutz, Lopes, and Sangorrín 2011). The results of that study indicated that the microbiota associated with pear fruits exhibited a better biocontrol efficacy against spoilage fungi than a commercial BCA based on *Cr. albidus* (Robiglio et al. 2011).

A strategy proposed by Wilson et al. (1993) and employed by several researchers, used fruit wounds to screen the biocontrol potential of washing waters obtained from fruit surfaces and containing a mix of unidentified yeast populations (Wisniewski, Droby, Chalutz, and Eilam 1995; Lima et al. 1998; Scherm et al. 2003; Zhang et al. 2010). Potential antagonist isolates were then obtained from active waters. This strategy allows the rapid selection of potential antagonists, with a minimal cost of time and money, and particularly favours the selection of fast growing antagonist

capable to colonise fruit wounds. The problem of that strategy used in several works is the fact that both isolation and bioassays are performed at room temperature (Wisniewski et al. 1995; Lima et al. 1998; Scherm et al. 2003; Zhang et al. 2010). This may partly explain the lack of correlation generally reported between laboratory tests with host/parasite systems at room temperature and the real performance of the BCAs/products under commercial cold conditions (Droby et al. 1993; Wisniewski, Wilson, El Ghaouth, and Droby 2001).

The objectives of this work were to isolate and to select antagonistic yeasts from pear fruit surfaces and wounds, adapted to low storage temperature. An improved selection strategy was used in this work for the first time. This strategy involves the isolation of BCAs from fruit wounds washing fluids showing biocontrol activity against *P. expansum* at −1/0°C. Additionally, a second *in situ* screening using the most promising yeast isolates against *B. cinerea* was also done to select BCAs' ability to control the two main regional postharvest pear pathogens.

Materials and methods

Source of spoilage fungi and biocontrol yeasts

Both spoilage fungi and epiphytic yeasts were isolated from pear fruits Beurre D'Anjou and Packham's Triumph cultivars after 6 months of storage at −1/0°C. Fruits were obtained from two packinghouses located in the Upper Valley of Río Negro and Neuquén provinces (Patagonia, Argentina) during 2008 season. Packinghouse 'B' has not used fungicides for the last two years. Packinghouse 'C' was characterised by the use of organic management, i.e. chemical fungicides have not been used for more than 5 years.

Pathogens

Isolation and identification

Fruits showing the typical symptoms of blue mould or grey mould were removed from storage and used for fungi isolation. Each isolate was grown at 24°C as a monoconidial culture on potato dextrose agar (PDA) and kept at 4°C until use. Fungal virulence was assured by periodic transfers through pears. *Botrytis* isolates were identified by phenotypic (cultural and morphological) features from cultures on PDA. *Penicillium* isolates were identified by phenotypic features from cultures on Czapek yeast autolysate agar plates and Czapek agar plates according to Frisvad and Samson (2004) and by ITS1-5.8S-ITS2 rDNA PCR-RFLP (Pianzzola, Moscatelli, and Vero 2004). All fungal isolates were conserved in PDA and stored at 4°C in the North Patagonian Culture Collection (NPCC), Neuquén, Argentina.

Selection

P. expansum and *B. cinerea* isolates were selected by their aggressiveness and sensitivity to fungicides thiabendazol (TBZ) [2-(4-Thiazolyl)-1H-benzimidazole as Tecto 50SC Syngenta Agro S.A.] and Captan [N-(triclorometiltio) phtalimida as CAPTAN S. Ando & Cía. S.A.] according to the following procedures.

Aggressiveness determination

The aggressiveness of each fungal isolate was determined by measuring the lesion diameters (mm) induced on pear fruits after wound inoculation with the respective isolate. Surface-sterilised pear fruits (70% ethanol) were wounded using a sterile tool (3 mm deep and 3 mm wide) and subsequently inoculated of an aqueous suspension of the respective pathogen isolate at 10^6 conidia/mL. *P. expansum* conidia were obtained from 7-days-old cultures in darkness at 20°C, and *B. cinerea* conidia were obtained from 14-days-old cultures at 20°C under a 12:12 h (light:dark) photo regime. Conidial suspensions were obtained by scraping the colony surface with a sterile scalpel, resuspended in sterile water and filtered to remove fungal mycelium. Conidia concentrations were adjusted by direct count with a Neubauer chamber. Treated fruits were placed in polyethylene bags and incubated at −1/0°C and 95% relative humidity (RH) under commercial storage room conditions. After 60 days, lesion diameters were measured and recorded. Each pear constituted a single replicate and a total of nine replicates were carried out.

MCC, defined as the minimal concentration of conidia that causes rot in fruit (100% incidence), were determined for the more aggressive *P. expansum* and *B. cinerea* isolates according to the methodology described earlier. Each wound was inoculated with 10 µl of a water suspension of the respective pathogen at different concentrations (10^2, 10^3, 10^4 or 10^5conidia/mL). Each experiment was conducted three times with three fruits per treatment.

Sensitivity to fungicides

Fungicide sensitivity of *P. expansum* and *B. cinerea* isolates was tested on PDA added with either TBZ or Captan. For this purpose, 10 µl of a conidial suspension (10^6 conidia/mL) was seeded as a drop on PDA plates amended with 1000, 500, 250, 100, 50, 10, 5 and 1 mg/L TBZ and 666, 333, 166, 88, 41, 20, 10, 5 and 1 mg/L Captan. After 72h of incubation at 20°C in darkness, fungal growth was visually determined. Minimum inhibitory concentration (MIC) of TBZ and Captan, defined as the lowest fungicide concentration that inhibited fungal growth, was determined for *B. cinerea* and *P. expansum* (Pianzzola et al. 2004). Experiments were carried out in duplicates.

Yeasts

Selection

In order to isolate highly effective antagonistic yeasts, a selective method partially adapted from Wilson et al. (1993) was employed. A total of 200 pear fruits were separated in two sets: 100 artificially wounded pears (3mm deep and 3 mm wide) and 100 healthy pears, and both set of pears were then stored at −1/0°C and 95% RH in commercial standard conditions. The same procedure was carried out in the two previously mentioned packinghouses (C and B). After 6 months of storage, 56 healthy pear wounds and 60 healthy surfaces from pears were selected for washing preparations. Both tissue blocks (5 mm deep × 5 mm wide) containing the healthy wounds and surfaces (2 cm diameter) from healthy pears were removed by using a sharp knife and immediately immersed in 1 mL water separately and subjected to

agitation at 150 rpm for 2 h at 1°C. The washing waters obtained were employed as a source of possible antagonists in the primary screening.

Primary screening

Packham's Triumph pear fruits stored for 6 months at −1/0°C were surface-sterilised with 70% (v/v) ethanol, and air dried prior to wounding. The artificial wounds (one wound – 3 mm deep and 3 mm wide – in the equatorial region of each fruit) were performed using a sterile cork borer. Each pear fruit was inoculated with 50 µl of the respective washing. After 2 hours at room temperature, 20 µl of a conidial suspension of *P. expansum* at the MCC (1×10^4 conidia/mL) were also added to the wounds. Pear wounds inoculated only with the pathogen were used as controls. After inoculation, the fruits were placed on trays packs in fruit boxes within polyethylene bags and stored at −1/0°C. The treatments were arranged in completely randomised design. Trials were conducted with five fruits per treatment. The wounds were examined for decay and lesion diameters (mm) every 15 days during the assays storage time. Both disease incidence (DI), calculated as the number of decay 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. Washings exhibiting a reduction in DI higher than 50% and a DR higher than 40% were selected as possible antagonist yeast source.

The potential antagonist yeasts were isolated from washing preparations from healthy wound and surface from fruits of primary screening. About 100 µl of each washing was spread on pear juice agar (85% v/v of fresh pear juice, 1.5% w/v agar-agar) supplemented with ampicillin (0.5 mg/L). Plates were incubated at −1/0°C until yeast colonies development (around 15 days). A representative number of yeast colonies were selected according to their frequencies and morphology and conserved at −20°C using glycerol (20% v/v) as a crioprotectant agent.

Secondary screening

Individual yeast isolates obtained from washings selected in the primary screening were used in the secondary screening. Two yeast isolates: *A. pullulans* NPCC 1281 and *Rhodotorula mucilaginosa* NPCC 1278 selected in a previous work by means of a different selection protocol (Robiglio et al. 2011) were also included in this assay. Antagonists were prepared by growing cultures on Glucosa, Peptona, Yeast extract (GPY) agar (g/L: yeast extract 5, glucose 20, peptone 5 and agar 20) for 24 h at 26°C. A loop of the respective young yeast culture was suspended in sterile water at a concentration of 10^6 cell/mL adjusted by direct counting using a Neubauer chamber. In this assay, surface-sanitised Packham's pears with one month of storage at −1/0°C were wounded (3 mm in diameter and 3 mm deep) and inoculated with 20 µl of the pure yeast culture suspension (10^6 cells/mL). After 2 hours, 20 µl of a *P. expansum* conidial suspension was inoculated at the MCC in the same wounds (1×10^4 conidia/mL). Fruits treated with synthetic fungicides TBZ and Captan were also evaluated in order to compare the control effects of both chemical and biological agents. These fungicides were applied by dipping the wounded pears in fungicide solution at commercial concentration (530 mg/L of TBZ and 660 mg/L of Captan)

for 30 s and inoculated with 20 µl of *P. expansum* conidial suspension (1×10^4 conidia/mL). Wounds inoculated only with spoilage fungi were used as controls.

After inoculation, fruits were placed on packing trays in plastic bags and stored for 200 days in boxes under standard conditions (−1/0°C and 95% RH). The treatments were arranged in completely randomised design. The wounds were periodically examined for decay incidence and lesion diameters (mm) every 15 days. The experiment was designed with five fruits per treatment and each treatment was repeated three times.

The most promising yeast strains against *P. expansum* were further tested in a second bioassay during the following postharvest period to determine their biocontrol activity against *B. cinerea*, according to the methodology described earlier. *B. cinerea* conidial suspension was inoculated at the MCC in the wounds (5×10^3 conidia/mL). Additionally, yeast suspensions (20 µl, 1×10^6 cells/mL) of each promising strains were individually inoculated into the wounds to evaluate the capacity of yeasts to successfully colonise the wound. After inoculation, fruits were placed on packing trays in plastic boxes and stored in boxes under standard conditions (−1/0°C and 95% RH) for 100 days. Both DI and DR were recorded during the assay storage time. The yeast populations were evaluated after 100 days of incubation by yeast cells counts (CFU) according to Vero, Garmendia, Gonzalez, Garat, and Wisniewski (2009). The experiment was designed with five fruits per treatment and each treatment was repeated three times.

Yeast identification

Yeasts obtained from washings selected in the primary screening were identified by ITS1-5.8S-ITS2 rDNA PCR-RFLP analysis as described by Esteve-Zarzoso, Belloch, Uruburu, and Querol (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). Yeast identity was confirmed by sequencing the D1/D2 domains of the 26S rRNA gene (Kurtzman and Robnett 1998). The sequences obtained for yeast isolates were compared with those published at GenBank database (available at the National Center for Biotechnology Information, NCBI) using BLAST.

Statistical analysis

Data were subjected to the analysis of variance (ANOVA) and Tukey test ($P = 0.05$) using STATISTICA data analysis software system, version 7 (Stat Soft Inc., 2004, France).

Results

Isolation, identification and characterisation of pathogens

A total of 32 isolates from pears exhibiting blue mould symptoms and 27 isolates obtained from pears exhibiting grey mould symptoms were identified by morphological methods as *B. cinerea* and *P. expansum*, respectively (Table 1). PCR-RFLP analysis of *P. expansum* isolates evidenced a same PCR product of about 600 pb and

Table 1. Origin, identification and characterization of *Penicillium expansum* and *Botrytis cinerea* isolates of pears with diseases symptoms, after 6 months at cold conservation.

Penicillium expansum						*Botrytis cinerea*					
	Origin			MIC[d] (mg L^{-1})			Origin			MIC[d] (mg L^{-1})	
NPCC	B/C[a]	D/P[b]	Lesion diameter (mm) ± SD[c]	TBZ	Captan	NPCC	B/C[a]	D/P[b]	Lesion diameter (mm) ± SD[c]	TBZ	Captan
2001	B	D	$21.4 \pm 2.5_{ek}$	50	20	2033	B	D	$24.8 \pm 1.8_{df}$	< 1	5
2002	B	D	$25.2 \pm 1.5_{ik}$	< 1	< 1	2034	B	D	$28.4 \pm 2.4_{eh}$	< 1	< 1
2003	B	D	$14.4 \pm 5.4_{bd}$	< 1	< 1	2035	B	D	$14.6 \pm 2.1_{ac}$	< 1	5
2004	B	D	$23.2 \pm 1.3_{gk}$	10	41	2036	B	D	$36.2 \pm 2.5_{hi}$	< 1	10
2005	B	D	$25.8 \pm 2.4_{jk}$	< 1	41	2037	B	D	$19.6 \pm 1.1_{bd}$	< 1	5
2006	B	D	$26.4 \pm 1.8_{k}$	> 1000	20	2038	B	D	$28.8 \pm 3.4_{fh}$	< 1	20
2007	B	D	$17.2 \pm 1.8_{cf}$	> 1000	41	2039	B	P	$10.4 \pm 1.1_{a}$	< 1	< 1
2008	B	D	$19.0 \pm 3.4_{dh}$	> 1000	< 1	2040	B	P	$36.0 \pm 2.3_{hi}$	< 1	10
2009	B	D	$22.6 \pm 2.6_{fk}$	> 1000	< 1	2041	B	P	$28.4 \pm 3.6_{eh}$	< 1	20
2010	B	D	$25.2 \pm 1.3_{ik}$	5	41	2042	B	P	$39.4 \pm 3.4_{i}$	< 1	41
2011	B	D	$21.4 \pm 3.5_{ek}$	< 1	10	2043	B	P	$20.0 \pm 1.6_{be}$	< 1	< 1
2012	B	D	$22.6 \pm 1.1_{fk}$	< 1	41	2044	B	P	$25.1 \pm 1.5_{dg}$	< 1	< 1
2013	B	D	$21.8 \pm 3.1_{ek}$	> 1000	41	2045	B	P	$10.0 \pm 1.6_{a}$	< 1	20
2014	B	D	$17.8 \pm 4.1_{ch}$	> 1000	41	2046	B	P	$64.2 \pm 9.8_{k}$	< 1	20
2015	B	D	$0.0 \pm 0.0_{a}$	> 1000	83	2047	B	P	$21.8 \pm 1.9_{bf}$	< 1	41
2016	B	D	$20.0 \pm 0.0_{ei}$	< 1	< 1	2048	B	P	$53.0 \pm 1.9_{j}$	< 1	41
2017	B	D	$23.3 \pm 1.0_{gk}$	> 1000	41	2049	B	P	$71.6 \pm 6.0_{k}$	< 1	41
2018	B	D	$9.6 \pm 1.1_{b}$	< 1	41	2050	B	P	$65.0 \pm 2.0_{k}$	< 1	41
2019	B	D	$20.8 \pm 3.8_{ej}$	< 1	10	2051	C	D	$34.0 \pm 5.9_{hi}$	< 1	41
2020	B	D	$20.0 \pm 2.1_{ei}$	< 1	< 1	2052	C	D	$24.6 \pm 1.5_{df}$	< 1	41
2021	B	D	$9.6 \pm 1.5_{b}$	> 1000	20	2053	C	D	$33.8 \pm 7.8_{gi}$	< 1	41
2022	B	D	$18.2 \pm 3.1_{ch}$	< 1	< 1	2054	C	D	$13.6 \pm 1.1_{ab}$	< 1	< 1
2023	B	P	$26.2 \pm 0.8_{k}$	> 1000	83	2055	C	D	$34.8 \pm 3.3_{hi}$	< 1	41
2024	B	P	$18.8 \pm 2.4_{ch}$	< 1	< 1	2056	C	D	$23.2 \pm 0.4_{cf}$	< 1	10

Table 1 (*Continued*)

Penicillium expansum						*Botrytis cinerea*					
	Origin			MIC[d] (mg L^{-1})			Origin			MIC[d] (mg L^{-1})	
NPCC	B/C[a]	D/P[b]	Lesion diameter (mm) ± SD[c]	TBZ	Captan	NPCC	B/C[a]	D/P[b]	Lesion diameter (mm) ± SD[c]	TBZ	Captan
2025	B	P	$13.4 \pm 1.1_{bc}$	>1000	20	2057	C	D	$24.4 \pm 3.3_{df}$	< 1	41
2026	B	P	$10.0 \pm 1.6_{b}$	< 1	41	2058	C	D	$48.8 \pm 4.3_{j}$	< 1	41
2027	B	P	$26{,}0 \pm 1{,}6_{jk}$	> 1000	41	2059	C	D	$14.2 \pm 1.5_{ab}$	< 1	< 1
2028	B	P	$24{,}8 \pm 1{,}3_{ik}$	< 1	166						
2029	B	P	$25{,}2 \pm 1{,}3_{ik}$	> 1000	41						
2030	C	P	$16{,}4 \pm 1{,}1_{ce}$	< 1	83						
2031	C	P	$17{,}4 \pm 2{,}4_{cf}$	< 1	83						
2032	C	P	$26{,}8 \pm 1{,}6_{k}$	< 1	41						

Note: Values within a column and pathogen followed by the same letter are not significantly different according to Tukey Tests ($P > 0.05$).
[a]Origin B/C. B: transition packinghouse; C: organic packinghouse.
[b]Origin D/P. D: D'Anjou cultivar; P: Packham's cultivar.
[c]The results are presented as mean of the independent experiments with three times with three fruit per treatment. ±SD: Standard devious.
[d]MIC: Minimal inhibitory concentration of TBZ and Captan.

a restriction pattern after digestion with *Hinf* I consisting on three bands of 300 + 180 + 120 pb. This pattern was coincident with that reported for the *P. expansum* type strain (Pianzzola et al. 2004).

All *P. expansum* and most *B. cinerea* isolates were recovered from the packing-house B (Table 1). Additionally Beurre D'Anjou cultivar was the main source of fungi isolates (Table 1). Results of the aggressiveness tests *in situ* indicated that all isolates of *P. expansum* and *B. cinerea* produced different lesion diameters on pear fruits (Table 1), the mean lesion diameters caused by *B. cinerea* and *P. expansum* were 31 mm and 19 mm, respectively, in assayed conditions. The isolates that showed the highest aggressiveness levels (the highest lesion diameters) were *P. expansum* NPCC 2006, NPCC 2023 and NPCC 2032 and *B. cinerea* NPCC 2046, NPCC 2049 and NPCC 2050 (Table 1).

Regarding resistance assays, MIC for TBZ was also a variable feature among the *P. expansum* isolates evaluated (from < 1 to > 1000 mg/L). Most isolates belonging to *P. expansum* species (86%) were capable to grow in plates containing 1000 mg/L of TBZ, concentration higher than that used in commercial conditions (530 mg/L). MIC for Captan was also variable; but all isolates were sensitive to the commercial doses of this fungicide (660 mg/L). The highest values of MIC for Captan were 83 and 166 mg/L; these values were only observed in 12.5% of the *P. expansum* isolates (Table 1).

B. cinerea isolates were not able to grow in plates containing 1 mg/L of TBZ (Table 1). As in *P. expansum*, MIC for Captan was variable among different isolates of *B. cinerea.* The highest MIC value, showed by 40% of the isolates, was 41 mg/L (Table 1). All *B. cinerea* isolates were sensitive to the commercial doses of both previously mentioned fungicides.

Due to the regional relevance of *P. expansum* as the main regional pear postharvest pathogen, one isolate of this species (*P. expansum* NPCC 2023) was selected to be used during both primary and secondary screenings because of its high aggressiveness and resistance to fungicides. Using the same criterion, the isolate *B. cinerea* NPCC 2049 was chosen to be used during the last step of this selection programme.

MCC was determined for these isolates on pear fruit at low temperature ($-1/0^{\circ}$C) and corresponded to 1×10^4 conidia/mL and 5×10^3 conidia/mL for *P. expansum* and *B. cinerea*, respectively.

Isolation, identification and selection of antagonists

Primary screening

Taking into account that both *P. expansum* and *B. cinerea* pathogens are able to grow at $-1/0^{\circ}$C (storage temperature), the complete process of isolation and selection of antagonist yeast was evaluated at this temperature.

A total of 116 washings were recovered from healthy wounds and pear surfaces obtained from both B and C packinghouses. These washings were tested in a primary screening against *P. expansum* NPCC 2023 in *in situ* assays on pear wounds in cold. More than 60% of the total number of washings tested showed some degree of antagonistic activity (at least 15% of reduction in lesion diameter compared to the control inoculated only with the pathogen). However, only 27 washings (23%),

Table 2. Origin and number of washing waters selected after primary screening against *P. expansum*.

	Number of washing waters (%)				
	Packinghouse B		Packinghouse C		
Washing waters origin	Packham's	D'Anjou	Packham's	D'Anjou	Total (%)
Wounds	10 (37.1)	3 (28.5)	1 (3.7)	4 (14.8)	18 (66.7)
Surface	1 (3.7)	2 (7.4)	2 (7.4)	4 (14.8)	9 (33.3)
Total	10 (37.0)	6 (22.2)	3 (11.1)	8 (29.6)	27 (100.0)

reached the criteria arbitrarily established for the primary screening to be selected as a potential source of antagonists; i.e., washings able to reduce the decay incidence by more than 50% and the lesion diameter by more than 40% (Table 2). Most washings with good antagonist activity (66.7%) were obtained from healthy wounds (Table 2).

According to both yeast colony morphology and frequency on plates incubated in cold, a total of 55 yeast cultures were obtained from the 27 selected washings. The yeasts were identified using the restriction analysis of the 5.8S-ITS region and identity confirmed by sequence analysis as belonging to 11 different species. In Table 3, we indicated the distribution of yeast species in each different washing waters. *A. pullulans* as well as three different species of *Cryptoccocus*: *Cryptoccocus victoriae*, *Cryptoccocus albidus* and *Cryptoccocus wieringae* were the most frequently detected species in the selected washings (Table 3). These yeast species were detected in washings, independently from the origin (packinghouse B/C, cultivar P/D, pear site wound/surface). All remaining yeast species were obtained from one particular washing (Table 3).

Four yeast species were only associated with pears under organic management (packinghouse C): *Cryptococcus tephrensis*, *Candida patagonica*, *Pichia membranifaciens* and *Rhodotorula glutinis*, while *Cystofilobasidium infirmominiatun* and *Rhodotorula laryngis* were only recovered from pears stored in the packinghouse B, under transition management (Table 3). In case of yeast genera that begin with the same initial, a second letter will be used in the abbreviation, to easily differentiate them.

The yeast *Cr. victoriae* was present in all washings that exhibited a DR higher than 60% (seven washing waters, Table 3), on its own (29%) or with other species (71%). In those cases, accompanying species were *Cr. albidus* and/or *A. pullulans*.

Secondary screening

A total of 26 isolates representative of the 11 yeast species identified (species in bold type letters in Table 3) were selected to be evaluated in a secondary screening on pear wounds.

The isolates were selected from active washings according to their frequencies in order to have a complete picture of the yeast diversity. In this bioassay, each yeast isolate was assessed individually against *P. expansum* NPCC 2023 (Table 4). Pears inoculated with TBZ and Captan fungicides at commercial concentrations were also considered with comparative purposes (Table 4).

Table 3. Yeast isolates from each selected washing waters (ww) in primary screening.[a]

		Transition packinghouse (B)			Organic packinghouse (C)		
Pear site	Cultivar	ww	DR (%)	Yeast Species	ww	DR (%)	Yeast Species
Surface	Beurre D'Anjou	1	60	*Cr. albidus,* ***Cr. victoriae***	17	52	*Cr. albidus,* ***R. mucilaginosa***
		2	53.2	*A. pullulans,* ***R. mucilaginosa****, Cr. wieringae*	18	41.3	*A. pullulans,* ***Cr. albidus***[b]*, Cr. tephrensis*
					19	43.3	*A. pullulans,* ***C. patagonica****,* ***Cr. tephrensis***
					20	45.3	*A. pullulans, Cr. albidus,* ***Cr. tephrensis***
	Packham's Triumph	3	85	*A. pullulans, Cr. victoriae*	21	42.1	***A. pullulans,*** *Cr. albidus, Cr. tephrensis,* ***R. glutinis***
					22	46.1	***A. pullulans***[b]*,* ***Cr. albidus***
Wounds	Beurre D'Anjou	4	100	***Cr. victoriae***	23	60	***Cr. victoriae***[b]
		5	43	***A. pullulans***	24	43	***A. pullulans***[b]*, Cr. albidus*
		6	40.3	***R. laryngis***	25	41.8	***A. pullulans***[b]
					26	40.4	*A. pullulans,* ***Cr. wieringae***
	Packham's Triumph	7	100	*A. pullulans, Cr. albidus,* ***Cr. victoriae***[b]	27	43.6	*A. pullulans,* ***P. membranifaciens***[b]
		8	69	*Cr. albidus, Cr. victoriae,* ***R. laryngis***[b]			
		9	60	*A. pullulans, Cr. victoriae, Cr. wieringae, R. laryngis*			
		10	53	***A. pullulans***			
		11	50	*A. pullulans*			
		12	50	***Cy. infirmominiatun***[b]			
		13	49.3	*Cr. wieringae, Cr. victoriae*			
		14	47.2	***A. pullulans***[b]			
		15	47	***Cr. victoriae***[b]***,*** *Cr. wieringae,* ***R. laryngis***			
		16	45.3	***Cr. victoriae***			

ww: washing waters; DR: decay reduction. *A.: Aureobasidium; C.: Candida; Cr.: Cryptococcus; Cy: Cystofilobasidium; R.: Rhodotorula; P.: Pichia.* Bold type: isolates selected to be evaluated in the secondary screening against *P. expansum.* Underline type: most promising yeast strains obtained in the bioassays against *B. cinerea.*

[a]Washing waters exhibiting a reduction in disease incidence (DI) higher than 50% and a DR higher than 40% in primary screening against *P. expansum.*

[b]Most promising yeast strains obtained in the secondary screening against *P. expansum.*

Table 4. Efficacy of yeast and chemical fungicides in secondary screening against pathogen on wound-inoculated pear at −1/0°C.

Treatment					*P. expansum* (200 days)		
Yeast species and strain (NPCC number)		Origin[a]			DI (%)	LD mm (± SD)	DR (%)
A. pullulans	1256	C	P	S	100_{c}	$50.33 \pm 1.52_{h}$	7_{a}
	1262	C	D	w	–	NI	100_{d}
	1269	B	D	w	60_{b}	$45.66 \pm 1.52_{d}$	15_{a}
	1273	C	P	S	–	NI	100_{d}
	1274	B	P	w	–	NI	100_{d}
	1277	C	D	w	100_{c}	$18.00 \pm 1.00_{c}$	67_{c}
	1281	**B**	**P**	**S**	**60b**	$\mathbf{38.66 \pm 3.45_{e}}$	**28ab**
C. patagonica	1249	C	D	S	100_{c}	$39.00 \pm 1.00_{e}$	28_{ab}
Cr. albidus	1247	C	P	S	60_{b}	$46.00 \pm 2.00_{fg}$	15_{a}
	1248	C	D	S	–	NI	100_{d}
Cr. tephrensis	1242	C	D	S	60_{b}	$18.00 \pm 1.00_{c}$	67_{c}
	1244	C	D	S	60_{b}	$43.00 \pm 1.00_{ef}$	20_{ab}
Cr. victoriae	1243	B	D	S	40_{a}	$29.50 \pm 0.50_{d}$	45_{b}
	1255	B	P	w	40_{a}	$23.66 \pm 0.57_{bc}$	56_{c}
	1259	C	D	w	–	NI	100_{d}
	1260	B	P	w	–	NI	100_{d}
	1263	B	P	w	–	NI	100_{d}
	1271	B	D	w	40_{a}	$28.00 \pm 1.00_{d}$	48_{bc}
Cr. wieringae	1268	C	D	w	40_{a}	$12.33 \pm 2.08_{a}$	76_{c}
Cy. infirmominiatun	1261	B	P	w	–	NI	100_{d}
P. membranifacien	1250	C	P	w	–	NI	100_{d}
R. glutinis	1246	C	P	S	80_{c}	$31.66 \pm 1.52_{d}$	41_{b}
R. laryngis	1251	B	D	w	80_{c}	$45.66 \pm 1.52_{d}$	15_{a}
	1253	B	P	w	60_{b}	$18.66 \pm 1.52_{ab}$	65_{c}
	1264	B	P	w	–	NI	100_{d}
R. mucilaginosa	1241	C	D	S	100_{c}	$51.33 \pm 1.52_{h}$	5_{a}
	1270	B	D	S	80_{c}	$41.00 \pm 1.00_{e}$	24_{ab}
	1278	**B**	**P**	**S**	**60b**	$\mathbf{18.00 \pm 1.00_{e}}$	**67c**
Chemical fungicide							
Tiabendazol (528 mg/L)					100_{c}	$52.66 \pm 0.57_{h}$	2_{a}
Captan (660 mg/L)					100_{c}	$41.66 \pm 1.52_{ef}$	23_{ab}
Fungal pathogen alone					100_{c}	$54.00 \pm 1.73_{h}$	

Note: Values within a column followed by the same letter are not significantly different according to Tukey Tests ($P > 0.05$).
[a]Origin B/C. B: transition packinghouse; C: organic packinghouse. D/P. D: D'Anjou cultivar; P: Packham's cultivar; S/H. S: Surface, W: Wounds.
[b]DI: disease incidence, rot percentage; LD: lesion diameter; DR: Decay reduction; NPCC: North Patagonian Culture Collection, Neuquén, Argentina; NI: no visual infection at inoculated wound sites.

All yeasts tested completely controlled the DI caused by *P. expansum* after 100 days of storage at −1/0°C. However, only 10 isolates (*A. pullulans* NPCC 1262, 1273 and 1274; *Cr. albidus* NPCC 1248, *Cr. victoriae* NPCC 1259, 1260 and 1263; *Cy. infirmominiatun* NPCC 1261, *P. membranifaciens* NPCC 1250 and *R. laryngis* NPCC 1264) maintained the complete control after 200 days of incubation (marked as [b] in

Table 5. Efficiency of selected yeast against *B. cinerea* in pear wound at −1/0°C.

Treatment		*B. cinerea* (100 days)[a]		
Yeast species and strain	NPCC	DI (%)	LD (mm ± SD)	DR (%)
A. pullulans	1273	100_b	$73.33 \pm 0.57_d$	19.34_a
	1274	100_b	$71.00 \pm 3.46_d$	23.08_a
	1262	100_b	$71.33 \pm 1.15_d$	22.72_a
Cr. albidus	1248	80_{ab}	$30.33 \pm 9.07_a$	62.08_c
Cr. victoriae	1259	60_a	$35.00 \pm 2.00_{ab}$	62.08_c
	1263	80_{ab}	$40.66 \pm 1.15_b$	55.94_c
	1260	100_b	$66.33 \pm 2.08_{cd}$	28.13_a
Cy. infirmominiatun	1261	100_b	$54.16 \pm 4.70_c$	40.23_b
P. membranifaciens	1250	60_a	$30.33 \pm 1.52_a$	67.13_c
R. laryngis	1264	100_b	$71.33 \pm 4.04_d$	22.72_a
Fungal pathogen alone		100_b	88.50 ± 5.46e	

Note: Values within a column followed by the same letter are not significantly different according to Tukey Tests ($P > 0.05$).
[a]DI: disease incidence, rot percentage; LD: lesion diameter; DR: Decay reduction; NPCC: North Patagonian Culture Collection, Neuquén, Argentina.

Table 3). Most isolates showing the best biocontrol performance were obtained from washing waters of wounds (80%), and from Packham's cultivar (70%) (Table 3).

A broad biocontrol effectiveness range was observed among yeast isolates belonging to the same species. For example, some strains of *A. pullulans* (NPCC 1262, 1273 and 1274) completely controlled DI while other strains of the same species (*A. pullulans* NPCC 1256 and 1277) only reduced the lesion diameter in a minimum percentage (Table 4).

The protection levels achieved by both *A. pullulans* NPCC 1281 and *R. mucilaginosa* NPCC 1278 yeast strains, selected as the best biocontrol yeasts in a previous work (Robiglio et al. 2011), were lower than those obtained for the best yeast isolates described in this work (Table 4). Finally, treatments carried out by TBZ and Captan demonstrated that these chemical fungicides were ineffective in controlling blue mould decay at the evaluated conditions. This result confirms the TBZ-resistance observed for *P. expansum* NPCC 2023 in *in vitro* assays.

B. cinerea *biocontrol assay*

The 10 most promising yeast strains (marked as [b] in Table 3) obtained in the bioassays against *P. expansum,* i.e. those exhibiting complete control of the decay incidence of blue mould, were further tested in a second bioassay to determine their biocontrol activity against *B. cinerea.* All yeast strains reduced lesion diameter caused by *B. cinerea* after 100 days of incubation at −1/0°C (Table 5). *Cr. albidus* NPCC 1248, *C. victoriae* NPCC 1259 and 1263, and *P. membranifaciens* NPCC 1250 reduced the decay incidence to 60–80% and the lesion diameter to 56–67% (Table 5). All effective yeast isolates were able to actively grow under storage condition, reaching maximum populations between 10^5 and 10^6 CFU per wound (data not shown). These four yeasts with the best antagonist activity were recovered from organic pear wounds.

Discussion

The results obtained in this work support the hypothesis that the best strategy to isolate potential antagonists against a particular etiological agent is to look in places where a disease caused by the pathogen can be expected, but it does not occur (Baker and Cook, 1974). The improved methodology proposed in this work is based on obtaining microorganisms from healthy wounds of pear fruits after 6 months of cold storage. These organisms are probably adapted to storage conditions (low temperatures, fruit-host and postharvest treatments) and could exhibit some antagonist activity due to the fact that they were isolated from healthy wounds. Other authors have isolated potential antagonists from fresh fruits before post-harvest storage; however, in that condition it became difficult to find cold-adapted yeasts able to live and compete against naturally occurring fungi (Chand-Goyal and Spotts 1996; Lima et al. 1998; Viñas et al. 1998; Sugar and Basile 2008). In our opinion, the ability to grow at low temperatures is a positive characteristic that must be prioritised from the initial stages of any yeast selection programme to be used as a BCA in cold storage. In the previously mentioned studies, even though a large number of yeasts were isolated from fruits, only 2.5–10% showed an acceptable biocontrol capacity on fruit at 1°C or 4°C (Lima et al. 1998; Viñas et al. 1998; Sugar and Basile 2008). Using our approach, 116 washing waters were tested in a primary screening *in situ* and more than 23% of them were pre-selected because of their biocontrol capacity against *P. expansum* at low temperature. Additionally, 10 out of 26 yeasts isolates obtained from these active washings, completely controlled *P. expansum* DI after 200 days at −1/0°C and four of them were also able to control, in different percentages, the incidence of *B. cinerea* after 100 days at the same temperature. Based on these data, our strategy to obtain BCAs seems to be much more effective than the previously reported methods. The fact that most isolates showing good antagonistic activity were obtained from wounds evidences that healthy wounds are a better BCA source than fruit surfaces.

Another aspect considered in our proposed strategy is the use of local pathogens for BCA selection reflecting the 'real world' in which the potential antagonists will act. The *P. expansum* and *B. cinerea* pathogen isolates used in this work were characterised and selected for their aggressiveness in pear fruits and their low sensitivity level to the two most used fungicides in postharvest storage. The uses of the most aggressive and resistant fungal strains will guarantee, to some extent, the success of the selected BCAs. In some previously published works, pathogen strains used in *in situ* assays during BCA selection were purchased from culture collections (Chand-Goyal and Spotts 1996; Yu et al. 2007). Other works make use of randomly chosen pathogen strains isolated from diseased fruits without testing their aggressiveness and fungicide resistance (Janisiewicz and Marchi 1992; Wilson et al. 1993; Zhang et al. 2008). Regarding resistance assays, and based on the discriminatory concentration of TBZ (10 mg/L) established by Fungicide Resistance Action Committee (FRAC) as the threshold to determine resistance to this fungicide (Smith, Trivellas, Johnson, and Joshi 1991), 50% of the *P. expansum* isolates showed resistant phenotype (Table 1), instead all *B. cinerea* isolates were sensitive to this fungicide. High levels of resistance to TBZ could be explained by the broad, long-term use of this fungicide in regional conventional packinghouses (Brent and Hollomon 2007; Dobra et al. 2008). A discriminatory concentration for Captan

sensitivity was not established by FRAC. The multi-site activity of this fungicide has been related to its full effectiveness despite their extensive and sometimes exclusive use over many years (Brent and Hollomon 2007). All isolates belonging to both pathogens were sensitive to the commercial concentration of Captan (660 mg/L) being *P. expansum* the species showing the lowest sensitivity levels (MIC 166 mg/L). Among the *P. expansum* and *B. cinerea* isolates analysed in this work, no direct relationship between aggressiveness and MIC for TBZ and Captan was detected.

The most frequently isolated yeast genera in active washings were *Aureobasidium*, *Cryptococcus* and *Rhodotorula.* These genera have already been reported as effective BCAs against a number of postharvest fruit pathogens under diverse conditions (Robert 1990; Chand-Goyal and Spotts, 1996; Yu et al. 2007; Sugar and Basile 2008; Vero et al. 2009). The identified biota also includes a single isolate of *Cy. infirmominiatun* whose anamorph *Cryptococcus infirmo-miniatum* was previously reported as pear epiphytic yeast (Chand-Goyal and Spotts 1996). The same researchers also reported the presence of *A. pullulans*, *Cr. albidus* and *R. glutinis* in pears and some isolates of these species showed capacity to reduce decay incidence caused by *P. expansum* in small-scale tests and in semi-commercial and commercial trials (Chand-Goyal and Spotts 1997; Bellows et al. 1999; Sugar and Basile 2008). Our work is the first report about the isolation of *Cr. victoriae, Cr. wieringae, Cr. tephrensis, R. laryngis* and *C. patagonica* from pear fruit surfaces (Table 3).

In accordance with the selective isolation method proposed in this work, most yeast species detected here has also been isolated from a variety of cold environments. *Cryptococcus, Pichia* and *Rhodotorula* genera include several species isolated from cold environment (Scorzetti, Petrescu, Yarrow, and Fell 2000; Starmer et al. 2005; Turchetti et al. 2008). *A. pullulans, Cy. infirmominiatun* and *R. mucilaginosa* have been isolated from glacial ice (Tosi, Casado, Gerdol, and Caretta 2002; Starmer et al. 2005; D'Elia, Veerapaneni, Theraisnathan, and Rogers 2009). On the other hand, yeast species *A. pullulans, C. patagonica, P. membranifaciens* and *R. mucilaginosa* are fairly ubiquitous in both food and natural environments (Querol and Fleet 2006; Sangorrín, Lopes, Giraudo, and Caballero 2007; Gholamnejad, Etebarian, and Sahebani 2010).

This is the first study in which yeast identification at species level has been performed to obtain the real mixed biota present in washings with biocontrol activity. In this sense, interactions between microorganisms in most of the active washings could also be involved in the biocontrol activity of each particular washing. This fact could be taken into account for future development of BCAs based on mixed cultures of yeasts (Viljoen 2006; Sharma et al. 2009). The most frequent mixtures in this work were those containing the species *A. pullulans, Cr. albidus* and *Cr. victoriae.*

In a previous work (Robiglio et al. 2011) a quite different microbiota was obtained from the same packinghouse B. That study was based on a different methodological approach for yeast isolation: the utilisation of washing waters only from the surface of healthy pears and the isolation on GPY agar at 26°C. Out of seven species identified in that work, only four were common to this work: *A. pullulans Cr. albidus, P. membranifaciens* and *R. mucilaginosa.* This difference could be due to the selective pressure applied in this work during yeast isolation: healthy wounds with a greater availability of nutrients than the surface might be antagonist yeasts because pathogens in the storage room do not grow. The fact that the most

promising yeast isolates obtained in the previous work (Robiglio et al. 2011): *A. pullulans* NPCC 1281 and *R. mucilaginosa* NPCC 1278 showed lower biocontrol activity than most yeast isolates obtained during this work (Table 4) is another evidence for the success of the improved methodology proposed.

Our results clearly indicate that there is a large diversity in the biocontrol capacity of different strains belonging to the same yeast species; however, a relationship between the biocontrol capacity and the origin was not observed. A broad biocontrol effectiveness range was particularly evident for isolates of *A. pullulans, Cr. victoriae, R. laryngis* and *R. mucilaginosa.* This diversity was also observed by other authors (Robert 1990; Chand-Goyal and Spotts 1996; Lima et al. 1998; Janisiewicz, Tworkoski, and Kurtzman 2001; Robiglio et al. 2011). Additionally, Janisiewicz et al. (2001) recommended that in order to find the best antagonist strain of particular yeast species, it would be worthwhile to first explore a single location (e.g., orchard) by making multiple isolations of the same species over time. The differences between different locations within a region or between different geographical regions may be even greater (Chand-Goyal and Spotts 1996).

This research represents the first evidence for the potential use of *Cr. victoriae* to control fruit postharvest diseases. Additionally, although other works report the use of *P. membranifaciens* (Masih and Paul 2002; Santos and Marquina 2004; Cao, Yuan, Hu, and Zheng 2010) and *Cr. albidus* (Fan and Tian 2001; Helbig 2002; Chan and Tian 2005) as BCAs for postharvest diseases on fruit, its potential use on pears has been reported in this work for the first time.

In addition, this work proposes an improved strategy based on the utilisation of fruit wounds at low temperatures as a selective step that overcomes the main limitations of other BCA selection protocols previously described (Wilson et al. 1993; Viñas et al. 1998). Several aspects of previously proposed methodologies were significantly improved in this work: (1) wounded pear fruits were stored for 6 months at $-1/0^{\circ}$C; (2) pear fruit blocks from healthy wounds were washed and these washings were used to co-inoculate fresh pear wounds with *P. expansum* at $-1/0^{\circ}$C; (3) regional isolates of *P. expansum* and *B. cinerea* selected for their aggressiveness and sensitivity to fungicides were used in BCA selection; (4) washings exhibiting a reduction in DI higher than 50% and a DR higher than 40% after 50 days at $-1/0^{\circ}$C were selected for yeast isolation and (5) yeast isolation was carried out on pear juice agar plates at $-1/0^{\circ}$C.

Following this improved strategy, four antagonistic microorganisms with potential exploitation as a.i.s for the development of products for postharvest control of blue and grey rot on pear were selected. Future research will focus on the elucidation of the mechanisms of action involved in biological control and on the adaptation of the microorganisms to the fermentation and formulation conditions requested by bioindustries to develop a formulated biofungicide with a potential market.

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