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Short communication

# Efficacy and putative mode of action of native and commercial antagonistic yeasts against postharvest pathogens of pear

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ABSTRACT

Putative mechanisms of action associated with the biocontrol capacity of four yeast strains (*Cryptoccocus albidus* NPCC 1248, *Pichia membranifaciens* NPCC 1250, *Cryptoccocus victoriae* NPCC 1263 and NPCC 1259) against *Penicillium expansum* and *Botrytis cinerea* were studied by means of *in vitro* and *in situ* assays. *C. albidus*$_{(YP)}$, a commercial yeast was also evaluated for comparative purposes. The yeast strains exhibited a variety of different mechanisms including: wound colonization, germination inhibition, biofilm formation, secretion of killer toxins, competition for nutrient and secretion of hydrolytic enzymes (protease, chitinase and glucanase). The relationship between strains (and their associated antagonist mechanisms) and *in situ* antagonist activity was also evaluated. Results indicate that mechanisms such as production of hydrolytic enzymes, the ability for colonization of wounds, production of killer toxin and inhibition of germination are the most important for biocontrol activity. Our study indicate that multiple modes of action may explain why *P. membranifaciens* NPCC 1250 and *C. victoriae* NPCC 1263 provided excellent control of postharvest pears disease.



## 1. Introduction

Postharvest decay of fruit accounts for significant levels of economic losses. It is estimated that in developed countries 20–25% of harvested fruits are decayed by pathogens during postharvest handling and 50% in underdeveloped countries (Korsten, 2006; Sharma et al., 2009). Among different biological approaches suggested in the literature, the use of biocontrol agents (BCAs) shows great potential as an alternative method of postharvest disease control (Droby et al., 2009).

Several mechanisms of action are thought to be involved in the biological control process although the functional role of most of them has not yet been fully explored (Wisnieswski et al., 1991; Sharma et al., 2009; Jamalizadeh et al., 2011). These mechanisms are generally based on: the ability of BCAs to adhere to specific sites, including both host and pathogen cells (Wisnieswski et al., 2007), the ability to secrete specific enzymes (Grevesse et al., 2003), the ability to induce resistance (Yao and Tian, 2005), the ability to regulate population density at specific sites (McGuire, 2000), the ability to secrete antimicrobial substances (water soluble or volatile) and the ability to form biofilms on the inner surface of wounds (Giobbe et al., 2007). One key mechanism that was addressed is the role of ROS (reactive oxygen species) or the resistance of BCAs to oxidative stress (Liu et al., 2011; Jamalizadeh et al., 2011). Biological control that relies on multiple mechanisms may be achieved by using either one biocontrol agent exhibiting several mechanisms or by applying more than one biocontrol agent in a mixture, provided that the component organisms exhibit different mechanisms of action (Guetsky et al., 2002; Sharma et al., 2009).

Blue mould and grey mould decays caused by *Penicillium expansum* and *Botrytis cinerea* respectively, are two of the most important postharvest pear diseases (\ Roitman, 1988; Zhang et al., 2005). Recently, we isolated and identified epiphytic yeasts during cold postharvest storage of pear fruits from two packinghouses in Argentinean North Patagonia and we tested their efficacy for controlling the postharvest diseases of pears caused by these two pathogens. Four yeast strains belonging to three different species were selected as the most promising BCAs: *Cryptococcus albidus* NPCC 1248, *Pichia membranifaciens* NPCC 1250, *Cryptococcus victoriae* NPCC 1259 and 1263 (Lutz et al., 2012).

In the present study potential mechanisms associated with commercial BCA and four newly isolated yeasts, against both *P. expansum* and *B. cinerea* were evaluated. The relationship between the different mechanisms detected among BCAs and *in situ* biocontrol capacity was also analysed.

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## 2. Materials and methods

### 2.1. Microorganisms and culture conditions

Both spoilage fungi (*B. cinerea* NPCC 2049 and *P. expansum* NPCC 2023) and epiphytic yeasts (*C. albidus* NPCC 1248, *P. membranifaciens* NPCC 1250, *C. victoriae* NPCC 1259 and 1263) were isolated from fruit of Packham's Triumph pear cultivars after six months of storage at −1/0 °C identified and selected in a previous study (Lutz et al., 2012). The commercial yeast used in this study was isolated from a commercial preparation of *C. albidus* YP (YieldPlus state supplier). All yeast and pathogen isolates were preserved in glycerol 20% (v/v) and stored at −20 °C in the North Patagonian Culture Collection (NPCC), Neuquén, Argentina.

### 2.2. In vitro antagonistic activity assays

#### 2.2.1. Production of diffusible and volatile substances on agar plate

Production of diffusible substances was evaluated using the dual culture method: 20 μL of yeast suspension ($10^6$ cells/mL) was seeded on plates containing 15 mL of sterile pear juice agar (PJA; 85% v/v of fresh pear juice, 1.5% w/v agar–agar) at 5 cm from a drop of pathogen conidia (10 μL; $10^4$ conidia/mL). The evaluation of volatile antifungal metabolites by yeast isolates was carried out according to Lillbro (2005). The two plates were placed "mouth to mouth", wrapped together with Parafilm. In both assays plates were incubated for 27 days in the dark at 0 ± 1 °C. After incubation, colony diameter was used to calculate inhibition (Etebarian et al., 2005). All experiments were performed in triplicate.

#### 2.2.2. Effects on spore germination

Suspensions of each antagonist yeast (100 μL; $1 \times 10^8$ cells/mL) and pathogen (100 μL; $5 \times 10^6$ conidia/mL) were added to sterile tubes (18 × 150 mm) containing 4.8 mL of sterile pear juice (85% v/v). After 24 h of incubation at 20 ± 1 °C on a rotary shaker (150 rpm), a total of 100 conidia were observed microscopically with a light microscope (NIKON) and the germination inhibition percentage was calculated. Conidia were considered germinated when the germ tube length was equal to or longer than the conidia length. Three replications were evaluated for each treatment and the experiment was repeated twice.

#### 2.2.3. Competition for nutrients

The role of competition for nutrients on the biological control activity of the antagonist yeasts was investigated. Yeast cells (100 μL; $1 \times 10^8$ cells/mL) and pathogen conidia (100 μL; $5 \times 10^6$ conidia/mL) were added to tubes (18 × 150 mm) containing 4.8 mL of sterile pear juice (85% v/v) supplemented with various carbon and nitrogen sources. Unsupplemented cultures served as controls. The concentrations of sugars employed were: 10% w/v glucose, 10% w/v fructose and 3% w/v sucrose to obtain concentrations three, two and three times higher than those reported for the same sugars in the pear tissue (USDA nutrient database for standard reference, release 22, 2009) respectively. Three nitrogen sources ($NH_4NO_3$, $NaNO_3$ and $NH_4Mo_7O_{24}$) were used at final concentration of 0.1% w/v. After 100 h of incubation at 4 °C on rotator shaker (150 rpm), the germination percentages were determined as detailed before. There were two replicate tubes per treatment, and the experiment was performed twice.

#### 2.2.4. Biofilm-forming capacity

Biofilm-forming capacity was evaluated by measuring yeast adherence to a polystyrene surface (Giobbe et al., 2007). Aliquots (10 μL) of the respective a yeast suspension ($10^6$ cells/mL), were inoculated in triplicate into wells of a 96-well polystyrene plate containing 200 μL of two different culture media: either GPY and or pear juice (85% v/v) and incubated at 0 ± 1 °C for 50 days. After incubation, 25 μL of crystal violet 0.1% (w/v) was added to each well. The plates were then incubated for 5 min and the excess dye was removed with sterile water. The adherence of cells was quantified by solubilising the retained crystal violet and measuring the optical density at 650 nm. A strain of *Candida albicans* NPCC 1367 was used as a positive control.

#### 2.2.5. Production of killer toxins

Yeast isolates were tested for killer activity using the reference sensitive yeast strain *Candida glabrata* NCYC 388 and the two pathogens. The assays were carried out by qualitative method (QLM) according to Lopes and Sangorrín (2010). If a particular selected yeast streak was surrounded by a clear zone of growth inhibition fringed with blue colour, this yeast was designated as producer of killer toxins. The assays were replicated three times.

#### 2.2.6. Extracellular lytic enzymes activity

In order to characterize the capability of the selected yeasts to produce and secrete fungal cell wall lytic enzymes (chitinase, protease, glucanase and pectinase), qualitative tests were performed on solid media (agar 15 g/L) containing the corresponding substrates according to previously reported techniques: 1) *chitinase*, on colloidal chitin (Sigma) pH = 7 and a mended with mineral salts (Sousa et al., 2009); 2) *β-1,3-glucanase*, on laminarin 5 g/L or 5 g/L curdulan or 5 g/L pustulan (Sigma) with YNB (Difco) 6.7 g/L, stained with Congo red (Renwick et al., 1991); 3) *protease*, on skim milk power 10 g/L (Dunne et al., 1997) and 4) *pectinase* on apple and citric pectin 140 g/L (Sigma) and YNB 6.7 g/L pH = 7 stained with hexadecyltrimethylammonium bromide (Buzzini and Martini, 2002). Prepared plates were inoculated with 5 μL of the respective 24 h yeast culture suspension ($10^6$ cells/mL). All enzymatic activities were evaluated after incubation in two different conditions: 30 days at 0 ± 1 °C or 7 days at 20 ± 1 °C. Enzymatic activity was detected by the presence of degradation halos developed around the colonies.

### 2.3. In situ antagonistic activity assays

#### 2.3.1. Wound site colonization

Artificial wounds (3 mm deep and 3 mm wide) were performed using a sterile tool on superficially sanitized pears (one wound per fruit). Yeast suspension (20 μL, $1 \times 10^6$ cells/mL) of each respective yeast were individually inoculated into the wounds. Inoculated fruits were placed on tray packs in boxes and incubated for 120 days at 0 ± 1 °C and 95% RH. The yeast growth at the wound site was monitored during the incubation time. Tissue samples containing the whole wound were extracted using a sterile knife. Each sample was placed in an Eppendorf tube containing 1 mL of sterile water, kept on a rotator shaker at 200 rpm for 60 min and homogenized by vortexing during 3 min. The obtained washing waters were serially diluted and seeded on GPY-agar plates. After incubation, at 0 ± 1 °C, the colony-forming units (CFU) were counted. Experiments were repeated three times, with ten fruits per treatment.

Growth parameters were calculated from each treatment by directly fitting CFU *versus* time to the reparametized Gompertz equation proposed by Zwietering et al. (1990). This task was accomplished using the non-linear models of the STATISTICA data analysis software system, version 8 (Stat-Soft, 2007, France).

#### 2.3.2. Biocontrol assays

Pears were wounded as described previously with one wound per fruit. Each wound was inoculated with 20 μL of a 24 h yeast culture suspension ($10^6$ cells/mL). After 2 h, the treated wounds were inoculated with 10 μL of a conidial suspension of $5 \times 10^3$ conidia/mL (*B. cinerea*) or $1 \times 10^4$ conidia/mL (*P. expansum*). After inoculation, the fruits were placed on trays packs in boxes with polyethylene bags and stored for 120 days at 0 ± 1 °C and 95% RH. The wounds were examined for decay (disease incidence; DI) and lesion diameters (mm) every 15 days. There were ten fruits per treatment and

each treatment was repeated three times; the whole experiment described above was conducted twice.

### 2.4. Statistical analysis

Data were subjected to the analysis of variance (ANOVA) and means were separated according to the Tukey test (P = 0.05) using STATISTICA. Data obtained from the different mechanisms of action (qualitative variables) were arranged in a matrix value of 1 or 0 to denote the presence or absence of a particular antagonistic ability respectively. The relationship among strains was evaluated using principal coordinates analysis (PCoA) and depicted in a 2D scatter plot by the Numerical Taxonomy System program (NTSYS). Minimum-length spanning tree (MST) from the simple matching coefficient matrices was calculated and superimposed on PCoA plots to help detect local distortions.

## 3. Results

### 3.1. Evaluation of antagonist mechanisms in vitro

*B. cinerea* growth inhibition in dual cultures attributed to the production of antifungal diffusible metabolites was particularly observed in the presence of *C. albidus*$_{(1248)}$ and *P. membranifaciens*$_{(1250)}$ antagonist yeasts, while no activity was observed against *P. expansum* (Table 1). Production of volatile antifungal compounds was mainly observed for the native yeast *C. victoriae*$_{(1259)}$ and *C. albidus*$_{(YP)}$ (62% and 100% growth inhibition *P. expansum respectively*) (Table 1).

Most antagonistic yeast isolates reduced partly spore germination of pathogens in liquid cultures (Table 1). Among native strains, *C. victoriae*$_{(1259)}$ exhibited the best antagonistic activity against *B. cinerea* (59%) while *C. victoriae*$_{(1263)}$ was the best against *P. expansum* (71%) (Table 1). *C. albidus*$_{(YP)}$ reduced the spore germination of both *B. cinerea* and *P. expansum* (66% and 87% respectively) (Table 1).

None of the five yeasts produced killer toxins at 20 ± 1 °C (Table 1). At 0 ± 1 °C all yeast strains except (*C. albidus*$_{(1248)}$) exhibited killer activity against the sensitive reference yeast *C. glabrata*, but only *P. membranifaciens*$_{(1250)}$ and *C. victoriae*$_{(1263)}$ were able to kill the two pathogens using the same method (Table 1).

All yeast strains were able to hydrolyse three polymer glucans (glucanase activity) at the two temperatures tested, except for both *C. victoriae*$_{(1259)}$ and *C. albidus*$_{(YP)}$ that only showed this activity at 20 ± 1 °C (Table 1). Additionally, *C. albidus*$_{(1248)}$, *P. membranifaciens*$_{(1250)}$ and *C. victoriae*$_{(1263)}$ also showed chitinase and protease activities at 0 ± 1 °C. Experiments of formation of biofilms demonstrated that the two strains of *C. victoriae* showed the best film-forming capacity on both pear juice and on GPY media (Table 1).

A total of six different nutrients including three carbon and three nitrogen sources were tested for their capacity to suppress the antagonist activity of the selected yeasts (Table 2). If both pathogen and BCA compete for a particular nutrient, the addition of this compound must suppress the biocontrol. Because, *C. victoriae*$_{(1263)}$ and *C. victoriae*$_{(1259)}$ yeasts did not show inhibitory effect on the germination of *B. cinerea* and *P. expansum* respectively (Table 1); these two yeasts/pathogen combinations were not evaluated in the competition assays (Table 2). In this analysis, we only considered the cases in which the germination percentage of the pathogen with the sugar in the absence of the biocontrol agent was higher than the same one with the respective yeast (data shadowed in grey in Table 2 were not analysed).

The inhibition of *P. expansum* germination caused by *C. victoriae*$_{(1263)}$ was significantly suppressed in the presence of sucrose and glucose (Table 2), and inhibition caused by *C. albidus*$_{(YP)}$ was partially reduced with glucose. The addition of all three nitrogen sources also decreased the biocontrol capacity of *C. victoriae*$_{(1263)}$ against this pathogen (Table 2). *P. expansum* germination inhibition caused by antagonistic

**Table 1**
*In vitro* antagonistic activity assays of four selected yeast and a commercial yeast.

| Antagonistic activity | | | Yeast strains | | | | |
|---|---|---|---|---|---|---|---|
| | | | *C. albidus* | *P. membra** | *C. victoriae* | *C. victoriae* | *C. albidus* |
| | | | 1248 | 1259 | 1250 | 1263 | YP |
| Inhibition of pathogen growth (%)[1] | Diffusible compounds | *B. c.* | $32_c$ | $17_b$ | $3_a$ | $0_a$ | $0_a$ |
| | | *P. e.* | 0 | 0 | 0 | 0 | 0 |
| | Volatile compounds | *B. c.* | $0_a$ | $3_a$ | $3_a$ | $0_a$ | $14_a$ |
| | | *P. e.* | $12_a$ | $12_a$ | $62_b$ | $0_a$ | $100_c$ |
| Inhibition of pathogen germination (%)[2] | | *B. c.* | $37_b$ | $40_b$ | $59_{cb}$ | $9_a$ | $66_c$ |
| | | *P. e.* | $59_c$ | $23_b$ | $7_a$ | $71_d$ | $87_e$ |
| Killer toxin activity[3] | *C. glabrata* | 20 ± 1 °C | − | − | − | − | − |
| | | 0 ± 1 °C | − | + | + | + | + |
| | *B. cinerea* | 20 ± 1 °C | − | − | − | − | − |
| | | 0 ± 1 °C | − | + | − | + | − |
| | *P. expansum* | 20 ± 1 °C | − | − | − | − | − |
| | | 0 ± 1 °C | − | + | − | + | − |
| Enzymatic activity | Glucanase[4] | 20 ± 1 °C | + | + | + | + | + |
| | | 0 ± 1 °C | + | + | − | + | − |
| | Chitinase | 20 ± 1 °C | − | − | − | − | − |
| | | 0 ± 1 °C | + | + | − | + | − |
| | Protease | 20 ± 1 °C | − | − | − | − | − |
| | | 0 ± 1 °C | + | + | − | + | − |
| | Pectinase[5] | 20 ± 1 °C | − | − | − | − | + |
| | | 0 ± 1 °C | − | − | − | − | − |
| Biofilms formation (%)[6] | | *PJ* | $22.66_a$ | $21.67_a$ | $52.00_b$ | $88.00_c$ | $13.30_a$ |
| | | *GPY* | $20.00_a$ | $15.00_a$ | $36.11_b$ | $45.14_b$ | $25.00_a$ |

*P. membra** = *P. membranifaciens*; *P. e.* = *Penicillium expansum*; *B.c.* = *Botrytis cinerea*; PJ = Pear Juice; GPY = glucose, peptone, yeast extract. 1—Inhibition of growth was calculated as the percentage of the ratio between growth of the dual-culture samples and control without selected yeasts. Values are mean growth from three Petri-dishes. 2—Inhibition of spore germination was calculated as the percentage between spore germination of each treatment and control without selected yeasts in pear juice (85%v/v). Values are mean spore germination from three replicate slides per treatment. 3—Killer activity at different temperatures of antagonist strains against target strains by QLM. 4—Equal data with 1-3β1-6β-glucan (laminarine), 1-3β-glucan (curdulan) and 1-6β-glucan (pustulan). 5—Equal data with apple and citric pectin. 6—Adhesion percentage with respect to *Candida albicans* NPCC 1367(positive control).

* Values in the same row followed by the same letter are not statistically different by Tukey's test ($p < 0.05$).

**Table 2**
Effect of direct interaction in juice and various nutrients on the germination of pathogens.

| Treatment | | Conidial germination (%)[a] | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Carbon sources | | | Nitrogen sources | | |
| Pathogen | Yeast | Control[b] | Glucose | Fructose | Sucrose | $NaNO_3$ | $NH_4NO_3$ | $NH_4Mo_7O_{24}$ |
| B. cinerea | – | $89.5_c$ | $68.5_b$ | $41_a$ | $67_b$ | $92_c$ | $88.5_c$ | $89.5_c$ |
| *B. c. + C. albidus*$_{(1248)}$ | | $56.5_c$ | $33_b$ | $20.5_a$ | $12_a$ | $56_c$ | $50_c$ | $51.5_c$ |
| *B. c. + P. membranifaciens*$_{(1250)}$ | | $54_b$ | $1_a$ | $2_a$ | $4_a$ | $53.5_b$ | $50.5_b$ | $51.5_b$ |
| *B. c. + C. victoriae*$_{(1259)}$ | | $37_{bc}$ | $34_b$ | $30.5_b$ | $19.5_a$ | $40.5_{bc}$ | $47_c$ | $33.5_b$ |
| *B. c. + C. albidus*$_{(YP)}$ | | $30.5_c$ | $0.1_a$ | $23.5_b$ | $7_a$ | $26.5_{bc}$ | $26.5_{bc}$ | $28_{bc}$ |
| P. expansum | – | $70_c$ | $32_b$ | $16_a$ | $69_c$ | $84_d$ | $81.5_d$ | $67.5_c$ |
| *P. e. + C. albidus*$_{(1248)}$ | | $29_{ac}$ | $27_{ac}$ | $22.5_{ab}$ | $23_{ab}$ | $15_a$ | $35_c$ | $48_d$ |
| *P.e. + P. membranifaciens*$_{(1250)}$ | | $54_d$ | $33.5_{ab}$ | $31_a$ | $43_{bc}$ | $28_a$ | $37_{ac}$ | $45.5_{cd}$ |
| *P.e. + C. victoriae*$_{(1263)}$ | | $20.5_a$ | $47_{bc}$ | $64_c$ | $41_b$ | $52.5_{bc}$ | $35.5_{ab}$ | $49_{bc}$ |
| *P. e. + C. albidus*$_{(YP)}$ | | $9_a$ | $65_c$ | $11_a$ | $16_a$ | $14.5_a$ | $45.5_b$ | $26_a$ |

The results are the mean of two independent experiments. Values in each line followed by the same letter are not statistically different by Tukey's test ($p < 0.05$). *P. e.* = *Penicillium expansum*; *B.c.* = *Botrytis cinerea*.
[a]Data shadowed in grey were not analysed.
[b]Controls were realised in pear juice (85%v/v) without supplemental nutrient. Nutrient treatments were realised in pear juice (85%) with supplemental nutrient according to Materials and methods.

activity of *C. albidus*$_{(1248)}$ was partially suppressed in the presence of $NH_4Mo_7O_{24}$ and $NH_4NO_3$; *C. albidus*$_{(YP)}$ showed a similar effect in the presence of $NH_4NO_3$ (Table 2). Neither carbon nor nitrogen compounds suppressed the antagonist activity of the tested yeasts against *B. cinerea*.

### 3.2. Colonization of fruit wound

Growth curves obtained after CFU counts during 120 days of incubation were fitted to describe the behaviour of the five yeasts in wounds (Fig. 1). After 7 days of yeast inoculation, the highest population density values were obtained with *C. albidus*$_{(1248)}$ and *C. victoriae*$_{(1263)}$, which increased by 4–6 orders of magnitude (Fig. 1) and showed μmax 0.40 $h^{-1}$and 0.19 $h^{-1}$, respectively (Table 3). At the same time, *C. albidus*$_{(YP)}$, *P. membranifaciens*$_{(1250)}$ and *C. victoriae*$_{(1259)}$ reached low CFU numbers increasing only 1.5 orders of magnitude. *C. victoriae*$_{(1259)}$ showed the lowest μmax value and its population maintained the same initial CFU numbers during the complete experiment. *C. albidus*$_{(YP)}$ was not effective in colonizing the pear fruit wounds in the cold, showing the highest λ value and a low maximum population (Fig. 1, Table 3). *C. albidus*$_{(1248)}$, *P. membranifaciens*$_{(1250)}$ and *C. victoriae*$_{(1263)}$ reached the highest maximum populations levels (Fig. 1).

### 3.3. Relationship between the putative mechanisms and the real antagonist activity in situ

In other to evaluate the potential relationship between the putative mechanisms and the real antagonist activity of each yeast *in situ*, the complete data set obtained from both *in vitro* and *in vivo* (wound colonization) antagonistic activity assays were used to carry out a principal coordinates analysis (PCoA). For that purpose, activity data were transformed into qualitative (1/0) binary information (presence/absence of: biofilm capacity, wound colonization capacity, killer activity,

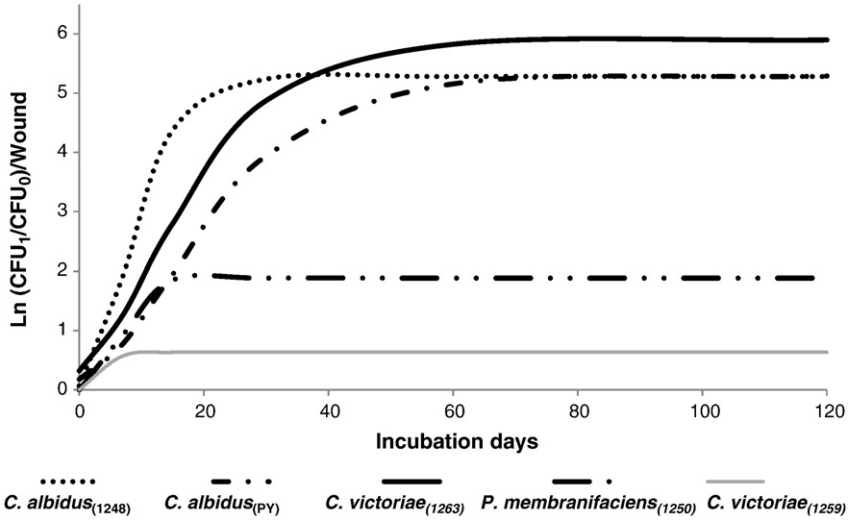


**Fig. 1.** Population dynamics of the four selected yeasts and *C. albidus*$_{(YP)}$ in the wounds of pear fruits at 0 ± 1 °C fitted with the Gompertz models.

**Table 3**
Gompertz derived parameters of antagonist yeasts grown in wounds of pear fruit. Fitted curves are represented in Fig. 1.

| Parameters | Yeast strains | | | | |
|---|---|---|---|---|---|
| | *C. albidus* 1248 | *P. membranifaciens* 1250 | *C. victoriae* 1259 | *C. victoriae* 1263 | *C. albidus* YP |
| A (LnCFU/wound) | $5.2803_{c}$ | $5.2905_{c}$ | $0.6340_{a}$ | $5.8984_{d}$ | $1.8857_{b}$ |
| μmax ($h^{-1}$) | $0.4091_{d}$ | $0.1585_{ab}$ | $0.1314_{a}$ | $0.1973_{b}$ | $0.3262_{c}$ |
| λ (days) | $2.3087_{c}$ | $2.7233_{d}$ | $1.1987_{b}$ | $0.7649_{a}$ | $4.1581_{e}$ |
| $R^2$ | 0.9959 | 0.9945 | 0.8736 | 0.9938 | 1.0000 |

A = maximum population value; μmax = maximum specific growth rate; λ = Lag phase period. R-square coefficient is shown. Values followed by different letters are significantly different according to Tukey's test at $p \leq 0.05$

enzymes, *etc.*). PCoA allowed us to generate a 2D plot explaining 79.1% of the total data variability (Fig. 3). In this plot, two strains clusters were observed: cluster I constituted by *C. albidus*$_{(YP)}$ and *C. victoriae*$_{(1259)}$ and Cluster II containing the native *C. albidus*$_{(1248)}$, *P. membranifaciens*$_{(1250)}$ and *C. victoriae*$_{(1263)}$. Cluster I group strains with a low number of antagonistic mechanisms while Cluster II includes strains with a high number of antagonistic mechanisms including hydrolytic enzymes and killer toxin production, the ability for wound colonization, germination inhibition and competition for ammonium molybdate against *P. expansum*.

The evaluation of the biocontrol capacity of each yeast strain in pear wounds, indicated that strains *P. membranifaciens*$_{(1250)}$ and *C. victoriae*$_{(1263)}$ (located in Cluster II) were the most promising strains, significantly reducing the incidence and lesion diameter caused by the two pathogens (Fig. 2). It is interesting to note that the yeast strains characterized as best BCAs against the two pathogens *in situ* (*P. membranifaciens*$_{(1250)}$ and *C. victoriae*$_{(1263)}$) segregated together in the PCoA plot, far apart from the strains *C. victoriae*$_{(1259)}$ and *C. albidus*$_{(YP)}$. Although *C. albidus*$_{(1248)}$, *P. membranifaciens*$_{(1250)}$ and *C. victoriae*$_{(1263)}$ shared similar antagonistic

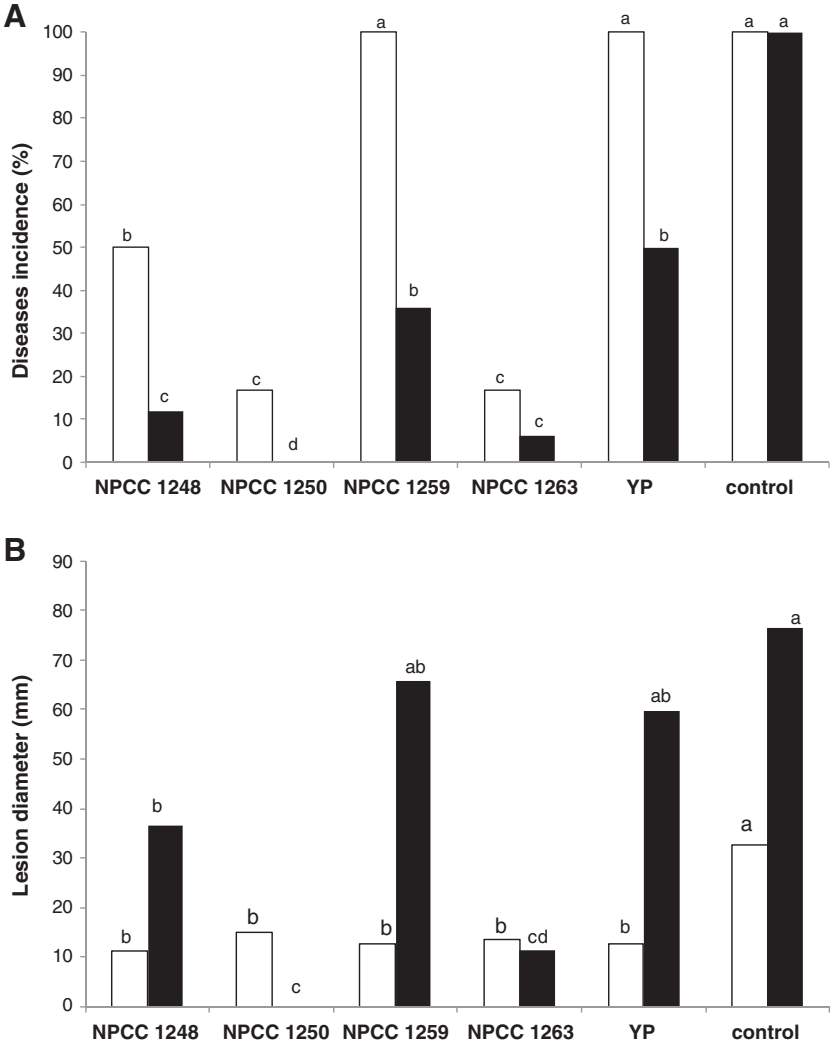


**Fig. 2.** Biocontrol efficacy of the four selected antagonistic yeasts and *C. albidus*$_{(YP)}$ on the decay of pear fruit caused by *P. expansum* (white bars) and *B. cinerea* (black bars): disease incidence (A) and lesion diameter (B) at cold conservation for 120 days. NPCC 1248 = *C. albidus*$_{(1248)}$, NPCC 1250 = *P. membranifaciens*$_{(1250)}$ NPCC 1259 = *C. victoriae*$_{(1259)}$ NPCC 1263 = *C. victoriae*$_{(1263)}$ and YP = *C. albidus*$_{(YP)}$. The data were based on ten replicates of each treatment. Values followed by different letters are significantly different according to Tukey's test at $p \leq 0.05$.

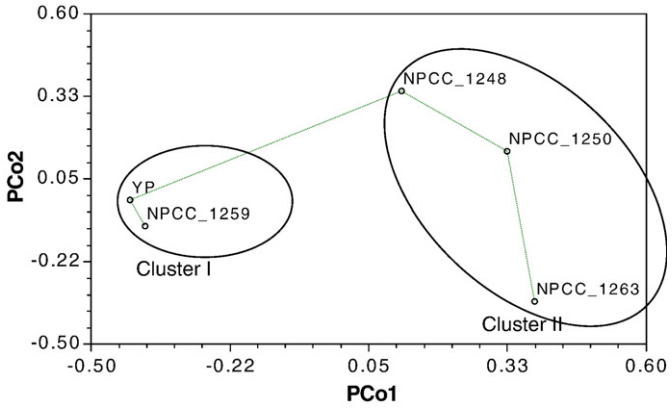


**Fig. 3.** Principal coordinates analysis of the individual pattern mechanism for five yeasts. NPCC 1248 = *C. albidus* NPCC 1248, NPCC 1259 = *C. victoriae* NPCC 1259, NPCC 1263 = *C. victoriae* NPCC 1263, NPCC 1250 = *P. membranifaciens* NPCC 1250 and YP = *C. albidus* commercial yeast. Mechanisms of action analysed: enzymatic activity, wound colonization, germination inhibition, competition for nutrients, production of diffusible and volatile compounds, biofilm-forming capacity and killer toxin production.

activities within cluster II (Fig. 3), these last two strains separated according to their killer activity against the pathogens (Fig. 3).

## 4. Discussion

Four yeast isolates with remarkable antagonistic properties against regional *P. expansum* and *B. cinerea* strains were obtained from pear fruits in a previous work carried out in our laboratory (Lutz et al., 2012). In the present study the mechanisms potentially involved in the antagonist activity of these selected regional yeasts were evaluated and compared with a culture of *C. albidus*$_{(YP)}$ obtained from the foreign commercial yeast. The relationship between the putative mechanisms and the *in situ* antagonist capacity of the strains was also evaluated.

All four regional yeasts assayed in this work have multiple mechanisms involved in their biocontrol effectiveness. *In vitro* results showed that for a particular yeast strain the putative mechanisms of action involved in the biocontrol of *P. expansum* are not necessarily the same as those involved in the biocontrol of *B. cinerea*, indicating a marked BCA/pathogen specificity in the antagonistic mechanism.

Several sugars and nitrogen sources have been tested for their capacity to suppress the antagonistic potential of the yeast strains in different studies of nutrient competition (Piano et al., 1997; Scherm et al., 2003; Chanchaichaovivat et al., 2008). As suggested by Janisiewicz and Korsten (2002), nitrogen is likely to represent a limiting factor in the carbon-rich environment of pear wounds. Our results support the hypothesis that nitrogen and simple sugars are likely to represent a limiting factor for *P. expansum* conidial growth, as the biocontrol capability of several yeast were significantly affected in presence of nutrient. Contrarily, the biocontrol activities of the five yeasts against *B. cinerea* were not decreased by the addition of a set of different nutrients. Interestingly, in our assays several sugars *per se* inhibited the germination of *P. expansum* and *B. cinerea*. Previous studies have also shown the inhibitory effect of fructose, glucose and 2-deoxi-D-glucose on *P. expansum* by reduction of spore germination (Piano et al., 1997; Scherm et al., 2003); and similar effects were reported for glucose, fructose and sucrose on *Botrytis* conidial germination (Guetsky et al., 2002; Card et al., 2009).

On the other hand, *C. albidus*$_{(1248)}$, *P. membranifaciens*$_{(1250)}$ and *C. victoriae*$_{(1263)}$, only exhibited a broad spectrum of diffusible hydrolytic enzymatic activities when experiments were carried out at the temperature of post-harvest storage (0 ± 1 °C). *P. membranifaciens*$_{(1250)}$ and *C. victoriae*$_{(1263)}$ were able to produce toxins against *C. glabrata*, *P. expansum* and *B. cinerea* only in cold (Table 1). The fact that these activities were not observed at 20 ± 1 °C, reinforces the need for evaluation of antagonist mechanisms at the temperatures in which the BCAs act during postharvest.

Regarding the ability of each yeast strains to multiply and survive in *in vivo* wounds, *C. albidus*$_{(1248)}$, *C. victoriae*$_{(1263)}$ and *P. membranifaciens*$_{(1250)}$ reached high and stable population levels in pear fruit wounds under storage conditions (Fig. 2). These results indicate that these yeasts are well adapted to the wound environment in pear fruits and have considerable potential as biological control agents. No relation between film-formation and wound colonization capacity of yeast strains was observed. *C. victoriae*$_{(1259)}$ showed high film-forming capacity in *in vitro* assays but was not the most effective yeast in wound colonization assays *in situ*.

The environment of the wound site seemed to be more favourable to the growth of most regional antagonistic yeasts than to *C. albidus*$_{(YP)}$ with this strain showing the long lag phase and the low numbers of cells at the end of experiment (Fig. 1 and Table 3). According to the data obtained, *C. victoriae*$_{(1263)}$ was the best colonizer yeast, showing high μmax, reduced λ and high A.

The accumulative effects of several control mechanisms detected in *in vitro* and *in situ* assays may explain the high biocontrol efficacy observed when these yeasts were applied to the wounds against pathogens in *in situ* assays. In order to detect which of the evaluated activities could be more related to the *in situ* results, we performed a PCoA. The three best strains identified according to their biocontrol ability (*C. albidus*$_{(1248)}$, *P. membranifaciens*$_{(1250)}$ and *C. victoriae*$_{(1263)}$) were also the same grouped together in the Cluster II of PCoA (Fig. 3). The putative mechanisms that characterized the Cluster II were: production of the three enzymes with glucanase activity, the ability for wound colonization, production of killer toxin, inhibition of germination and competition nitrogen. These results suggest that one or all the previously mentioned mechanisms seem to play a decisive role in the biocontrol *in situ* against both pathogens. *C. albidus*$_{(1248)}$ (located in Cluster II, Fig. 3) possesses similar mechanisms observed for *P. membranifaciens*$_{(1250)}$ and *C. victoriae*$_{(1263)}$; however *C. albidus*$_{(1248)}$ showed less efficacy in *in situ* biocontrol assays. Based on the mechanisms evaluated here, this difference could be determined by the lack of killer toxin activity in comparison with *P. membranifaciens*$_{(1250)}$ and *C. victoriae*$_{(1263)}$; although other mechanisms not evaluated in this work could also be involved in the difference.

Although further investigations using scaled-up conditions are necessary, the results of this study highlight the potential application of *P. membranifaciens*$_{(1250)}$ and *C. victoriae*$_{(1263)}$ as biological agents for postharvest protection of pear fruits against *P. expansum* and *B. cinerea*, with the aim of increasing the storage time of the fruit and of reducing the economic losses due to grey and blue mould decays. More detailed studies of the antagonist mechanisms potentially used by these two selected yeast for biocontrol are currently being undertaken in our laboratory

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