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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; 1×10^8 cells/mL) and pathogen (100 μL; 5×10^6 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; 1×10^8 cells/mL) and pathogen conidia (100 μL; 5×10^6 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, 1×10^6 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 5×10^3 conidia/mL (*B. cinerea*) or 1×10^4 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