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A new strain of *Metschnikowia fructicola* for postharvest control of *Penicillium expansum* and patulin accumulation on four cultivars of apple

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ABSTRACT

The efficacy of three antagonistic yeasts, *Metschnikowia pulcherrima* strain MACH1, *M. pulcherrima* strain GS9, and *Metschnikowia fructicola* strain AL27, against *Penicillium expansum* and patulin accumulation was evaluated on apples stored at room (22 ± 1 °C for 7 days) and cold temperatures (1 ± 1 °C for 56 days). To increase the potential range of application of the biocontrol agents (BCAs), their efficacy was evaluated on four cultivars of apple, *i.e.* 'Golden Delicious', 'Granny Smith', 'Red Chief' and 'Royal Gala'. AL27 was more effective than MACH1 and GS9 in the control of blue mold rot and in the reduction of patulin accumulation. The efficacy of AL27 was in most cases similar to the chemical control used, making the antagonist as competitive as chemical fungicides. *In vitro* experiments showed that AL27 reduced the conidial germination and germ tube length of *P. expansum* more than the other strains. The three BCAs were more effective in the control of blue mold rot on 'Golden Delicious' apples than on the other tested cultivars.

1. Introduction

Postharvest losses of fruit and vegetables are mainly due to attacks of pathogens during harvest, storage, transport and marketing (Snowdon, 1990). Some species of *Penicillium* are important plant pathogens causing decay on various fruit and vegetables, through their antioxidant proteins and hydrolytic enzymes (Bertolini and Tian, 1996; Qin et al., 2007). *Penicillium expansum* can particularly cause blue molds and blue rots on several plant species (Stange et al., 2002).

Besides its pathogenic activity, *P. expansum* is able to produce patulin, a highly reactive unsaturated lacton, that may cause acute and chronic toxicity, including carcinogenic, mutagenic, and teratogenic effects (Beretta et al., 2000; Hasan, 2000; McCallum et al., 2002). The mycotoxin causes impairment of kidney functions, oxidative damage, and weakness to the immune system. It also has a negative impact on reproduction in males *via* interaction with hormone production (Selmanoglu and Kockaya, 2004; Fuchs et al., 2008). Patulin can be found in several typologies of fruit-derived food, including apple, pear, peach and apricot juices and nectars (Spadaro et al., 2007, 2008a). The Joint FAO/WHO Expert Committee on Food Additives (JEFCA) established a provisional maximum tolerable daily intake (PMTDI) of 0.4 μg kg^{-1} body weight (bw) day^{-1}, based on a no observable effect level of 43 μg kg^{-1} bw day^{-1} and a safety factor of 100 (World Health Organization, 1995). Based on this PMTDI, patulin is regulated in the European Union at levels of 50 mg kg^{-1} in fruit juices and fruit nectars, 25 mg kg^{-1} in solid apple products, and 10 mg kg^{-1} in apple-based products for infants and young children (European Commission, 2006).

The use of chemical fungicides is an important strategy for controlling *P. expansum* in harvested commodities (Eckert and Ogawa, 1990; Janisiewicz and Korsten, 2002; Zhou et al., 2002). However, during the last decades, some fungicides have lost their efficacy due to the development of resistant strains. Several studies demonstrated resistance of *P. expansum* to the most common fungicides used in postharvest (Sholberg et al., 2005; Errampalli et al., 2006). Moreover, concern for public safety has resulted in the cancellation of some of the most effective fungicides in Europe (European Parliament, 2009) and the United States (United States Congress, 1996; Dayan et al., 2009). Therefore, research focused on the development of alternative control that should be both effective and economically feasible. The use of microbial antagonists to control postharvest diseases of fruit and vegetables is one of the most promising alternatives to fungicides (Qin et al., 2004; Droby et al., 2009). Some components of the microbial community present on the surface of fruit and vegetables, such as bacteria and yeasts, have been shown to have significant antagonistic activity against *P. expansum* (Usall et al., 2001; Janisiewicz and Korsten, 2002).

Different yeasts are also able to reduce the patulin level *in vitro* (Coelho et al., 2008; Reddy et al., 2011). Fermentative yeasts reduce patulin contamination during production of cider from

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apple juice (Harwig et al., 1973). Moss and Long (2002) showed that *Saccharomyces cerevisiae* metabolizes patulin to the less toxic E-ascladiol, whereas there are few studies on the effect of biological control yeasts on patulin accumulation in stored pome fruit (Castoria et al., 2005; Morales et al., 2008a; Lima et al., 2011).

Several studies have revealed that fruit cultivars may differ in their susceptibility to blue mold rots and to patulin accumulation (Neri et al., 2010; Konstantinou et al., 2011). Therefore, the apple cultivar should be considered a critical factor influencing the biocontrol of *P. expansum* and its patulin accumulation on fruit. Morales et al. (2008b) found that the pH value of the apple varieties was a determining factor in patulin accumulation only under cold storage: 'Golden Delicious' apples, characterized by a lower pH, were more prone to patulin accumulation at 1 °C. At room temperatures, varieties of apple with higher amounts of organic acids, such as 'Golden Delicious' and 'Fuji', accumulated more patulin. Another study showed that patulin accumulation was significantly higher in 'Golden Delicious' and 'Red Delicious' than in 'Granny Smith' and 'Fuji' apples, due to the lower acidity of the fruit (Konstantinou et al., 2011).

The specific *P. expansum* strain may be another important factor in its pathogenicity and in its ability to synthesize patulin in the fruit (Neri et al., 2010). Sommer et al. (1974) found that different *P. expansum* strains produced differing patulin levels, and the levels were not related to the virulence of the *P. expansum* strains (Neri et al., 2010; Reddy et al., 2010). Beretta et al. (2000) similarly found that the patulin content in apples was not always related to the diameter of the rotten areas, since very high levels were sometimes detected in fruit with small rots.

The aims of the present study were to evaluate the efficacy of three antagonistic yeasts *Metschnikowia pulcherrima* strain MACH1 (Saravanakumar et al., 2008), *M. pulcherrima* strain GS9 (Spadaro et al., 2008b), and *Metschnikowia fructicola* strain AL27, in the control of *P. expansum* and patulin accumulation in apples stored at room and cold temperatures. To increase the potential range of application of the biocontrol agents (BCAs), their efficacy was evaluated on four cultivars of apple, *i.e.* 'Golden Delicious', 'Granny Smith', 'Red Chief' and 'Royal Gala'.

2. Materials and methods

2.1. Microorganisms

M. pulcherrima strain MACH1 (Saravanakumar et al., 2008), *M. pulcherrima* strain GS9 (Spadaro et al., 2008b) and *M. fructicola* strain AL27 were isolated from the carposphere of 'Golden Delicious' apples harvested in unsprayed orchards located in Northern Italy. The microorganism culture was stored at −20 °C in cell suspension with 65% (v/v) glycerol and 35% (v/v) of a solution of 100 mM $MgSO_4$ and 25 mM Tris (pH 8.0). The strain AL27 was deposited within the Industrial Yeasts Collection (DBVPG) on March 29, 2011 with deposit designation 30P and its uses were patented with the Italian patent application TO2011A000534, deposited on June 20, 2011. The strains were grown in YEMS (30 g L^{-1} yeast extract, 5 g L^{-1} D-mannitol, 5 g L^{-1} L-sorbose; Spadaro et al., 2010).

Inocula of the antagonists for all experiments were prepared by subculturing in 250 mL Erlenmeyer flasks containing 75 mL YEMS and incubated on a rotary shaker (100 rpm) at 22 °C for 48 h. Yeast cells were collected by centrifugation at 1500 rpm for 10 min, washed and resuspended in sterilized Ringer solution (pH 6.9 + 0.1; Merck, Darmstadt, Germany) and brought to a standard concentration of 10^8 cells mL^{-1} by direct counting with a hemacytometer.

Four isolates of *P. expansum* (PEX06, PEX12, PEX25 and PEX27), each obtained from rotted apples harvested in Piedmont, Northern Italy, and selected for their virulence (Reddy et al., 2010), were used as a mixture during the experiments to ensure a high level of disease. Each strain belongs to the AGROINNOVA collection and were stored in tubes with potato dextrose agar (PDA; Merck) and 50 mg L^{-1} of streptomycin (Merck) at 4 °C. Conidial suspensions used for fruit inoculation were prepared by growing the pathogens on Petri dishes on PDA containing 50 mg L^{-1} of streptomycin. After a week incubation at 22 °C, conidia from the four strains were collected and resuspended in sterile Ringer's solution. After filtering through eight layers of sterile cheese-cloth, conidia were counted and brought to a final concentration of 10^5 mL^{-1}. The resultant suspensions were shaken using a vortex mixer for 30 s before inoculation.

2.2. Molecular and morphological identification

The yeast antagonist *Metschnikowia fructicola* strain AL27 was identified by sequencing the internal transcribed spacer 1 (ITS1), 5.8S ribosomal RNA gene, and internal transcribed spacer 2 (ITS2) according to White et al. (1990) and the D1/D2 domain at the 5′ end of the LSU rRNA gene according to Kurtzman and Robnett (1998). The DNA, coming from antagonist cell suspensions grown in YPD for 48 h, was extracted using NucleoMag 96 Plant Kit (Macherey Nagel, Oensingen, Switzerland) and Kingfisher magnetic particle processor (Thermo Labsystems, Basingstoke, United Kingdom) following the manufacturers' protocols. The ITS regions were amplified using genomic DNA as a template and universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The D1/D2 domains were amplified using the primers NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′) on the genomic DNA. PCRs were performed using a TGradient thermal cycler (Biometra, Göttingen, Germany). Each 20 μL PCR contained 1 μL of DNA template (50 ng), 200 mM of each deoxynucleotide triphosphate, 2 μL of 10× buffer (Taq DNA Polymerase, Qiagen, Chatsworth, CA, USA), 0.7 mM each primer, and 1.0 U Taq DNA Polymerase (Qiagen). PCR program for ITS regions was: 95 °C, 3 min; 34 cycles: 94 °C, 15 s; 55 °C, 45 s; 72 °C, 55 s; 72 °C, 7 min; 4 °C. PCR program for D1/D2 domain was: 95 °C, 10 min; 30 cycles: 94 °C, 30 s; 55 °C 30 s; 72 °C, 45 s; 72 °C, 7 min; 4 °C. A 10 μL aliquot of PCR products from each reaction was electrophoresed in 2.0% agarose gel in TBE buffer, and then stained with SYBR SAFE (Invitrogen, Eugene, OR, USA). Gel images were acquired with a Gel Doc 1000 System (Bio-Rad Laboratories, Hercules, CA, USA). PCR amplification products were cloned into the PCR4 TOPO vector (Invitrogen) using the TOPO TA cloning kit following the manufacturer protocol and sequenced by BMR Genomics (Padova, Italy) using an ABI PRISM 3730XL DNA Sequencer (AME Bioscience, Sharnbrook, United Kingdom). The sequences were analyzed by using the software BLASTn (Basic Local Alignment Search Tool; Altschul et al., 1990) for similarity. The microscope observation of the cell and colony morphology was complementary to the molecular analysis. *M. pulcherrima* strain MACH1 and *M. pulcherrima* strain GS9 were previously identified (Saravanakumar et al., 2008; Spadaro et al., 2008b).

2.3. Antagonism in vitro

The effect of the isolates of *Metschnikowia* spp. on conidial germination and on germ tube length of *P. expansum* was assessed in 5 mL of potato dextrose broth (PDB, Merck). A conidial suspension (100 μL; 5×10^6 conidia mL^{-1}) of *P. expansum* strain PEX06 was added to a 10 mL test tube. Living cells of each antagonistic yeast (100 μL of a suspension containing 5×10^7, 5×10^8, or 5×10^9 cells mL^{-1}), were added to the test tube. As control, 100 μL of the conidial suspension (5×10^6 conidia mL^{-1}) of the pathogen in Ringer's solution was added to 5 mL of PDB. After