Biological Control 54 (2010) 172–180

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Efficacy of the antagonist *Aureobasidium pullulans* PL5 against postharvest pathogens of peach, apple and plum and its modes of action

Dianpeng Zhang [a], Davide Spadaro [b,*], Angelo Garibaldi [a], Maria Lodovica Gullino [a]

[a] AGROINNOVA – Centre of Competence for the Innovation in the Agro-Environmental Sector, University of Torino, Via L. da Vinci 44, 10095 Grugliasco (TO), Italy
[b] DiVaPRA – Sector of Plant Pathology, University of Torino, Via L. da Vinci 44, 10095 Grugliasco (TO), Italy

ARTICLE INFO

Article history:
Received 10 November 2009
Accepted 7 May 2010
Available online 13 May 2010

Keywords:
Apple
Aureobasidium pullulans
β-1,3-Glucanase
Biological control
Botrytis cinerea
Chitinase
Monilinia laxa
Peach
Penicillium expansum
Plum

ABSTRACT

The efficacy of *Aureobasidium pullulans* PL5 against different postharvest pathogens of fruits (*Monilinia laxa* on plums and peaches, *Botrytis cinerea* and *Penicillium expansum* on apples) were evaluated under storage conditions when applied at 10^8 cells ml^{-1} and their interactions were studied *in vitro* and *in vivo* to discover the possible modes of action. Under 1.2 °C and 95% relative humidity (RH) for 28 days, the efficacy of PL5 against *M. laxa* on plums was 45%, reducing disease incidence from 78% to 43%. Under 1 °C and 95% RH for 21 days, the efficacy against *M. laxa* on peaches was 63%, reducing disease incidence from 79% to 29%. Under 4 °C and 95% RH for 45 days, the efficacy against *B. cinerea* and *P. expansum* on apples was 56% and 46%, respectively. In Lilly–Barnett minimal salt medium with the fungal cell walls of pathogens as sole carbon source, PL5 produced β-1,3-glucanase, exo-chitinase and endo-chitinase. Nutrient concentrations had significant effect on pathogen growth reduction by PL5. No attachment was observed in antagonist–pathogen interactions *in vitro* or *in vivo*. PL5 completely inhibited pathogen spore germination in PDB at 10^8 cells ml^{-1}, whereas at 10^6 cells ml^{-1} the efficacy was significantly decreased. However, inactivated cells and culture filtrate of PL5 had no effect on pathogen spore germination and germ tube elongation. Our results showed that *A. pullulans* PL5 could be introduced in commercial formulations to control postharvest pathogens on fruits and its activity was based on secretion of lytic enzymes and competition for nutrients.

1. Introduction

Fruit and vegetables are highly perishable products, especially during the postharvest phase and major losses are caused by postharvest pathogens (Chan and Tian, 2005; Zhang et al., 2008). *Monilinia laxa*, *Botrytis cinerea* and *Penicillium expansum* are among the most important postharvest pathogens on fruit and vegetables (Snowdon, 1990). Among them, three species of *Monilinia* can cause severe losses on stone fruits (Karabulut et al., 2002; Pellegrino et al., 2009), but *M. laxa* is the most dangerous in European countries; *B. cinerea* could cause gray mold on pome and stone fruits, and *P. expansum* can cause blue mold decay, which is one of the most destructive disease of pear and apples and it is accompanied by the production of patulin, a mycotoxin with immunosuppressive effects on humans (Spotts and Chen, 1987; Spadaro et al., 2007). Chemical treatment is an important method for controlling postharvest diseases of fruits (Eckert and Ogawa, 1988). However, pathogen resistance to fungicides (Holmes and Eckert, 1999) and concern for public safety have resulted in the cancelation of some of the most effective fungicides in Europe (Directive 91/414 CE and Regulation) and the United States (Food Quality Protection Act). In addition, the use of synthetic fungicides to control postharvest diseases of peaches and plums is prohibited in European Union countries. Therefore, researches have been focused on the development of alternative control that should be both effective and economically feasible (El-Ghaouth et al., 1998). Biological control is an effective alternative to fungicidal treatment in controlling postharvest diseases of fruits (Jijakli and Lepoivre, 1998; Spadaro and Gullino, 2004). In 1995, the first commercial products were registered in the United States by the US Enviromental Protection Agency (EPA) and are sold under the names BioSave 100 and 110 to control postharvest rots of pome and citrus fruit. In 2007, the biofungicide "Shemer" (based on a strain of *Metschnikowia fructicola* Kurtzman and Droby) was registered in Israel, and is commercially used for the control of sweet potato and carrot storage diseases (Blachinsky et al., 2007). In addition, a commercial formulation of *Candida sake* was recently developed and registered for use on pome fruit in Spain under the name Candifruit® (Droby et al., 2009).

The comprehension of the modes of action of an antagonist is an important prerequisite both for enhancing their biocontrol activity and establishing screening criteria in search for new antagonists (Qin et al., 2003). Elucidation of the mechanisms of action is often

* Corresponding author. Fax: +39 011 6709307.
E-mail address: davide.spadaro@unito.it (D. Spadaro).

1049-9644/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.biocontrol.2010.05.003

hampered by the complex interaction among host–pathogen–antagonist (Jijakli and Lepoivre, 1998). The mode of action of antagonists generally involves antibiotics (Bull et al., 1998), nutrient competition and site exclusion (Bencheqroun et al., 2007), induced host resistance (El-Ghaouth et al., 1998), and direct interactions between the antagonist and the pathogen (Castoria et al., 1997). Additional modes of action including the production of lytic enzymes *viz.*, β-1,3-glucanase and chitinase were also reported (Ippolito et al., 2000; Saligkarias et al., 2002; Yu et al., 2008).

Aureobasidium pullulans De Bary (Arnaud) is widely distributed in different environments. Different strains of *A. pullulans* can produce amylase, proteinase, lipase, cellulase, xylanase, mannanase, transferases, pullulan, siderophore, and single-cell protein. Therefore it is a biotechnologically important yeast that can be used in different fields (Chi et al., 2009). Moreover, different strains of *A. pullulans* showed wide efficacy against *B. cinerea*, *P. expansum* and *Rhizopus stolonifer* on apple, sweet cherry, grapes, strawberry and peach (Lima et al., 1997; Ippolito et al., 2000; Schena et al., 2003; Bencheqroun et al., 2007). In particular, the strain PL5 of *A. pullulans* showed a high efficacy in the control of postharvest diseases of peaches (Zhang et al., 2010). Strains of *A. pullulans* have been reported to act against fungal pathogens through competition for nutrients (Bencheqroun et al., 2007), secretion of exo-chitinase and β-1,3-glucanase (Castoria et al., 2001), or induction of defence responses (Ippolito et al., 2000). Understanding the modes of action is essential for developing appropriate commercial formulations and application methods to maximize the potential use of microbial biocontrol agents.

The aim of this research was to evaluate the efficacy of *A. pullulans* PL5 against *B. cinerea*, *P. expansum*, and *M. laxa* on postharvest fruits under storage conditions. Generally, biocontrol agents are selected and optimized for their efficacy just against one pathogen on one fruit. In our experiment, the antagonist was tested against three pathogens on three fruit species under standard storage conditions. A second aim was to evaluate the production of hydrolytic enzymes by *A. pullulans* PL5 *in vitro*, by studying the β-1,3-glucanase (EC 3.2.1.39), exo-chitinase or *N*-acetyl-β-glucosaminidase (EC 3.2.1.52) and endo-chitinase (EC 3.2.1.14) activities. A third aim was to investigate the effects of different nutrient concentrations on the interactions with three postharvest pathogens (*M. laxa, B. cinerea* and *P. expansum*) *in vitro* and *in vivo* to reveal their possible modes of action.

2. Materials and methods

2.1. Microorganisms and fruit

The yeast-like fungus *Aureobasidium pullulans* De Bary (Arnaud) PL5 was isolated from a plum cv. Angeleno produced in Piemonte (Northern Italy) and maintained on potato dextrose agar plates (PDA; 39 g l^{-1}, Merck) at 4 °C for further studies. The antagonist, selected for its efficacy (Zhang et al., 2010), was identified through microscopic observation of cell and colony morphology, and 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). The sequence was deposited in GenBank (FJ919775). The strain was grown at 25 ± 1 °C in 300 ml YPD (10 g l^{-1} of yeast extract; 20 g l^{-1} of triptone–peptone of casein and 20 g l^{-1} of D(+)-glucose) on a rotary shaker (250 rpm) for 48 h. Cells were harvested after centrifugation at 5000*g* for 10 min, resuspended in sterile Ringer's solution (pH 6.9 ± 0.1; Merck) and adjusted to the desired cell concentration (10^8 cells ml^{-1}) with a Bürker chamber.

The pathogens *Monilinia laxa* (Aderhold and Ruhland) Honey, *Botrytis cinerea* (de Bary) Whetzel, and *P. expansum* Link were isolated from infected fruit, and pure cultures were maintained on PDA plates at 4 °C. The spore suspensions of the pathogens were prepared from 7 days old mycelia by scraping the conidia on PDA plates with a sterile Ringer's solution and adjusting to the desired conidial concentration with a Bürker chamber.

Fruit used throughout the experiments were apples (*Malus* × *domestica* Borkh.) cv. Golden delicious, plums (*Prunus domestica* L.) cv. Angeleno, and peaches [*P. persica* (L.) Batsch] cv. Redhaven harvested at commercial maturity and kept at 1 °C until use. Fruit were disinfected with 2% sodium hypochlorite for 2 min, washed with tap water and air-dried prior to wounding.

2.2. Efficacy of the antagonist PL5 against M. laxa on plums and peaches under storage conditions

The cells of the antagonist PL5 was harvested by centrifugation at 5000*g* for 10 min after being grown in 300 ml YPD medium in 1000-ml Erlenmeyer flasks for 48 h at 25 °C on a rotary shaker at 250 rpm, and were diluted with 30 l tap water in 50 l tank into a final concentration of 1×10^8 cells ml^{-1}. Thirty liters of tebuconazole solution (2.5 ml/l of Folicur, Bayer Crop Science; 25.0% a.i.) was prepared according to the manufacturer. The plums and peaches were surface sterilized with 1% commercial sodium hypochlorite solution for 1 min followed by rinsing with tap water. After 2 h air-drying at 25 °C, fruits were treated with the antagonist suspension by dipping in tank for 1 min. Fruit surfaces were then air-dried at 25 °C for 2 h and 1 ml of a conidial suspension of *M. laxa* (5×10^4 spores ml^{-1}) was sprayed universally onto each fruit. The fruits treated with tebuconazole constituted the chemical control, while the fruits simply inoculated with the pathogen served as inoculated control. Three replicates of 25 fruits were prepared for each treatment. Two hours after inoculation of the pathogen on fruit surface, the plums were stored at 1.2 °C and 95% RH and the peach fruits were stored at 1 °C and 95% relative humidity (RH) under storage conditions. After 21 and 28 days of storage, incidence of the rotten peaches and plums were measured, respectively. The experiments were repeated twice.

2.3. Efficacy of the antagonist PL5 against B. cinerea and P. expansum on apples under storage conditions

To evaluate the efficacy of the antagonist PL5 against *B. cinerea* and *P. expansum* on apples, the trials were prepared in a similar way, as described above. Briefly, the cells of antagonist *A. pullulans* PL5 were diluted with 30 l tap water in 50 l tank into a final concentration of 1×10^8 cells ml^{-1}. The apples were surface sterilized with 1% commercial sodium hypochlorite solution for 1 min followed by rinsing with tap water. After 2 h of air-dry at 25 °C, fruits were treated with the antagonist suspension (1×10^8 cells ml^{-1}) by dipping in tank for 1 min. Fruit surfaces were air-dried at 25 °C for 2 h and 1 ml of a conidial suspension of *B. cinerea* (5×10^4 spores ml^{-1}) or *P. expansum* (5×10^4 spores ml^{-1}) were sprayed onto each fruit according to the different trials. The fruits treated with 2.5 ml/l of Folicur (Bayer Crop Science; tebuconazole: 25.0%) played as chemical controls, while the fruits only inoculated with the pathogen served as inoculated controls. Three replicates of 25 fruits were prepared for each treatment. Two hours after inoculation of the pathogen on fruit surface, the apples were stored at 4 °C and 95% RH under storage conditions. After 45 days of storage, the rotten apples were counted and the infected percentage of the apples was recorded. The experiment was repeated twice.

2.4. Pathogen cell wall preparation

Cell wall preparations (CWP) of each pathogen were prepared as described by Saligkarias et al. (2002) with small modifications. Briefly, the pathogens were grown in potato dextrose broth media