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Postharvest biological control of blue mold of apple by *Pseudomonas fluorescens* during commercial storage and potential modes of action

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ABSTRACT

Three *Pseudomonas fluorescens* isolates, 1–112, 2–28 and 4–6, isolated from the rhizosphere of pulse crops were tested for their ability to suppress *Penicillium expansum* (blue mold) on 'McIntosh' and 'Spartan' apples in commercial cold storage, and their possible mechanisms of action were investigated *in vitro*. On 'McIntosh' apples the decay incidence and lesion diameter of blue mold were significantly reduced by isolates 1–112 and 4–6 compared with control fruits after 15 weeks storage at 1 °C. On 'Spartan' apples only isolate 2–28 provided significant levels of disease control after 15 weeks of storage at 1 °C. In dual culture and in volatile tests all three isolates of *P. fluorescens* significantly inhibited conidial germination and mycelial growth of *P. expansum in vitro*. All three isolates were positive for the production of protease, but negative for cellulase, chitinase and glucanase. Molecular evidence for the potential for synthesis of the antibiotic, phenazine-1-carboxylic acid, in isolates 1–112 and 4–6 and of hydrogen cyanide in isolate 2–28 was obtained by polymerase chain reaction of *phzCD* and *hcnBC* genes, respectively. Genes for 2,4-diacetylphloroglucinol, pyoluteorin and pyrrolnitrin production were not detected in any of the *P. fluorescens* isolates. Scanning electron microscopy indicated that all three *P. fluorescens* isolates adhered to the fungal hyphae and colonized the wounds of apples, but only isolate 1–112 was able to colonize conidia of the fungal pathogen. *P. fluorescens*' ability to compete for nutrients and space and produce inhibitory metabolites that target conidial germination and mycelial growth may be the basis for its control of *P. expansum* on apple.

1. Introduction

Pome fruit are highly perishable products and become particularly susceptible to postharvest disease caused by fungal pathogens during packing, storage and transportation. To date over 90 fungal species have been identified as causal agents of postharvest decay of apple during storage (Li et al., 2011). Blue mold caused by the psychotrophic fungal pathogen *Penicillium expansum* Link. is the most important postharvest disease of apples and can result in fruit losses of up to 50% (Quaglia et al., 2011; Vilanova et al., 2014). *P. expansum* also produces the mycotoxin patulin which can have acute and chronic effects on human health (Etebarian et al., 2005; Quaglia et al., 2011). Traditionally, the pome fruit industry has controlled postharvest disease with chemical fungicides (Chan and Tian, 2005; Janisiewicz and Korsten, 2002). Synthetic fungicides such as Mertect® (a.i. thiabendazole) and Scholar® (a.i. fludioxonil) have been applied extensively to tree fruits to reduce postharvest loss, but pathogen resistance is emerging (Errampalli et al., 2006). Public pressure to reduce fungicide use and for produce free of synthetic fungicides, has led to research for safer alternatives such as biological control agents (Chan and Tian, 2005; Janisiewicz and Korsten, 2002).

The majority of new fungicides have site-specific targets with a lower potential for negative impacts on the environment, but these fungicides are at high risk for development of resistance by fungal pathogens (Brent and Hollomon, 2000). Biological control using microbial antagonists is a promising alternative to fungicides as biocontrol agents have many modes of action to combat fungal pathogens, making pathogen resistance unlikely (Brent and Hollomon, 2000). Modes of action utilized by microbial antagonists include direct parasitism (Li et al., 2016), competition for nutrients or space (Janisiewicz et al., 2000; Bencheqroun et al., 2007), production of lytic enzymes (Zhang et al., 2010) or antibiotics (Janisiewicz and Roitman, 1988), and induction of host defences (Ippolito et al., 2000; Li et al., 2011). Elucidating the mechanisms of action of microbial antagonists may allow for enhanced disease control. Many yeast antagonists have shown promise in controlling blue mold of apple, including *Pichia caribbica* (Cao et al., 2013), *Aureobasidium pullulans* (Ippolito et al., 2000; Mari et al., 2012), *Metschnikowia fructicola* (Spadaro et al., 2013) and *Cryptococcus laurentii*

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(Lima et al., 2010); several bacterial antagonists have also shown potential including *Rahnella aquatilis* (Calvo et al., 2007), *Pseudomonas cepacia* (Janisiewicz and Roitman, 1988), *P. syringae* (Janisiewicz and Jeffers, 1997) and *Burkholderia gladioli* (Scuderi et al., 2009).

Pseudomonas fluorescens is a Gram-negative bacterium that naturally inhabits water, soil and plant surfaces (Pujol et al., 2005; Raaijmakers et al., 1999). The three isolates of *P. fluorescens* used in this study were isolated from the rhizosphere of pulse crops in Saskatchewan, Canada (Hynes et al., 2008), and previously had shown potential as biocontrol agents (Nelson et al., 2010; Wallace et al., 2016). Adaptation of these bacteria to cold Canadian soils makes them ideal candidates for control of postharvest disease of apple during commercial storage. *P. fluorescens* has been studied extensively as a biocontrol agent for plant diseases in the rhizosphere (Bull et al., 1991; Raaijmakers et al., 1999; Van Wees et al., 1997; Wang et al., 2000), but little is known of its potential as a biocontrol agent in postharvest disease of apple (Etebarian et al., 2005; Peighami-Ashnaei et al., 2009). Antagonistic mechanisms of action used by *P. fluorescens* against fungal pathogens include production of volatile compounds (Kai et al., 2007), competition for iron (Loper, 1988), production of antibiotics such as pyrrolnitrin and 2,4-diacetylphloroglucinol (Ligon et al., 2000; Nowak-Thompson et al., 1994) and induction of host systemic resistance (reviewed by Pieterse et al., 2014). The objectives of this study were: (i) to compare the ability of *P. fluorescens* 1–112, 2–28 and 4–6 to control *P. expansum,* on 'McIntosh' and 'Spartan' apples during commercial storage, to that of commercial controls, Scholar® and Bio-Save®; (ii) to investigate the possible modes of action utilized by the bacteria to inhibit *P. expansum in vitro*.

2. Materials and methods

2.1. Antagonists

P. fluorescens isolates 1–112, 2–28 and 4–6, previously shown to be positive for the production of siderophores, were obtained from the rhizosphere of pulse crops in Western Canada (Hynes et al., 2008). Each isolate was maintained on half strength tryptic soy agar (7 g tryptic soy broth (TSB), 15 g agar in 1000 ml of water) at 4 °C and grown in half strength TSB prior to inoculation. Bacterial inocula were prepared by incubating the three strains of *P. fluorescens* in TSB for 2 d at 28 °C on a rotary shaker set at 220 rpm. The optical density of the incubated culture was measured with a spectrophotometer and the colony forming units (CFU) per ml were determined using standard calibration curves and adjusted to the desired concentration depending on the experiment.

2.2. Pathogen

P. expansum Link strain 1790 was obtained from Dr. P. Sholberg, Agriculture and Agri-Food Canada, Summerland Research and Development Centre, Summerland, BC and was maintained on half strength potato dextrose agar (PDA: 15 g potato dextrose broth (PDB), 15 g agar in 1000 ml of water) at 4 °C. A conidial suspension was prepared according to the method used by Errampalli (2004). The conidia were enumerated with a Petroff-Hauser counting chamber and conidial suspensions were adjusted to the appropriate concentration with sterile distilled water.

2.3. Fruit

Apple (*Malus domestica* Borkh.) fruit of cv. 'McIntosh' and 'Spartan' were harvested at commercial maturity in the Okanagan Valley, British Columbia, Canada and provided by the British Columbia Tree Fruits Cooperative (BCTFC) (9751 Bottom Wood Lake Road, Lake Country, BC V4 V 1S7) for this study. Fruit were selected for their uniform size and absence of blemishes or visible rot. Harvested fruit were stored at 1 °C prior to treatment. Fruit were surface disinfected with 6% sodium hypochlorite and 0.01% Tween 20 for 4 min, rinsed with tap water for 4 min, and dried before wounding.

Physiological fruit quality parameters were assessed on healthy apples after 15 weeks in commercial cold storage. Firmness was measured on each apple at two opposite sites along the equatorial region with a Güss Fruit Texture Analyzer (Güss, Strand, South Africa) with an 11-mm probe. The probe descended towards the apple at 1.0 mm s^{-1} and the maximum force (lbs) required to penetrate the apple was defined as firmness. Total soluble solids (TSS) were determined by measuring the refractive index of pressed juice using a digital hand-held pocket refractometer PAL-1 (Atago, California, U.S.A.) (Spadaro et al., 2013). The starch index was determined by slicing the apples in half equatorially followed by spraying with an iodine solution (KI: 8.6 g KI, 2.2 g I_2 in 1000 ml of water). After drying for one minute the apples were visually compared to the Cornell Starch chart, where 1 indicates high levels and 9 indicates low levels of starch (Blanpied and Silsby, 1992). Titratable acidity was determined by titration with 0.1 N NaOH to pH 8.1 and 15 ml of pressed juice were diluted with 60 ml of distilled water. The final volume of NaOH added when the endpoint of the titration was reached was used to determine the mg of malic acid per 100 ml of juice (Toivonen and Hampson, 2014).

2.4. Biocontrol activity on apples

Each apple was wounded (2 × 2 × 7 mm) twice with a sterile nail and then inoculated by submersing the bag of fruit into 1 × 10^8 CFU ml^{-1} of *P. fluorescens* for one minute, allowed to sit for one minute, followed by drenching for one minute in 1 × 10^4 conidia ml^{-1} of *P. expansum*. Similarly apples were drenched in commercial controls, Bio-Save® (JetHarvest Solutions, Longwood, Florida, USA) with the active ingredient *Pseudomonas syringae* or Scholar® 50 WG (Syngenta, Guelph, Ontario, Canada) with the active ingredient (a.i.) fludioxonil, as per manufacturers' instructions, allowed to sit for one minute, followed by drenching for one minute in 1 × 10^4 conidia ml^{-1} of *P. expansum*. The drenching method of inoculation was used to mimic commercial practices where packinghouses apply fungicides or biocontrol agents as a drench prior to storage. Each replicate consisted of a bag of ten apples and each treatment had three replicates. After inoculation bags of apples were placed in large plastic totes and transferred to a 1 °C commercial cold storage room at the BCTFC in Winfield, BC. Three holes (40 mm in diameter) were cut in the lid of each tote and covered with 0.45 µm filters to allow for aeration and prevent microbial dispersal in the storage facilities. Treatments included non-inoculated controls (NIC), positive controls of the fungal pathogen alone, negative controls of each bacterial isolate alone, the pathogen in combination with each *P. fluorescens* isolate, or the fungicide Scholar®, or a biocontrol agent, Bio-Save®. The lesion diameters and disease incidence were determined after 15 weeks in 1 °C commercial cold storage. Disease incidence of each apple was determined by the number of wounds that had visible fungal decay. Two independent experiments were conducted in 2015, one on 'McIntosh' and one on 'Spartan' apples.

2.5. Biocontrol activity in vitro

The three isolates of *P. fluorescens* were tested for their antagonistic effect on the mycelial growth of *P. expansum* as described by Tolba and Soliman (2013) with slight modifications. A fungal lawn of *P. expansum* was created on ¼ TSA/PDA (7 g PDB, 7 g TSB, 15 g agar in 1000 ml of water) by spreading 100 µl of 10^6 conidia ml^{-1} with a sterile glass rod. Concomitantly, the three different isolates of *P. fluorescens* (10 µl of a solution of 10^8 CFU ml^{-1}) were inoculated onto sterile 6-mm filter discs (VWR 415 filter paper). The inoculated filter discs were allowed to air dry before being transferred onto the middle of the fungal lawn and incubated at 20 °C for 5 d. Negative controls consisted of a fungal lawn with a filter disc inoculated with 10 µl of sterile water. After 5 d the efficacy of the different isolates of *P. fluorescens* was determined by measuring the diameter of fungal inhibition from the centre of the filter