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

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\textbf{A B S T R A C T}

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 \textit{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* \textit{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 phiCD and \textit{hcnBC} genes, respectively. Genes for 2,4-diacylphloroglucinol, 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.

\textbf{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 psychrotrophic 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*.
(Lima et al., 2010); several bacterial antagonists have also shown potential including *Rhahnella 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-Asnaei 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 inoculated 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 V4V 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 Guss Fruit Texture Analyzer (Guss, 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 I₂ 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 submerging the bag of fruit into 1 × 10⁴ CFU ml⁻³ of *P. fluorescens* for one minute, allowed to sit for one minute, followed by drenching for one minute in 1 × 10⁶ 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 × 10⁶ 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 10⁶ conidia ml⁻¹ with a sterile glass rod. Concomitantly, the three different isolates of *P. fluorescens* (10 μl of a solution of 10⁶ 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.
disc to the closest edge of the fungal lawn. Each treatment was replicated five times and the experiment performed three times.

The effect of *P. fluorescens* isolates on conidial germination of *P. expansum* was assessed as described by Spadaro et al. (2013) with slight modifications. Conidial germination was assessed in 50% filter-sterilized apple broth (FSAB) pH 6.5. FSAB was made using Ambrosia apples obtained from the BCTFC, Winfield, BC. Whole apples were cored, and then juiced using a Breville Juice Fountain Plus (JE98XLS, Breville, England). Apple juice was centrifuged at 2000 rpm for 5 min at 4 °C and filtered through Whatman No. 2 filter paper (8 μm particle retention). The pH of the apple juice was adjusted to 6.5 using 1.0 M sodium hydroxide followed by filtration through a Nalgene Sterile Filtration System (0.22 um pore size) (ThermoFisher Scientific, Waltham, MA, U.S.A.). The FSAB was stored at 4 °C until use (Leelasuphakul et al., 2008). Five hundred microliters of FSAB were transferred into each well of a 24-well tissue culture plate (Nunc™, ThermoFisher Scientific, Waltham, MA, U.S.A.). A conidial suspension (250 μl of a solution containing 10⁶ conidia ml⁻¹) of *P. expansum* alone, or in combination with living cells (250 of volatile organic compounds (VOCs) produced by *P. expansum* was completed in triplicate and the experiment was performed three times.

Conidial germination was considered when the size of germ tubes. The conidia were considered germinated when the size of spores were observed per replicate with a light microscope and percent cubation at 20 °C on a rotary shaker (100 rpm), a minimum of 100 colonies grown on 1% (w/v) skim milk agar (10 g skim milk powder, 5 g TSB, 15 g agar) were considered as chitinase producers. Proteolytic activity of the bacteria was assessed clearing after 7 d of incubation at 28 °C on chitin agar were considered as positive controls. DNA was extracted from each isolate of *P. fluorescens* using the E.Z.N.A.® Bacterial DNA Kit and centrifugation protocol (Omega-Bio Tek, Norcross, U.S.A.). DNA was stored at −20 °C until use for subsequent analysis.

All PCR reactions were performed in a volume of 25 μl containing 1 μl of bacterial DNA, 1 X PCR buffer (New England Biolabs, Ipswich, U.S.A.), 5% dimethyl sulfoxide (Fluka, Buchs, Switzerland), 100 μM each of dATP, dCTP, dGTP, and dTTP, 0.4 μM of each primer, and 1.25 U of Taq DNA polymerase (Amersham Pharmacia) (Kim et al., 2013). All primers were obtained from Integrated DNA Technologies (IDT)- UBC Vancouver. PCR was performed on an Applied Biosystems Veriti 96-Well Thermocycler (ThermoFisher Scientific, Waltham, U.S.A.). The method for amplification of the gene encoding for hydrod cyanide (HCN) production, *hcnBC*, was performed as described by Ramette et al., 2003.

Following amplification, detection of PCR products was determined on a 1% agarose gel stained with SYBR® Safe DNA Gel Stain (Invitrogen, Carlsbad, U.S.A.). The DNA Clean and Concentrator®-5 (Zymo Research, Irvine, U.S.A.) kit was used to purify the PCR products, except for *P. fluorescens* 4–6 where multiple bands were observed on the agarose gel for the *Aca/Acb* primer set. When multiple bands were amplified with the respective primer set the bands were excised from the agarose gel using a sterile scalpel and purified using the E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, U.S.A.). The PCR products were quantified using a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, U.S.A.) and sent to the Fragment Analysis and DNA Sequencing Services (FADSS) at the University of British Columbia-Okanagan campus for sequencing. Sequences were assembled and proof-read with Sequencher™ 4.7 software (Gene Codes Corp., Ann Arbor, U.S.A.). BLASTn was used to confirm the identity of the PCR product.

2.7. Production of lytic enzymes

In order to assess the ability of *P. fluorescens* to produce and secrete lytic enzymes (chitinase, protease, cellulase and glucanase), qualitative tests were performed on solid agar amended with the various substrates as described by Lutz et al. (2013). To assess chitinolytic activity bacteria were cultured on medium amended with 0.5% (w/v) colloidal chitin (7 g (NH₄)₂SO₄, 1 g K₂HPO₄, 0.1 g MgSO₄·7H₂O, 0.5 g TSB, 15 g agar, 5 g chitin in 1000 ml of water). Isolates that showed zones of clearing after 7 d of incubation at 28 °C on chitin agar were considered as chitinase producers. Proteolytic activity of the bacteria was assessed by observing zones of precipitation of paracasein around bacterial colonies grown on 1% (w/v) skim milk agar (10 g skim milk powder, 5 g TSB, 15 g agar in 1000 ml of water) and incubated at 28 °C for 2 d

**Table 1.** Target antibiotic genes and primers used in PCR analysis of *P. fluorescens* DNA.

<table>
<thead>
<tr>
<th>Target gene &amp; product</th>
<th>Target group</th>
<th>Primer</th>
<th>Sequence</th>
<th>Length</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>phdB (2,4-diacetylphloroglucinol)</td>
<td><em>f. Pseudomonas</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Phb2a</td>
<td>GAGGACGTCGAGAACACACCA</td>
<td>745 bp</td>
<td>Ramette et al. (1997)</td>
</tr>
<tr>
<td>phbCD (phenazine-1-carboxylic acid)</td>
<td><em>f. Pseudomonas</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Phb2b</td>
<td>ACCGGACGGTCTGGTATGAG</td>
<td>1150 bp</td>
<td>Ramette et al. (1997)</td>
</tr>
<tr>
<td>prnD (pyrrolnitrin)</td>
<td><em>Pseudomonas</em></td>
<td>PCA2a</td>
<td>TGGCAGGCTGCGCGACAC</td>
<td>476 bp</td>
<td></td>
</tr>
<tr>
<td>prnC (pyoluteorin)</td>
<td><em>Pseudomonas</em></td>
<td>PCA2b</td>
<td>GGGCGGGCGCTGGTGATGGA</td>
<td>786 bp</td>
<td>de Souza and Raaijmakers (2003)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fluorescent Pseudomonas.
were removed from the plate and transferred to 10-ml glass vials (Morris et al., 2012). Cellulolytic activity of the bacteria was assessed in 0.1 M sodium cacodylate buffer solution, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2, and gently agitated at 40 rpm on a rotary shaker for 1 h then 5 mm2 of solid medium containing both bacteria and fungi were removed from the plate and transferred to 10-ml glass vials filled with 3 ml of fixative and gently agitated at 50 rpm on a rotary shaker for 3 h. Fixative was removed from the vials and samples were washed in 0.1 M sodium cacodylate buffer solution, followed by washes in distilled water. Distilled water was removed from the vials and samples were dehydrated in graded series with ethanol (25, 50, 75, 90 and 100% once for concentrations up to 90% and twice for the 100% concentration). Samples were dried with a critical point dryer (CPD 020, Balzers Union, Aktiengesellschaft, Balzers, Liechtenstein) and affixed to aluminum stubs with carbon tape followed by sputter coating with 20 nm of gold. The samples were then observed with a Tescan Mirano3 XMU Field Emission Scanning Electron Microscope (Tescan Orsay Holding a.s., Brno, Czech Republic).

2.8. Scanning electron microscopy (SEM) in vitro

The possible physical interaction of the bacteria and fungal pathogen was assessed in mini Petri dishes (60 mm in diameter) containing ¼ PDA/TSA. Mycelial discs (5 mm in diameter) of 7 d old cultures were inserted into the middle of the Petri dish. Bacteria were cultured in ½ strength TSB for two days at 20 °C and streaked in parallel 15 mm from the centre of the fungal disc. Cultures were incubated at 20 °C for 5 d. Samples were prepared for Scanning Electron Microscopy (SEM) observation using the routine protocol described by Alves et al. (2013) with slight modifications. Culture plates were flooded with fixative solution, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2, and gently agitated at 40 rpm on a rotary shaker for 1 h then 5 mm2 of solid medium containing both bacteria and fungi were removed from the plate and transferred to 10-ml glass vials filled with 3 ml of fixative and gently agitated at 50 rpm on a rotary shaker for 3 h. Fixative was removed from the vials and samples were washed in 0.1 M sodium cacodylate buffer solution, followed by washes in distilled water. Distilled water was removed from the vials and samples were dehydrated in graded series with ethanol (25, 50, 75, 90 and 100% once for concentrations up to 90% and twice for the 100% concentration). Samples were dried with a critical point dryer (CPD 020, Balzers Union, Aktiengesellschaft, Balzers, Liechtenstein) and affixed to aluminum stubs with carbon tape followed by sputter coating with 20 nm of gold. The samples were then observed with a Tescan Mirano3 XMU Field Emission Scanning Electron Microscope (Tescan Orsay Holding a.s., Brno, Czech Republic).

2.9. Scanning electron microscopy (SEM) in vivo

The possible physical interaction of the bacteria and fungal pathogen was assessed in apple wounds. Apples were sliced so that four faces were obtained from a single apple, allowing a large surface area of peel to remain on each slice. Apple slices were placed in a petri dish containing 2 ml of sterile water with the peel side facing up. A 2 mm deep 4 × 4 × 4 mm wide triangular wound was made at the equatorial region using a scalpel. The peel from the wound was removed and 25-μl aliquots of P. fluorescens 1–112, 2–28 or 4–6, at 1 × 10^8 CFU ml\(^{-1}\) were inoculated into each wound site. After 2 h, a 25-μl suspension of P. expansum at 1 × 10^5 spores ml\(^{-1}\) was inoculated into each wound, plates were sealed with parafilm to maintain high humidity and incubated at 20 °C for 7 d. Wounded tissue (8 mm3) was excised from the centre of the fungal disc. Cultures were incubated at 20 °C for 7 d. Wounded tissue (8 mm3) was excised from the centre of the fungal disc. Cultures were incubated at 28 °C (Lutz et al., 2013; Renwick et al., 1991).

3. Results

3.1. Biocontrol activity in vivo

The antifungal activity of P. fluorescens isolates was tested against P. expansum on ‘McIntosh’ and ‘Spartan’ apples during commercial cold storage at 1 °C and compared to commercial controls, Bio-Save® and Scholar® (Fig. 1). After 15 weeks in cold storage, isolate 1–112 (1.3 mm), 4–6 (4.1 mm), Bio-Save® (1.1 mm) and Scholar® (0.4 mm) reduced the size of the lesion of blue mold on ‘McIntosh’ apples in

![Fig. 1. Blue mold lesion diameter and disease incidence of 'McIntosh' (A & B) and 'Spartan' (C & D) apples inoculated with the fungal pathogen P. expansum after 15 weeks in commercial cold storage at 1 °C. Apples treated with P. expansum were also subjected to treatment with each isolate of P. fluorescens, 1–112, 2–28 or 4–6, or Bio-Save® or Scholar®. Control apples were treated with the pathogen only. Each bar represents the mean of three replicates of 10 apples each ± standard error. Different letters indicate significant differences according to Tukey’s test (P < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image-url)
Table 2
Physiological fruit quality characteristics of untreated ‘McIntosh’ and ‘Spartan’ apples after 15 weeks commercial cold storage trials, 2015-16.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Weight (g)</th>
<th>Firmness (lbs)</th>
<th>Starch Index</th>
<th>Total soluble solids (%)</th>
<th>Titratable acidity (% malic acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>McIntosh</td>
<td>157.4 ± 7.3 b</td>
<td>10.0 ± 0.2 a</td>
<td>9.0 ± 0.0 a</td>
<td>14.2 ± 0.2 a</td>
<td>0.604 ± 0.016 a</td>
</tr>
<tr>
<td>Spartan</td>
<td>189.8 ± 9.6 a</td>
<td>8.6 ± 0.2 b</td>
<td>9.0 ± 0.0 a</td>
<td>14.4 ± 0.2 a</td>
<td>0.391 ± 0.010 b</td>
</tr>
</tbody>
</table>

* Weight, firmness, starch and total soluble solids are the means ± the standard error of ten apples.
* Titratable acidity data is the mean ± the standard error of 12 apples or 4 replicates.
* Means followed by a common letter within a column are not significantly different according to Tukey’s test ($P < 0.05$).

Table 3
Effect of *P. fluorescens* isolates, 1–112, 2–28 or 4–6 (10^6 CFU ml^{-1}) on conidial germination of *P. expansum* (10^8 conidia ml^{-1}) in FSAB.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conidial germination (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>92.1 ± 3.7 a</td>
</tr>
<tr>
<td>1–112</td>
<td>1.2 ± 0.6 c</td>
</tr>
<tr>
<td>2–28</td>
<td>7.8 ± 0.9 b</td>
</tr>
<tr>
<td>4–6</td>
<td>0.3 ± 0.3 c</td>
</tr>
</tbody>
</table>

* Each value is the mean of three replicates ± standard error. Means followed by a common letter are not significantly different according to Tukey’s test ($P < 0.05$).

Table 4
Effect of VOCs produced by three isolates of *P. fluorescens*, 1–112, 2–28 and 4–6, on the germination of conidia of *P. expansum* on 1/4 TSA/PDA plates.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>58 ± 5.4 a</td>
</tr>
<tr>
<td>1–112</td>
<td>0 ± 0 b</td>
</tr>
<tr>
<td>2–28</td>
<td>0 ± 0 b</td>
</tr>
<tr>
<td>4–6</td>
<td>0 ± 0 b</td>
</tr>
</tbody>
</table>

* Each value is the mean of five replicates ± standard error. Means followed by a common letter are not significantly different according to Tukey’s test ($P < 0.05$).

3.2. Biocontrol activity in vitro

All three *P. fluorescens* isolates inhibited *P. expansum in vitro* (Fig. 2). *P. fluorescens* isolate 1–112 yielded the highest inhibition zone (15.1 mm), followed by isolate 4–6 (10.1 mm) and *P. fluorescens* isolate 2–28 produced the smallest inhibition zone (4. 0 mm) (Fig. 2). A filter disc inoculated with sterile water served as the control and did not result in inhibition of *P. expansum* (data not shown).

*P. fluorescens* 1–112, 2–28 and 4–6 inhibited conidial germination of *P. expansum* by 98.7, 91.5 and 99.7%, respectively (Table 3). In the control, 92.1% of the conidia germinated. *P. fluorescens* isolates 1–112 and 4–6 produced the greatest inhibition of *P. expansum* conidial germination in FSAB after 14 h incubation at 20 °C.

In the sealed plate assay there was no physical contact between *P. fluorescens* and *P. expansum*; thus, the antifungal effect on conidial germination could be attributed to the VOCs. The VOCs generated by all three isolates of *P. fluorescens* inhibited conidial germination of *P. expansum* by 100% in comparison to the control (Table 4). Although VOCs produced by *P. fluorescens* inhibited germination of *P. expansum* conidia, the fungus recovered when transferred to new PDA plates.

3.3. Presence of antibiotic biosynthesis genes in *P. fluorescens*

In order to assess if *P. fluorescens* 1–112, 2–28 and 4–6 have the potential to produce common pseudomonad antibiotics, total DNA was amplified by PCR with specific primers for genes for 2,4-diacytylphloroglucinol (DAPG), phenazine-1-carboxylic acid (PCA), pyrrolnitrin (Prn), pyoluteorin (Plt) and hydrogen cyanide (HCN) biosynthesis. Only isolate 2–28 was positive for the hydrogen cyanide biosynthesis gene (Fig. 3A). Both isolate 1–112 and 4–6 amplified 1050 bp fragments for the gene encoding the production of PCA (Fig. 3B). All three isolates were negative for the genes encoding the production of Plt, DAPG and Prn.

3.4. Lytic enzyme production by *P. fluorescens*

All three biocontrol isolates were positive for protease, but negative for the lytic enzymes cellulase, chitinase and glucanase (Table 5).

3.5. Scanning electron microscopy (SEM), in vitro assay

The interaction of *P. fluorescens* isolates 1–112, 2–28 and 4–6 with the fungal pathogen *P. expansum in vitro* was examined with SEM after 5 d of incubation (Fig. 4). Untreated hyphae of *P. expansum* were smooth, healthy and bearing numerous conidia on phialides (Fig. 4). All three isolates of *P. fluorescens* colonized the fungal hyphae (Fig. 4B–D), while only isolate 1–112 was observed to colonize the conidia of *P. expansum*.
observed to colonize the conidia in apple wounds (Fig. 5B).

**3.4. Scanning electron microscopy (SEM)**, in vivo assay

(Fig. 4B). Large amounts of an extracellular matrix accumulated around the hyphae in the interaction with 2–28 (Fig. 4C), and smaller amounts in the interaction with isolate 4–6 (Fig. 4D). Although isolate 4–6 appeared to produce smaller amounts of an extracellular matrix, it may produce toxic compounds capable of degrading the hyphae as multiple holes were observed in the hyphal cell wall (Fig. 4D). In some areas, *P. expansum* hyphae were completely surrounded by *P. fluorescens* cells (Fig. 4C).

**3.6. Scanning electron microscopy (SEM)**, in vivo assay

The interaction of the antagonist with the fungal pathogen in apple wounds was examined with SEM after 7 d incubation (Fig. 5). In the control, apple wounds inoculated with only a conidial suspension of *P. expansum*, a dense mycelium with numerous branched conidiophores with intact conidia was observed (Fig. 5A). After co-culturing the pathogen with the antagonist, numerous individual un-germinated conidia of *P. expansum* were observed (Fig. 5B–D). In the wounds of apple fruits inoculated with *P. fluorescens* and *P. expansum*, the antagonist adhered to the fungal hyphae (Fig. 5B–D), but to a lesser extent than was observed *in vitro* (Fig. 4B–D). Although all three isolates of *P. fluorescens* colonized the hyphae of *P. expansum*, only isolate 1–112 was observed to colonize the conidia in apple wounds (Fig. 5B). *P. fluorescens* isolates 1–112, 2–28 and 4–6 colonized the apple wound, decreasing colonization by the fungal pathogen, and produced an extracellular matrix (Fig. 5B–D). They also appeared to reduce colonization of the wound by the fungal pathogen, but no quantitative assessment was made.

**4. Discussion**

Over the past 30 years many scientists and several commercial companies have focused their research efforts on the use of biological control agents as an alternative to chemical fungicides. The development of resistant fungal pathogens, de-registration of key fungicides, as well as environmental and human health concerns have been the main driving forces towards alternative control strategies (Droby et al., 2016; Wilson and Wisniewski, 1989). The carposphere has been extensively investigated for new antagonists effective at controlling common postharvest pathogens (Mari et al., 2012), but these microorganisms are often poorly adapted to the commercial storage environment (Hu et al., 2015; Lutz et al., 2012). Our study examined the potential of three cold-adapted isolates of *P. fluorescens*, previously isolated from the rhizosphere of pulse crops in Saskatchewan, Canada (Hynes et al., 2008), to control *P. expansum* on ‘Spartan’ and ‘McIntosh’ apples in 1 °C commercial cold storage. To elucidate the possible mechanisms of action of *P. fluorescens* 1–112, 2–28 and 4–6 to control *P. expansum* we studied the interaction of the antagonist and the pathogen as well as the antagonist’s ability to produce inhibitory metabolites such as VOCs, antibiotics and lytic enzymes capable of inhibiting conidial germination and mycelial growth.

This study indicates that all three isolates of *P. fluorescens* were able to provide some control against *P. expansum* during commercial cold storage. On ‘McIntosh’ apples isolates 1–112 and 4–6 reduced the lesion size and incidence of blue mold decay and were both comparable to Bio-Save® and Scholar®. On ‘Spartan’ apples isolate 2–28 provided disease control comparable to Bio-Save® and Scholar®. Isolate 1–112 reduced the incidence of blue mold on ‘Spartan’ apples but not the size of the lesion. Our findings are consistent with Etebarian et al. (2005), who showed *P. fluorescens* 1100-6 provided control of *Penicillium* spp. comparable to Bio-Save® on ‘Gala’ apples after two months storage at 1 °C. More recently, Mikani et al. (2008) showed that 10 strains of *P. fluorescens* were effective at controlling *Botrytis cinerea* on apples stored at 5 °C for 25 d. The variability in the level of disease control provided by each isolate of *P. fluorescens* is in agreement with other studies that have shown biocontrol capabilities can vary among different strains of the same antagonist species (Mikani et al., 2008; Spadaro et al., 2013). Spadaro et al. (2013) attributed the higher level of control provided by yeast antagonists on ‘Golden Delicious’ apples, compared to ‘Granny Smith’, ‘Red Chief’ and ‘Royal Gala’ apples, to their higher soluble solids content, as one of the main modes of action of yeast biocontrol agents is competition for nutrients, particularly carbon sources. In our study ‘McIntosh’ and ‘Spartan’ apples did not differ significantly in their total soluble solids content, but did differ in weight, firmness and malic acid content. Janisiewicz et al. (2000) investigated the competition between *A. pullulans* and *P. expansum* for limited nutrients and showed that the antagonist inhibited conidial germination and depleted amino acids. A more comprehensive understanding of the fruit quality parameters, particularly carbon and nitrogen compounds, of ‘McIntosh’ and

Table 5

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Cellulase</th>
<th>Protease</th>
<th>Chitinase</th>
<th>Glucanase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-112</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2-28</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>4-6</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
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</table>
Spartan apples may help explain why better control of blue mold was observed on 'McIntosh' apples in our study.

All three isolates of \textit{P. \textit{fluorescens}} reduced conidial germination and mycelial growth of \textit{P. \textit{expansum}} \textit{in vitro}. \textit{In vitro}, zones of inhibition were observed between the bacteria and the mycelial lawn of \textit{P. \textit{expansum}}, where isolate 1–112 produced the largest inhibition zone. The inhibition provided by the three isolates could be due to diffusible inhibitory compounds produced by \textit{P. \textit{fluorescens}} or the direct interaction with \textit{P. \textit{expansum}} resulting in suppressed growth of the pathogen. Fungal spores are a major source of postharvest disease. As a result, when investigating a new biocontrol agent it is imperative to determine the effect of the antagonist on the ability of the fungal pathogen to germinate. Our \textit{in vitro} experiments showed that \textit{P. \textit{fluorescens}} 1–112, 2–28 and 4–6 were highly effective at inhibiting conidial germination of \textit{P. \textit{expansum}} in FSAB by over 90% in comparison to the control. Isolates 1–112 and 4–6 were the most effective, inhibiting conidial germination by more than 98%. Our findings are in agreement with other studies that have shown the ability of microbial antagonists to inhibit spore germination of postharvest fungal pathogens in liquid culture (Calvo et al., 2007; Lee et al., 2012; Spadaro et al., 2013). Similar to Janisiewicz et al. (2000), we chose sterile apple juice or FSAB as our culture medium, as the substrate closely resembles that found under natural conditions. All three isolates of \textit{P. \textit{fluorescens}} produced volatile organic compounds (VOCs) that inhibited the conidial germination of \textit{P. \textit{expansum}} \textit{in vitro}. Zhou et al. (2014) reported that \textit{P. \textit{fluorescens}} ALEB 7 B produced volatiles with fungicidal activity and dimethyl disulphide (DMDS) played a major role. Similarly, Hernández-León et al. (2015) showed that several \textit{Pseudomonas} biocontrol strains produced an...
abundance of sulfur-containing volatiles with antifungal activity, including DMDS. Preliminary work on fumigation by a biocontrol agent (Mercier and Jiménez, 2003) showed that fumigation of apples with Muscodor albus cultures for 7 d gave complete control of blue mold and grey mold in wound-inoculated fruits. More recently, Di Francesco et al. (2015) demonstrated that VOCs produced by A. pullulans L1 and L8 gave excellent control of P. expansum, Botrytis cinerea and Colletotrichum acutatum on ‘Golden Delicious’ apple. Whether the antifungal VOCs produced by P. fluorescens 1–112, 2–28 and 4–6 contribute to blue mold suppression on apple remains to be demonstrated. Our findings demonstrated that direct contact of P. fluorescens with P. expansum is not required for antagonism in vitro and bio-fumigation with P. fluorescens volatiles could be a potential alternative strategy for controlling postharvest diseases.

The primary mechanism of action of bacterial biocontrol agents has commonly been attributed to the production of toxic metabolites such as antibiotics (Haas and Keel, 2003; Raaijmakers et al., 2002; Sharma et al., 2009). The importance of the production of DAPG, PCA, Prn, Plt and HCN by fluorescent pseudomonads in biological control of plant diseases has been well documented (Haas and Keel, 2003; Mavrodi et al., 2006; Santoyo et al., 2012). For example, the antifungal activity of P. fluorescens 2–79 against plant pathogens in the rhizosphere has been linked to the production of phenazine antibiotics (Thomashow and Weller, 1988). Selin et al. (2010) showed that PCA is not responsible for the biocontrol activity of Pseudomonas chlororaphis PA23, but it is essential for biofilm formation. Genetic modification of P.
fluorescens F113 by Delany et al. (2001) elucidated the importance of DAPG in the control of the oomycete Pythium ultimum. The main modes of action of the commercially available biocontrol agent Bio-Save® (i.e. P. syringae) are competition for nutrients and space, but the production of syringomycin E may also play a role in its ability to control post-harvest decay (Bull et al., 2008). Janisiewicz and Roihman (1988) reported the principal mode of action of Pseudomonas cepacia against P. expansum and B. cinerea was through antagonism by the production of Prn. Pyoluteorin is another antibiotic that is commonly produced by fluorescent pseudomonads and is characterized by its bactericidal, herbicidal and fungicidal activities (Vinay et al., 2016). Altering the expression of genes for the production of Plt by P. fluorescens Pf-5 resulted in enhanced disease control compared to the wild type strain (Kraus and Loper, 1995). Voisard et al. (1989) concluded that the production of the volatile compound HCN by P. fluorescens CHAO is important in the control of black root rot of tobacco. To assess if the production of antifungal metabolites could be a mechanism of antagonism utilized by our isolates of P. fluorescens, we screened them for the presence of biosynthesis genes encoding the production of antibiotics commonly associated with pseudomonad biocontrol agents. Molecular evidence for the antibiotic, PCA, in isolates 1–112 and 4–6 as well as for HCN production in isolate 2–28 was obtained by PCR. None of the isolates was positive for the primers specific for the genes involved in Plt, DAPG and Prn production. Further work is needed to assess if the production of the antibiotic PCA or volatile compound HCN play a role in the antagonism of P. expansum by P. fluorescens on apple. Our findings suggest that P. fluorescens 1–112, 2–28 and 4–6 may rely on other mechanisms of antagonism, as they appear to lack some of the genes necessary for the production of common antibiotics.

The physical interaction of the antagonist with the pathogen has been shown to play a major role in the biocontrol capabilities of the antagonist (Cook, 2002; Francesco et al., 2016). Chen et al. (2016) found that Bacillus amyloliquefaciens inhibited mycelial growth and altered the morphology of the fungal pathogen Botrytis dothidea. Earlier work by Hashem et al. (2014) showed that Cryptococcus albidus heavily colonized the hyphae of P. expansum in vitro and formed an extracellular matrix. Chan and Tian (2005) observed that the potential biocontrol agents C. albidus and Pichia membranefaciens loosely adhered to the hyphae of P. expansum in the wounds of apple. In our study scanning electron microscopy was used to elucidate the interaction between the fungal pathogen, P. expansum, and the antagonist, P. fluorescens, in vitro and in the wounds of apples. We found that P. fluorescens colonized the fungal hyphae of P. expansum to varying degrees depending on the growth medium (Figs. 4 and 5). In vitro, the fungal hyphae were heavily colonized by P. fluorescens, but in the apple wound little colonization of the hyphae was observed. All three isolates colonized the wounds of apples, excluding the pathogen from much of the wound site. The colonization of the fungal hyphae and wound site by P. fluorescens may inhibit P. expansum mycelial growth and conidial germination by restricting access of the pathogen to essential nutrients. Preliminary work by Hynes et al. (2008) characterizing P. fluorescens 1–112, 2–28 and 4–6 confirmed their ability to produce siderophores, extracellular, low-molecular weight, iron (III) transport agents. Fruit wounds are nutrient rich, but microelements critical for fungal development such as iron are limited (Francesco et al., 2016). Numerous reports have shown the significance of siderophores produced by fluorescent Pseudomonas spp. in biological control of plant pathogens (Cabrera et al., 2007; Francesco et al., 2016; Loper, 1988; Thomashow and Weller, 1990). The biocontrol capabilities of our P. fluorescens isolates may be attributed to their ability to chelate iron, making it less available to the competing fungal pathogen. Our findings that all three isolates failed to produce chitinases, cellulases or glucanases is supported by the fact that little degradation of the fungal hyphae was observed in vitro and in the apple wound. Inhibition of P. expansum conidial germination by P. fluorescens 1–112, 2–28 and 4–6 can be inferred from the numerous individual conidia that were not germinated in the apple wounds. This is in agreement with our in vitro findings, which showed that all three isolates of P. fluorescens inhibited conidial germination of P. expansum in sterile apple juice. Both in vitro and in vivo all three isolates were observed to form an extracellular matrix that resembled a biofilm. Due to the nature of sample preparation for electron microscopy, further work is needed to confirm the ability of P. fluorescens isolates to form a biofilm. Interestingly, only isolate 1–112 was observed to colonize the conidia of P. expansum. Our findings suggest that mechanisms, such as competition for nutrients and space at the wound site, may play a larger role in P. fluorescens biocontrol capabilities than direct parasitism on P. expansum. The nature of the interaction of the antagonistic bacteria with the fungal pathogen was observed to vary from isolate to isolate and is highly influenced by the medium on which the organisms were cultured.

5. Conclusion

In conclusion, the results of this study showed that P. fluorescens isolates 1–112 and 4–6 were effective agents for control of P. expansum on ‘McIntosh’ apples stored at 1 °C for 15 weeks. Similarly, isolate 2–28 was effective at controlling P. expansum on ‘Spartan’ apples. These results suggest that P. fluorescens may have the ability to control blue mold during commercial cold storage. Potential modes of action may include competition for nutrients and space, production of inhibitory metabolites, and biofilm formation, targeting conidial germination and mycelial growth. These data suggest that development of resistance by fungal pathogens to these biocontrol agents is unlikely. In order to achieve efficacy similar to that of chemical fungicides, which is in the range of 98–100%, P. fluorescens may need to be applied in combination with other physical or chemical controls or lower doses of fungicides.

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