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Mechanisms of action of three isolates of *Pseudomonas fluorescens* active against postharvest grey mold decay of apple during commercial storage

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ABSTRACT

The antagonistic activity of three isolates of *Pseudomonas fluorescens*, 1–112, 2–28 and 4–6, to control *Botrytis cinerea* (grey mold) under commercial cold storage conditions with two varieties of apple, 'Ambrosia' and 'Spartan', and possible mechanisms involved were investigated. All three isolates of *P. fluorescens* significantly reduced the size of the lesion and the incidence of grey mold on 'Ambrosia' apples after 15 weeks in cold storage, in comparison to control fruits, and disease control was comparable to that of the commercial biofungicide BioSave® (*Pseudomonas syringae*). On 'Spartan' apples only isolates 1–112 and 4–6 significantly reduced the size of the lesion and the incidence of grey mold, after 15 weeks in cold storage, and both isolates were comparable to the synthetic fungicide Scholar® (fludioxonil). Living cells of *P. fluorescens* or their metabolites markedly inhibited spore germination of *B. cinerea* in filter-sterilized apple juice (FSAJ). Cell-free supernatant and volatile organic compounds (VOCs) produced *in vitro* by *P. fluorescens* inhibited the mycelial growth of *B. cinerea* by 58.9 and 82.2%, respectively. Scanning electron microscopy (SEM) observations indicated that *P. fluorescens* colonized the wounds of apples and isolates 1–112 and 4–6 formed a biofilm at the wound site. Competition for nutrients and space, production of toxic metabolites and biofilm formation may play a role in the antagonism of *P. fluorescens* 1–112, 2–28 and 4–6 to *B. cinerea*.

1. Introduction

Botrytis cinerea (anamorph) and its teleomorph (*Botryotinia fuckeliana*), a necrotrophic fungal pathogen affecting more than 200 plant species worldwide (Williamson et al., 2007), was recently reported as the second most important pathogen in plant pathology (Dean et al., 2012). It causes grey mold decay on apples and is one of the most destructive postharvest pathogens of pome fruit (Xiao, 2014). Postharvest losses from fungal decay have been reported as high as 25 and 50% in developed and developing countries, respectively (Janisiewicz and Korsten, 2002; Sharma et al., 2009). Chemical fungicides such as thiabendazole (Mertect®) and fludioxonil (Scholar®) have been applied extensively to fruit to reduce postharvest loss, but pathogen resistance is emerging (Errampalli et al., 2006; Panebianco et al., 2015a; Jurick et al., 2017). The declining effectiveness of fungicides due to the development of fungal resistance, the de-registration of key fungicides, and public demand for produce free of chemicals have led to heightened interest in finding more eco-friendly and sustainable alternatives. The use of microbial antagonists or biological control agents is a promising alternative as they pose less risk to human health and the environment (Janisiewicz and Korsten, 2002; Manso and Nunes, 2011).

Seminal work by Pusey and Wilson (1984) showed that bacteria isolated from soil could be used to control brown rot of stone fruit caused by *Monilinia fructicola*. These novel findings stimulated more research into the potential use of microorganisms to control postharvest diseases of fruit (reviewed by Janisiewicz and Korsten (2002)). Janisiewicz (1987) first showed that the fruit surface is a potential source of antagonistic microorganisms when he reported that yeast and bacteria isolated from the surface of fruit were effective at controlling postharvest decay on Golden Delicious apples. Many candidate biological control agents isolated from fruit or plant surfaces have since been reported to be effective in controlling grey mold decay on pome fruit including: *Metschnikowia pulcherrima* (Piano et al., 1997), *Pichia guilliermondii* (Zhang et al., 2011), *Rhodotorula mucilaginosa* (Li et al., 2011), *Aureobasidium pullulans* (Mari et al., 2012), *Rahnella aquatilis* (Calvo et al., 2007), and *Bacillus licheniformis* (Jamalizadeh et al., 2008). Although much of the research done in the past two decades has focused on the potential of yeast antagonists to control postharvest fungal pathogens (Sharma et al., 2009; Spadaro and Droby, 2016), one of the first and longest commercially registered postharvest

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biofungicides, Bio-Save® (JetHarvest Solutions, Longwood, Florida, USA), is based on the bacterium *Pseudomonas syringae*. The efficacy of *P. syringae* has been well documented in laboratory and storage trials for controlling common postharvest fungal pathogens (Janisiewicz and Jeffers, 1997; Errampalli and Brubacher, 2006; Xiao and Boal, 2013; Panebianco et al., 2015b). Also, one of the more recently registered synthetic fungicides for managing postharvest decay, Scholar®, has the active ingredient fludioxonil which is a derivative of pyrrolnitrin, an antibiotic first isolated from *Pseudomonas* sp. (Arima et al., 1964). Janisiewicz and Roitman (1988) were the first to show that the bacterial antagonist *Pseudomonas cepacia* was highly inhibitory to postharvest apple decay and pryrrolnitrin, isolated from the antagonist, was also very effective in controlling apple decay. Although pyrrolnitrin showed strong antifungal activity, it was sensitive to light decomposition (Kilani and Fillinger, 2016) and two synthetic analogs, fenpiclonil and fludioxonil, were developed by Ciba-Geigy (now Syngenta) and serve as active ingredients in several commercial fungicides registered for pre- or post-harvest treatment (Kilani and Fillinger, 2016).

Microbial antagonists are living organisms that may possess an array of mechanisms to combat postharvest fungal pathogens. Understanding the mechanisms of action of biological control agents is not only of importance to enhance their viability and efficacy in disease control, but, also as a prerequisite of product development and registration (Droby et al., 2016). The primary mechanism of action of microbial antagonists has been often attributed to competition with the fungal pathogen for limited nutrients, such as iron or nitrogen, at the wound site or for space (Janisiewicz et al., 2000; Janisiewicz and Korsten, 2002; Bencheqroun et al., 2007). Additional modes of action such as antibiosis, biofilm formation, production of volatile organic compounds (VOC), quorum sensing, lytic enzyme production, parasitism and induction of host defenses have also been suggested (Di Francesco et al., 2016; Spadaro and Droby, 2016). The main modes of action of the commercially available biological control agent, Bio-Save® (a.i. *P. syringae*) are competition for nutrients and space. The production of syringomycin E *in vitro* by *P. syringae* ESC-10 suggests another potential mechanism to control postharvest decay, but has not yet been demonstrated *in vivo* (Bull et al., 1998). Biological control agents can exhibit a wide range of mechanisms of action that may be influenced by a number of factors including the fruit host, fungal pathogen and storage environment, and a single mechanism may not be responsible for complete disease control (Chan and Tian, 2005; Li et al., 2011).

In our earlier studies, three isolates of the bacterium *Pseudomonas fluorescens*, 1–112, 2–28 and 4–6, isolated from the rhizosphere of pulse crops in Saskatchewan, Canada (Hynes et al., 2008) showed strong inhibitory activity against common postharvest pathogens (Nelson et al., 2011; Wallace et al., 2016). The aims of the present study were to: (i) evaluate the potential of three isolates of *P. fluorescens* to control *B. cinerea* on 'Ambrosia' and 'Spartan' apples during commercial cold storage at 0 °C and compare them to commercial controls, Scholar® and Bio-Save®; (ii) investigate the effect of *P. fluorescens* isolates or their metabolites on the growth of *B. cinerea in vitro*; (iii) observe the interaction of *P. fluorescens* with *B. cinerea* in apple wounds; and (iv) assess the ability of *P. fluorescens* to form a biofilm *in vitro*.

2. Materials and methods

2.1. Antagonist

Pseudomonas fluorescens isolates, 1–112, 2–28 and 4–6, were obtained from the rhizosphere of pulse crops in Saskatchewan Canada by Hynes et al. (2008). Bacterial isolates were maintained on half strength tryptic soy agar (TSA: 15 g tryptic soy broth (TSB) (BD, Franklin Lakes, NJ, U.S.A.), 15 g agar (Bacto™, BD, Franklin Lakes, NJ, U.S.A.) in 1000 mL of water) at 4 °C for short-term use. For long-term storage, the bacteria were preserved at -80 °C in 20% glycerol. Before the experiment, bacterial inocula were prepared by incubating each isolate of *P. fluorescens* in TSB at 20 °C on a rotary shaker set at 185 rpm. The optical density (OD) of the incubated culture was measured with a spectrophotometer (ThermoFisher Scientific, Waltham, MA, U.S.A.). The colony forming units (CFU) per mL of each bacterial isolate were determined using standard calibration curves and adjusted to the desired concentration depending on the experiment.

2.2. Pathogen

Botrytis cinerea Pers.:Fr strain 27 was kindly provided by Dr. P. Sholberg, Agriculture and Agri-Food Canada, Summerland Research and Development Centre, Summerland, BC and was maintained on 1/2 strength potato dextrose agar (PDA: 12 g potato dextrose broth (PDB), 15 g agar in 1000 mL of water) at 4 °C. A spore suspension was prepared according to the method of Errampalli (2004). The spores were enumerated with a Petroff-Hauser counting chamber and spore suspensions were adjusted to the appropriate concentration with sterile distilled water.

2.3. Fruit and physiological quality parameters

Apple (*Malus domestica* Borkh.) fruit of cv. 'Ambrosia' and 'Spartan' were harvested from orchards in the Okanagan Valley, British Columbia, Canada and kindly 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 wounds and subsequently stored at 0 °C (no longer than 1 month) prior to the experiments.

Physiological fruit quality parameters were assessed on healthy untreated apples prior to commercial 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 (N) required to penetrate the apple was defined as firmness (Spadaro et al., 2013). 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.). 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 1 min 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. Antagonism in vivo

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. The fruit were wounded (2 × 2 × 7 mm) by puncturing midway with a sterile nail between the stem and calyx end on opposite sides. After wounding, fruit were placed in mesh bags (10 apples per bag) and then inoculated by submersing the bag of apples into 1×10^8 CFU mL^{-1} of *P. fluorescens* for 1 min, allowed to sit for 1 min, followed by drenching for 1 min in 1×10^4 spores mL^{-1} of *B. cinerea*. Similarly apples were drenched in commercial controls, Bio-Save® (JetHarvest Solutions, Longwood, Florida, USA) with the active ingredient (a.i.) *Pseudomonas syringae* or Scholar® 50 WG (a.i. fludioxonil, Syngenta, Guelph, Ontario, Canada), as per manufacturers' instructions, allowed to sit for 1 min, followed by drenching for 1 min in 1×10^4 spores mL^{-1} of *B. cinerea*. The drenching method of inoculation was chosen as it closely resembles commercial operations where packinghouses apply fungicides or bio-fungicides as a drench prior to