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Research article

Development of a novel biological control agent targeting the phytopathogen *Erwinia amylovora*

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ABSTRACT

Antibiotics are used extensively to control animal, plant, and human pathogens. They are sprayed on apple and pear orchards to control the bacterium *Erwinia amylovora*, the causative agent of fire blight. This phytopathogen is developing antibiotic resistance and alternatives either have less efficacy, are phytotoxic, or more management intensive. The objective of our study was to develop an effective biological control agent colonizing the host plant and competing with *Erwinia amylovora*. It must not be phytotoxic, have a wide spectrum of activity, and be unlikely to induce resistance in the pathogen. To this end, several bacterial isolates from various environmental samples were screened to identify suitable candidates that are antagonistic to *E. amylovora*. We sampled bacteria from the flowers, leaves, and soil from apple and pear orchards from the springtime bloom period until the summer. The most effective bacteria, including isolates of *Pseudomonas poae*, *Paenibacillus polymyxa*, *Bacillus amyloliquefaciens* and *Pantoea agglomerans*, were tested *in vitro* and *in vivo* and formulated into products containing both the live strains and their metabolites that were stable for at least 9 months. Trees treated with the product based on *P. agglomerans* NY60 had significantly less fire blight than the untreated control and were statistically not different from streptomycin-treated control trees. With *P. agglomerans* NY60, fire blight never extended beyond the central vein of the inoculated leaf. The fire blight median disease severity score, 10 days after inoculation, was up to 70% less severe on trees treated with *P. agglomerans* NY60 as compared to untreated controls.

Importance

Increasing resistance to antibiotics represents a growing challenge. One factor contributing to global antibiotic resistance is the large-scale application of antibiotics in agricultural fields. The bacterial plant pathogen *Erwinia amylovora* is responsible for a disease called fire blight in different hosts, including apple and pear trees. Biological control agents against *E. amylovora* were developed through a screening approach specifically tailored to isolate bacteria effective against this phytopathogen. The cell-free supernatants of various bacteria demonstrated inhibitory activity against three different *E. amylovora* strains, including two streptomycin-resistant ones. A few isolates were even capable of completely killing the target phytopathogen by direct interaction. The developed products, based on both live bacteria as well as their metabolites, were less phytotoxic than traditional treatment and also as effective as antibiotics in controlling the fire blight disease on apple trees.

1. Introduction

Resistance to antibiotics of human bacterial pathogens is a huge problem. The Center for Disease Control and Prevention (CDC) in the United States considers this as "one of the most pressing public health problems in the world" [1]. Many bacteria evolve resistance to the most commonly prescribed antibiotics, resulting in increasingly prolonged infections and higher medical costs.

Although the risk is small, a possible factor contributing to antibiotic resistance is the large-scale application of antibiotics in agricultural fields, for instance on fruit trees such as apple and pear orchards [2, 3]. The more that fields (trees and soil) are exposed to antibiotics, the higher is the likelihood that the environmental microbiota will develop resistance to antibiotics, which can lead to the transfer of resistance determinants to pathogens of animals, including humans [1].

The bacterial plant pathogen responsible for fire blight is *Erwinia amylovora* [4, 5, 6] a rod-shaped Gram-negative bacterium capable of

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infecting different hosts in the *Rosacea* family, including all species of the *Maloideae* subfamily, containing apple and pear trees [7, 8]. Primary infections caused by this pathogen affect initially the flowers, which constitute the main entry point of the bacterium into the host tree. However, the presence of *E. amylovora* does not necessarily cause an infection of apple and pear unless the bacterial population exceeds 10, 000 bacteria per flower, and infection conditions are favorable [8], including: 1) wetting of flowers by rain or dew and 2) a higher temperature which favors bacterial multiplication. In general, the temperature must exceed 18 °C during the flowering period for the infection by *E. amylovora* to lead to fire blight [9, 10]. In addition, the wetting of the flowers allows the formation of an aqueous film which transports the bacteria from the stigma where they multiply to the nectariferous glands. These nectaries are the entry point of infection in the tree. Effective disease control therefore focuses on preventing the growth of the pathogen on flower surfaces before infection occurs [11].

Antibiotics sprayed on apple and pear orchards were banned in European countries to reduce bacterial resistance development and eliminate the traces of antibiotics contaminating foods. For instance, honey produced by bees was often found to be contaminated with the antibiotic sprayed to fight fire blight disease on apple and pear trees [12]. Moreover, the National Organic Standards Board (NOSB) in the United States has banned the use of streptomycin in organic apples and pears orchards in 2014 [13]. Further, the use of antibiotics in agriculture is not sustainable because it inevitably leads to resistance [11, 14]. In the long term, this use could potentially lead to health problems for everyone if the consumption of antibiotics keeps increasing in humans, animals and plants [15].

While the most widely used formulations to control bacterial phytopathogens in crop protection are based on copper, e.g. copper hydroxide and copper sulfate, these products cannot be used on all plants because of phytotoxicity issues [16, 17] and copper accumulation in soils is detrimental. Thus, there are major restrictions on the use of copper in Europe.

This phytotoxicity can be partially alleviated with "fixed" copper, designed to leave a poorly soluble residue on the surface of the leaves [18]. When the leaves are wet, copper ions slowly escape from these deposits to attack the bacteria. The greater the seasonal application of copper, the higher the risk of phytotoxicity on flowers and the possibility of unacceptable russeting of the fruits of certain apple cultivars, such as McIntosh and Empire, as well as a decrease in fruit yields [17]. Since copper efficiency and phytotoxicity are closely related, it is difficult to maximize the former while limiting the latter. For this reason, antibiotics are considered by growers as the standard bacterial control method on some crops [17]. Thus, antibiotics such as streptomycin and oxytetracycline are widely used on apple and pear trees to fight bacterial diseases [15, 19].

Annual losses due to fire blight can be significant in many countries [15]. In the United States, losses are estimated at over $ 100 million per year [20]. In Michigan, during the year 2000, a fire blight epidemic resulted in economic losses of approximately $ 42 million due to the elimination of 350,000 to 400,000 apple trees [21]. In 1998, apple and pear producers in Washington and northern Oregon suffered a loss of an estimated $ 68 million due to fire blight [11].

Antagonism between bacteria can be based not only on antibiosis, but also on competition for niche and nutrients as well as on parasitism. A few biological products have shown commercial antagonistic potential against fire blight, but they all suffer from limitations. For instance, the =*Aureobasidium* yeast marketed as Blossom protect™ is incompatible with many fungicides and can sometimes result in unacceptable russetting of fruits. The timing of application must also be very precise to insure efficacy. Many bacteria products are now commercially available based on *Bacillus* spp. (*B. amyloliquefaciens, B. subtilis*) as well as *Pseudomonas fluorescens* and *Pantoea agglomerans* but these registered products in Canada are of limited efficacy [22].

Thus, the objective of this research was to develop a more ecologically acceptable and ethically responsible product as compared to copper and antibiotics in combating fire blight in apple and pear orchards. In addition, this biological product should possess a wider spectrum of activity and, ideally, multiple modes of actions against *E. amylovora* in order to reduce the likelihood of resistance development.

Using a screening approach tailored to isolate bacteria effective against *E. amylovora*, we present the development of a biological control to fire blight based on both bacterial active strains as well as their metabolites, which are less phytotoxic than copper but also as effective as antibiotics in controlling the disease caused by *E. amylovora*.

2. Materials and methods

2.1. Media

Two nonselective media (Tryptic Soy Agar (TSA), and Plate Count Agar (PCA)) and three selective media (Benedict (for isolation of *Streptomyces* spp.) [23], BCSA (for isolation of *Burkholderia* spp.) [24] and Gould (for isolation of *Pseudomonas* spp.) [25] supplemented with 50 mg mL^{-1} cycloheximide (to limit the growth of fungi) were used to isolate bacteria from environmental samples.

2.2. Isolation of microorganisms from plants

Ten seeds and three segments (0.5 cm^2) randomly excised from each leaf, stem, root, and fruit were vortexed in 5 mL sterile 0.85% (w/v) NaCl. To isolate sporulating bacteria, the suspensions were preheated at 80 °C for 30 min. Aliquots (100 μL) of each suspension were spread onto the nonselective- and selective media plates (Table 1). To isolate bacteria, the plates were incubated in the dark for 2–5 days at room temperature (~21 °C) [26, 27, 28, 29, 30].

2.3. Isolation of bacteria from soil

One gram of soil was added to 9 mL sterile phosphate-buffered saline (PBS) then agitated for 30 min. Sample suspensions were serially diluted (Table 1). One hundred microliters of each dilution (10^{-2}, 10^{-3}, and 10^{-4}) were spread onto nonselective- and selective media. Plates were incubated under the same conditions as above.

2.4. Storage and culture of isolated bacteria

Colonies of bacteria were purified first and then colonies with different morphological characteristics were transferred to tubes containing 3 mL TSB and incubated overnight at 30 °C. Bacteria were stored at -80 °C in TSB amended with 20% v/v glycerol.

2.5. Strains used to evaluate antimicrobial activity

The pathogenic strains used as indicators for antimicrobial activity were *E. amylovora* S435, a strain isolated from an infected apple tree at the IRDA (Quebec, Canada), a research center conducting studies on fire blight; streptomycin-resistant *E. amylovora* S153 (Botany and Plant Pathology, Oregon State University, USA) and streptomycin-resistant *E. amylovora* S1605 (MAPAQ, Quebec, Canada).

2.6. First screening step: antagonistic activity assays

The antagonistic activities of the bacterial isolates against *E. amylovora* were determined by an agar plate assay.

2.6.1. Method

Aliquots of bacterial colonies were selected from each bacterial glycerol-frozen stock solution and incubated overnight at 30 °C in 3 mL TSB. Five microliters of each bacterial culture were deposited on lawns of *E. amylovora* S435 growing on TSA plates. These lawns were first made by incubating 50 μL of *E. amylovora* S435 overnight in 3 ml TSB at 30 °C and

Table 1. Environmental samples collected from various locations for bacterial isolation.

Sample	Location source and sampling date	Number of samples
Agricultural field soil	[A]: Apple and pear orchards, Mont-Saint-Bruno (IRDA) Québec, Canada, September 2014	4
Agricultural field soil	[B]: Apple and pear orchards, Oka, Québec, Canada, September, 2014	3
Agricultural field soil	[C]: Sherrington, Québec, Canada, September, 2014	4
Agricultural field soil	[D]: Wimauma, Florida, USA, April, 2013	1
Agricultural field soil	[E]: Sherrington, Québec, Canada, November, 2011	4
Agricultural field soil	[F]: Wimauma, Florida, USA, July, 2012	2
Strawberry leaves	[G]: Dover, Florida, USA, April, 2013	1
Tomato leaves and fruits	[H]: Wimauma, Florida, USA, July, 2012	3
Apple (McIntosh and Honeycrisp cultivars) and pear (Beauté Flamande cultivar) leaves, stems, and fruits	[I]: Apple and pear orchard, Mont-Saint-Bruno (IRDA), Québec, Canada, September, 2014	4
Tomato leaves and fruits	[J]: Sherrington, Québec, Canada, November, 2011	4
Leaves, stems, and fruits of various plants	[K]: Orchard, Laval, Québec, Canada, September, 2014	4
Flowers, leaves, stems, and soil of Empire, Marshall McIntosh, Cortland, Mcintosh, Paula Red, Honeycrisp, Royal Court and Lobo apple cultivars	[L]: Apple and pear orchards, Mont-Saint- Bruno (IRDA), Québec, Canada, May–September, 2015	30
Apple leaves, stems, and soil	[M]: Apple trees, Laval, Québec, Canada, June, 2015	5
Rome apple leaves and stems	[N]: Geneva, New York, USA, July, 2015	5
Pear flowers, leaves, and stems	[O]: Laval, Québec, Canada, May, 2015	5
Total		**79**

then resuspending them in sterile water to get an OD_{620} of 0.2. Finally, 100 µL of the suspensions were spread onto TSA plates. The plates were incubated at room temperature (~21 °C) for 2 d. Bacterial isolates forming clear haloes (inhibition zones) on the *E. amylovora* S435 lawns were selected for the second screening step.

2.7. Second screening step: antimicrobial activity assays

For evaluation of the extracellular antimicrobial activity, bacterial isolates produced clear inhibition zones in at least one of the previous assays were inoculated into 3 mL TSB at 30 °C and 150 rpm on a rotary shaker for 2 d. The cultures were then centrifuged at 18,000 *x* g for 10 min at 20 °C and the supernatants were passed through a 0.22-µm pore diameter filter to obtain sterile supernatant.

Antimicrobial activity against *E. amylovora* S435 was assessed using a well-diffusion inhibition assay. First, lawns of the test bacteria were grown on agar plates. Fifty microliters of *E. amylovora* S435 were incubated overnight in 3 mL TSB at 30 °C and 150 rpm and then resuspended in sterile water (OD_{620} = 0.2). The suspensions were spread onto TSA plates and left to air-dry. Wells were bored into the agar with a sterile glass tube (d = 10 mm) and filled with 200 µL cell-free bacterial culture supernatant. The plates were then incubated at room temperature (~21 °C) and the diameters of the inhibition zones around the wells were measured after 2 d. For the control, 200 µL TSB were added to one well. Plates were incubated under the same conditions described in previous sections. Each treatment was performed in triplicate.

2.8. In vitro growth and co-culture competition

The growth and competition of the active strains were measured by co-culturing them with *E. amylovora* S435, S153 or S1605 in liquid media. Using TSB cultures incubated overnight, 50 µL of each test bacterium (diluted to OD_{620} = 0.02) were mixed with 50 µL *E. amylovora* strains (OD_{620} = 0.02) and cultivated in 3 mL TSB at 30 °C with agitation at 150 rpm for 24 h. Serial dilutions up to 10^{-6} were then prepared. One hundred microliters of each co-culture dilution were spread onto TSA plates and incubated at room temperature (~21 °C). After 2–3 d, the colony-forming units (CFUs) were counted (*Erwinia* colonies were always distinct from the test isolates). For the control, 50 µL of pure *E. amylovora* S435 (OD_{620} = 0.02) was cultured.

2.9. Identification of bacterial isolates

2.9.1. DNA extraction

DNA of active bacterial isolates were extracted according to Fastprep™ procedures and instruments (MP Biomedicals, Solon, OH, USA) The dry DNA pellet was resuspended in 50 µL sterile ddH_2O and maintained at -20 °C [31, 32, 33].

2.9.2. 16S rRNA gene sequence analysis

To identify the isolates of interest, PCR amplification of the gene encoding the 16S rRNA was performed (Supplemental Table 1). PCR was carried out in a 50-µL reaction mixture consisting of 1X Taq buffer, 200 µM dNTPs mix, 0.4 µM pA-27f-YM, 0.4 µM pH, 1 unit Feldan Taq DNA Polymerase (BioBasic Canada Inc., Markham, Ontario, Canada), and 50 ng extracted DNA. The amplifications were performed in a C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada) using an initial DNA denaturation step of 5 min at 95 °C followed by 29 cycles of 30 s at 95 °C, primer annealing for 40 s at 55 °C, primer elongation for 1.5 min at 72 °C, and a final extension step for 10 min at 72 °C. After DNA amplification, the PCR products were analyzed by agarose gel electrophoresis (1.0% w/v agarose, 100 V, 60 min). The DNA was stained with ethidium bromide (0.5 µg mL^{-1}) and visualized under UV illumination [34].

2.9.3. 16S rRNA gene sequence analysis via Resphera Insight v 2.2

Resphera Insight v. 2.2 (Resphera Biosciences, Baltimore, MD, USA), which provides ultra-high-resolution taxonomic assignment of 16S rRNA sequences down to the species level, was used as illustrated by the manufacturer [35] to predict an accurate consensus lineage of the active isolates.

2.9.4. Amplification of specific Bacillus and Paenibacillus genes

The *rpoB*, *gyrA*, and *gyrB* gene fragments were used as molecular diagnostic markers to identify isolates within the *Bacillus subtilis* group. To this end, specific primers for the amplification of each gene were used (Supplemental Table 1). PCR amplifications were carried out in a 25-µL reaction mixture as described above, using the appropriate forward- and reverse primers. The amplifications were performed using specific PCR temperature protocols. After DNA amplification, the *rpoB*, *gyrA*, and *gyrB*

fragments were analyzed by agarose gel electrophoresis (Supplemental Tables 1 and 2).

To refine the identification of the *Paenibacillus* spp. isolates, the *rpoB* gene was amplified (Supplemental Table 1) [36,37]. PCR amplifications were carried out in a 25-μL reaction mixture with 1X Taq buffer, 200 μM dNTPs mixture, 0.4 μM of each primer, 1 unit Feldan Taq DNA Polymerase, and 50 ng bacterial DNA. The amplifications were performed as described above except that the primer was elongated for 35 s at 72 °C. After DNA amplification, the *rpoB*fragments (240 bp) were analyzed by agarose gel electrophoresis, All PCR products were excised and purified from the agarose gel using a gel extraction kit (Bio Basic Canada Inc., Markham, Ontario, Canada) and sequenced at *Institut de recherches cliniques de Montréal* (IRCM). The same primers were used for the initial PCR reaction and the sequencing reactions with 16S rRNA and the *rpoB* gene from the *Paenibacillus* sp. isolates. The *rpoB* fragments from the *Bacillus* sp. isolates were cloned into a pGEM-T-Easy Vector™ (pGEM-t Easy Kit, Promega, Madison, WI, USA) and sequenced using the universal primers Sp6 and T7. The *gyrA* fragments were sequenced with the same primers used in the initial PCR. The *gyrB* fragments were amplified with the universal primers UP-1 and UP-2r and sequenced with the UP-1S and the UP-2Sr primers.

The sequences obtained for each isolate were processed with the BioEdit™ sequence alignment editor (Ibis Therapeutics, Carlsbad, CA, USA) and analyzed with Ribosomal Database Project RDP (https://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) If the sequence identity was >99%, we concluded that the bacterial isolates belonged to the same species. If the sequence identity was >97%, then the strains were classified in the same genus or family.

2.10. Biological control products formulation

Six strains were selected from the antagonistic and antimicrobial activity assays based on their ability to control *E. amylovora in vitro*. Each of these products was formulated to suppress the pathogen via the active live strains and the secreted metabolites. The final formulation for each product was broth-based as illustrated below.

2.11. Formulation of spore-forming bacteria

To formulate two products based on strains of *P. polymyxa* 273 and *B. amyloliquefaciens* subsp. *plantarum* FL50S, Schaeffer's sporulation medium (SSM) was used [38]. After incubation for 72 h at 30 °C with agitation at 200 rpm, the cultures were heated for 10 min at 80 °C to kill any vegetative bacteria. These two products, each containing sporulated bacteria and supernatant extracellular metabolites, were prepared in triplicate and stored in polypropylene bottles at room temperature (~21 °C). The effect of heating on the activity of cell-free supernatants (CFS) was evaluated as described above in the antimicrobial activity assays section.

2.12. Formulation of vegetative bacteria

For the formulation of products based on vegetative bacteria, *P. poae* FL10F, *P. fluorescens* IRDA4F, *P. agglomerans* NY60 and NY130 were cultivated in Difco Nutrient Broth (Becton, Dickinson and Co., Franklin Lanes, NJ, USA) supplemented with 0.5% v/v glycerol to promote the production of secondary metabolites [39]. Each culture was incubated for 2 d at 30 °C with agitation at 200 rpm. Then, NaCl was added at a final concentration of 4% w/v to preserve the bacterial products [40]. Each formulated product, consisting of both vegetative bacteria and their supernatant extracellular metabolites, was prepared in triplicate and stored in polypropylene bottles at room temperature (~21 °C). The same products were formulated as described above but without the addition of NaCl to assess the residual antibacterial action of the isolates (bacteria and metabolites) without preservative.

2.13. Stability and survival of stored isolates in vivo

Apple leaves (McIntosh) were disinfested by soaking them in 70% v/v ethanol for 1 min. They were then air-dried under a sterile hood for 30 min. Formulation of test bacteria that had been stored at room temperature (~21 °C) for 3 mo were diluted at a rate of one part product in twenty parts sterile water. One milliliter of this product was applied to each leaf with a trigger sprayer. The leaves were incubated on wet filters for 7 d at 25 °C and 40% RH. Three leaves were selected at t = 0 and another three were selected after 7 d to count CFUs. Each leaf was cut with sterile scissors, soaked in 5 mL NaCl (0.85% w/v), vortexed for 1 min, and incubated for 30 min with shaking at 200 rpm. Dilution series were prepared and used to inoculate TSA count plates. Three replicates were performed for each treatment described above.

2.14. Potted tree validation

A trial was conducted at the IRDA research station in Saint-Bruno-de-Montarville, Québec to evaluate the efficacy of the best isolates previously screened. Inoculum was obtained by suspending a 5-d culture of the virulent strain *E. amylovora* S435 in potassium phosphate buffer (pH = 6.5) in King's B agar which favors the growth of this phytopathogen [41, 42]. The bacterial density was adjusted to ~1×10^9 CFU mL^{-1}. Actively growing shoots of potted trees (cv. McIntosh grafted onto M26) were inoculated by transversely bisecting the two youngest leaves with scissors dipped in the inoculum. Shoots without at least the last leaf unfurled were not used to ensure uniformity. One or two shoots were inoculated per tree. Two hours after inoculation, candidate biological control agent suspensions were sprayed to runoff onto five trees per treatment using a low-pressure sprayer. The experimental design was completely randomized. Nine treatments were applied in the potted-tree experiment and 5 trees per treatment. Thus, a total of 45 potted trees were used in this experiment. These treatments were sprayed with the following formulations respectively: 1) streptomycin (100 ppm); 2) water only; 3) sterile culture medium; 4) *P. polymyxa* 273; 5) *P. poae* FL10F; 6) *B. amyloliquefaciens* FL50S; 7) *P. fluorescens* IRDA 4F; 8) *P. agglomerans* NY130 and 9) *P. agglomerans* NY60.

Inoculated controls were sprayed with the medium used to formulate the candidate biocontrol products. Uninoculated controls were sprayed only with water. The medium applied in Treatment 3 was the same one used to cultivate *P. poae* FL9F, *P. poae* FL10F, *P. agglomerans* NY60, *P. agglomerans* NY130, and *P. fluroescens* IRDA4F. It consisted of Nutrient Broth NB supplemented with 0.5% glycerol (w/w) and a 4% NaCl (w/w). The trees were incubated in a growth chamber at 25 °C and observed 2, 4, 7, and 10 d after treatment. Disease severity (DS) was rated as follows: 0 = absence of necrosis; 1 = necrosis limited to central vein of inoculated leaves; 2 = necrosis extending to petiole; 3 = necrosis reaching shoot; 4 = necrosis reaching other leaves on the inoculated shoot [43]. Fire blight severity scores were reported only 10 d after inoculation. No disease was found on the uninoculated controls. Severity scores were analyzed with a cumulative link mixed model (clmm) in the "ordinal" package of R [44]. Treatment and observation date were used as fixed effects. Individual trees were used as a random effect.

3. Results

3.1. Characteristics of the most efficient bacterial strains isolated from the screening

3.1.1. First screening for bacterial isolates

To isolate bacteria antagonistic against *E. amylovora*, the leaves, flowers, stems, and fruits of apples, pears, tomatoes, and strawberries, and agricultural field soil samples, were collected from various locations over a few years period (2011–2015) (Table 1).

3.2. Isolation of microorganisms from environmental samples

A total of 79 environmental samples were analysed. About 5,000 isolates were tested against *E. amylovora* S435 using a direct antagonistic activity assay as the first screening step. Of all the isolates assayed, 205 strains produced inhibition zones of varying diameters on a lawn of *E. amylovora* S435 (Figure 1A). These 205 selected isolates were stored at -80 °C, and the antagonistic screening process was repeated three times with similar results.

For the second screening step, the extracellular antimicrobial activities of the 205 isolates were determined and 32 isolates were retained for their cell-free supernatants (CFS) activities (Figure 1B and Table 2). The CFS of strains FL10F, 273, and FL50S displayed the strongest activity against *E. amylovora* S435. They also inhibited the growth of streptomycin-resistant *E. amylovora* S153 *and E. amylovora* S1605 (Table 3). Different strains of *E. amylovora* resistant and susceptible to streptomycin antibiotic were used in this screening in order to develop a biological control agent with a wide spectrum of activity against the phytopathogen regardless of the trait variations of each strain of *E. amylovora*. Cell-free supernatants from these three isolates formed inhibition halos 25.0–35.0 mm in diameter on *E. amylovora* S435 cultures and were kept for further analyses.

Interestingly, a subgroup of five isolates determined to be strongly inhibitory in the direct antagonism assay (step 1 of screening, Table 4) produced CFS with no visible inhibitory activity against *E. amylovora* S435 (Table 2, bottom).

3.3. Metabolite activity at various dilutions

To increase agar diffusion assay sensitivity and address relative differences in metabolite solubility, CFS of the strains most active against *E. amylovora* (with the largest inhibitory zones, Table 2) were diluted by 2-fold and 10-fold and the *E. amylovora* S435 inhibition zone assays were repeated. Certain CFS displayed no inhibition against *E. amylovora* S435 when diluted by 10-fold. Nevertheless, the CFS from six of the isolates retained some inhibitory activity even at a 10-fold dilution (Table 2).

3.4. Identification of active isolates

3.4.1. 16S rRNA

A 1.6-kb 16S rRNA DNA fragment was amplified and sequenced for each bacterial isolate. Thirty-seven bacterial isolates were identified by gene sequencing (Table 2), including 21 isolates belonging to the *Bacillus subtilis* group and 2 isolates belonging to the *Paenibacillus* genus. Analysis of pairwise 16S rDNA sequence alignments were highly similar to each at the genus level but could not be used to distinguish different but closely related bacterial species such as *P. polymyxa*, *P. peoriae*, *P. jamilae*, and *P. kribbensis*. Nine isolates belonging to the fluorescent *Pseudomonas* group and five isolates belonging to the *Pantoea agglomerans* species were also identified.

3.4.2. Further identification of Pseudomonas and Pantoea strains using Resphera Insight™ (v2.2)

Resphera Insight™ (v2.2) which is a clinical-grade proprietary analysis protocol developed to provide ultra-high-resolution taxonomic assignment of 16S rRNA sequences to species-level membership was used to further identify *Pseudomonas* and *Pantoea* strains. This computationally intensive procedure maintains the capacity for 99.9% sensitivity and >99.5% species-level specificity for hundreds of bacterial strains, and in cases of ambiguous membership, predicts an accurate consensus lineage. The results Resphera Insight™ (v2.2) were supportive to the 16S rRNA findings (Table 5).

3.4.3. Identification of isolates via gyrA, gyrB and rpoB gene sequences

The *gyrA* and *gyrB* sequences were used to discriminate the isolates belonging to the *Bacillus subtilis* group. Isolate 50S most closely resembled *Bacillus amyloliquefaciens*; their sequence similarity was 99%. Partial sequencing of the gene encoding the B protein subunit of DNA gyrase (*gyrB*) was performed on the FL3F, FL4F, FL9F, and FL10F isolates, all of which most nearly resemble a *Pseudomonas fluorescens* group strain with sequence similarities of 99–100%. Based on the *rpoB* gene sequence, isolate 273 was 99% similar to a *P. polymyxa* strain.

3.4.4. In vitro competition in co-cultures

The *in vitro* competition was assessed by co-culturing the active strains in liquid with *E. amylovora* S435, S153, and S1605 strains. Not all active strains completely killed each *E. amylovora* strains. However, all three of *E. amylovora* strains were below the detection limit of the enumeration technique following co-cultivation with either *Pantoea agglomerans* NY60 or NY130.

3.4.5. Formulations of products: stability and activity

The objective of this research was to formulate biological control products effective against fire blight on apple and pear trees. Based on the above data, six strains were selected based on their excellent activity against *E. amylovora* for the development of formulations. Each of these products was formulated to have a dual mode of action via 1) live strains and 2) metabolites present in extracellular CFS. When the product is sprayed on the trees, the active metabolites would control *E. amylovora*, while the live bacteria in the formula would grow, colonize, and outcompete any remaining or *forthcoming E. amylovora*.

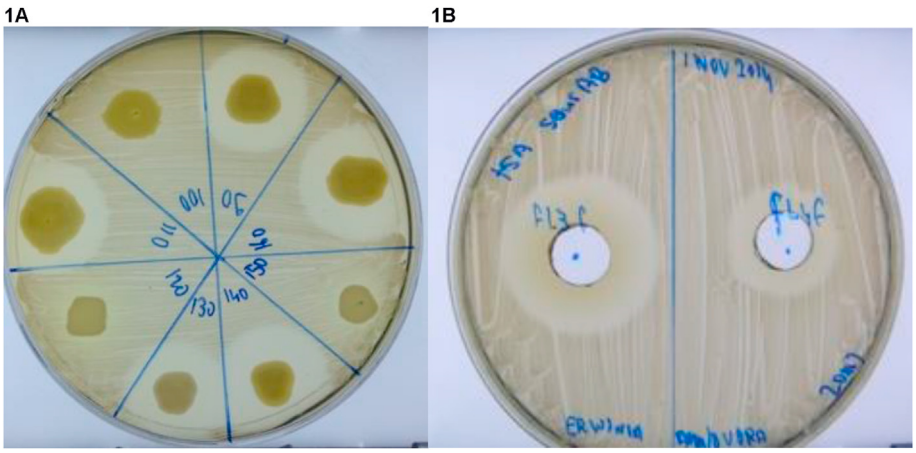

Figure 1. Antagonistic activity of several bacterial isolates against *E. amylovora* S435. A: Isolates 90, 110, 130, 140, and 160 form clear haloes (inhibition zones) on the *E. amylovora* S435 lawn. **B:** Antimicrobial activity of two bacterial isolate supernatants CFS against *E. amylovora* S435. Left: isolate FL3F; Right: isolate FL4F.

Table 2. Activities of cell-free culture supernatants against *Erwinia amylovora* S435.

Bacterial isolates/16S rRNA gene sequence identification = 99% similarilty	Source	Medium of isolation	Diameter of growth inhibition zone (including well diameter)*, mm	2-fold diluted CFS	10-fold diluted CFS
P. polymyxa 273	[E]	Benedict	26.66 ± 0.34	21.00 ± 0.36	17.58 ± 0.50
P. polymyxa 344	[J]	TSA	23.67 ± 0.21	17.08 ± 0.27	no inhibition
B. amyloliquefaciens subsp. *plantarum* FL50S	[F]	PCA	28.58 ± 0.33	27.16 ± 0.21	19.08 ± 0.27
B. amyloliquefaciens 304	[E]	Benedict	27.25 ± 0.30	20.16 ± 0.30	No inhibition
Bacillus sp. FD308	[A]	TSA	27.42 ± 0.41	25.16 ± 0.27	No inhibition
Bacillus sp. 331	[J]	TSA	28.30 ± 0.42	20.50 ± 0.43	No inhibition
Bacillus sp. FD402	[B]	TSA	24.50 ± 0.43	20.16 ± 0.47	No inhibition
B. amyloliquefaciens 417	[E]	TSA	27.33 ± 0.60	20.16 ± 0.60	No inhibition
Bacillus sp. 418	[E]	TSA	26.75 ± 0.31	19.58 ± 0.25	No inhibition
B. subtilis 421	[E]	TSA	25.16 ± 0.21	22.66 ± 0.42	No inhibition
B. amyloliquefaciens 431	[E]	TSA	27.16 ± 0.40	20.16 ± 0.30	No inhibition
Bacillus sp. FD604	[A]	TSA	27.41 ± 0.20	21.33 ± 0.49	No inhibition
Bacillus sp. IRDA27	[L]	TSA	25.50 ± 0.34	20.16 ± 0.30	No inhibition
Bacillus sp. IRDA63	[L]	TSA	23.50 ± 0.34	17.08 ± 0.27	No inhibition
Bacillus sp. IRDA618	[L]	TSA	23.50 ± 0.50	19.50 ± 0.18	No inhibition
Bacillus sp. IRDA619	[L]	TSA	21.50 ± 0.50	16.50 ± 0.22	No inhibition
Bacillus sp. IRDA627	[L]	TSA	25.50 ± 0.34	20.16 ± 0.30	No inhibition
Bacillus sp. IRDA672	[L]	TSA	24.00 ± 0.50	21.50 ± 0.34	No inhibition
Bacillus sp. IRDA675	[L]	TSA	23.50 ± 0.50	16.50 ± 0.22	No inhibition
Bacillus sp. IRDA683	[L]	TSA	29.16 ± 0.30	25.16 ± 0.27	No inhibition
Bacillus sp. IRDA684	[L]	TSA	24.00 ± 0.50	21.50 ± 0.34	No inhibition
Bacillus sp. IRDA685	[L]	TSA	25.50 ± 0.34	21.00 ± 0.36	No inhibition
Bacillus sp. IRDA687	[L]	TSA	22.50 ± 0.50	17.08 ± 0.27	No inhibition
Pseudomonas poae FL3F	[G]	PCA	28.10 ± 0.27	25.16 ± 0.30	19.50 ± 0.18
P. poae FL4F	[G]	PCA	29.92 ± 0.20	23.75 ± 0.31	21.50 ± 0.34
P. poae FL9F	[G]	PCA	26.00 ± 0.26	23.50 ± 0.34	16.50 ± 0.22
P. poae FL10F	[G]	PCA	35.08 ± 0.27	30.41 ± 0.37	25.10 ± 0.27
Pseudomonas fluorescens IRDA4F	[L]	TSA	29.16 ± 0.30	19.50 ± 0.18	No inhibition
Pseudomonas sp. 41	[L]	TSA	23.25 ± 0.36	20.16 ± 0.30	No inhibition
Pseudomonas sp. 42	[L]	TSA	23.33 ± 0.35	17.08 ± 0.27	No inhibition
Pseudomonas sp. 43	[L]	TSA	23.50 ± 0.34	19.58 ± 0.25	No inhibition
Pseudomonas sp. NY1238	[L]	TSA	21.17 ± 0.40	16.50 ± 0.22	No inhibition
P. agglomerans NY50	[N]	TSA	No activity	No activity	No activity
P. agglomerans NY60	[N]	TSA	No activity	No activity	No activity
P. agglomerans NY130	[N]	TSA	No activity	No activity	No activity
P. agglomerans IRDA 36	[L]	TSA	No activity	No activity	No activity
P. agglomerans IRDA 59	[L]	TSA	No activity	No activity	No activity

Tryptic Soy Agar (TSA), Plate Count Agar (PCA). Benedict, ± Standard error of the mean (SEM) of three replicates, * Well diameter = 10 mm.

The long-term survival of bacteria and stability of the antagonistic activities of the six biological control formulations were assessed over a period of 9 months. The products were stored at room temperature (~21 °C) in plastic bottles and were found to be relatively stable during the period. Based on CFU/ml counts, the densities of both fluorescent *Pseudomonas* spp. strains and both *Pantoea* strains decreased by ~2 log, and those of those of *P. polymyxa* 273 and *B. amyloliquefaciens* FL50S by ~1 log (Table 6). The mean results of three replicates are presented in

Table 3. Efficacies of the extracellular metabolites of *P. polymexa* 273, *B. amyloliquefaciens* FL50S and *Pseudomonas poae* FL10F against *E. amylovora* S153 and S1605.

N	Bacterial isolates	Diameter of growth inhibition zone of *E. amylovora* strains (including well diameter)*, mm	
		S153	S1605
1	*Paenibacillus polymyxa* 273	25.00 ± 0.25	25.58 ± 0.37
2	*Bacillus amyloliquefaciens* subsp. *plantarum* FL50S	27.30 ± 0.42	27.83 ± 0.40
3	*Pseudomonas poae* FL10F	29.58 ± 0.52	33.08 ± 0.27

± Standard error of the mean (SEM) of three replicates, * Well diameter = 10 mm.

Table 4. Direct antagonistic activity of *Pantoea* isolates against *E. amylovora* S435.

N	Bacterial isolates	*Pantoea* colony diameter (mm)/inhibition zone on *E. amylovora* S435 lawn (including *Pantoea* diameter in mm)
1	*P. agglomerans* NY50	11.33 ± 0.33/28.33 ± 0.33
2	*P. agglomerans* NY60	10.00 ± 0.57/30.66 ± 0.33
3	*P. agglomerans* NY130	10.66 ± 0.66/31.33 ± 0.66
4	*P. agglomerans* IRDA 36	10.66 ± 0.66/25.33 ± 0.66
5	*P. agglomerans* IRDA 59	10.33 ± 0.66/26.33 ± 0.88

All strains were isolated on TSA plates. ± Standard error of the mean (SEM) of three replicates.

Table 5. Summary of further *Pseudomonas* and *Pantoea* strains identification for isolates FL10F, FL9F, FL3F, IRDA 4F, NY60 and NY130.

Isolates	FL10F CFSAN034337	FL9F CFSAN055119	FL3F CFSAN034336	IRDA 4F CFSAN055120
Isolates identification (% similarity)				
Resphera Insight	*Pseudomonas poae* 99%	*Pseudomonas poae* 99%	*Pseudomonas poae* 98%	Fluorescent *Pseudomonas* sp. 99%

Summary of further *Pantoea* strains identification for isolates NY60 and NY130

Isolates	NY60 CFSAN047153	NY130 CFSAN047154
Isolates identification (% similarity)		
Resphera Insight	*Pantoea agglomerans* 99%	*Pantoea agglomerans* 99%

Table 6. Stability of the biological control product formulations.

N	biological control product	Concentration of bacteria (CFU/ml)		
		Time:Zero	3 months	9 months
1	*Paenibacillus polymyxa* 273	1.10 ± 0.02 x 10^7	1.01 ± 0.01 x 10^7	2.00 ± 0.11 x 10^6
2	*Bacillus amyloliquefaciens* subsp. *plantarum* FL50S	2.10 ± 0.10 x 10^7	2.00 ± 0.10 x 10^7	1.03 ± 0.06 x 10^7
3	*Pseudomonas poae* FL10F	1.06 ± 0.08 x 10^9	1.96 ± 0.14 x 10^8	1.96 ± 0.12 x 10^7
4	*Pantoea agglomerans* NY60	2.63 ± 0.06 x 10^9	1.93 ± 0.06 x 10^9	1.00 ± 0.10 x 10^7
5	*Pantoea agglomerans* NY130	2.56 ± 0.03 x 10^9	1.96 ± 0.03 x 10^9	0.96 ± 0.13 x 10^7
6	*Pseudomonas fluorescens* IRDA4F	2.00 ± 0.10 x 10^9	1.96 ± 0.03 x 10^9	1.96 ± 0.12 x 10^7

± Standard error of the mean (SEM) of three replicates.

Table 7. Antimicrobial activity of CFS of the biological control product formulations against *E. amylovora* S435.

N	biological control product	Diameter of growth inhibition zone (including well diameter) *, mm		
		Time Zero	3 months	9 months
1	*Paenibacillus polymyxa* 273	26.66 ± 0.34	23.33 ± 0.35	21.67 ± 0.21
2	*Bacillus amyloliquefaciens* subsp. *plantarum* FL50S	28.58 ± 0.33	26.67 ± 0.16	23.50 ± 0.34
3	*Pseudomonas poae* FL10F	35.08 ± 0.27	33.00 ± 0.50	29.16 ± 0.30
4	*Pseudomonas fluorescens* IRDA 4F	29.16 ± 0.30	27.66 ± 0.42	23.25 ± 0.36

± Standard error of the mean (SEM) of three replicates, * Well diameter = 10 mm.
Pantoea strains were not tested for lack of CFS activity.

Table 8. Antagonistic activity of the biological control product formulations.

N	biological control product	Time Zero*	3 months*	9 months*
1	*Paenibacillus polymyxa* 273	10.00 ± 0.57/24.66 ± 0.33	11.00 ± 0.00/22.66 ± 0.33	11.33 ± 0.66/22.00 ± 0.00
2	*Bacillus amyloliquefaciens* subsp. *plantarum* FL50S	11.00 ± 0.57/26.00 ± 0.00	11.00 ± 0.57/26.66 ± 0.33	11.00 ± 0.00/24.30 ± 0.33
3	*Pseudomonas poae* FL10F	10.66 ± 0.33/29.66 ± 0.88	9.00 ± 0.00/28.33 ± 0.66	11.00 ± 0.57/28.00 ± 0.57
4	*Pseudomonas fluorescens* IRDA 4F	11.00 ± 0.57/29.66 ± 0.33	10.00 ± 0.00/28.30 ± 0.66	9.00 ± 0.00/26.00 ± 0.57
5	*Pantoea agglomerans* NY60	10.00 ± 0.57/30.66 ± 0.33	10.66 ± 0.33/29.50 ± 0.28	10.00 ± 0.57/29.30 ± 0.16
6	*Pantoea agglomerans* NY130	10.66 ± 0.16/32.33 ± 0.15	10.00 ± 0.57/30.66 ± 0.33	10.00 ± 0.57/28.50 ± 0.28±

*Biological control product colony diameter (mm)/inhibition zone on *E. amylovora* S435 lawn (mm). ± Standard error of the mean (SEM) of three replicates.

Table 9. Survival of the biological control product formulations on leaves.

N	Microbial-based products	Time Zero*CFU per leaf	After 7 days*CFU per leaf
1	*Paenibacillus polymyxa* 273	$1.25 \pm 0.006 \times 10^3$	$1.53 \pm 0.033 \times 10^3$
2	*Bacillus amyloliquefaciens* subsp. *plantarum* FL50S	$1.90 \pm 0.057 \times 10^4$	$1.76 \pm 0.003 \times 10^4$
3	*Pseudomonas poae* FL10F	$1.20 \pm 0.088 \times 10^5$	$1.04 \pm 0.000 \times 10^5$
4	*Pseudomonas fluorescens* IRDA 4F	$1.35 \pm 0.005 \times 10^5$	$1.35 \pm 0.005 \times 10^5$
5	*Pantoea agglomerans* NY60	$2.03 \pm 0.066 \times 10^4$	$2.20 \pm 0.000 \times 10^4$
6	*Pantoea agglomerans* NY130	$1.95 \pm 0.009 \times 10^4$	$2.00 \pm 0.000 \times 10^4$
7	Control Leaves (disinfected with ethanol)	0.00 ± 0.000	0.00 ± 0.000

± Standard error of the mean (SEM) of three replicates.

Table 6. Furthermore, the inhibitory activity of the various CFS and the direct cell-to-cell antagonism of bacteria in the formulations remained active after >9 mo storage at room temperature as shown in Tables 7 and 8. Heating during the formulation of spore-forming bacteria based products had no effect on the activity of CFS (Table 7).

Survival of formulated bacteria sprayed on apple leaves (McIntosh) was then assessed. Table 9 describes the mean of the three replicates which were performed for each treatment, showing that formulated products remained viable on apples leaves for 7 d.

3.4.6. Potted tree validation

Formulations based on *P. polymyxa* 273 and *B. amyloliquefaciens* 50S were prepared as described in "Formulation of spore-forming bacteria". Those based on *P. poae* FL9F, *P. poae* FL10F, *P. fluorescens* IRDA 4F, *P. agglomerans* NY60, and *P. agglomerans* NY130 were formulated as described in "Formulation of vegetative bacteria". They were diluted to 20% (v/v) before application. Trees treated with *P. agglomerans* strains NY60 and NY130 had much less fire blight than the control after 10 days, with Disease Severity (DS) scores not significantly different from those treated with streptomycin (Table 10). The median DS score was up to 70% less severe on trees treated with *P. agglomerans* NY60 as compared to untreated ones (Table 10). Fire blight never extended beyond the central vein of the inoculated leaf in trees receiving *P. agglomerans* NY60. No evidence of phytotoxicity appeared in either treatment.

4. Discussion

From 2011-2015, bacteria suppressing the fire blight pathogen *E. amylovora* were isolated from 79 environmental samples. On lawns of *E. amylovora* S435 cultures, 205 bacterial isolates created inhibition zones of various diameters and were, therefore, considered antagonistic against this phytopathogen. Culture supernatants from only 32 of these bacteria clearly inhibited the growth of S435 cultures on agar plates. These were retained for further experimentation as well as five isolates of *P. agglomerans* displaying strong cell-cell antagonistic activity against the phytopathogen, but not through extracellular metabolites. These most active strains were identified by 16S rRNA, *gyrA*, *gyrB* and *rpoB* and were found to be belonging to the *Bacillus*, *Paenibacillus*, *Pseudomonas* and *Pantoea* genera. Interestingly there are many publications reporting microorganisms belonging to the above genera for their ability to control *E. amylovora*. An experiment on a strain of *P. agglomerans* which produces, by antibiosis, a complex zone of growth inhibition of *E. amylovora* was performed. This activity was attributed to two antibiotics produced, called pantocin A and B [45]. It was also reported that polymyxin P is the main active ingredient in the suppression of *Erwinia* species by *Paenibacillus polymyxa* M-1 [46]. It was also found that culture filtrates of a strain of *Pseudomonas aeruginosa* strongly inhibited the growth of *E. amylovora* [47]. A strain of *Bacillus amyloliquefaciens* subsp. *plantarum* with a high activity against *E. carotovora* which infects vegetables in post-harvest was isolated [48]. Another study on a *Pantoea vagans* strain indicated two main factors contributing to the biological activity against *E. amylovora*: 1) competition for limiting substrates and 2) production of antibacterial metabolites. They also demonstrated that the genes encoding that antibacterial pantocin A were found on a 28 kb chromosomal genomic island [49].

Among the 32 strains with active CFS, *P. polymyxa* 273, *B. amyloliquefaciens* subsp. *plantarum* FL50S, and four *P. poae* strains (FL10F, FL3F, FL4F, FL9F) all retained their anti-*E. amylovora* inhibition activity even at 10-fold dilution indicating that either the anti-*Erwinia* metabolites are produced at high concentrations or are very effective at low concentrations.

The CFS of *P. poae* FL10F, *P. polymyxa* 273, *and B. amyloliquefaciens* subsp. *plantarum* FL50S demonstrated the strongest antimicrobial activity against three different *E. amylovora* strains, including two streptomycin-resistant strains. The activity of CFS is expected to be attributed to more than a single metabolite; thus, resistance is less likely to develop.

Although many strains produce extracellular inhibitory metabolites, the *Pantoea* isolates demonstrated the strongest antagonistic activity against *E. amylovora* S435. And only *Pantoea* strains NY60 and NY130 when they were co-cultured with *E. amylovora* S453, S153, and S1605 were capable of completely killing the target phytopathogens, presumably by direct interaction. Such effective inhibitory activity of *P. agglomerans* against *E. amylovora* has never been previously reported [41, 49, 50, 51].

Six strains were chosen based on their abilities to control *E. amylovora* *in vitro* and *in vivo* for the development of formulations. They were selected from different bacterial genera to increase variability in the formulated strains and to increase their efficacies on apple trees. Each of these formulations consisted of both whole cultures stabilized by heating (for the sporulating species) or NaCl addition. Both the unidentified active metabolites present in the extracellular milieu maintained activity and a significant proportion of the bacteria remained alive after storage of 9 months at room temperature indicating that these strains can be probably formulated and stored at room temperature with a 9-month shelf life.

A trial was conducted to evaluate the effectiveness of the bacterial formulations against fire blight. Although the formulations were applied two hours after trees inoculation with *E. amylovora*, formulations based on *P. poae* FL10F, *P. agglomerans* NY60 or NY130 were effective and trees had significantly less fire blight than the controls. Importantly, trees treated with formulations based on *P. agglomerans* NY60 or NY130, strains isolated from an apple orchard, were similar to those treated with streptomycin, the standard treatment for fire blight. This suggests that *P. agglomerans* NY60 or NY130 formulations have a probable curative effect in controlling *E. amylovora* [52].

Our bacteria did not have to be exposed to phytopathogenic bacteria such as *E. amylovora* in order to produce cell-free supernatant (CFS) with efficient inhibitory activity. These active CFS are produced when our bacteria are cultivated in TSB even in the absence of a phytopathogen. The activity of ths CFS is attributed to the presence of secondary metabolites, not hydrolytic enzymes (Dagher and Déziel, unpublished).

A single formulation of consortia based on the combination of the formulations contained CFS from several active bacteria could be

Table 10. Effects of various treatments on potted McIntosh apple trees experimentally inoculated with *E. amylovora* S435.

N	Treatment	Median score	Score range	Tukey**
1	Streptomycin (100 ppm)	0	[0, 2]	a
2	Water	3.5	[2, 4]	c
3	Sterile culture medium	3	[2, 4]	c
4	*P. polymyxa* 273	3	[1, 4]	bc
5	*P. poae* FL10F	2	[1, 3]	bc
6	*B. amyloliquefaciens* FL50S	3.75	[3, 4]	c
7	*P. fluorescens* IRDA 4F	3	[2, 4]	c
8	*P. agglomerans* NY130	1	[0, 3]	ab
9	*P. agglomerans* NY60	1	[0, 1]	ab

** Values followed by the same letter are not significantly different ($P \leq 0.05$) according to lsmeans [53] with an adjustment for Tukey's HSD to control for family-wise error.

developed in future experiment. Here, we initially developed and compared the activity of separate formulations for two main reasons. First, we wanted to be closer to a real situation, so using bacterial formulations instead of only live bacteria was the best option. Second, and more importantly, we needed to identify which formulation/biocontrol strain was the most efficient. The results obtained will inform future experiments, including possible synergistic mixtures. However, this is a complex process, because biocontrol bacteria can also be antagonistic to each other.

Details on antimicrobial mechanisms will require further experimentation, ongoing investigations are being conducted. Although our active strains are natural isolates, more work is required on the safety of the biocontrol formulations, which will involve analyses of the whole genome sequences of the most active strains.

5. Conclusions

A major screening was conducted to select bacteria with strong inhibitory activity against *E. amylovora*. Bacterial control agents were isolated from various North American environmental samples and tested for their efficacy against this phytopathogen. Candidate bacteria were isolated from the flowers, leaves, and soil in apple- and pear orchards from springtime bloom to the summer season. In this way, the likelihood of isolating bacteria capable of colonizing the trees and competing against *E. amylovora* would be optimized. The most effective strains were tested *in vitro* and *in vivo*. They were formulated into stable products containing both living cells and their cell-free supernatants. A formulated biological control formulation based on *P. agglomerans* NY60 was not phytotoxic and was highly effective against fire blight relative to the antibiotic streptomycin. Further research is necessary to identify and isolate the gene products responsible for anti-*Erwinia* activity. Other studies are also required to elucidate the modes of action of the *Pantoea* strains whose intact cells are antagonistic against fire blight but whose CFS are not. Finally, field trials must be run to refine and perfect biological control agents which could successfully replace antibiotics and copper for fire blight control in commercial orchards.

Declarations

Author contribution statement

Fadi Dagher: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Snizhana Olishevska, Vincent Philion, Jie Zheng: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Eric Déziel: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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