Insecticidal effect of *Bacillus pumilus* PTB180 and *Bacillus subtilis* PTB185 used alone and in combination against the foxglove aphid and the melon aphid (Hemiptera: Aphididae)

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Abstract

The foxglove aphid, *Aulacorthum solani* (Kaltenbach) (Hemiptera: Aphididae), and the melon aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), are among the serious insect pests found in greenhouses. The efficacy of microbial control against these insects has been demonstrated and can be enhanced by the combination of different microbial agents. This study evaluated the efficacy of *Bacillus pumilus* Meyer and Gottheil PTB180 and *Bacillus subtilis* (Ehrenberg) Cohn PTB185, used alone and together, to control these two aphids both in the laboratory and in greenhouse on tomato, *Solanum lycopersicum* Linnaeus (Solanaceae), and cucumber, *Cucumis sativus* Linnaeus (Cucurbitaceae), plants. The results from the laboratory tests showed an increase in mortality induced by all biological treatments. In the greenhouse, all treatments induced mortality rates significantly higher than that of the control for *A. solani*. Similarly, all treatments performed better than the control against *A. gossypii*, significantly reducing its reproduction. Furthermore, we found no additive effects when mixing products nor negative interactions affecting survival for the bacteria investigated. These microorganisms therefore have potential for use in biological control.

Résumé

Les pucerons de la digitale, *Aulacorthum solani* (Kaltenbach) (Hemiptera : Aphididae), et du melon, *Aphis gossypii* Glover (Hemiptera : Aphididae), font partie des ravageurs les plus nuisibles en serres. L'efficacité de la lutte microbienne contre ces insectes a été démontrée et pourrait être améliorée par la combinaison de différents agents microbiens. Cette étude a évalué l'efficacité de *Bacillus pumilus* Meyer and Gottheil PTB180 et *Bacillus subtilis* (Ehrenberg) Cohn PTB185, utilisés seuls ou ensemble, pour lutter contre ces deux pucerons en laboratoire et en serre sur des plants de tomate, *Solanum lycopersicum* Linnaeus (Solanaceae), et de concombre, *Cucumis sativus* Linnaeus (Cucurbitaceae). En laboratoire, les résultats ont montré une augmentation de la mortalité induite par tous les traitements biologiques. En serres, pour *A. solani*, tous les traitements ont induit un taux de mortalité nettement supérieur au témoin. Tous les traitements ont aussi significativement réduit la reproduction de *A. gossypii*. De plus, nous ne montrons aucun effet additif lors du mélange des produits ni aucune interaction négative affectant la survie des bactéries. Ces microorganismes ont donc un potentiel intéressant pour la lutte biologique.

Introduction

In Canada, the greenhouse vegetable industry is growing steadily, and tomatoes, *Solanum lycopersicum* Linnaeus (Solanaceae), and cucumbers, *Cucumis sativus* Linnaeus (Cucurbitaceae), are among the most produced crops (Agriculture and Agri-Food Canada 2016). In fact, in 2015, greenhouse tomatoes and cucumbers accounted for 60% of the value of Canadian greenhouse vegetable exports, worth CAD$311.31 million and CAD$186.24 million, respectively (Agriculture and Agri-Food Canada 2016). Overall, Quebec provides 4% of the national production of greenhouse vegetables, ranking third among Canadian provinces.

Aphids are among the insects that are most harmful to cultivated crops (Blackman and Eastop 2017). These biting sap-sucking insects attack a wide range of plants, causing large yield losses. For greenhouse crops, the melon aphid, *Aphis gossypii* Glover, and the foxglove aphid, *Aulacorthum solani* (Kaltenbach) (Hemiptera: Aphididae), are among the most damaging aphid species (Jandricic et al. 2014). In Canada, the melon aphid is a major pest of Cucurbitaceae and causes damage to greenhouse cucumber plants by inducing a reduction in photosynthesis rate (Hu et al. 2017; Razmjou et al. 2006). The foxglove aphid is also a particularly problematic pest in greenhouse crops (Jandricic 2013), attacking many plants such as ornamentals and tomatoes (Jandricic et al. 2010).

Chemical control is still used against both species (Wang et al. 2002). However, excessive and repeated use of chemical insecticides has led some aphids to develop

resistance. The first incidence of insecticide resistance was recorded in Europe in 1964 in the melon aphid (Foster et al. 2017). Since then, several studies carried out in different locations around the world have demonstrated that this aphid pest has acquired and developed resistance to many groups of chemical insecticides, including carbamates (Furk et al. 1980), organophosphates (Gubran et al. 1993), pyrethroids (Wang et al. 2002), and even some neonicotinoids (Matsuura and Nakamura 2014; Wang et al. 2020). Although some studies in Japan have demonstrated low resistance to an organophosphorus insecticide in a few clones of the foxglove aphid (Takada et al. 2006), no case of resistance to a chemical insecticide has been documented for this pest in North America (Jandricic 2013). The extent of the damage and economic losses caused by aphids and the emergence of resistance have led to the misuse of chemicals (Foster et al. 2017). These products represent a potential threat to human health and the environment; hence the importance of developing new, more environmentally friendly means of control.

Microbial biological control that is based on the use of entomopathogenic microorganisms is driven by a demand for residue-free, more environmentally safe agricultural products (Mascarin and Jaronski 2016) and for a way to complement strategies using arthropods' natural enemies in greenhouse crops (Gonzalez et al. 2016). Entomopathogenic bacteria are microbial agents used in biological control programmes against certain insect pests, including the melon aphid (Iqbal et al. 2020). Because of their ability to produce beneficial secondary metabolites, some *Bacillus* species have successfully replaced synthetic products in insect management in

agriculture (Mia et al. 2016). Products developed from *Bacillus thuringiensis* (Bt) account for 98% of bacterial microbial pesticides (Lacey et al. 2015). Indeed, this bacterium has clearly demonstrated efficacy against a wide range of insect pests. However, some cases of insect resistance to this bacterium have been observed (Peralta and Palma 2017). Consequently, some studies have taken a new approach and have sought to identify other *Bacillus* species with insecticidal capacity. Recently, metabolites contained in biosurfactants produced by various *Bacillus* strains showed aphicidal properties (Rodríguez et al. 2018; Yang et al. 2017). Moreover, strains of *Bacillus pumilus* Meyer and Gottheil and *Bacillus amyloliquefaciens* showed strong insecticidal effects (López-Isasmendi et al. 2019; Molina et al. 2010), whereas *Bacillus subtilis* (Ehrenberg) Cohn and *B. thuringiensis* can induce systemic resistance to aphids in wheat (Veselova et al. 2019). *Bacillus pumilus* and *B. subtilis*, which are great producers of enzymes and secondary metabolites, are already widely used against some pathogenic fungi and thus are suitable for industrial contexts (Cossus et al. 2021; Molina et al. 2010; Weber and Marahiel 2002).

The objective of this study was to evaluate the effect of *B. pumilus* PTB180 and *B. subtilis* PTB185, used alone and in combination, against the foxglove aphid and the melon aphid in laboratory and greenhouse trials. We hypothesised that (1) the two strains, PTB180 and PTB185, would induce mortality of both aphid species; (2) the combination of both bacteria would induce additive or synergistic insecticidal effects against both aphids; and (3) the two bacteria will have a good survival rate on plants, regardless of whether they are applied alone or in combination.

Materials and methods

Biological materials

Insects. Insect rearing was initiated during the fall of 2016. Specimens of the foxglove aphid were supplied by the Biological Control Laboratory of the Department of Biological Sciences, Université du Québec à Montréal, where they were reared on potato seedlings. We transferred them onto potato, *Solanum tuberosum* Linnaeus, cultivar Norland (Solanaceae) (Norseco, Laval, Québec, Canada) or tomato plants (hybrid Celebrity; Norseco) upon arrival and allowed them to acclimate on these host plants for a minimum of three months before using them in bioassays. Potato plants were used only for the rearing of the first cohorts of the foxglove aphid and were replaced by tomato plants for the subsequent rearing. Specimens of the melon aphid were supplied by the Quebec Institute for Horticultural Development (Saint-Hyacinthe, Québec, Canada) and were maintained on cucumber plants (cultivar Marketmore 70). All plants infested with aphids were kept in a growth chamber adjusted to a 16:8-hour light:dark photoperiod, a temperature of 23 °C, and a relative humidity of 65%.

To obtain cohorts of the same age for both aphid species, 10 or 15 wingless parthenogenetic adult females were introduced with a fine brush on uninfested three-week-old plants, potato or tomato for the foxglove aphid and cucumber for the melon aphid. Adults were removed after 48 hours, and the resulting nymphs were considered synchronous cohorts.

Bacteria. Spore suspensions of both strains, *Bacillus pumilus* PTB180 (GenBank accession number MW036295.1) and *Bacillus subtilis* PTB185 (GenBank accession number MW246959.1), were provided by Premier Tech (Rivière-du-Loup, Québec, Canada). These solutions contained 1×10^9 colony-forming units per millilitre and were always kept at 4 °C. For the laboratory tests, a concentration of 1×10^8 colony-forming units per millilitre was applied for the two strains. For the greenhouse tests, the bacteria were applied according to the manufacturer's recommendations, with a concentration of 1×10^7 colony-forming units per millilitre. All dilutions were made with water.

Treatments

Four treatments were applied to foliage in all trials conducted during the present study to evaluate the effect of both *Bacillus* spp. and their combinations on both aphids: (1) control (spraying with sterile distilled water); (2) *B. pumilus* PTB180; (3) *B. subtilis* PTB185; and (4) *B. pumilus* PTB180 + *B. subtilis* PTB185. For the treatment involving the two bacteria, combinations were prepared by mixing an equal volume of solution of each microorganism. The total microbial concentration of the resulting combined preparation was therefore similar to treatments applied alone, but each microorganism taken separately had a lower concentration than when applied alone.

Laboratory test

Foxglove aphid. Three independent trials repeated over time were conducted with the foxglove aphid in the laboratory. The experimental units used were Petri dishes (100 mm × 15 mm; Fisher Scientific Company, Ottawa, Ontario, Canada) containing 1.5% agar (BD Difco, Mississauga, Ontario, Canada) melted and cooled to 45–50 °C. For each trial, a total of 20 experimental units (20 Petri dishes per trial; five replicates × four treatments; 60 Petri dishes in total over all independent trials; 15 per treatment) distributed according to a completely randomised design were established. Ten second-instar aphid nymphs were transferred onto an agar-attached tomato leaf in each Petri dish and sprayed with bacterial preparations (1 mL/Petri dish). Each Petri dish therefore received 1×10^8 colony-forming units (log 8 colony-forming units) of either strain or of the mixture of both strains.

Following the application of the treatments, to avoid excessive humidity, the dishes containing the leaves and insects were placed in a laminar flow hood for 30 minutes until the spray droplets dried. The Petri dishes were then maintained in a growth chamber adjusted to a 16:8-hour light:dark photoperiod, a temperature of 23 °C, and a relative humidity of 71% ± 5%. After 24 hours, the aphid nymphs were transferred to Petri dishes containing newly agar-attached plant leaves aerated by piercing 3-cm-diameter holes on the plate covers and sealing fine-mesh muslin over each hole to avoid water-vapour condensation. Due to rapid leaf senescence, the aphids were transferred every three days to freshly cut agar-attached leaves. Aphid mortality was evaluated daily for one week. Because aphids multiply rapidly, new nymphs produced by adults during the experiment were removed. The mortality percentage after one week was used for statistical analysis.

Melon aphid. The protocol used for the foxglove aphid was used also with the melon aphid, with the following modifications: two independent trials were conducted instead of three, and 3-cm-diameter cucumber leaf discs cut from three-week-old plants were used.

Greenhouse tests

The tests were conducted in a greenhouse at Université Laval (Québec, Québec, Canada), adjusted to a 16:8-hour light:dark photoperiod, a temperature of 25 °C during the day and 21 °C at night, and a relative humidity of 65%. Environmental parameters were continuously recorded during the experiments with HOBO data loggers (Onset Computer Corporation, Bourne, Massachusetts, United States of America). Tomato and cucumber plants were watered daily using a drip irrigation system and fertilised weekly with 150 mL of a solution containing 200 mg/L of the 20–20–20 fertiliser (Plant Prod 20–20–20 Classic, Plant Prod, Brampton, Ontario, Canada; 5.9% nitrate nitrogen, 3.9% ammoniacal nitrogen, 10.2% urea nitrogen, 8.7% soluble phosphorus, 16.6% soluble potassium). The experimental design was a completely randomised block design with four treatments and five replicate blocks, for a total of 20 plants.

Foxglove aphid. Two trials were repeated over time using the four treatments described above. Each experimental unit consisted of a three-week-old (four-leaf stage) tomato plant growing in a 946-mL pot containing PRO-MIX® BX Mycorrhizae (Premier Tech). Twenty wingless parthenogenetic adult female aphids (24-48 hours old) were gently introduced on the first fully expanded leaves of each plant assigned to

each treatment. Entire plants were then enclosed in insect cages (34.29 × 34.29 × 60.96 cm, screen mesh 106 holes/cm^2; BioQuip Products, Compton, California, United States of America) to enclose aphids but also to allow them to move freely on the whole plant. They were allowed one week to produce offspring before the treatments were applied. For each treatment, about 40 mL of bacterial preparation were sprayed per plant, using a hand sprayer and ensuring that both sides of the foliage were sufficiently and uniformly wet. Therefore, each plant received 40×10^7 colony-forming units (log 8.6 colony-forming units) of either bacterial strain or of the mixture of the two strains. After nine days, dead and live aphids were counted per whole tomato plant. The cages were also checked, and aphids present were included in the total. When counts could not be completed in one day, the plants were kept at 4 °C for one night, a process that does not affect the survival of aphids.

Melon aphid. Three trials were repeated over time using the four treatments described above. The same experimental protocol was followed for the melon aphid as for the foxglove aphid, but using cucumber plants (three-leaf stage). Also, the number of introduced aphids differed from the foxglove aphid trials. Only 10 wingless parthenogenetic adult female individuals of the melon aphid were introduced at the beginning of the trial. In addition, compared to the foxglove aphid tests, the time allowed for aphids to produce offspring was reduced from one week to only three days. However, even with fewer aphids at the initial introduction, the final density exceeded 1000 aphids per cucumber plant. Thus, only three leaves per plant were sampled for dead and live aphid counts, corresponding to older leaves that initially received adults

and sprays. As the plants reached the six-leaf stage only by the end of the trial, this sampling still represents half of the plant.

Survival of bacteria. The survival of bacilli (*B. pumilus* and *B. subtilis*) on the phyllosphere of cucumber and tomato plants was evaluated in the greenhouse trials. Aphid-free plants were sprayed with the same bacterial preparations applied to the infested plants, including a sterile distilled-water control. For each host plant species, survival tests were repeated twice with four replicas each time. Tomato and cucumber leaves were collected twice, at 24 hours and at nine days after spraying, and kept overnight at 4 °C. Leaf discs measuring 1 cm in diameter were cut from these leaves using a cork borer and weighed.

Each leaf disc was ground with 1 mL of sterilised distilled water and diluted. The 10^{-2} and 10^{-3} dilutions were kept for enumeration. To prevent other bacteria from developing, all tubes containing the selected dilutions were heated in a water bath at a temperature of 55 °C for 15 minutes to induce sporulation of bacilli. Then, 100 µL of each dilution was spread on the medium Nutriment Agar Oxoid (Fisher Scientific) and incubated at 37 °C for 24 hours.

To be able to count the colonies of each of the two strains of *Bacillus*, it was essential to distinguish one from the other. Because the morphology of the colonies of these two strains is quite distinct, it was easy to identify each by simple macroscopic observation of the colonies formed on Nutriment Agar Oxoid. *Bacillus pumilus* PTB180 is characterised by a hunched-up circular shape with coiled margins and an opaque-looking whitish coloring (Fig. 1A), whereas *B. subtilis* PTB185 is

characterised by an irregular shape with jagged margins, a creamy or slightly yellowish colour, and a flat, shiny surface (Fig. 1B). The final population at each measurement time was reported as log colony-forming units per gram of fresh leaf, and the number of colonies was calculated for a given sample according to the following formula:

$$\frac{\text{number of colonies} \times \text{dilution factor} \times \text{volume of solution}}{\text{leaf fresh weight}}$$

Statistical analysis

All statistical analyses were performed using the Mixed procedure of SAS software, release 9.4 (SAS Institute Inc., Cary, North Carolina, United States of America) at the 0.05 level of significance. For the laboratory tests, the mortality rates of the two aphid species were used as the response variables. These data were analysed using a generalised randomised block analysis of variance model, in which the treatments were considered a fixed factor and the repeated trials and the replication of each treatment within each block were considered random factors. Following a significant treatment effect, multiple pairwise least significant–difference comparisons were used to determine which treatments differ. The normality assumption was verified using the Shapiro Wilk's test, and the homogeneity of the variances was verified by the residual plot.

For the greenhouse tests, the same statistical analyses described previously were applied. The response variable was the mortality rate for both aphids, but for the

248 melon aphid, the number of live aphids found on the sampled cucumber leaves was
249 also examined. Finally, for the tests on the persistence of bacteria, the concentrations
250 calculated at the two measurement times (day 1 and day 9) were analysed as a function
251 of the different treatments with an analysis of variance with repeated measures using
252 the Mixed procedure (SAS software, release 9.4).
253

254 Results

255 Laboratory tests

256 **Foxglove aphid.** A significant effect of treatment was found on aphid mortality
257 ($F_{3,54} = 8.45$, $P < 0.0001$). Post-hoc pairwise comparisons among treatments suggest
258 that all products (used alone or in combination) significantly increased mortality
259 compared to the control (Fig. 2). With 38%, PTB180 induced the highest mortality rate
260 and differed from PTB185, which induced 22% mortality. In between these, PTB180
261 + PTB185 induced 29% mortality, and thus differed only from the control.

262 **Melon aphid.** Treatments had a significant effect on aphid mortality
263 ($F_{3,35} = 18.11$, $P < 0.0001$). Post-hoc pairwise comparisons revealed that the mortality
264 of the melon aphid in laboratory tests was similar and higher than the control for all
265 treatments (Fig. 3), ranging between 39% and 50%.
266

Greenhouse tests

Foxglove aphid. Our results showed a significant effect of treatment ($F_{3,27} = 111.45$; $P < 0.0001$), namely significant differences between the control and all other treatments (Fig. 4), with mortality rates ranging from 43% to 46%. No other differences were observed between treatments.

Melon aphid. Treatments had no significant effect on the melon aphid mortality in greenhouse tests ($F_{3,42} = 1.81$; $P = 0.1594$). Mortality was indeed very low, varying between 4% and 6% between treatments (Fig. 5). We therefore looked at the number of live aphids on three cucumber leaves as a function of the treatments instead of mortality. A significant effect of treatment was found on the number of live aphids ($F_{3,42} = 5.15$, $P = 0.0040$). Post-hoc pairwise comparisons suggest that all treatments reduced the number of live aphids present on cucumber plants nine days after application (Fig. 6). For the control, an average of 916 live aphids per three leaves was obtained. Overall, treatments reduced the number of live aphids by an average of 26% compared to the untreated control, an average reduction of 240 aphids per three cucumber leaves. No differences were observed between treatments.

Tests on survival

Bacteria survival. Survival tests demonstrated that both bacilli were able to persist for up to nine days on tomato and cucumber leaves when initially applied at a concentration of 40×10^7 colony-forming units per plant. On tomato leaves, the

288 survival of bacterial was not affected by days ($F_{1,21}$ = 1.03, P = 0.3218), treatments
289 ($F_{2,2.02}$ = 3.64, P = 0.2142), or the interaction between days and treatments
290 ($F_{2,21}$ = 1.45, P = 0.2581). The populations of both *B. pumilus* PTB180 and *B. subtilis*
291 PTB185 applied alone or in a combined mixture did not significantly differ after one
292 or nine days, reaching an average of 2.8×10^6 colony-forming units per gram of fresh
293 leaf on the ninth day after application (Table 1). On cucumber leaves, the survival of
294 bacterial differed between days ($F_{1,21}$ = 18.36, P = 0.0003) but was not influenced by
295 treatments ($F_{2,3.07}$ = 0.07, P = 0.9320) or by the interaction between days and
296 treatments ($F_{2,21}$ = 1.25, P = 0.3079). The numbers of *B. pumilus* PTB180 and
297 *B. subtilis* PTB185 used alone or in a mixture significantly dropped from
298 2.1×10^7 colony-forming units per gram of fresh leaf on day one to 1.5×10^7 colony-
299 forming units per gram of fresh leaf after nine days. No bacterial colonies grew for the
300 control treatments.
301

302 # Discussion

303 To our knowledge, this study is the first to report on the aphicidal potential of
304 *B. pumilus* strain PTB180 and *B. subtilis* strain PTB185. Previous studies had
305 confirmed the insecticidal effect of other strains of these two bacteria against different
306 insects (Chandrasekaran et al. 2012; Chandrasekaran et al. 2014; Molina et al. 2010)
307 and even an aphicidal effect for *B. subtilis* (Rajendran et al. 2011; Veselova et al. 2019).

Bacillus pumilus PTB180 and *B. subtilis* PTB185 showed pathogenicity against the foxglove aphid, *A. solani*. Indeed, our results are the first to reveal mortality of *A. solani* following the application of the two *Bacillus* strains. The insecticidal capacity of the two *Bacillus* species studied can be explained by their ability to produce substances and enzymes that attack specific sites on the host insect. *Bacillus* species, including *B. subtilis*, are known to produce chitinase, an extracellular enzyme that degrades chitin, a component of the cuticle of most insects during their developmental changes (Kilani-Feki et al. 2016; Moussa et al. 2014). For instance, chitinase purified from different strains of *B. subtilis* caused increased mortality of the tobacco cutworm, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) (Chandrasekaran et al. 2012; Chandrasekaran et al. 2014). It is therefore possible that the strain *B. subtilis* PTB185 used in our study produces this enzyme, facilitating its penetration into the body cavity of the foxglove aphid by perforating the cuticle and the intestinal membrane. Although *B. pumilus* may act on aphids in a similar way, this does not necessarily suggest that the two bacteria have the same mode of action (Molina et al. 2010). In fact, little is known about the insecticidal potency of *B. pumilus*, but strain 15.1 was shown to perform well, causing up to 94% mortality of the larvae of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) (Molina et al. 2010).

For the melon aphid, *A. gossypii*, our results showed different patterns than with the foxglove aphid. For instance, under laboratory conditions, we found that both PTB180 and PTB185 strains caused significant mortality of the melon aphid. Under greenhouse conditions, however, our results suggest that both bacterial strains had little

effect on the melon aphid's mortality but instead caused a significant reduction of aphid reproduction. The discrepancy between laboratory and greenhouse results may be explained by the different experimental conditions between the two environments (e.g., lighting, temperature, etc.). In addition, in the greenhouse tests, a hypothetical interaction between cucumber plants, microorganisms, and aphids may have occurred. We have not measured this interaction but hypothesise that it may have reduced the reproduction of the melon aphid by impacting its physiology or by inducing systemic resistance to the pest in the cucumber plants (Veselova et al. 2019). Such interaction has been observed by Rajendran et al. (2011), who found a decrease in the incidence of *A. gossypii* on cotton following the application of EPCO 102 and EPCO 16, two endophytic strains of *B. subtilis*. These strains act by improving the production and accumulation of defense enzymes (e.g., peroxidase, polyphenol oxidase, etc.), chitinase, and phenolic compounds in treated plants (Rajendran et al. 2011). These enzymes act against the target insects in different ways, including interrupting their diet. For example, chitinase blocks the activity of the majority of intestinal enzymes essential to feeding in the tobacco cutworm, *S. litura* (Chandrasekaran et al. 2014). The hypothetical interruption of *A. gossypii* feeding may slow down its development, therefore increasing the periods separating its moults, extending the total time required to complete its life cycle, and possibly reducing the number of offspring it produces. Also, although unlikely, a behavioural effect possibly impacted our results. Even though we sampled half of the plants and younger leaves had been deployed for only a few days, aphids might have moved to younger leaves. If this behavioural effect

happened, it would have made our counts slightly inaccurate; however, its effect would be expected to be consistent among treatments. Based on this, we believe our conclusion to remain valid.

Our study is the first to investigate the interaction between *B. pumilus* PTB180 and *B. subtilis* PTB185 and the possible synergistic or additive effects between these two entomopathogens. Such effects have been observed for entomopathogens when they are combined with chemical insecticides, toxins, and other entomopathogens (Konecka et al. 2020; Wraight and Ramos 2005). However, our results suggest that applying a mixture of both *B. pumilus* PTB180 and *B. subtilis* PTB185 against both the foxglove and the melon aphids did not improve their efficacy, as we found no clear additive effects. Indeed, the treatment involving both bacteria never differed from any treatment involving either bacterium species alone, regardless of the aphid species or of the setting (laboratory or greenhouse). Similarly, Xu et al. (2011) showed that synergistic effects between biocontrol agents for the management of plant diseases are unusual and that experimental demonstrations of such effects are rare. Likewise, neither Lewis and Bing (1991) nor Costa et al. (2001) have detected any additive effect or increased mortality caused by *B. bassiana* GHA applied with a toxin produced by *Bacillus thuringiensis* (Bt) against the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), or the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae). However, several studies have demonstrated a synergistic interaction between different strains of *B. bassiana* and B. *thuringiensis* against different insect pests (Wraight and Ramos 2005; 2017;

374 Yaroslavtseva et al. 2017). As the presence or absence of synergy between two
375 microorganisms varies according to the time of their application (Szczech and Shoda
376 2004; Wraight and Ramos 2005), additional research is recommended to test the
377 synergistic interaction between strains PTB180 and PTB185, depending on the time
378 and manner (simultaneously or successively) of their application against aphids, as well
379 as to their formulation.

380 Our greenhouse tests on the survival and compatibility of the two
381 microorganisms used in this study showed that they both demonstrated good survival
382 over time. We observed concentrations higher than 10^6 colony-forming units per gram
383 of fresh leaf after nine days for both microorganisms on both plants. Such high counts
384 are considered to indicate excellent survival (Collier et al. 2005; Jurkevitch and Shapira
385 2000; Wang et al. 2014; Wei et al. 2016).

386 In conclusion, this work revealed the aphicidal properties of *B. pumilus*
387 PTB180 and *B. subtilis* PTB185 on two common aphid pests in greenhouse crops.
388 These two bacterial strains caused mortality of *A. solani* and decreased *A. gossypii*
389 reproduction. No synergy or additive effect was observed following application of the
390 different combinations of the two bacterial strains. Overall, we detected mortality rates
391 ranging from 30% to 50%. Those rates represent significant increases in mortality but
392 are inferior to most optimal mortality rates experimentally reported, often ranging
393 between 50% and 100% (Iqbal et al. 2020; López-Isasmendi et al. 2019; Molina et al.
394 2010; Saif Ur et al. 2019; Trinh et al. 2020; Yang et al. 2017). Nevertheless, they still
395 indicate some aphicidal potential for the two bacterial strains. Achieving high mortality

rates depends on many factors, such as the concentration, formulation, timing of application, delay between applications, and temperature (Prince and Chandler 2020; Saif Ur et al. 2019). Variations in each of these factors can cause success or failure of a microbial biocontrol programme. As such, more research needs to be done regarding those parameters. Still, *B. pumilus* PTB180 and *B. subtilis* PTB185 appear to be potential candidates for biological control of the foxglove aphid in greenhouse. They also seem to influence the melon aphid, but assessment of effective application parameters is needed before these two strains could be used as biological pest control agents. If used, both products should be integrated into integrated pest management strategies alongside other available and compatible tools.

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Table

Table 1. Concentrations of *B. pumilus* PTB180 and *B. subtilis* PTB185 one day and nine days after application to tomato and cucumber leaves, alone and in combined mixture. Trials conducted in greenhouse following a completely randomised design plan with four replicates and two independent, time-repeated trials *(N* = 8). SE, standard error; CFU, colony-forming units.

		Bacterial concentration (± SE) (log CFU/gram of fresh leaf)	
	Treatments	1 day	9 days
Tomato leaves	PTB180	6.38 (± 0.14)	6.41 (± 0.14)
	PTB185	6.42 (± 0.14)	6.41 (± 0.14)
	180 + 185*	6.73 (± 0.14)	6.52 (± 0.14)
Cucumber leaves	PTB180	7.26 (± 0.07)	7.19 (± 0.08)
	PTB185	7.34 (± 0.07)	7.14 (± 0.08)
	180 + 185*	7.34 (± 0.07)	7.18 (± 0.08)

* For the PTB180 + PTB185 mixtures, the indicated colony-forming units per gram are the sum of both bacterial colony-forming units identified according to the morphology of the colonies.

Figure captions

Fig. 1. Morphology of bacterial colonies on Nutriment Agar Oxoid medium (photos from Premier Tech, Quebec, Canada): **A,** *Bacillus pumilus* PTB180; **B,** *B. subtilis* PTB185.

Fig. 2. Cumulative mortality of the foxglove aphid second-instar nymphs seven days after application of *Bacillus pumilus* PTB180 and *B. subtilis* PTB185, alone and in combination, on tomato leaves in laboratory testing. Trials conducted in laboratory following a completely randomised design plan with five replicas and three independent, time-repeated trials ($N = 15$). Treatments with same letters are not significantly different (least-significance difference, $\alpha = 0.05$, $F_{3,54} = 8.45$, $P < 0.0001$).

Fig. 3. Cumulative mortality of the melon aphid second-instar nymphs seven days after application of *Bacillus pumilus* PTB180 and *B. subtilis* PTB185, alone and in combination, on cucumber leaves in laboratory testing. Trials conducted in laboratory following a completely randomised design plan with five replicas and two independent, time-repeated trials ($N = 10$). Treatments with same letters are not significantly different (least-significance difference, $\alpha = 0.05$, $F_{3,35} = 18.11$, $P < 0.0001$).

Fig. 4. Cumulative mortality of the foxglove aphid adults nine days after application of *Bacillus pumilus* PTB180 and *B. subtilis* PTB185, alone and in combination, on greenhouse tomato plants. Trials conducted in greenhouse following

a completely randomised design plan with five replicas and two independent, time-repeated trials ($N = 10$). Treatments with same letters are not significantly different (least-significance difference, $\alpha = 0.05$, $F_{3,27} = 111.45$; $P < 0.0001$).

Fig. 5. Cumulative mortality of the melon aphid adults on three cucumber leaves sampled nine days after application of *Bacillus pumilus* PTB180 and *B. subtilis* PTB185, alone and in combination, on greenhouse cucumber plants. Trials conducted in greenhouse following a completely randomised design plan with five replicas and three independent, time-repeated trials ($N = 15$). No treatment effect was found ($F_{3,42} = 1.81$; $P = 0.1594$).

Fig. 6. Variation in the number of live melon aphid found on three cucumber leaves sampled nine days after application of *Bacillus pumilus* PTB180 and *B. subtilis* PTB185, alone and in combination, on greenhouse cucumber plants. Trials conducted in greenhouse following a completely randomised design plan with five replicas and three independent, time-repeated trials ($N = 15$). Treatments with same letters are not significantly different (least-significance difference, $\alpha = 0.05$, $F_{3,42} = 5.15$, $P = 0.0040$).