Egyptian Journal of Biological Pest Control

RESEARCH

Open Access

New insights on entomopathogenic bacteria isolated from soil of citrus crops to combat the polyphagus aphid pest *Hyalopterus pruni* (Geoffroy 1762) (Hemiptera, Aphididae)

Mohamed Hafsa[1*] and Leila Allal Benfekih[1]

Abstract

Background The employment of entomopathogenic microorganisms is a promising approach for ensuring optimal phytosanitary protection in the framework of biological management of insect crop pests. Among these microbes, entomopathogenic soil-borne bacteria are preferred over pesticides because they help successfully in the natural regulation of arthropod populations, as soil has a favorable ecology for the availability and richness of many beneficial bacterial species. In this study, it was focused on the isolation, identification and characterization of entomopathogenic bacteria isolated from cultivated citrus soils and on the evaluation of their insecticidal potential in the laboratory against the mealy plum aphid, *Hyalopterus pruni* (Geoffroy 1762) (Hemiptera: Aphididae), a polyphagous and major pest attacking and virus-transmitting of several *Prunus* crops.

Results Isolation results gave 11 bacterial isolates, which were more than 50% effective in selection tests on *H. pruni* aphids at 1×10^8 CFU/ml. A total of seven isolates generated significant insecticidal potential at different concentrations, and their molecular identification based on 16S rRNA genome sequencing yielded the following results: *Lysinibacillus fusiformis* (B4), *Bacillus thuringiensis* (B13), *B. thuringiensis* (B22), *B. thuringiensis* (B23), *B. thuringiensis* (B24), *Pseudomonas sp*. (P2) and *Enterococcus gallinarum* (P4). The most pathogenic potential of these strains was for *E. gallinarum* (P4), which induced 100% *H. pruni* mortality after 72 h of treatment at the concentration (C3 = 1×10^6 CFU/ml), followed by *B. thuringiensis* (B23), (B22), (B4), (P2), (B13) and (B24) isolates, which caused 96, 91, 85, 83, 65 and 50% mortality rates, respectively, at the lowest concentration (C4 = 1×10^5 CFU/ml). The LC_{50} and LT_{50} values were calculated for the entomopathogenic isolates of (P2), (P4), (B23) and (B24). A lowest LC_{50} value was 1.08×10^2 CFU/ml for (P4) *E. gallinarum* after 72 h of treatment, whereas (P2) *Pseudomonas* sp. presented the shortest LT_{50} of 33.6 h at concentration (C4 = 1×10^5 CFU/ml).

Conclusions The present study's outcomes have confirmed the existence, abundance, and variety of entomopathogenic bacteria at the soil level in citrus groves. Interestingly, these bacteria could be useful for aphids' population control on a wide scale through the utilization of their toxins and enzymes, even against insect pests of a broad order.

Keywords Biological control, Entomopathogenic bacteria, Soil, Insect pests, *Hyalopterus pruni*

*Correspondence:
Mohamed Hafsa
hafsamohamed560@gmail.com
[1] Laboratory for Research on Medicinal and Aromatic Plants, Faculty of Nature Sciences and Life, Saad Dahlab University, Blida 1, Road of Soumâa, 09000 Blida, Algeria

Background

Insect pests are part of bio-aggressors that pose a significant risk to agricultural output, impacting the production level, quality, and aesthetic value of the crop. Aphids belong to the "sap-sucking" pests which about

100 species are considered severe agricultural and horticultural insect pests, directly responsible for destroying many economically significant crop plants. Population growth rate, considering the aphids' age-specific development, survival, and fecundity, accurately characterizes this pests' impact (Blackman and Eastop 2000). Aphids can cause not only direct damage by sucking sap, but also indirect damage as vectors for various viral diseases. *Prunus* crops, such as peaches, are considered to be the best-known cultures in terms of their economic impact and production (Rousselin et al. 2017) worldwide. The mealy plum aphid *Hyalopterus pruni* (Geoffroy 1762) (Hemiptera: Aphididae), is a major polyphagous pest to several crops, including the genus *Prunus* globally growing under temperate and Mediterranean climates and in Europe and North America (Rousselin et al. 2017). *H. pruni*, causes damage directly to *Prunus* trees by absorbing phloem sap, reducing fruit yield quality and causing chlorosis which consequently affects growth in the long term (Lozier et al. 2009). This aphid species is also the main vector for plum pox virus (PPV) transmission which is responsible for shark disease, causing major economic losses on *Prunus* genus trees (Hazir et al. 2021).

Chemical insecticides are still widely used in peach orchards to control the aphid population. Despite those chemical substances targeted effect, insect populations may swiftly acquire resistance, unfortunately making these insecticides useless and impeding long-term management (Paliwal et al. 2022). Furthermore, detrimental effects on human health due to the indiscriminate pesticide usage, inhalation poisoning, toxicity in the food chain, contamination of surface and groundwater, and other environmental issues pose a serious obstacle to the ability to safeguard the quantity and quality of numerous important crops.

Exploring soil-borne bacteria diversity remains an attractive alternative to develop biological control of insect pests using these natural microorganisms (Meddas et al. 2020). Indeed, the soil is a site of microbial competition because of its vast biological diversity, including rhizospheric bacteria that influence plant development and most importantly control insect pests in a variety of crops. Bacteria of the *Bacillus* and *Pseudomonas* genera are considered to be the most widely used rhizospheric bacteria for potential biological control since they are capable of efficiently combating a wide variety of commercially important insect pests (Oulebsir-Mohand kaci et al. 2015a, b).

Bacteria of the *Bacillus* genus such as *Bacillus thuringiensis (Bt)* constitute approximately 95% of the global biopesticide economy, due to their extensive utilization as microbial control agents. The bacterium *B. thuringiensis* produces insecticidal proteins in the form of crystalline inclusions during the sporulation phase, known as Cry or Cyt toxins, which have proved effective against important lepidopteran pests. Some *Bt* strains produce a binary toxin called Vip2Ae-Vip1Ae, in addition to new Cry proteins from the *Bt* strain H1.5 named Cry41Ab1 and Cry41Aa1, respectively (Palma et al. 2014). Likewise, *Pseudomonas* is recognized for its entomopathogenic activity against a variety of insect pests, such as aphids (Manjula et al. 2018). *P. fluorescens* can lessen the severity of numerous fungal illnesses and improve Plant Growth Promotion Rhizobacteria (PGPR) by the synthesis of several secondary metabolites, such as hydrogen cyanides, siderophores, and antibiotics (O'Sullivan and O'Gara 1992).

Sustainable methods that use beneficial microorganisms' natural insect diseases to reduce environmental harm, still become more meaningful to date. It has been determined that more than 100 bacterial species exhibiting entomopathogenic activity are exo- and endopathogens of arthropods. Aphid populations have been effectively controlled by entomopathogenic fungi, while bacterial control methods have achieved not much focus. This present investigation is based on the isolation and identification of rhizospheric bacteria from different soils cultivated on citrus, in order to investigate the insecticidal potential of effective isolates on the most damaging insect pest of *Prunus*, *Hyalopterus pruni*.

Methods

Soil sampling

Sampling techniques consisted of collecting sufficient soil volumes from four randomly selected locations in citrus groves. Soil samples were collected from the localities of Beni Mered, Mouzaïa, Oued El Alleug, Bougara, and Tabainat surrounding citrus area in the province of Blida (Central Mitidja plain, Algeria). Soils specimens were gathered from a depth of 10 to 15 cm near the root system, to isolate bacteria of the *Pseudomonas* genus (Bikram et al. 2018), and from a distance of 5 to 10 cm in the soil's uppermost stratum, in order to separate *Bacillus* strains (Mihir et al. 2017). Every soil sample collected was placed in hermetically sealed sterile plastic bags, until usage in the laboratory.

Bacteria isolation

Soil samples were dried separately, sieved, and finely ground. After that, 10 g of soil was suspended in 90 ml of sterile physiological water and then homogenized with a magnetic stirrer for 30 min. Successive dilutions were then prepared to obtain the final dilution of 10^{-5} from a 10^{-1} one. The dilutions used to isolate *Bacillus* bacteria were heated at 80 °C for 10 min to eradicate non-spore-forming bacteria, each prepared dilution was applied in

0.1 ml nutrient agar (NA). *Pseudomonas*, was incubated in a King B medium, which allows the production of fluorescein (Oulebsir-Mohand kaci et al. 2015a, b). Incubation was carried out at 30 °C for 24 h for *Bacillus* and at 28 ± 2 °C for 24 to 72 h in the case of *Pseudomonas*.

Identification of bacterial isolates

Morphological and biochemical characterization

The identification and taxonomic characterizations of isolated bacteria were based on several morphological characteristics corresponding mainly to the type of bacterial colony (size, shape, color, margin, opacity, and pigmentation), the shape of the bacterial cells, Gram staining and their mobility. Afterward, according to Bergey's Manual of Systematic Bacteriology using morphological, physiological, and biochemical methods (Buchanan and Gibbons 1974), the colonies that seem to meet the criteria of the desired genera (*Bacillus* and *Pseudomonas*) were successively transplanted in adequate medium to obtain a pure culture.

Molecular identification

Molecular identification focused on the sequencing of 16S rRNA genes for the performing strains being most efficient against the mealy plum aphid *H. pruni* at different selected concentrations. Bacterial genomic DNA was extracted using the GF-1 Nucleic Acid Extraction Kit (Vivantis Technologies Sdn Bhd, Selangor DE, Malaysia). Isolated DNA was kept at 4 °C until needed for PCR. The amplification of the bacterial genomic extract was achieved using the primer set for the 16S rRNA gene (27F: 5′-AGA GTT TGA TCC TGG CTC AG-3′ and 1492R 5′-CCG TCA ATT CCT TTG AGT TT-3′) (Edwards et al. 1989) using a thermocycler (iCycler Bio-Rad, USA). The runs were as follows: initial denaturation at 94 °C for 12 min, followed by other denaturation for 30 s at 94 °C, 30-s annealing at 55 °C, and 1 min 40 s for a primer extension at 72 °C. The amplification was repeated in 30 cycles, followed by a final extension at 72 °C (7 min).

PCR products were separated into a 1.5% agarose gel (Sigma-Aldrich, USA), and the gel was viewed under UV light after staining with Midori Green Advance (Nippon Genetics, Japan) and inspected using a UV trans-illuminator. PCR electrophoresis products were purified (Clean-Upkit, Vivantis) for sequencing onto a 3130 Genetic Analyzer Capillary Array for detection (Applied Bio systems) in the forward and reverse directions in separate reactions and duplicates. A BLAST analysis was conducted on the 16S rRNA sequence data by comparing similarity percentages using the NCBI GenBank database (NCBI GenBank; http://blast.ncbi.nlm.nih.gov) (Altschul et al. 1990). A phylogenetic tree was created using Molecular Evolutionary Genetics Analysis (MEGA 11) (Tamura et al. 2021).

Pathogenicity bioassays on *H. pruni*

Insect collection

The mealy plum aphid, *H. pruni* was collected from a non-treated peach orchard located in the experimental station at Saad Dahlab University Blida-1 (USDB-1), at the end of April 2022.

Preparation of bacterial suspensions

The isolated and characterized bacteria strains were purified on nutrient agar plates to get pure colonies for each isolate. After 24 h of incubation at 30 °C, a few colonies were collected and inoculated into 5 ml of nutrient broth to prepare the bacterial suspensions. The tubes containing bacterial solutions were incubated at 30 °C with 200 rpm shaking for 48 h to allow sporulation and crystal formation (Karungu et al. 2018). A volume of 1 ml of this bacterial solution was then seeded in tubes containing 5 ml of sterile distilled water. Bacterial concentrations used for bioassays were established by adjusting the optical density of the bacteria dilution at 625 nm to 0.1 (about 1×10^8 CFU/ml) (Agbe et al. 2020).

Screening tests and bioassays

Adults *H. pruni* were firstly treated in a preliminary test in triplicate with a bacterial suspension concentration of 1×10^8 CFU/ml, for all bacterial isolates (twenty-nine) in order to determine the most efficient isolate on aphids. Fresh peach leaves not infested with aphids were sterilized for two minutes with 2° chlorine bleach, then rinsed three times with sterile distilled water, dried with sterile absorbent paper and soaked on both sides for 2 min in the previously prepared bacterial suspension. Pathogenicity tests are based on soaking leaves in bacterial solutions, the way aphids feed by using their perforating mouthparts to penetrate plant tissues and extract sap from the phloem, ensuring that bacterial cells pass directly into the intestine. A number of 15 aphid individuals were then placed on the soaked leaves and put into sterile Petri dishes with wet piece cotton with sterile distilled water to maintain leaf vitality. The bacterial suspension was replaced with sterile distilled water for negative controls.

In further bioassay, isolates (11 isolates) that expressed more than 50% aphids mortality in the preliminary screening test were used at four different concentrations (1×10^8, 1×10^7, 1×10^6 and 1×10^5 CFU/ml). Treatments were performed with 15 aphid individuals per replicate and three replicates per concentration. Positive controls leaves were soaked in the same concentrations solutions of referenced strains *Bacillus* sp. HF911367 (*Bt*) and

Pseudomonas sp. HF911366 (*Pf*) provided by laboratory of Valorization and Conservation of Biological Resources (VALCORE), M'Hamed Bougara University – Boumerdes (Algeria) (Oulebsir-Mohandkaci et al. 2015a, b) and negative controls infested leaves, were treated with sterile distilled water.

Mortality rate

Mortality rate was recorded after 24, 48, and 72 h, for all replicates both in screening tests and bioassays at different concentrations, based on the ratio of the total dead aphids number over the total aphids' number used in treated and controls. The observed mortality was corrected using the ABBOT formula, (Abbott 1925): Percentage of corrected mortality MC% = 100 * (M1–M2/100-M1), where M1 was the percentage of mortality observed in controls and M2 was the percentage of mortality observed among treated.

Statistical analysis

To determine differences among treatment means, analysis of variance was performed using XLSTAT 2023V 1.6.1410 for Windows and The IBM SPSS Statistics 20 software. In the event of significant differences, Tukey's HSD test and Student's t test were used to separate the means of the different treatments. All parameters were analyzed at the 5% significance level. For controls *Bt* and *Pf* strains and bacteria isolates potentially efficient at different concentrations on *H. pruni* individuals, probit analysis was used to calculate median lethal concentrations and times ($LC_{50/90}$, $LT_{50/90}$) using Fisher and Yates method (1971) (Finney 1971).

Results

Morphological and biochemical characterizations

Isolation from the soil of citrus orchards in the Blida region resulted in a total of 90 isolates, including initially the two desired genera *Bacillus* and *Pseudomonas*. After 24 and 48 h of incubation for the respective isolates, some colonies were observed that developed on nutrient agar (NA) and King B. Colonies that probably belonged to the desired genus were selected. All isolates belonging to the *Bacillus* genus were large 2–4 mm colonies, cream-beige color, dry with irregular contour while being opaque (Fig. 1a). *Pseudomonas*, colonies were characterized by a regular contour, opaque, rather large, and round (Fig. 1b), with the typical production of fluorescent pigment.

Molecular identification

GenBank accession number of the 16S rRNA gene sequences for strains: B4, B13, B22, B23, B24, P2 and P4 is OR915493, OR915490, OR915491, OR915495, OR915489, OR915494 and OR915492, respectively. BLAST analysis of the five bacterial strains coded, B1, B2, B3, B4, and B5, indicated that they belong to the genus *Bacillus* (Bacillaceae family) with very similar sequence (100%) to *Bacillus thuringiensis* and *Bacillus cereus* (Table 1) (Fig. 2). The other isolates showed a 100% similarity sequence with other bacteria species. Strain B23 showed 100% similarity in his sequence with *Bacillus proteolyticus*, B24 and B13 with *B. wiedmannii* and B22 with strain *B. anthracis*. However, only two type strains, *Lysinibacillus fusiformis* and *L. sphaericus*, were closely linked to strain B6, which showed substantial sequence identity (100%). In other cases, P2 has a total similarity (100%) with *Pseudomonas sp*, whereas

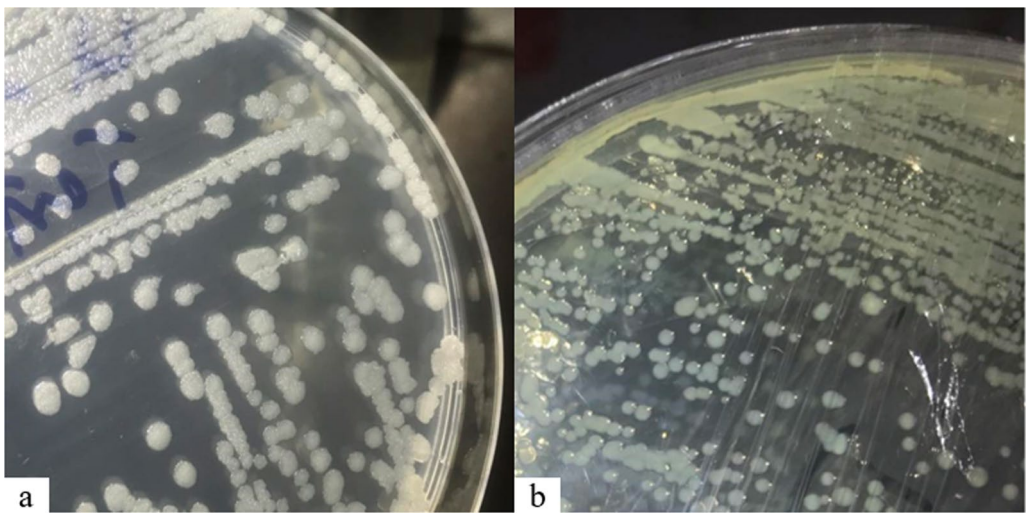

Fig. 1 Colony morphological of *Bacillus* in nutrient agar medium (**a**) and *Pseudomonas* in King B (**b**)

Table 1 Suggested identification of bacterial isolates, based on BLAST analysis of their 16S rRNA gene sequences

Isolate	GenBank identification suggestion	Similarity (%)	GenBank accession numbers
B23	*Bacillus thuringiensis* serovar tenebrionis strain NB176-1	100	CP114399.1
	Bacillus cereus strain LAM 30	100	EU019990.1
	Bacillus proteolyticus strain MRC_ZO3_41	100	OK605866.1
B24	*Bacillus thuringiensis* strain HER1410	100	CP050183.1
	Bacillus cereus strain HZ-01	100	MT328556.1
	Bacillus wiedmannii G071	100	LC515603.1
B13	*Bacillus thuringiensis* strain NBAIR_Bt104	100	OQ600809.1
	Bacillus wiedmannii strain SN2-2	100	MT071682.1
	Bacillus cereus strain ER5	100	MT124530.1
B22	*Bacillus thuringiensis* strain FDAARGOS 791	100	CP054568.1
	Bacillus anthracis strain FDAARGOS 695	100	CP054816.1
	Bacillus cereus strain 65gite	100	MT378165.1
P4	*Enterococcus gallinarum* strain GI13	100	MT158590.1
	Enterococcus sp. CSQRZN3.4.9	100	LC484830.1
B4	*Lysinibacillus fusiformis* strain Uyi_38	100	MT507231.1
	Lysinibacillus sphaericus strain TB-22	100	KC540952.1
P2	*Pseudomonas* sp. strain BIS1097	100	MN810183.1
	Pseudomonas sp. strain P111-L04pd	100	MN043751.1

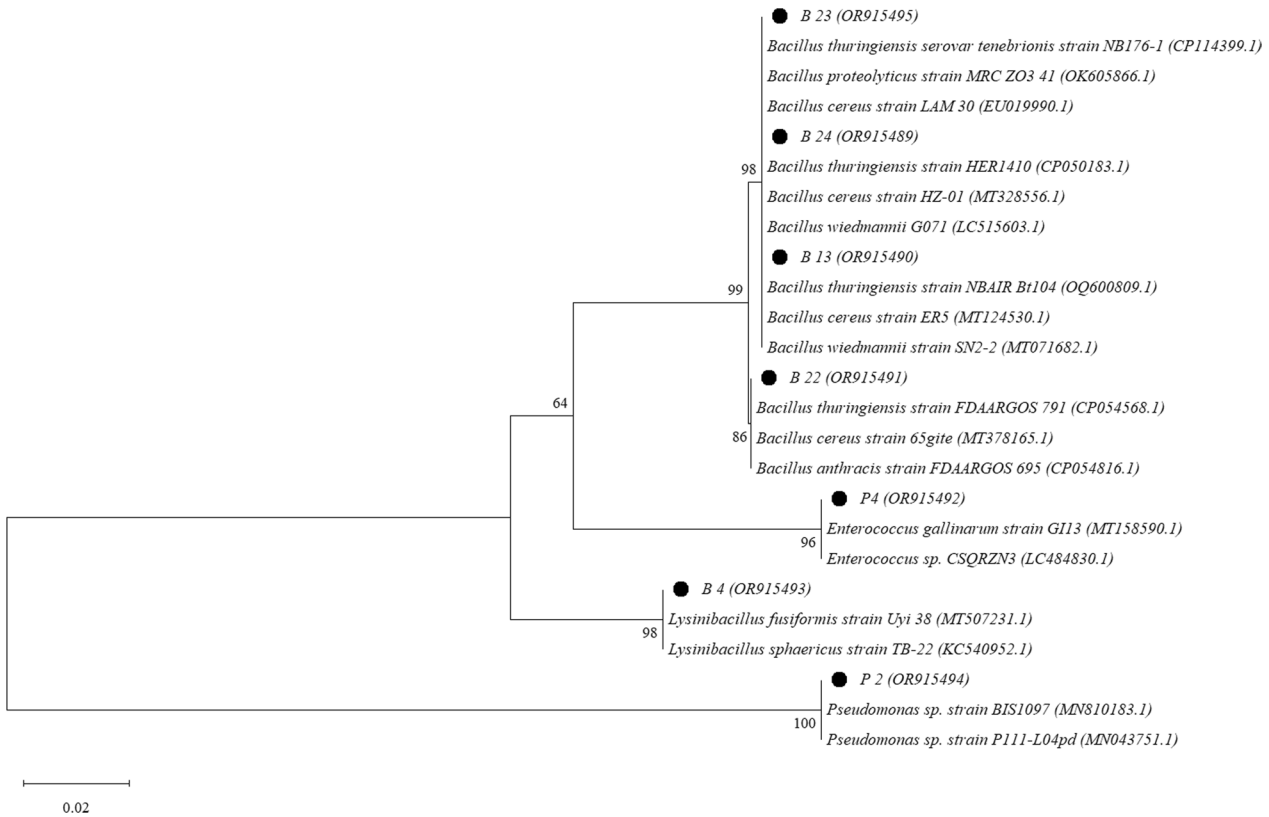

Fig. 2 Phylogenetic tree of selected isolates using neighbor-joining method based on 16S rRNA gene sequencing

P4 belongs to the *Enterococcus* genus, and presented a high sequence similarity (100%) with the strain *Enterococcus gallinarum* strain GI13 (MT158590.1) and *Enterococcus* sp. (Table 1) (Fig. 2).

In conclusion, bacterial isolates were identified as follows: *B. thuringiensis* for B23, B24, B13 and B22 isolate, *Enterococcus gallinarum* (P4), *Lysinibacillus fusiformis* (B4) and *Pseudomonas* sp. (P2) (Table 2). The phylogenetic position of bacterial isolates (B23, B24, B13, B22, P4, B4, and P2) with closely related species based on 16S rRNA gene sequencing is shown in the phylogenetic tree created using the neighbor-joining method (Fig. 2).

Table 2 Accession numbers of isolated bacteria in GenBank database

Isolates	Strains	Accession number
B4	*Lysinibacillus fusiformis*	OR915493
B13	*Bacillus thuringiensis*	OR915490
B22	*Bacillus thuringiensis*	OR915491
B23	*Bacillus thuringiensis*	OR915495
B24	*Bacillus thuringiensis*	OR915489
P2	*Pseudomonas* sp.	OR915494
P4	*Enterococcus gallinarum*	OR915492

Screening tests

Corrected aphid mortality rates showed a highly significant difference at 72 h ($df = 30$, Fr = 3.5, $p < 0.0001$) under the effect of the 1×10^8 CFU/ml concentration between *Bacillus* and *Pseudomonas* characterized isolates, including 5 *Pseudomonas* (P) and 22 *Bacillus* (B) isolates compared to controls *Bt* (*Bacillus* sp.) HF911367 and *Pf* (*Pseudomonas* sp.) HF911366 (Fig. 3).

Isolate P4 and *Bt* control strains produced very high mortality levels (95.83 and 93.81%, respectively) significantly different from those of strains B24, B17, B23, B14, P2, and B22 (24% of the total) which have generated fewer high effects on *H. pruni* mortality, ranging from 71.42 to 85.71%. The control *Pseudomonas* strain *Pf* and isolates B4, B13, B2, B15, P3, B16, P1, and B21 (31% of the total), have caused aphid mortalities ranging between 40 and 53%, whereas B18, B9, B20, B5, B19, B7, B8, B1, B3, B6, B12, and B11 strains (41.3% of the total) have induced much low mortality percentages varying from 12 to 34% (Fig. 3). Contrariwise, B10 isolate did not affect aphid mortality.

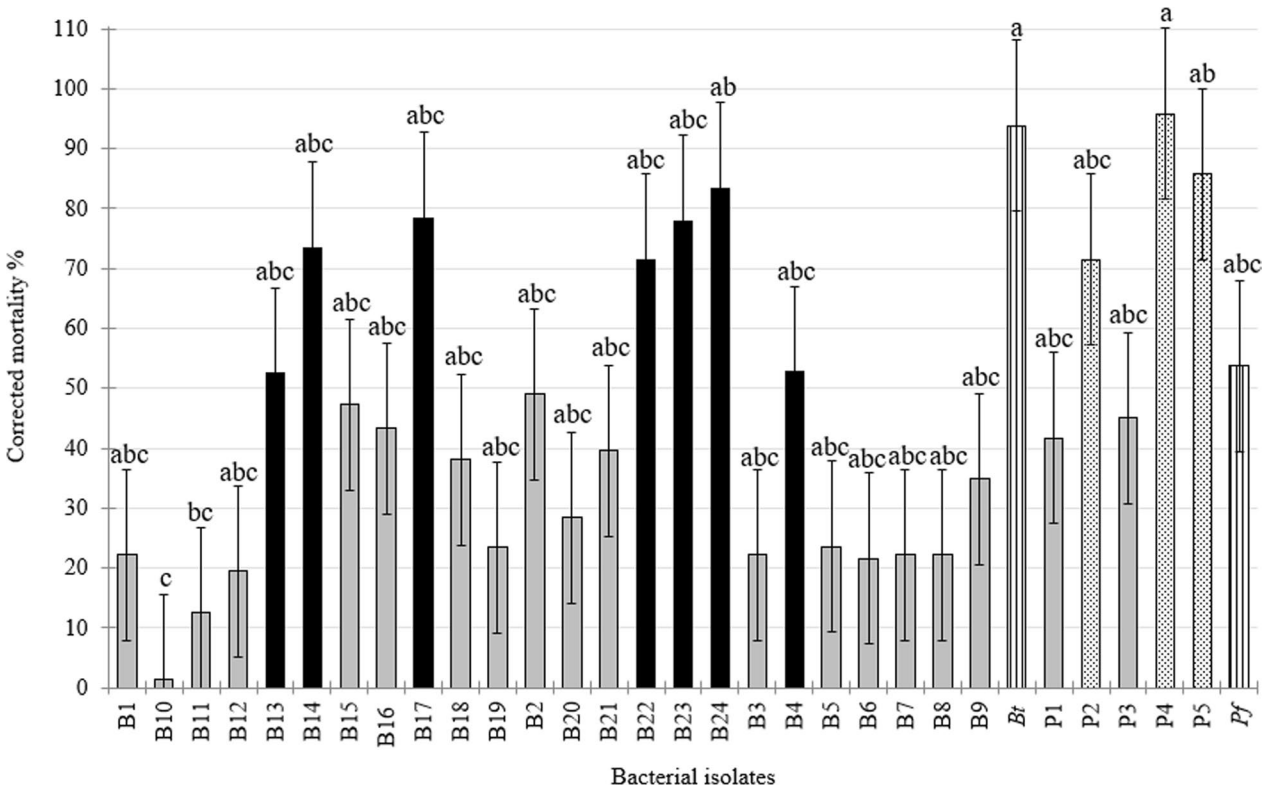

Fig. 3 Effect of all bacterial isolates against *Hyalopterus pruni*, 72 h after application at concentration 1×10^8 CFU/ml

Evaluation of the selected isolates effect on *H. pruni* Global effect with applied concentrations after 24, 48 and 72 h

The estimated mean effect of the 4 applied concentrations between selected isolates ($n=10$ isolates) was significantly different ($df=22$, Fr = 3.886, $P<0.0001$). Aphid *H. pruni* average mortality observed with all 10 selected isolates (B13, B14, B17, B22, B23, B24, control *Bt* and P2, P4, P5, control *Pf*) was less than 20% after 24 h of treatment with the fourth concentrations (C1 to C4). The four isolates (B22, B23, P2 and P4) demonstrated after 72 h greater than 50% mortality rate, but the three isolates (B23, P2 and P4) showed a mortality rate over 50% after 48 h (Fig. 4). At 72 h after treatment, isolate P4 showed the highest cumulative mortality rate (85.81%) on aphid *H. pruni*, compared to that of P2 and B23 isolates (76.98 to 79.15%), B22 isolate (71.94%) and to that of controls 61.62% (*Pf*) and 51.02% (*Bt*). Relatively even at 48 h, P4, P2, and B23 showed high mortality rates of 62.68, 64.15, and 60.20%, respectively, whereas *Bt* (22%) and *Pf* (32.39%) controls have induced much low mortality rates (Fig. 4). However, average *H. pruni* mortality rates were recorded 48.68% (B13), 48.21% (B24), 47.73% (B4), 39.45% (B14), 30.72% (P5), and 30.72% (B17) after 72 h and only counted between 15.84 and 25.60% for B13, B24, and B4, respectively, at 48 h.

Globally, increasing *H. pruni* aphid mortality percentages was time dependent ($df=2.000$, Fr = 189,669, $p<0.0001$). The best mortality rate (56.24%) was recorded after 72 h after treatment, followed by those recorded at 48 h (34.31%), then at 24 h after application of the treatments (9.62% only) (Table 3).

All the tested bacterial isolates concentrations (C1 to C4) have achieved a similar and low effect (6–14%) at 24 h, and a high mortality rate at the end of 72 h (Fig. 5).

Concentration C1 induced the highest mortality rate at 48 h (45.63%) and at 72 h (74.11%). Nevertheless, concentrations (C2, C3 and C4) had a convergent effect throughout the treatment period, with no significant difference between C2 (31.91%), C3 (28.92%) and C4 (30.77%) at 48 h, and also between C2 (48.05%), C3 (49.29%) and C3 (53.51%) after 72 h of treatment (Fig. 5). Tukey post hoc comparison showed a significant difference ($df=3$, Fr = 16.149, $p<0.0001$) between the applied concentrations and within the time of treatment with the concentrations used ($df=6$, Fr = 2.529, $p=0.021$).

Table 3 Average aphid mortality (%) under selected isolates effect after 24, 48, 72 h of treatment

Time after bacterial treatments (h)	Average aphid mortality rate
72	56.24a
48	34.31b
24	9.92c

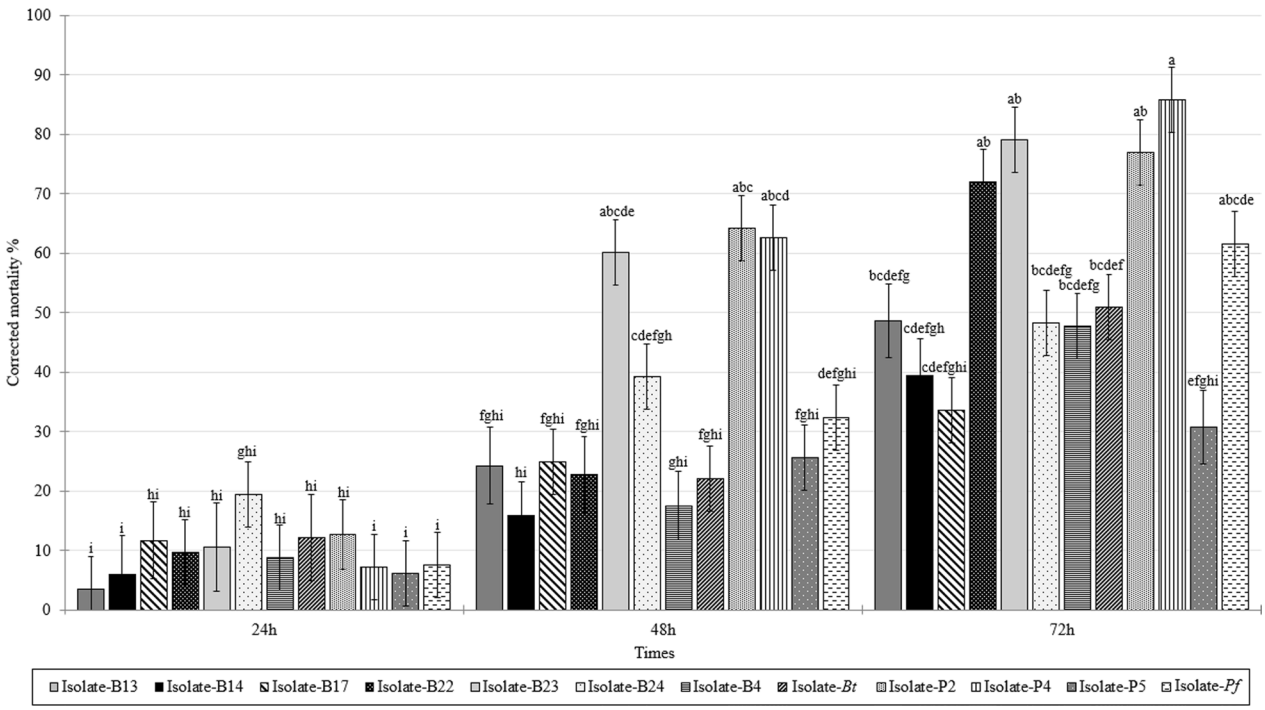

Fig. 4 Estimated means mortality of selected isolates 24, 48, 72 h after application at used concentrations

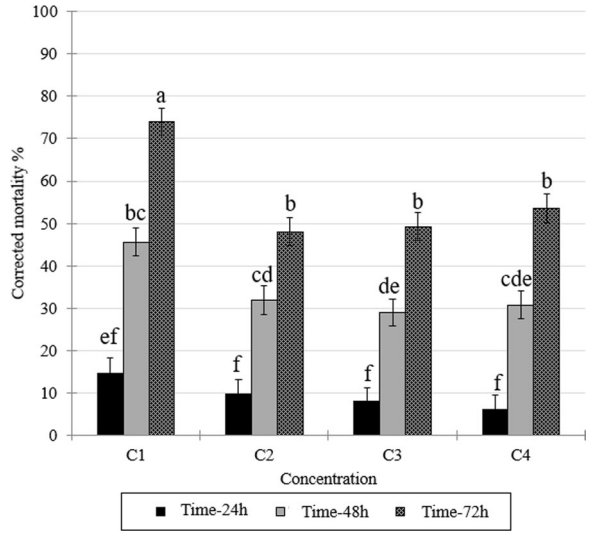

Fig. 5 Aphid *H. pruni* mortality after 24, 48 and 72 h with each concentration

Effect of the concentrations of selected isolates on *H. pruni* after 72 h of treatment

Accordingly, isolates P4, P5, B24, B17, B23, B14, P2, B22, B4, and B13 (Fig. 3), which have exhibited 50% of aphid mortality and more, were therefore tested at the concentrations ($C1 = 1 \times 10^8$ UFC/ml, $C2 = 1 \times 10^7$ UFC/ml, $C3 = 1 \times 10^6$ UFC/ml, $C4 = 1 \times 10^5$ UFC/ml), for a duration time of 72 h. Evaluation of pathogenicity at these concentrations showed variable mortality rates on *H. pruni* (Fig. 6), ($df = 33$, $Fr = 2{,}368$, $p = 0.001$). At the lowest concentration (C4), bacteria strains B23, B22, B4 and P2 induced high mortalities in *H. pruni* aphids 96, 91, 85 and 83%, respectively (Fig. 6). Moreover, these mortality rates were higher than those recorded in P4 (65.92%) and B13 (65.61%). With concentrations C2 and C3, P4 isolate caused as well very significant mortality of *H. pruni* aphids ranging from 80 to 100%, and even rather similar death rates (79 and 73%) with the isolate P2 were detected at these same concentrations. B23 and B22 also recorded high mortality rates at C3 and C2 but were less effective than those of P2 and P4, with rates of 74% (B22), 65% (B23), and 77.42% (B23) at C3, and 50.31% (B22) at C2, where B24 was 50% effective at this concentration. In contrast, isolate B4 recorded high mortality at the lowest concentration while showing low mortality at the other concentrations (C2 and C3). In comparison with the two referenced strains, *Bt* and *Pf,* the mortality rate decreased according to the concentrations prepared by *Bt,* where 45% (C2), 36% (C3), and 24% for the lowest concentration (C4) were recorded. In contrast, the *Pf* strain gave mortality rates of over 60% at treatments with low concentrations of C3 (73.61%) and C4 (61.80%), with a rate of 55.32% for C2. All isolates tested gave effective mortalities of between 51.37% and 95% at the highest concentration (C1) (Fig. 6).

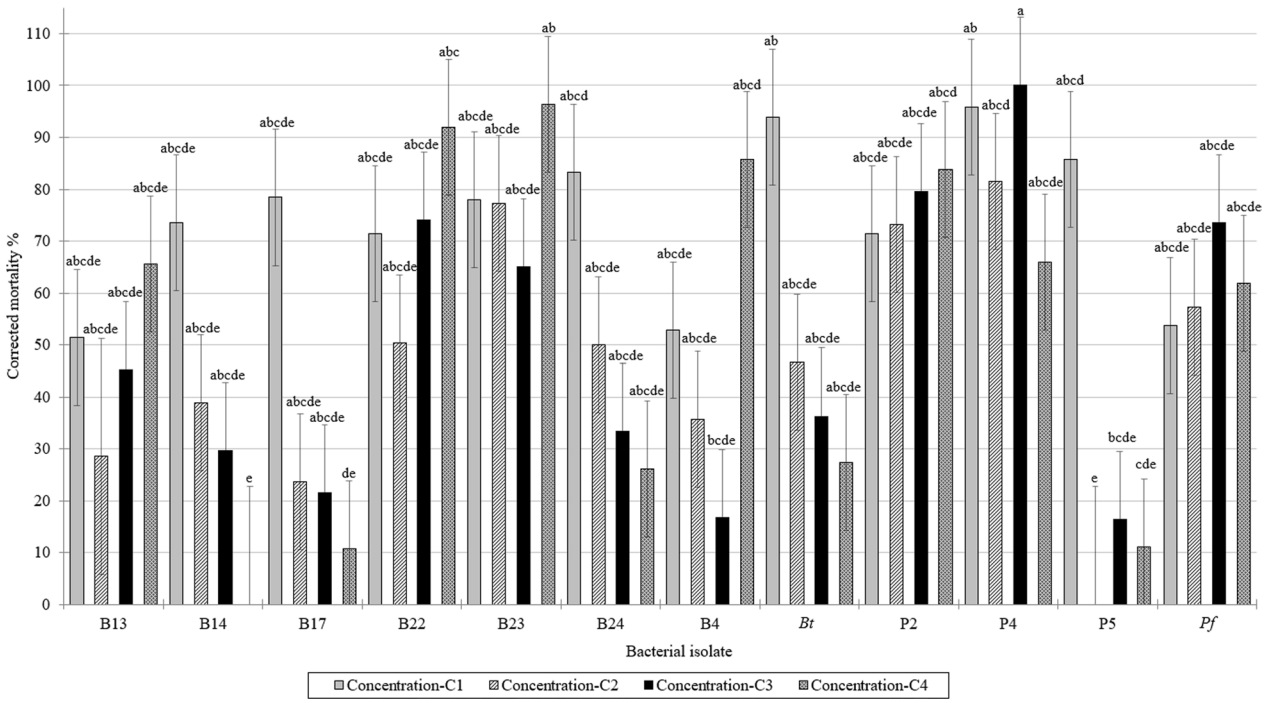

Fig. 6 Effect of selected isolate at different doses after 72h of application against *Hyalopterus pruni*

Determination of lethal concentration (LC_{50}, LC_{90}) values

LC_{50} of isolate P4 (*Enteroccocus galinarum*) was the lowest (1.08×10^2 CFU/ml) among all the other potentially most efficient isolates after 72 h of treatment and the positive controls *Bt* and *Pf* (Table 4). Notably, strain P2 showed the lowest LC90 (1.16×10^3 CFU/ml) after 72 h of treatment (Table 4).

Determination of lethal time (LT_{50}, LT_{90}) values

The lowest LT_{50} value (33.6 h) for P2 strain effect was recorded after treatment with concentration C4 (1×10^5 CFU/ml). In contrarily, P4 isolate exhibited the lowest LT_{90} (50.4 h) after treatment with concentration C3 (1×10^6 CFU/ml) (Table 5).

Discussion

Obtained results have shown that B4, B22, B23, B24, B13, P2, and P4 isolates have generated high mortality rates against *H. pruni*. These isolates were identified as *Bacillus*, *Pseudomonas*, and *Enterococci* genera, using 16S rRNA gene sequence analysis. Bacillaceae and Pseudomonadaceae were already known to occur in soil (Oulebsir-Mohand kaci et al. 2015a, b). Enterococcaceae have also been isolated from soil (Falcone et al. 2017).

P4 strain (*Enteroccocus galinarum*) was the most effective to kill *H. pruni* as the average recorded 85.5% of mortality which was obtained for the four tested concentrations, and still total aphids mortality (100%) has been reached after 72 h with a concentration of 1×10^6 CFU / ml. Several studies have focused on isolating *Enterococci* species from various gut insects to using these bacteria for biological control, as they cause sepsis and rapid death when trans-locating toward the hemolymph, (Mason et al. 2011). According to the literature, *Enterococcus* genus has not been tested against aphids (Hemiptera) but in the present study, the species *E. gallinarum* isolated from soil and tested against *H. pruni* aphids, has proven an aphicidal effect.

Compared to *Pseudomonas,* the P2 strain (*Pseudomonas* sp.) also caused 83% mortality rate after 72 h of treatment with the lowest concentration ($C4=1\times10^5$ CFU/ml). *P. fluorescens* strains have insecticidal activity against agricultural pests, including aphids (Kupferschmied et al. 2013). Our results corroborate those of Paliwal et al (2022), who successfully demonstrated that *P. fluorescens* presented 100% mortality after 72 h of treatment against the green peach aphid *Myzus persicae*, but at the concentration of 1×10^7 CFU/ml. Furthermore, Manjula et al (2018) confirmed *P. fluorescens* efficacy against the cotton aphid, *Aphis gossypii*, causing 65.3% reduction after 1% foliar application. On the other hand, Hashimoto et al. (2002) identified *P. fluorescens* and other bacteria as possible pathogens for aphid *Myzus persicae*.

In this study, the difference in aphid mortalities recorded with P2 isolate (*Pseudomonas* sp.) in comparison with those of the referenced strain *pf* (HF911366) may be explained by host insect specificity, given that the referenced strain was tested for its insecticidal effect on *L. migratoria* larvae and *G. mellonella* (Oulebsir-Mohand kaci et al. 2015a, b). Thus, Paliwal et al (2022) found different mortality rates having been caused by *P. fluorescens* (PfR37) on two different species of aphids (*Myzus persicae* and *Aphis fabae*).

B. thuringiensis strains B23 and B22 were responsible for the highest *H. pruni* aphids mortality at the lowest concentration ($C4=1\times10^5$ CFU/ml), (96% and 92%, respectively). Our results match with those of

Table 4 Lethal concentration values (CFU/ml) for B22, B23, P2, P4 isolates compared to *Bt* (HF911367) *Pf* (HF911366)

Bacterial isolates	LC_{50}	LC_{90}
B22	3.30×10^8	3.50×10^4
B23	1.05×10^{10}	1.21×10^5
P2	2.10×10^{11}	1.16×10^3
P4	1.08×10^2	1.27×10^5
Bt	2.07×10^6	1.62×10^8
Pf	2.09×10^9	3.78×10^3

Table 5 Median lethal times values (hours) for B22, B23, P2, P4 isolates compared to *Bt* (HF911367) and *Pf* (HF911366)

Bacterial isolates	C1(1×10^8 CFU/ml)		C2 (1×10^7 CFU/ml)		C3 (1×10^6 CFU/ml)		C4 (1×10^5 CFU/ml)	
	LT_{50}	LT_{90}	LT_{50}	LT_{90}	LT_{50}	LT_{90}	LT_{50}	LT_{90}
B22	55.2	134.4	69.6	235.2	60	105.6	48	84
B23	48	100.8	43.2	88.8	50.4	153.6	36	57.6
P2	55.2	98.4	45.6	91.2	43.2	96	33.6	76.8
P4	40.8	60	45.6	91.2	36	50.4	48	96
Bt	40.8	86.4	124.8	2215.2	292.8	16,845.6	199.2	2102.4
Pf	69.6	249.6	55.2	134.4	57.6	91.2	64.8	134.4

Alamuratov et al. (2023) who demonstrated that *Bt* has an entomopathogenic effect against *H. pruni* with a 69.5% mortality as the best efficiency obtained at concentration 2×10^8 CFU/ml after 72 h in laboratory conditions. The same authors observed similarly different mortality rates due to other *Bt* strains using the same concentration. These results are similar with our finding related to variable aphid's mortalities observed with *Bt* isolates B13 and B24 that generated low insecticidal potential compared to *Bt* strains (B23, B22). Previous works also found this difference in insecticidal potential of multiple soil-borne *B. thuringiensis* strains on lepidopteran and dipteran insects (Astuti et al. 2018) and on the pea aphid *Acyrthosiphon pisum* (Porcar et al 2009). This difference can be explained by the host specificity of many *B. thuringiensis* sub-species related to the variation of insecticidal activity of the endotoxins they produce (Yamagiwa et al. 2002).

P4 isolate (*Enterococcus gallinarum*) was found to be the most potent among the tested isolates. As the *Enterococcus*' genus is a component of insects' gut microbiota (Cox and Gilmore 2007) ingesting a small quantity of these bacteria may thus cause intestinal disturbances with commensal bacteria of the same genus, which explains why this low concentration could cause mortality in populations of *H. pruni* aphids.

In the present study, results highlighted that both strains P4 and P2 displayed the best insecticidal potential against *H. pruni* aphids. Paliwal et al (2022) mentioned that a low concentration (5.24×10^1 CFU/ml) for this same bacteria species was sufficient to cause 50% mortality of several aphid species after 72 h of treatment.

Compared to the other potentially effective strains (P2, P4), *Bt* strains B22 and B23 showed however high levels of LC and LT against *H. pruni* aphids. Alamuratov et al. (2023) have found a *Bt* concentration of 2×10^8 CFU/ml that induced mortality range varying between 63.5 and 35.2% after 72 h of treatment. So, according to our finding, selected *Bt* soil-borne strains do not have the best insecticidal effects after 72 h of treatment against *H. pruni* as *Pseudomonas* and *Enterococci* strains have. *Bt* strains used as controls showed lifted LC and LT. This could be due to variations in the insecticidal genes availability, the aphids species utilized, or the applied bioassay methodology and more precisely host specificity (Meddas et al. 2020).

Conclusion

The study involves isolating and characterizing bacteria from cultivated citrus soil, followed by an evaluation of their pathogenic potential against the mealy plum aphid, *Hyalopterus pruni*. The selected bacterial isolates showed high mortality in *H. pruni* populations after 72 h of treatment revealed the presence of three different genera of entomopathogenic bacteria. This ensures that citrus soils contain a high diversity of entomopathogenic bacteria. Also, *Enterococcus gallinarum* species of *Enterococci* genus was available in cultivated soil ecology and generates the best mortality rate compared to other genera, highlighting the greatest importance of this genus in aphid control applications. Moreover, *Bacillus* and *Pseudomonas* genera had a pathogenic effect. The pathogenic effect of a new species, *Lysinibacillus fusiformis*, of the *Bacillus* genus, was already known for its insecticidal effect against another order of insects (Lepidoptera). Ultimately, this work will provide an opportunity to develop and study the active molecules and toxins of *B. thuringiensis* and *Pseudomonas* sp., which their entomopathogenic effects have already proved and to exploit the impact of the new species, *E. gallinarum* and *L. fusiformis*, as microbial bioinsecticides against aphids and various other insect pests.

Abbreviations

<	Less than
>	Greater than
±	Plus or minus
°	Degree
°C	Degree centigrade
16S rRNA	Small subunit ribosomal RNA molecules
ANOVA	Analysis of variance
BLAST	Basic local alignment search tool
Bt	*Bacillus thuringiensis*
C	Concentration
CFU	Colony-forming units
cm	Centimeter
df	Degree of freedom
DNA	Deoxyribonucleic acid
e.g.	Example
Etc	And the rest
FAO	Food and Agriculture Organization
h	Hours
H. pruni	*Hyalopterus pruni*
H_2S	Sulfure d'hydrogène
LC	Lethal concentrations
LSD	Least significant difference
LT	Lethal time
MEGA	Molecular evolutionary genetics analysis
ml	Milliliter
NA	Nutrient agar
NCBI	National Center for Biotechnology Information
nm	Nanometer
PCR	Polymerase chain reaction
Pf	*Pseudomonas fluorescens*
PGPR	Plant growth promotion *Rhizobacteria*
RNA	Ribonucleic acid
rpm	Revolutions per minute
spp	Species
UV	Ultraviolet

Acknowledgements

Not applicable.

Author contributions

MH conducted the experiments and wrote the paper. The results were evaluated and processed by MH and LAB. The manuscript was reviewed and modified by LAB. For final submission, the work was read and approved by all authors.

Funding
Not applicable.

Availability of data and materials
The data generated and/or analyzed during the current study are available from the corresponding author.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Received: 22 April 2024 Accepted: 17 August 2024
Published online: 24 August 2024

References

Abbot WS (1925) A method of computing the effectiveness of an insecticide. J Econ Entomol 18:265–267. https://doi.org/10.1093/jee/18.2.265a
Agbe H, Sarkar DK, Chen XG (2020) Tunable superhydrophobic aluminum surfaces with anti-biofouling and antibacterial properties. Coatings 10(10):1–10. https://doi.org/10.3390/coatings10100982
Alamuratov R, Abdimurot U, Pulatov ZA (2023) Evaluation of efficacy of *Bacillus thuringiensis* strains against plum aphis-*Hyalopterus pruni* (Geoffr) (Hemiptera: Aphididae) in laboratory and field conditions. Sci Innov Int Sci J 2(6):155–160. https://doi.org/10.5281/zenodo.8092393
Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215(3):403–410. https://doi.org/10.1016/S0022-2836(05)80360-2
Astuti DT, Pujiastuti Y, Suparman SHK, Damiri N, Nugraha S, Sembiring ER, Mulawarman (2018) Exploration of *Bacillus thuringiensis* Berl. from soil and screening test its toxicity on insects of Lepidoptera order. IOP Conf Ser Earth Environ Sci 102:1–7. https://doi.org/10.1088/1755-1315/102/1/012063
Bikream N, Shrestha J, Bhattarai S (2018) Indentifiation of *Pseudomonas fluorescens* using different biochemical tests. Int J Appl Biol 2(2):27–32. https://doi.org/10.20956/ijab.v2i2.5260
Blackman RL, Eastop VF (2000) Aphids on the world's crops: an identification and information guide, 2nd edn. Wiley, Chichester
Buchanan RE, Gibbons NE (1974) Bergey's manual of determinative bacteriology, 8th edn. Williams and Wilkins, Baltimore
Cox CR, Gilmore MS (2007) Native microbial colonization of *Drosophila melanogaster* and its use as a model of *Enterococcus faecalis* pathogenesis. Infect Immun 75(4):1565–1576. https://doi.org/10.1128/IAI.01496-06
Edwards U, Rogall T, Blöcker H, Emde M, Böttger EC (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res 17(19):7843–7853. https://doi.org/10.1093/nar/17.19.7843
Falcone N, She Z, Chen C, Dong B, Yi D, Kraatz HB (2017) Demonstration of a tailorable and PCR-free detection of Enterococcus DNA isolated from soil samples. Anal Methods 9(10):1–7. https://doi.org/10.1039/c6ay03309a
Finney DJ (1971) Probit analysis, 3rd edn. Cambridge University Press, Cambridge
Hashimoto Y (2002) Study of the bacteria pathogenic for aphids: isolation of bacteria and identification of insecticidal compound. (Hokkaido Prefect Agric Exp Stn), Hokkaido, Japan
Hazir A, Yurtmen M, Fidan H (2021) Potential aphid (Hemiptera: Aphididae) vectors of plum-pox virus (Virus: Potyviridae) and status of sharka disease in stone fruit orchards in the east Mediterranean Region of Turkey. J Agric Sci 7(4):484–492. https://doi.org/10.15832/ankutbd.722483
Karungu S, Huang D, Atoni E, Waruhiu C, Agwanda B, Hu X, Yuan Z (2018) Isolation, identification and evaluation of mosquito entomopathogenic *Bacillus* species and related genera from randomly selected sites in Kenya. Afr J Microbiol Res 12(12):290–299. https://doi.org/10.5897/AJMR2018.8824
Kupferschmied P, Maurhofer M, Keel C (2013) Promise for plant pest control: root-associated pseudomonads with insecticidal activities. Front Plant Sci 4(287):1–17. https://doi.org/10.3389/fpls.2013.00287
Lozier JD, Roderick GK, Mills NJ (2009) Tracing the invasion history of mealy plum aphid, *Hyalopterus pruni* (Hemiptera: Aphididae), in North America: a population genetics approach. Biol Invasions 11(2):299–314. https://doi.org/10.1007/s10530-008-9248-8
Manjula TR, Kannan GS, Sivasubramanian P (2018) Field efficacy of *Pseudomonas fluorescens* against the cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae) in *Bt* and Non Bt Cotton. Int J Curr Microbiol App Sci Special 1(6):11–24
Mason KL, Stepien TA, Blum JE, Holt JF, Labbe NH, Rush JS, Raffa KF, Handelsmana J (2011) From commensal to pathogen: translocation of *Enterococcus faecalis* from the Midgut to the Hemocoel of *Manduca sexta*. Mbio 2(3):1–7. https://doi.org/10.1128/mbio.00065-11
Meddas S, Oulebsir-Mohand kaci H, Hadjouti R, Reghmit N, Houas Y, Amirat D, Nait-Bouda L, Doumandji-Mitiche B (2020) Caractérisation des *Bacillus* spp. Isolées du sol d'olivier et étude de leurs potentialités de biocontrole contre la mouche de l'olive Dacus oleae (Gmel) au nord de l'Algérie. Agrobiologia. 10(1):1872–1882
Mihir R, Shaswat S, Sumita A, Bidyut Kumar S (2017) Isolation and characterization of *Bacillus thuringiensis* strains native to Assam soil of north east India. 3 Biotech. 7:303. https://doi.org/10.1007/s13205-017-0935-y
O'Sullivan DB, O'Gara F (1992) Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. Microbiol Rev 56(4):662–676. https://doi.org/10.1128/mr.56.4.662-676.1992
Oulebsir-Mohand kaci H, Doumandji-Mitiche B, Behidj N (2015a) Evaluation de la toxicité de *Pseudomonas fluorescens* et *Bacillus sphaericus* à l'égard du criquet migrateur *Locusta migratoria* (Linné, 1758). Revue Nat Technol 12(1):100–109
Oulebsir-Mohand Kaci H, Khemili-Talbi S, Benzina F, Halouane F (2015b) Isolation and of entomopathogenic bacteria from algerian desert soil and their effects against the migratory locust, *Locusta migratoria* (Linnaeus, 1758) (Orthoptera: Acridiidae). Egypt J Biol Pest Control 25(3):739–746
Paliwal D, Hamilton AJ, Barrett GA, Alberti F, Van Emden H, Monteil CL, Mauchline TH, Nauen R, Wagstaff C, Bass C, Jackson RW (2022) Identification of novel aphid-killing bacteria to protect plants. Microb Biotechnol 15(4):1203–1220. https://doi.org/10.1111/1751-7915.13902
Palma L, Munoz D, Berry C, Murillo J, Caballero P (2014) Draft genome sequences of two *Bacillus thuringiensis* strains and characterization of a putative 41.9-kDa insecticidal toxin. Toxins 6(5):1490–1504. https://doi.org/10.3390/toxins6051490
Porcar M, Grenier AM, Federici B, Rahbe Y (2009) Effects of *Bacillus thuringiensis* α-endotoxins on the pea aphid (*Acyrthosiphon pisum*). Appl Environ Microbiol 75(14):4897–4900. https://doi.org/10.1128/AEM.00686-09
Rousselin A, Bevacqua D, Sauge MH, Lescourret F, Mody K, Jordan MO (2017) Harnessing the aphid life cycle to reduce insecticide reliance in apple and peach orchards. A review. Agron Sustain Dev 37(38):1–13. https://doi.org/10.1007/s13593-017-0444-8
Tamura K, Stecher G, Kumar S (2021) MEGA11: molecular evolutionary genetics analysis version 11. Mol Biol Evol 38(7):3022–3027. https://doi.org/10.1093/molbev/msab120
Yamagiwa M, Ogawa R, Yasuda K, Natsuyama H, Sen K, Sakai H (2002) Active form of dipteran-specific incticidal protein Cry 11A produced by *Bacillus thuingiensis* subsp. Israelensis Biosci Biotechnol Biochem 66(3):516–522. https://doi.org/10.1271/bbb.66.516

Publisher's Note