Eur J Plant Pathol (2020) 156:867–883
https://doi.org/10.1007/s10658-020-01938-6

Molecular characterization of Algerian *Erwinia amylovora* strains by VNTR analysis and biocontrol efficacy of *Bacillus* spp. and *Pseudomonas brassicacearum* antagonists

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Accepted: 16 January 2020 / Published online: 25 January 2020
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Abstract Samples of pear shoots, blossoms and leaves showing typical fire blight symptoms were collected from diseased pear trees during spring and summer over a period of 3 years from orchards situated in the Mitidja region, a main pear producing area in Northern Algeria. From all collected plant samples, thirty-seven putative *Erwinia amylovora* strains were isolated and identified by biochemical, serological and molecular tests. Molecular typing of these strains was performed using six variable number of tandem repeats sequences (VNTRs). Minimal spanning trees showed that four different haplotypes were present within the bacterial population analyzed. The efficacy of twenty potential bacterial antagonists was evaluated in vitro against two *E. amylovora* pathogenic strains. Four antagonists (*Bacillus amyloliquefaciens, Bacillus methylotrophicus* and two *Pseudomonas brassicaceaurum*) were selected to perform a biocontrol assay on immature pear fruits. Results showed that the two *Pseudomonas* strains were the most effective for preventive treatment on pear fruits, leading to a necrosis reduction of up to 90%. No curative effect was observed. The four selected antagonists were characterized for features linked to biocontrol activity, such as biofilm, siderophores and biosurfactant-production, motility, and effect of environmental conditions on bacterial growth.

Keywords Biocontrol · *Erwinia amylovora* · Fire blight · MLVA- VNTR

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10658-020-01938-6) contains supplementary material, which is available to authorized users.

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Introduction

Erwinia amylovora, the pathogen responsible for fire blight disease of many *Rosaceae* plants, is ranked among the most destructive bacterial plant pathogens (Mansfield et al. 2012). This quarantine pest, assigned to the A2 list of the European and Mediterranean Plant Protection Organisation (EPPO), belongs to enterobacteria which includes Gram-negative, mobile, and facultative aero-anaerobic bacteria (EPPO 2013).

E. amylovora was the first bacterium described as the causative agent of a plant disease (Burrill 1883).

E. amylovora enters hosts mainly through the nectarthodes of flowers and establishes systemic infections (Norelli et al. 2003). The pathogen moves through the intercellular spaces of parenchyma, then along the xylem vessels, and incites necrosis (Dellagi et al. 1998; Piqué et al. 2015). The symptoms include dieback and blight of inflorescences, shoots, leaves, fruits, trunks as well as production of bacterial exudate (van der Zwet and Beer 1995). Symptoms resembling those of fire blight were observed in several pear orchards in different provinces in north Algeria and the presence of *E. amylovora* in these orchards was confirmed in 2011 by the national official control services (The Agriculture Ministry and National Institute of Plant Protection INPV). Phytosanitary measures have been introduced to control the disease in these areas (Laala et al. 2012).

Treatment of plants with antibiotics could be employed for the control of fire blight, but their use in plant protection within the European Union has only been permitted on an emergency basis, under tightly restricted conditions (Fried et al. 2013). In Algeria, antibiotics are also prohibited. Their use is linked to an increased risk for selection of resistant strains of *E. amylovora* and other bacteria inhabiting orchards and the possible negative impact on the environment. Because of these limitations, the research interest in finding new solutions for fire blight control has increased, including the use of bacterial biocontrol agents (Mikiciński et al. 2016).

Several biopesticides using bacteria are available, such as *Bacillus subtilis* QST 713 (Aldwinckle et al. 2002), *B. subtilis* BD170 (Broggini-Schärer et al. 2005), *Pseudomonas fluorescens* A506 (Wilson and Lindow 1993) and *Lactobacillus plantarum*, however; they have a low efficacy against *E. amylovora* (Roselló et al. 2013). The main mechanism of action of these strains to control fire blight is based on competition through nutrient depletion and site colonization (Cabrefiga et al. 2007). Research is also being carried out with the aim of improving the efficacy of these biopesticides as well as the selection of new effective strains with different mechanisms of action (Pusey and Wend 2012).

In this context, the aim of this work was to study the molecular diversity of Algerian *E. amylovora* population in pear orchards and identify possible biocontrol agents against them. To this end, we isolated and identified *E. amylovora* strains from different pear orchards of Northern Algeria. Analysis of genomic sequences of *E. amylovora* isolates was performed using VNTR markers, which have high discriminatory power that allows distinguishing different strains within a population (Bühlmann et al. 2014). Multiple-Locus Variable number tandem repeat Analysis (MLVA) using VNTRs have been already used successfully to perform molecular typing of *E. amylovora* (Bühlmann et al. 2014) and several other bacterial species such as *Staphylococcus aureus* (Schouls et al. 2009), *Acinetobacter baumannii* (Pourcel et al. 2011), *Mycobacterium tuberculosis* (Aandahl et al. 2012) and *Pseudomonas aeruginosa* (Lashgarian et al. 2018). For these species, the VNTR technique has proved to be highly valuable in ecological, epidemiological and evolutionary studies. Knowledge of the *E. amylovora* population structure contributes to our understanding on how fire blight spreads over a geographical region.

Fire blight is difficult to control, as *E. amylovora* is able to rapidly spread in the plant and effective control methods are still lacking. Various mechanisms were proposed to explain the inhibitory effect of different bacterial antagonists against *E. amylovora*, including the production of toxic secondary metabolites and competition for nutrients and space (Cabrefiga et al. 2007). Moreover, *Pseudomonas fluorescens* and *Pantoea agglomerans* strains showing high potential as biological control agents against fire blight, were shown to inhibit growth of *E. amylovora* through a mechanism related to the production of antibiotics (Anderson et al. 2004; Temple et al. 2004). With the aim to select effective biocontrol agents of fire blight, native Algerian strains that were isolated as endophytes of different spontaneous plants (Krimi et al. 2016) were tested in vitro as well as in vivo on pear fruits in this study to evaluate their efficacy against *E. amylovora*.

Material and methods

Field surveys and collection of samples from diseased plants

Surveys were carried out during spring and summer from 2014 to 2017 in orchards of *Pyrus communis* cv. Santa Maria located in an area of pear fruit production in the Mitidja zone (Northern Algeria) which was officially declared affected by fire blight in 2011 by the Ministry of Agriculture and National Institute of Plant Protection.

In these orchards, about 70% of trees, 15 to 20 years old, showed various typical fire blight symptoms, such as necrosis of shoots, flowers, leaf petioles and fruits, oozed shoots, and mummified fruits.

Oozing shoots, blossoms and leaves were collected from 14 orchards (farms) located in seven different provinces: Sidi Yahia, Oued El Alleug, Bensalah, L'Arbaa, Birtouta, Tessala El Merdja and Attatba (nine farms at Blida, three at Algiers and two at Tipaza) (Fig. 1). Plant material was placed in sterile plastic bags, labeled, transported to the laboratory and stored at 4 °C until isolation of bacteria that was performed during the same week of sampling.

Isolation and purification of *E. amylovora* strains

The collected samples of infected shoots and twigs were examined for the presence of exudates that developed during storage on the plant surface allowing easy isolation. Part of these exudates were suspended in 2 ml of sterile distilled water (SDW) and streaked on Levan and King B media (Schaad et al. 1988). Plates were kept for 1 week at 27 °C and observed daily for bacterial growth. Single colonies of putative *E. amylovora* isolates showing mucoid, circular, white and *domed* morphology (Yaich et al. 2011) were selected and transferred to Levan medium (Schaad et al. 1988).The purification step was repeated three to four times in order to obtain pure cultures (Jones and Geider 2001). Pure isolates were maintained at 4 °C on Levan agar medium slants for further testing and in 30% glycerol solution at −80 °C for long term storage.

Biochemical characterization of bacterial isolates

Gram staining (Suslow et al. 1982) and a series of assays to determine biochemical features of the bacterial isolates, i.e. gelatin liquefaction, fluorescent pigment production on King's B medium, reducing substances from sucrose, utilization of citrate, oxidation/fermentation of glucose (O/F), catalase production, oxidase production, growth at 39 °C and indole prodution were carried out using the EPPO identification protocol (EPPO 2013). The phenotypic properties were determined according to the methods of Jones and Geider (2001).

Serological test

The isolates were tested by the commercially available rapid lateral flow immunosorbent assay named 'AgriStrip test' (BIOREBA). For this assay, strips were inserted into tubes containing bacterial suspensions at the recommended concentration (10^6 cfu ml^{-1}). Strains

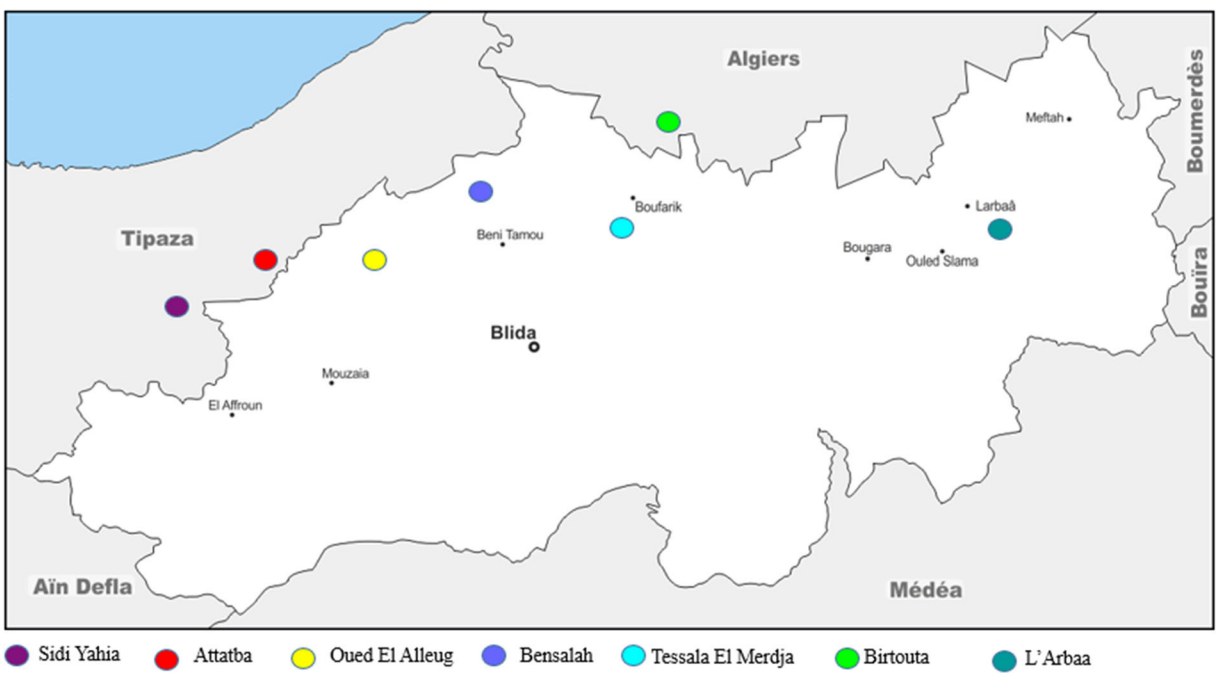


Fig. 1 Geographical location of pear orchards in North Algeria

producing a red band on the strip were considered positive and identified as *E. amylovora* species.

Tobacco hypersensitivity test

The tobacco hypersensitivity (HR) test was performed on young leaves of *Nicotiana tabacum* cv. *xanthi*. The test was carried out using 24 h old bacterial cultures grown on Levan medium. For each isolate, a bacterial suspension (10^8 cfu ml^{-1}) was infiltrated using a needle into parenchymatic tissues of tobacco leaves (Vanneste et al. 1990). Tobacco plants were kept at room temperature. A complete collapse of the infiltrated tissue within 24–48 h was considered a positive response.

Pathogenicity test on immature pear fruits

Immature fruits collected from symptomless pear trees were inoculated following the protocol described by Zhao et al. (2005) with some modifications. Pear fruits were surface sterilized using 75% ethanol, wounded with a sterile scalpel (5 wounds/fruit) and inoculated with 5 μl of suspension (10^6 cfu ml^{-1}) of the putative *E. amylovora* 37 pathogenic strains. As negative control, pear fruits were inoculated with 5 μl of SDW. Fruits were placed in plastic boxes containing sterile Whatman paper on the bottom, daily sprayed with SDW and incubated at 25–26 °C. The experiment was repeated four times. Symptoms were recorded over a period of 5 to 7 days. Production of bacterial exudates and/or development of necrotic areas around or underneath the inoculation point was recorded as a positive response (Billing et al. 1960).

Duplex PCR

Thirty-seven strains with *Erwinia amylovora* biochemical features, identified as HR positive, and pathogenic on pear fruits, were tested by duplex PCR (Hannou et al. 2013). Forty-eight hour-old bacterial colonies were picked-up from Levan agar medium, transferred into Levan broth and incubated at 28 °C overnight on a rotary shaker. The bacterial cultures were centrifuged for 10 min at 14000 rpm and the pelleted bacterial cells were resuspended in SDW. Cells suspensions in SDW were lysed by heating for 15 min at 95 °C, quickly cooled on ice and centrifuged for 5 min at 7000×g. The obtained supernatants were used to perform amplification by duplex PCR. The duplex PCR protocol was carried out using two pairs of primers: a) AJ75 (5-CGTATTCACGGCTTCGCAGAT) and AJ76 (5-ACCCGCCAGGATAGTCGCATA), a pair that amplifies an 844-bp fragment of the 29-kb plasmid pEA29 of *E. amylovora* (McManus and Jones 1995), and b) AMSbL (5-GCTACCAGCAGGGTGAG-3) and AMSbR (5- TCATCACGATGGTGTAG - 3), a pair that amplifies a 1.6-Kb fragment of the chromosomic *ams*-region of *E. amylovora* (Bereswill et al. 1995). Sterile water and genomic DNA extracted from the French *E. amylovora* strain CFBP 1430 were used as negative and positive amplification controls, respectively. PCR amplification was performed as described in Hannou et al. (2013).

The PCR program included an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 45 s at 94 °C for denaturation, 45 s at 52 °C for annealing, and 1 min and 45 s at 72 °C for extension; with a final extension at 72 °C for 10 min (Hannou et al. 2013). Amplified fragments were loaded on 1,5% agarose gel to be separated by electrophoresis. VNTR analyses.

Twenty-five selected *E. amylovora* strains isolated in this study were analyzed using six VNTRs primers described in Bühlmann et al. (2014) applying a Multiple-Locus VNTR Analysis (MLVA). Bacterial DNA templates were obtained using a direct lysis method, as previously described in Bühlmann et al. (2014), of overnight cultures grown on King's B medium at 28 °C. DNA-containing supernatants (1 μl) were used as the template in the PCR reaction mixtures containing 12.8 μl ultrapure water, 4 μl GoTaq Flexi 5X buffer (Promega, Fitchburg, WI, USA), 1.2 μl $MgCl_2$ 25 mM, 0.5 μl dNTP 10 mM, 0.25 μl each primer 10 μM, 0.05 μl (0.25 u) GoTaq Flexi DNA polymerase (Promega) in a final volume of 20.05 μl. The amplification was performed in a GeneAmp PCR system model 9700 (Applied Biosystems, Courtaboeuf, France) following the steps: 95 °C for 5 min, 32 cycles denaturation at 95 °C for 30 s, annealing for 30 s at a temperature depending on the VNTR primer, elongation at 72 °C for 30 s, and a final elongation step at 72 °C for 10 min.

The VNTR primers described by Bühlmann et al. (2014) were used at the following annealing temperatures: 60 °C for VNTR A, B and H, 50 °C for VNTR C, 52 °C for VNTR D and 56 °C for VNTR F (Online Resource 1).

The VNTR primers were labelled with one of the following fluorescent dyes: Atto550 for VNTR A, HEX for VNTR B and D, FAM for VNTR C and H and

Atto565 for VNTR F (Eurofins Genomics, France) at the 5′ end. Up to four differently labelled PCR products were diluted 1/30 and pooled to prepare the DNA samples. Then, 2.4 μL aliquots of the DNA samples were mixed with 9.35 μL HiDi formamide (SigmaAldrich, Saint Quentin, France) and 0.15 μL GeneScan 500 Liz internal lane size standard (Applied Biosystems) and denatured at 95 °C for 5 min prior to capillary electrophoresis analysis with an ABI PRISM 3130 analyzer (Applied Biosystems).

Peak data were analyzed using Peakscanner™ Software V1.0 (Life Technologies) to calculate the repeat number for each VNTR locus on the basis of fragment length and fluorescent label (Bühlmann et al. 2014). Simpson's index of diversity (Hunter and Gaston 1988) was calculated using Bionumerics Software V7.6.3 (Applied Maths). Minimum-spanning-trees (MST) were generated using Bionumerics Software V7.6.3 using the standard categorical coefficient and the maximum number of single-locus variants as a priority rule.

The categorical coefficient discriminates between different repeat numbers, however it does not quantify the difference. Equal weight was assigned to each of the retained VNTR loci.

Selection of effective antagonistic strains against *E. amylovora* and their characterization

Potential antagonistic *Pseudomonas* and *Bacillus* species evaluated in this study belong to the collection of the phytobacteriology laboratory of Blida University, Algeria. All strains were endophytes isolated from various spontaneous weeds (*Calendula arvensis*, *Euphorbia helioscopia*, *Euphorbia peplus*, *Plantago lanceolata* and *Urtica dioica)* showing antagonistic activity against different phytopathogenic bacteria (Krimi et al. 2016).

Twenty *Pseudomonas* and *Bacillus* strains from the previously mentioned bacterial collection (Blida University, Algeria) were screened for in vitro antagonistic activity against pathogenic *E. amylovora* strains by the dual culture assay, i.e. a plate-spraying technique (Stonier 1960) as modified by Cooksey and Moore (1980). A volume of 100 μl of suspension (10^7cfu ml^{-1}) of each potential antagonistic strain was spotted at the center of YPGA medium (Hildebrand et al. 1988). The plates were incubated for 48 h at room temperature (RT). After incubation, the plates were sprayed with cell suspensions of two *E. amylovora* strains E1 and E5, isolated and characterized in this study. Inoculum of pathogens was prepared from late log-phase cultures grown in YPG broth on a rotary shaker at 25 ± 2 °C and diluted to about 1×10^8 cells ml^{-1} in SDW. The plates were let again at RT for 24–48 h. Generally in this kind of assay, the antibacterial agent from the antagonistic strain diffuses into the agar and inhibits growth of the test microorganism. Antagonism is detected as the presence of a zone of growth inhibition of the test microorganism around the growth of the spotted antagonistic strain (Cabrefiga et al. 2007), and then the diameter of growth inhibition zones are measured using a graduated precision rule (mm) (Lindow 1988).

The test was repeated three times for each of the twenty antagonistic strain tested. Antagonistic strains able to inhibit both pathogenic strains E1 and E5 were tested for biocontrol of fire blight on immature pear fruits. For the negative control, the plates were sprayed with cell suspensions of *E. amylovora* strains E1 and E5 without any antagonistic strain.

Bacteria motility, siderophore, biofilm and biosurfactant production

Motility helps bacteria to reach the most favorable environment and to successfully compete with other microorganisms (Harshey 2003) and then may represent a remarkable competitive advantage for putative biocontrol agents. Tests to evaluate swimming and swarming motility of the four selected antagonistic strains (CR2, EHF5, PF3, and PS1) were performed according to the protocol of Déziel et al. (2001). A volume of 2 μl of each bacterial suspension (10^8 cfu ml^{-1}) was spotted in the center of plates containing tryptone swim and swarm agar media, respectively (Déziel et al. 2001). For each strain, three plates per medium were seeded. After 24 and 48 h of incubation at 25 ± 2 °C, two radius measurements per colony were made. *Pseudomonas chlororaphis* M71 and *E. coli* DH5α were used as positive and negative controls, respectively. The experiment was carried out twice.

To evaluate the ability to produce siderophores, a volume of 200 μl of overnight cultures of the four selected antagonistic bacterial strains (10^8 cfu ml^{-1}) was added to 50 ml of iron-free succinate and KB broth media respectively (Sasirekha and Srividya 2016). The flasks were incubated at room temperature for 72 h on a

horizontal shaker. After centrifugation for 10 min at 10,000 rpm, the absorbance of supernatants were measured at 400 nm using spectrophotometer (Meyer and Abdallah 1978). The experiment was performed twice. The medium without bacteria was used as negative control. Bacterial growth and siderophore production in KB medium were compared to those in succinate medium containing ammonium sulphate (Tailor and Joshi 2012). The negative control was used as reference to quantify the difference between absorbance in KB medium and succinate medium. To characterize the nature of siderophores, 5 ml of 2% aqueous solution of FeCl3 were added to each supernatant and OD was determined at 490–515 nm for catecholate (Jalal and Vander Helm 1990) and at 420 to 450 nm for hydroxamate (Neilands 1981). Non-inoculated media were used as reference.

The ability of the antagonistic strains to produce biofilm was tested by adding 15 μL of an overnight culture of strains EHF5, PF3, CR2 and PS1 (10^8 cfu ml^{-1}) to 0.5 ml of Luria-Bertani (LB) broth. Suspensions were incubated without agitation at 28 °C and biofilm was quantified after 3, 5 and 20 h. LB medium without bacteria was used as negative control. The experiment was repeated 3 times. Cultures were stained with 1 ml of 0.4% (*w*/*v*) crystal violet (CV) for 20 min at RT and then rinsed thoroughly and vigorously with distilled water to remove unattached cells and residual dye. One volume of ethanol (70%) was used to solubilize the dye. The absorbance of the solubilized dye was determined by a spectrophotometer at 570 nm wavelength (O'Toole and Kolter 1998; Stepanovic et al. 2000).

The ability to produce biosurfactant compounds was determined by the drop collapsing test described by Bunster et al. (1989) with some modifications. Strains were grown in KB broth amended with 1% of yeast extract for 48 h at 28 °C on a rotary shaker. Bacterial cultures were then centrifuged and the supernatant was filter-sterilized. An amount of 10 μl of each supernatant was then placed on a parafilm strip and mixed with 1 μl of 1% bromophenol blue solution. *P. chlororaphis* M71 strain and sterile KB broth were used as positive and negative controls respectively. The strip was covered with a Petri dish and kept in humid and sterile conditions. The diameter, size, and shape of drops were observed after 24 and 36 h. Flat drops with a diameter size wider than negative control indicates biosurfactant production by the tested strains.

Effect of environmental factors on bacterial growth

The effect of temperature, pH and NaCl concentration was determined by growing the antagonists on LB agar plates. Bacteria were streaked on agar surface and incubated at 4 °C, 30 °C, 35 °C, 42 °C and 45 °C for 24 to 48 h and then checked for growth (Rhodes 1958). Bacterial growth at 27 °C was used as reference temperature. To determine the effect of pH, bacteria were streaked on LB agar plates with pH 4, 6, 8 and 10. PH 7 was considered as the reference value. To test salt tolerance, the following amounts of NaCl were added to LB agar: 0.3 M (17.58 g l^{-1}), 0.5 M (29.3 g l^{-1}), 0.8 M (46.88 g l^{-1}) and 1 M (58.6 g l^{-1}) NaCl. Three replicates were used per sample. LB medium supplemented with 0.17 M (10 g l^{-1}) of NaCl (Rabhi 2011) was used as reference.

To study the effect of osmotic stress on antagonist growth, nutrient broth (NB) supplemented with different concentrations of PEG 8000 (20%, 40% and 60%) was used. Tubes containing 5 ml of supplemented NB were inoculated with 100 μl of each bacterial suspension and incubated at RT on a horizontal shaker for 72 h. Three replications per sample were maintained. Non-supplemented NB broth was used as negative control. Strain growth was determined by measuring the O.D. at 600 nm with a spectrophotometer (Manjunatha et al. 2017).

Fire blight control by *Bacillus* and *Pseudomonas* strains

Immature pear fruits cv. Santa Maria were used to determine the effectiveness of the bacterial antagonists in controlling locally pathogenic *E. amylovora* strains. Fruits were washed under running tap water, superficially disinfected with ethanol and then rinsed three times in SDW. Five wounds of 10 mm long and 3 mm deep were made on each fruit by using a sterile syringe needle. The fruits for each antagonist/pathogen combination were inoculated with 50 μL of 10^8 cfu ml^{-1} suspension of each antagonistic strain and 50 μl of 10^7 cfu ml^{-1} of the suspension of the pathogenic strains. Pear fruits wounded and treated with SDW were used as negative control. Treatments were arranged in a completely randomized design with two replications per treatment, five pear fruits per replicate and five wounds per fruit. Disease incidence was assessed for each repetition.

The biocontrol activity was tested by treating the wounds with the antagonists 24 h before or 24 h after inoculation with two strains of *E. amylovora* (E1, E5), to evaluate their preventive and curative activity, respectively. After inoculation, pear fruits were placed in sterile containers at RT. One week after inoculation the presence or absence of the necrotic lesions and exudate production were recorded. Disease severity was evaluated considering the size of fruit necrosis and production of bacterial exudates according to the following disease index: 0: no necrosis, 1: necrosis formation around the wound without ooze production, 2: necrosis of less than 50% of fruit surface with ooze production, 3: necrosis of 50%–80% of fruit surface with ooze production, 4: 100% fruit necrosis and ooze production (Ohike et al. 2018).

Results

Isolation of *E. amylovora* strains and their characterization by biochemical, serological and pathogenicity assays

Thirty-seven bacterial strains isolated from the samples of pear shoots and twigs collected during the field survey, which formed *E. amylovora*-like colonies, were selected for further study. The colonies were mucoid, white, shiny, domed, with radial stripes and dense flocculent centers on Levan medium (Billing et al. 1961; Ashmawy 2010),. The isolates were Gram-negative, oxidase-negative, catalase-positive, citrate utilizers, gelatin degraders, non-fluorescent on King's B medium and sucrose reducers. Moreover, they did not grow at 39 °C (Online Resource 2). Serological test of the 37 isolates were positive for *E. amylovora*, and all isolates produced a positive HR reaction on tobacco leaves. Pathogenicity test on all isolates induced necrosis and brownish coloration of tissues surrounding the artificially inoculated wounds. Within two to three days after inoculation, symptoms spread to the whole fruit and ooze production was observed. SDW negative control did not cause any symptom on the pear fruits.

Molecular identification of *E. amylovora* isolates

There was amplification of the 844-bp fragment of pEA29 plasmid with AJ75/AJ76 primers (McManus and Jones 1995) in all 37 isolates, suggesting the ubiquitous presence of this plasmid in the tested strains. In addition, all strains (Online Resource 3) produced a clearly visible 1.6-kb band corresponding to the *ams* chromosomal region with the AMSbL/AMSbR primers (Bereswill et al. 1995).

A variable number of tandem repeat sequences (VNTR) assay was used successfully to genotype 25 selected *E. amylovora* strains. Only two loci (A and F) were polymorphic with repeats per locus ranging from five to seven for locus A and from six to seven for locus F (Table 1).

Minimal spanning tree analysis (MST) showed that four haplotypes were present within the 25 tested isolates that differed for one VNTR repeat number. The analysis did not originate distinct groups on the basis of year of isolation. (Fig. 2). One distinct haplotype was instead found only from one farm of Attatba province when the analysis was performed considering the geographical origin of strains (Fig. 3).

Compared to data published in Bühlmann et al. (2014), strains 40b, 44, 45, 46 shared the same haplotype with strain NIBZ-1058 isolated from *Pyrus* in 2007 in Slovenia. Other Algerian strains harboured new haplotypes when compared to Bühlmann's data.

Table 1 Results of MLVA analysis performed on twenty-five isolates of *E. amylovora*

loci	Number of different alleles	Number of repeats min	Number of repeats max	Diversity index (Simpson)
A	3	5	7	0.4432
B	1	3	3	0
C	1	7	7	0
D	1	9	9	0
F	2	6	7	0.8528
H	1	8	8	0

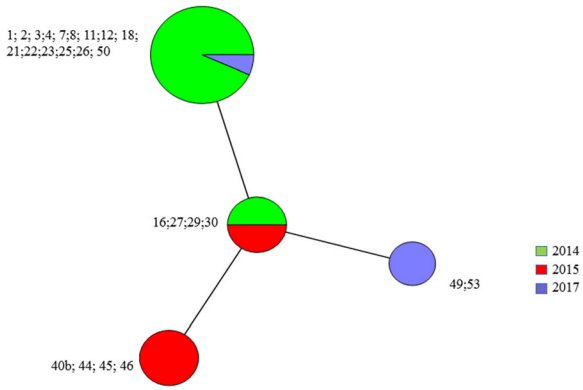


Fig. 2 Minimal spanning tree of Algerian *E. amylovora* isolates colored according to year of isolation. Each circle represents an MLVA type with a size corresponding to the number of strains that share an identical MLVA type. MLVA types connected by a thick solid line differ by at most one VNTR locus

In vitro selection of bacteria antagonistic to *E. amylovora* strains

All twenty potential antagonistic strains screened in this study by the dual culture assay inhibited the growth of both *E. amylovora* strains (E1 and E5) in vitro, with inhibition zones ranging from 18 mm for *Bacillus amyloliquefaciens* CR1 to 53 mm for *B. methylotrophicus* PF3 (Online Resource 4. Among this group of antagonistic strains, four were selected for further study, including a) two *Bacillus* strains (*B. amyloliquefaciens* EHF5 and *B. methylotrophicus* PF3) that produced wide inhibition zones as well as presenting traits such as rapid growth and colonization, aggressiveness towards other microorganisms and adaptation to environmental stress, making them good potential biocontrol agents, and b) two *Pseudomonas* strains (*P. brassicacearum* CR2 and PS1) that had been shown also in previous work (Krimi et al. 2016) to be interesting beneficial strains with potential biocontrol and growth promotion activities.

Characterization of antagonists

The swimming ability of EHF5, PF3 and CR2 strains at 24 h was comparable to that of the negative control (*E. coli*), while PS1 showed a swimming activity significantly higher than the negative control but lower than the positive control (*P. chlororaphis*) (Fig. 4a). After 48 h of incubation all four strains were able to swim, even though *Pseudomonas* strains were slower than *Bacillus* and *P. chlororaphis*. There were no significant differences in swarming ability among the four antagonistic strains at 24 h of incubation, however; after 48 h of incubation, *Bacillus* colonies covered the whole agar surface while *Pseudomonas* colonies did not show any swarming ability (Fig. 4b).

The four strains showed a different ability to produce siderophores on KB and succinate media. Strain EHF5 was the most active siderophore producer on KB medium without ferric chloride while strains CR2 and PS1 produced catecholate and hydroxamate siderophore type on KB medium amended with $FeCl_3$ respectively (Fig. 5). On succinate medium amended with $FeCl_3$, all strains produced fewer siderophores than on succinate without ferric chloride (Online Resource 5).

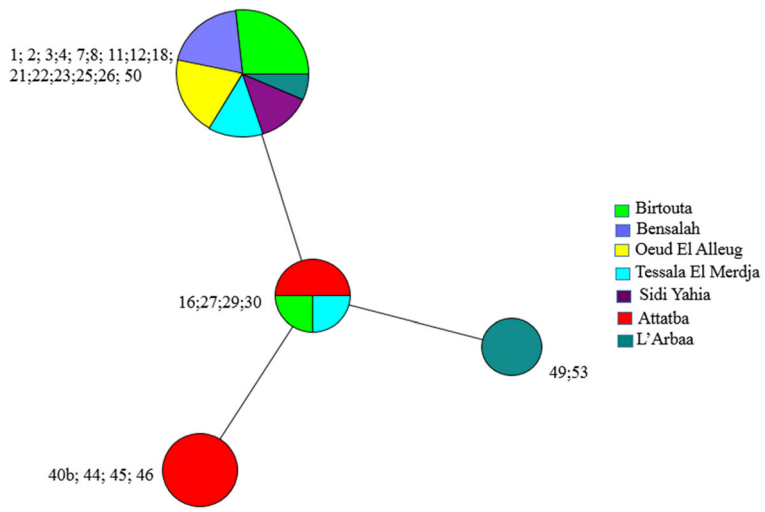
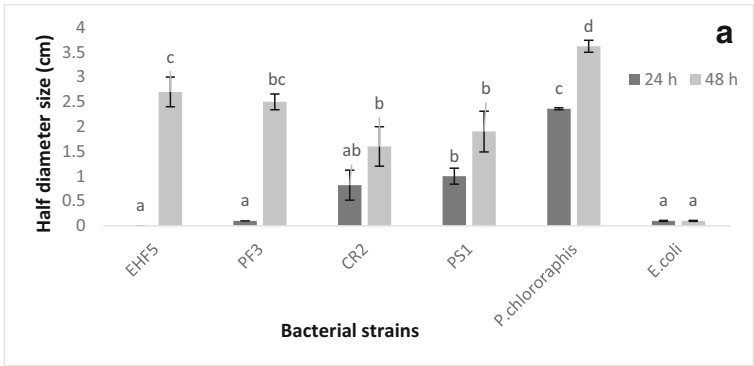


Fig. 3 Minimal spanning tree on Algerian *E. amylovora* isolates colored according to geographical origin. Each circle represents an MLVA type with a size corresponding to the number of strains that share an identical MLVA type. MLVA types connected by a thick solid line differ by at most one VNTR locus



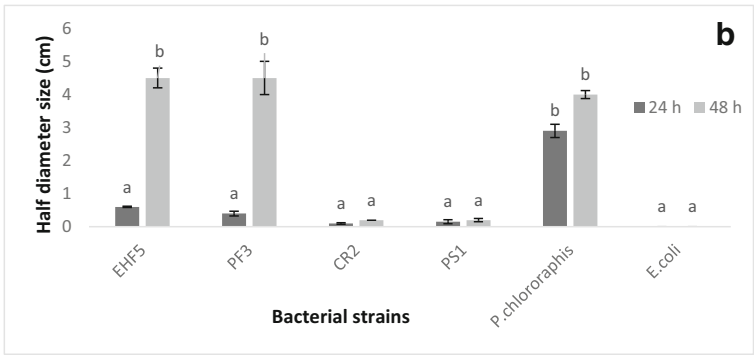


Fig. 4 Swimming (**a**) and swarming (**b**) activity of antagonists at 24 and 48 h of incubation. Data represent the average measure of the half diameter of the bacterial colonies. Two half diameters for each colony were measured on three repetitions. The experiment was carried out twice. The bars represent the standard error. Significant differences ($p \leq 0{,}05$) at 24 and 48 h were determined by Tukey test

The four antagonistic strains produced biofilm in vitro. No significant difference was observed among the strains after 3 and 5 h of incubation. EHF5 was the most active after 5 h of incubation and did not differ from PS1 strain at 20 h of incubation. After 20 h of incubation, strain CR2 was the most active biofilm producer. Conversely, PF3 showed a low ability to produce biofilm (Fig. 6).

The drop collapsing test showed that only the two *Bacillus* strains were able to produce biosurfactant compounds determining the spread of bacterial suspension over the solid surface (results not shown). The drops for these two strains appeared larger and flatter than the negative control (up to 7–8 mm diameter vs 4–5 mm of the negative control). On the contrary, both *Pseudomonas* strains did not produce biosurfactant compounds in this test since the diameter and shape of their drops were similar to those of the negative control.

Effect of environmental factors on the growth of antagonists

The antagonistic strains grew at pH values 4–8 (except for CR2 strain that did not grow at pH 4.0) and did not grow at pH 10. All strains grew at temperature ranging between 30 and 42 °C, while at the temperatures of 4 °C

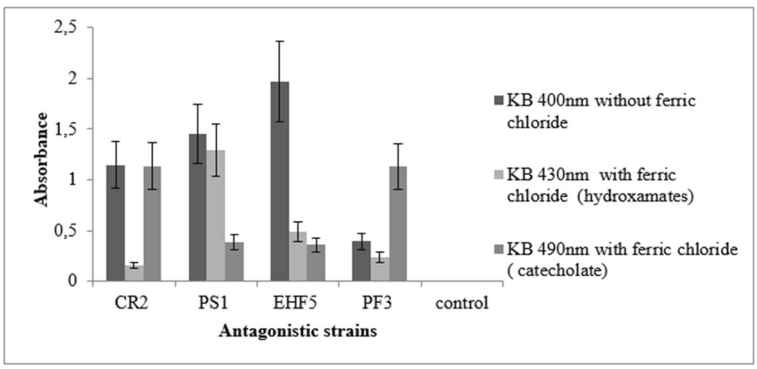


Fig. 5 Type of siderophores detected in cultural filtrates of the antagonistic strains grown on KB medium. Bacterial suspension at 10^8 cfu ml^{-1}. The columns represent the average of 2 repeats and the bars represent standard error

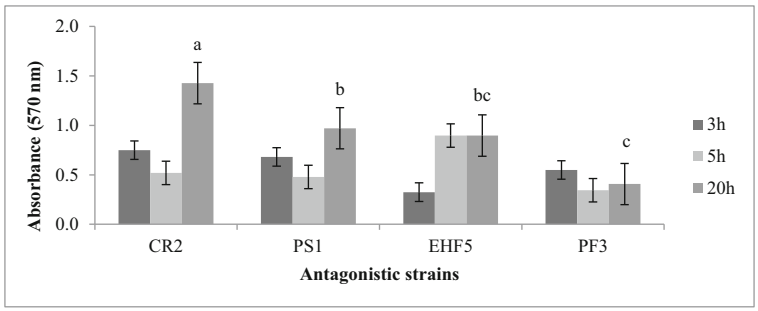


Fig. 6 Biofilm production by the four antagonistic strains at 3, 5 and 20 h post incubation. Bacterial suspension had a concentration of 10^8 cfu ml^{-1}. The columns represent the average of 3 repeats and the bars represent standard error. Significant differences ($p \leq 0{,}05$) were obtained by Tukey test only for results obtained after 20 h of incubation

and 45 °C, no growth was recorded. The two *Pseudomonas* strains where not inhibited by concentrations of NaCl ranging between 0,3 M and 1,0 M, while both *Bacillus* strains did not grow at 1.0 M NaCl (Table 2).

Strain tolerance to osmotic stress decreased with increasing concentrations of PEG. In the presence of 20% PEG, growth of the two pseudomonads was reduced more than 80% while growth of *Bacillus* spp. was reduced by approximately 70%, indicating that all strains were very sensitive to osmotic stress. At a concentration of 40% PEG, bacterial growth was further reduced, and at a concentration of 60% PEG, absorbance values of broth cultures were close to zero. No significant differences were found among the different strains (Fig. 7).

Fire blight control by Bacillus and Pseudomonas strains

The strains *Bacillus methylotrophicus* PF3, *B. amyloliquefaciens* EHF5, *P. brassicacearum* CR2 and PS1 were selected to test their ability to control fire blight. These strains were selected based on results for the in vitro evaluation of their antagonistic activity, their bio-control linked performances, and their resistance to abiotic factors.

A significant reduction of disease symptoms ($P \leq 0.05$) was recorded when *E. amylovora* was inoculated on pear fruits after wound protection with the *Bacillus* and *Pseudomonas* strains under study (Fig. 8). *Pseudomonas* strains CR2 and PS1 were the most effective against both *E. amylovora* strains used, with symptoms on pear fruits reduced to a necrotic area of few millimeters surrounding the wounds and without bacterial exudate (disease index 1) (Fig. 9). *Bacillus* strains also caused a significant reduction of disease symptoms, but the efficacy was lower than *Pseudomonads* (disease index 2). The effect of antagonists on the necrosis spread and ooze production was not significant when the *E. amylovora* strains (E1 and E5) were inoculated 24 h before the wound protection (data not shown).

Discussion

Fire blight has been reported since 2010 in Tipaza and Oued El Alleug provinces of Algeria on pear, apple and

Table 2 Effect of different pH, temperature and salt concentrations on the growth of antagonistic strains

Antagonistic Strain	pH					Temperature (°C)					NaCl concentration (M)			
	4	6	7	8	10	4	30	35	42	45	0,3	0,5	0,8	1,0
CR2 (*P. brassicacearum*)	–	+	+	+	–	–	+	+	+	–	+	+	+	+
PS1 (*P. brassicacearum*)	+	+	+	+	–	–	+	+	+	–	+	+	+	+
EHF5 (*B. amyloliquefaciens*)	+	+	+	+	–	–	+	+	+	–	+	+	+	–
PF3 (*B. methylotrophicus*)	+	+	+	+	–	–	+	+	+	–	+	+	+	–

+: bacterial growth occurred

-: no bacterial growth occurred

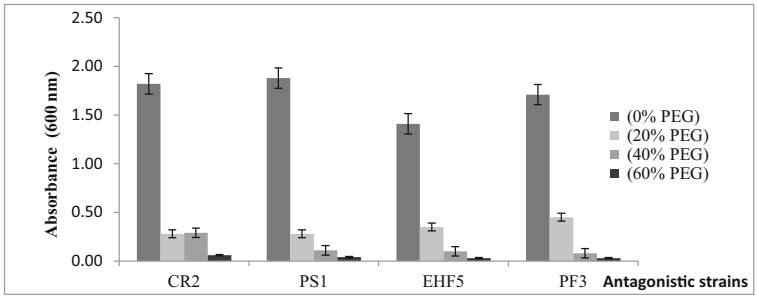


Fig. 7 Effect of osmotic stress on the growth of the antagonistic bacteria. Bacterial suspension had a concentration of 10^8 cfu ml^{-1}. The columns represent the average of 3 repeats and the bars represent standard error. No significant differences were obtained by Tukey test

quince trees. The disease spread quickly to other fruit growing regions in the north of Algeria such as Blida and Algiers. Infected areas in Blida province reached 400 Ha 1 year after the first *E. amylovora* record in this area (Laala et al. 2012).

Several plasmids (pEA29, pEI70, pEA34, pEA8.7, pCPP60, pEA72, pEL60, pEA2.8, and pEU30) have been identified in *E. amylovora* strains (McGhee and Jones 2000; Foster et al. 2004). However, pEA29 is the plasmid playing a significant quantitative role in pathogenicity (Laurent et al. 1989). Indeed, sequences of this plasmid are used for genetic differentiation of *E. amylovora* strains (Sebaihia et al. 2010).

In this study, 37 strains isolated from diseased pear trees in orchards located in different areas of North Algeria were identified as *E. amylovora* species by biochemical tests, PCR based methods, a serological identification test (Agristrip) and pathogenicity assay. AJ75 /AJ76 primers annealing to plasmid pEA29 and

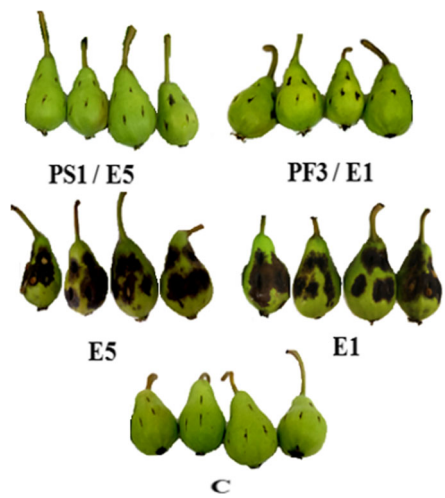


Fig. 8 Effect of selected antagonistic strains (PS1, PF3) in preventing fire blight on immature pear fruits artificially inoculated with *E. amylovora* (E1, E5 strains). Symptom evaluation was recorded 7 days after the pathogen inoculation. C: Negative Control

AMSbL /AMSbR primers annealing to *ams* chromosomal region were used for molecular identification of these strains. At the same time, the ubiquitous presence of the plasmid pEA29 in the Algerian strains tested was confirmed.

Genetic diversity of *E. amylovora* is low given the relatively recent global spread of this pathogen. Indeed, at genome level, 99.4% identity was found within the Rubus-infecting strains and 99.98% within the Spiraeoideae-infecting strains (Mann et al. 2013). The genetic diversity of *E. amylovora* has been studied in Morocco, Tunisia and Iran by VNTR analysis (Hannou et al. 2013; Dardouri et al. 2017; Refahi et al. 2017) using the same VNTRs as described in Hannou et al. (2013). Additional MLVA analysis (Alnaasan et al. 2017) was performed with the primers described by Bühlmann et al. (2014) to study strains from the Mediterranean area. No database is publicly available for MLVA on *Erwinia amylovora*. In this study, VNTR analysis differentiated four haplotypes within the Algerian *E. amylovora* strains identified. Previous analysis performed on strains isolated in North Africa showed that five different genotypes were present within the Tunisian collection of *E. amylovora* (Dardouri et al. 2017) while two distinct patterns were found among the Moroccan *E. amylovora* strains (Hannou et al. 2013).

Bühlmann et al. (2014) showed the presence of 227 haplotypes by studying a total of 833 *E. amylovora* strains of worldwide origin in a work based on MLVA analysis. One of these strains originated from Slovenia shared the same haplotype as four Algerian isolates of the present study. According to Alnaasan et al. (2017), Italian isolates of *E. amylovora* showed a high diversity which let hypothesize multiple events of introduction of the bacterium from several countries. Two of these isolates were closely related to strains from Algeria and Egypt (Alnaasan et al. 2017). This suggested that *E. amylovora* could have been introduced in Italy and

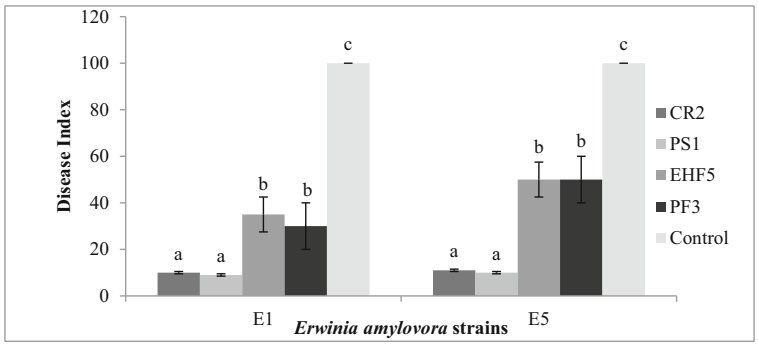


Fig. 9 Disease symptoms on pear fruits developed around wounds protected with the antagonistic strains (CR2, PS1, EHF5, PF3) and inoculated with *E. amylovora* (E1 or E5 strain). The bars represent standard error. Values were elaborated by using McKinney disease index (DI) and significance among the differences were calculated by Tukey test ($p \leq 0{,}01$)

Algeria during the same period even though no official report from Algeria is available. It is possible that the high volume of traded plants and the inherent difficulty to intercept latently infected plants, have contributed to the introduction and spread of *E. amylovora* in these Mediterranean countries. Further comparisons with isolates from other countries in North Africa are needed to conclude on the routes and the number of introductions in Algeria.

Regardless of the *E. amylovora* diversity present in each country and the pathways of pathogen's introduction in new areas, sustainable management measures to address plant infections by this disastrous pathogen are urgently needed. To this respect, research on biological control agents has been initiated in the past, including various *Pseudomonas* and *Bacillus* strains.*Pseudomonas* spp. are very ubiquitous, colonize a wide variety of ecological niches, and can be isolated from water, soil and plants. They grow in temperatures ranging from 4 °C to 42 °C (Chakravarty and Gregory 2015). In this study, the *Pseudomonas* strains did not grow at 4 °C and 45 °C, which are extreme temperatures, apart from CR2 (*P. brassicacearum*) which grew at 4 °C. Species of the genus *Bacillus* possess significant physiological and metabolic diversity that allow them to survive in extreme habitats, they can be thermophilic and psychrophilic and are capable at growing at pH, temperatures, and salt concentrations (Awais et al. 2007).

Pseudomonas accumulate from the external environment or by synthesis of several varieties of compatible solutes. Glutamate and trehalose are used by *Pseudomonas* species during growth in a medium of high osmolarity (D'Souza-Ault et al. 1993; Pocard et al. 1994). Pseudomonads are able to survive and multiply in pH between 6 and 8. However, their survival is low in acidic medium such as pH 4 (Garrity 2005). The *Bacillus* have physiological properties and varied habitats (Awais et al. 2007) shown with PF3 and EHF5 (acidophilic) but alkaline-sensitive at basic pH 10 for all strains.

Strains of *Bacillus* were moderately halotolerant, tolerating up to 0.8 M NaCl, However, *Psedomonas* strains were more halotolerant they grow at 1 M NaCl. *Psedomonas* are particularly resistant to hyper-osmotic shock in LB medium. There is a synergistic effect between the different osmoprotective agents of the LB medium which explains the high yield of growth at high osmolarity (Ghoul 1990). For *Bacillus*, the sudden decrease in the activity of cytoplasmic water induces severe plasmolysis. It inhibits cellular functions such as nutrient absorbtion, DNA replication and biosynthesis of macromolecules (Csonka 1989).

In this study, four strains were identified that strongly inhibited *E. amylovora* in vitro (dual culture) and in vivo (on pear fruits), and their biocontrol performances characterized. Even though several studies have shown that the best performing strains in the in vitro assay to evaluate antagonistic effect, are not always the most effective biocontrol agents in vivo (Ülke and ınar 1999; Mikiciński et al. 2008), this in vitro assay still represents the best technique to screen among large number of putative antagonistic bacterial strains. The two *P. brassicacearum* strains as well as the *B. amyloliquefaciens* and *B. methyloptrophicus* strains, selected based on their in vitro antagonistic activity and other known characteristics, showed a variable biocontrol efficacy against *E. amylovora* on artificially inoculated immature pear fruits. This pathogenicity test is a very reliable tool both for biocontrol and virulence studies (Cabrefiga and Montesinos 2005). Both

P. brassicacearum strains were highly effective (more than the *Bacillus* strains) in reducing symptom development (small necrosis size and suppression of bacterial exudates production) on pear fruits, even though in dual culture test the *Bacillus* strains. Had shown the best inhibiting activity.

Gerami et al. (2013) also showed that the bacterial antagonists *Pseudomonas putida* and *Serratia marcescens* were effective *in planta* but not in vitro. The discrepancy may be related to biocontrol mechanisms other than antibiosis such as competition for space and nutrients, siderophore and nonvolatile inhibitory metabolites production (Stockwell et al. 2001; Temple et al. 2004). Competition is a mechanism effective against *E. amylovora* reported both for *P. fluorescens* A506 (Wilson and Lindow 1993) as well as *P. fluorescens* EPS62e (Cabrefiga et al. 2007). Siderophores produced by strain *Pseudomonas graminis* 49 M strongly inhibited *E. amylovora* growth in vitro, but clear evidence of their role in the biocontrol of the pathogen in vivo have not yet been found (Mikiciński et al. 2016). Similarly to our results, there are several reports on the ability of antagonistic bacteria of *Pseudomonas* spp. in suppressing fire blight disease (Mercier and Lindow 2001; Ait Bahadou et al. 2018).

In this research line, further work is under way to determine the mechanisms of action underlying the in vivo biocontrol of *E. amylovora*. *Pseudomonas* CR2 and PS1 strains, that were more effective than *Bacillus* in controlling the pathogen on immature pear fruits, produced hydroxamate and catecholate siderophores on KB medium, were able to swim but not swarm on appropriate agar media and were more active producers of biofilm than *Bacillus* strains. The high siderophore production in succinate medium may be related to the production of pyoverdine in which the three amino moieties of the chromophore are replaced by various acyl groups derived from succinate, malate, α-keto-glutarate (Linget et al. 1992; Sasirekha and Srividya 2016). This kind of siderophore may act also as an antibiotic (Kraus and Loper 1995). Biofilm formation is another important feature for bacteria to succeed during colonization of plant surfaces and intercellular spaces since it increases their survival and activity and allows water retention (Zhang et al. 1998). Biofilms are made up mostly of exopolysaccharide (EPS), proteins, lipids and nucleic acids (Davey and O'toole 2000) and protects bacteria against unfavorable environmental conditions (Flemming 1993). The significantly higher ability of the two *Pseudomonas* strains to form biofilm in comparison with the *Bacillus* spp. strains may be one important trait linked to its biocontrol activity. Biofilm formation, production of siderophores and antibiotic synthesis are regulated by the quorum sensing (QS) system. This is a mechanism of intercellular signaling among bacteria that operates through small molecular weight QS signals to regulate the transcription of target genes (Maddula et al. 2006).

QS also affects motility and biosurfactants production of bacteria that are considered two important activities for a bacterial biocontrol agents. In particular, swarming motility is involved in colonization process of leaf and root surface and is described as a movement of bacteria in groups of tightly bound cells (Harshey 2003). Both *Bacillus* strains showed higher swimming and swarming activity than the two *Pseudomonas* strains and were able to produce biosurfactants; nevertheless their in vivo biocontrol activity was less effective than that of *Pseudomonas* strains. The in vivo biocontrol efficacy of microbial antagonists is the result of the activation of several mechanisms which interact with the target pathogen, the autochthonous microflora, the host genotype and the environmental conditions. Given the complexity of these interactions, the result is not always predictable on the basis of the information provided by the experimental in vitro trials. On the other hand, it is also possible that further powerful mechanisms as 2,4-diacetylphloroglucinol (DAPG) and hydrogen cyanide (HCN), not investigated in this work, are involved in biocontrol of *E. amylovora* by *P. brassicacearum* strains (Paulin et al. 2017).

Extreme conditions of pH, temperature, salt concentration and osmolarity were equally tolerated by the *Bacillus* spp. and *P. brassicacearum* strains showing their ability to face stressing environmental conditions.

In the light of the results obtained during this study, it is reported here that *E. amylovora* is widespread in Northern Algeria orchards and that *P. brassicacearum* could constitute a potential biocontrol agent to be exploited against this pathogen. *P. brassicacearum* has been already reported as an effective biological control agent against *Sclerotinia sclerotiorum, Phytophthora cactorum*, *Verticillium dahliae* and also *Clavibacter michiganensis* subsp. *michiganensis*. (Loewen et al. 2014; Novinscak et al. 2016). Further studies are needed to identify the mechanisms involved in *P. brassicacearum* interaction with *E. amylovora* and to assess its practical use in field. In addition, there is a

need to optimize the formulation for maximum activity and survival of the bacterial cells, and to assess the vegetative stage and rate of application. To this end, monitoring viable cells *in planta* could aid the design of a suitable application scheme (Daranas et al. 2018). Likewise, the improvement of ecological fitness of *P. brassicacearum* PS1 and CR2 could be investigated and further studies under different agricultural and climatic conditions are needed to confirm their performance in the field.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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