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Brief Report

Plant-associated microbiota as a source of antagonistic bacteria against the phytopathogen *Erwinia amylovora*

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Summary

Control of bacterial plant diseases is a major concern, as they affect economically important species and spread easily, such as the case of fire blight of rosaceous caused by *Erwinia amylovora*. In the search for alternatives to the use of agrochemicals and antibiotics, this work presents a screening of natural bacterial antagonists of this relevant and devastating phytopathogen. We recovered bacterial isolates from different plant tissues and geographical origins and then selected those with the strongest ability to reduce fire blight symptoms *ex vivo* and remarkable *in vitro* antagonistic activity against *E. amylovora*. None of them elicited a hypersensitivity reaction in tobacco leaves, most produced several hydrolytic enzymes and presented other biocontrol and/or plant growth-promoting activities, such as siderophore production and phosphate solubilization. These isolates, considered as biocontrol candidates, were identified by 16S rRNA sequencing as *Pseudomonas rhizosphaerae*, *Curtobacterium flaccumfaciens, Enterobacter cancerogenus*, *Pseudomonas azotoformans, Rosenbergiella epipactidis* and *Serratia plymuthica*. This is the first time that the last five bacterial species are reported to have biocontrol potential against *E. amylovora*.

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Introduction

The plant health risk posed by plant pathogens is significant and growing continuously due to rampant globalization and climatic change (Spence *et al*., 2020). Plant microbial diversity is a key factor in preventing plant diseases (Berg *et al*., 2017). Microorganisms that colonize plants without causing them any damage are relatively little studied as a source of potential biocontrol agents for serious plant diseases. They can not only suppress pathogens but also promote plant growth, contribute to the removal of pollutants, solubilize phosphate or help plants assimilate nitrogen (Rosenblueth and Martínez-Romero, 2006). One of the most serious disease affecting economically important rosaceous plants worldwide, such as apple and pear, as well as ornamental and wild plants is the fire blight, caused by the bacterium *Erwinia amylovora* (Kharadi *et al*., 2021). This disease is devastating due to its challenging control. Pruning of symptomatic parts and agrochemicals is not sufficiently effective against this highly adaptable pathogen (Ordax *et al*., 2006), and these products may be phytotoxic or have side effects for the environment. Besides, application of antibiotics poses a negative impact due to the risk of emergence of resistant strains (Förster *et al*., 2015). The search for alternative management strategies that are effective and eco-friendly, such as the use of microorganisms with biocontrol activities, has been triggered by the growing importance of the fire blight, the moderate effectiveness of existing control measures and the social demand to improve the safety and sustainability of agricultural production systems (Sundin and Wang, 2018).

Currently, there are several commercial products against fire blight that use antagonistic bacteria, such as *Pseudomonas fluorescens* A506 (Wilson and Lindow, 1993), *Pantoea vagans* C9-1 (Ishimaru *et al*., 1988), *Pantoea agglomerans* P10c (Vanneste *et al*., 2002) and E325 (Pusey, 1999), and *Bacillus subtilis* QST713 (Aldwinckle *et al*., 2002) and BD170 (Broggini

et al., 2005). The application of these organisms is highly dependent on the environmental conditions (Ngugi *et al*., 2011). To improve the biocontrol against fire blight, it is necessary to enhance a versatile selection of possible antagonists and to understand their mode of action (Zengerer *et al*., 2018; Santos Kron *et al*., 2020). Currently, different mechanisms in antagonistic bacteria are known (Köhl *et al*., 2019). It is generally assumed that most biocontrol agents do not use a unique mechanism but a combination of several ones, with a synergic effect that promotes pathogen inhibition (Whipps, 2001). In fire blight, best-known mechanisms are those based on competition for nutrients and colonization of the niche and to a lesser extent on the production of toxic secondary metabolites (Cabrefiga *et al*., 2007). Progress in biocontrol of fire blight has been driven by the selection of effective antagonistic strains, by enhanced knowledge of the mechanisms by which these strains suppress disease, and by increased understanding of the ecology of bacterial epiphytes on plant surfaces (Thomson, 2000; Bonaterra *et al*., 2012; Llontop *et al*., 2020). An ideal biocontrol strategy introduces the antagonists only when and where they are considered necessary or are expected to be effective (Bonaterra *et al*., 2003).

The aim of this work was to explore the native microbiota from different plant sources, host or non-host of *E. amylovora*, for new bacterial strains antagonistic to this pathogen, in order to obtain a versatile bacterial collection that can be screened subsequently for potential biocontrol agents against the causal agent of fire blight.

Results and discussion

Some plant bacterial isolates showed antagonistic activity against E. amylovora *on immature fruits*

A collection of bacterial isolates from loquat, pear, potato tubers and rhizosphere was screened on detached immature loquat fruits to check their antagonistic potential against *E. amylovora*. The severity of symptoms was periodically monitored according to a visual scale (0–3) (Fig. 1A). Regarding the protective effect of isolates, applied 24 h before pathogen inoculation, the percentages of incidence and severity of infection were significantly higher at 9–10 days than at 7–8 days (Tukey HSD, $P < 0.001$) and 4–5 days post-inoculation (dpi) (Tukey HSD, $P < 0.01$). At 9–10 dpi, an incidence of infection of 80%–100% was observed in the fruits pre-treated with most of the isolates tested, while an incidence of less than 40% was observed only in those pre-treated with a few isolates (Fig. 1B). At the same time, 9–10 dpi, the severity of infection was greater than 60% with most of the isolates tested (Fig. 1C). Thus, a subset of bacterial isolates successfully delayed the development of fire blight symptoms in fruits.

In terms of the efficacy on inhibiting or delaying *E. amylovora* necrosis, isolates were grouped as isolates as: very active (VA) (100%–90% efficacy), active (A) (89%–70%), moderately active (MA) (69%–40%), soft active (SA) (39%–20%) and non-active (NA) (19%–0%), according to the extent of symptoms (Fig. 2). After 9–10 dpi, 0.8% of isolates continued to be VA, 1.6% A, 7% MA and 14.8% SA. Interestingly, the percentage of incidence and severity of infection was significantly different between the five groups of the efficacy scale defined in this work (Fig. 3), with the VA isolates leading to the lowest percentages of incidence and severity of infection (ANOVA, $P < 0.001$ for incidence and severity). The different levels of efficacy observed may depend on the antagonistic abilities of isolates, either by the competition in the colonization of plant surface or by the production of substances with activity against *E. amylovora*, all leading ultimately to the appearance of necrosis to varying degrees (Paternoster *et al*., 2010; Roselló *et al*., 2013; Mikiciński *et al*., 2020).

Some plant bacterial isolates also showed in vitro *antagonism against* E. amylovora

The 10 isolates that produced the best results in the *ex vivo* screening assay on loquat fruits (IVIA T1-42, IVIA T1-56, IVIA T2-27, IVIA T3-27, MB-IVIA-Nd, MB-IVIA-Nf, MB-IVIA-Nh, MB-IVIA-Ni, MB-IVIA-MAI MB-IVIA-SOL; from here: T1-42, T1-56, T2-27, T3-27, Nd, Nf, Nh, Ni, MAI and SOL), were also tested for *in vitro* antagonistic activity. Growth inhibition of *E. amylovora* was obtained with the isolates T2-27, T3-27, Nd, MAI and SOL, with halos after 24 h of 0.5 ± 0.1, 1.9 ± 0.3, 2.8 ± 0.8, 2.4 ± 0.4 and 2.5 ± 0.1 cm diameter, respectively. The other tested isolates did not produce inhibition halos in the assayed conditions or their halos were of very small diameter. The isolates that produced the greatest inhibition halos could potentially be better antagonists (Cao *et al*., 2018). To determine the inhibition mechanism of Nd, SOL, MAI, T3-27 and T2-27 isolates, *in vitro* antagonistic trials were repeated by using cultures previously inactivated with chloroform, comparing them to live cultures. In all cases, the growth inhibition halos of *E. amylovora* appeared only with live cultures (Fig. S1). Therefore, under the conditions assayed the antagonism was due more to a competition for the scarce nutrients available in the diluted KB than to the production of substances with effect against *E. amylovora*. These results support the hypothesis that the impact of antagonism may be greater under starvation conditions, which may occur in the early stages of infection and/or colonization of the host plant by *E. amylovora* (Roselló *et al*., 2013).

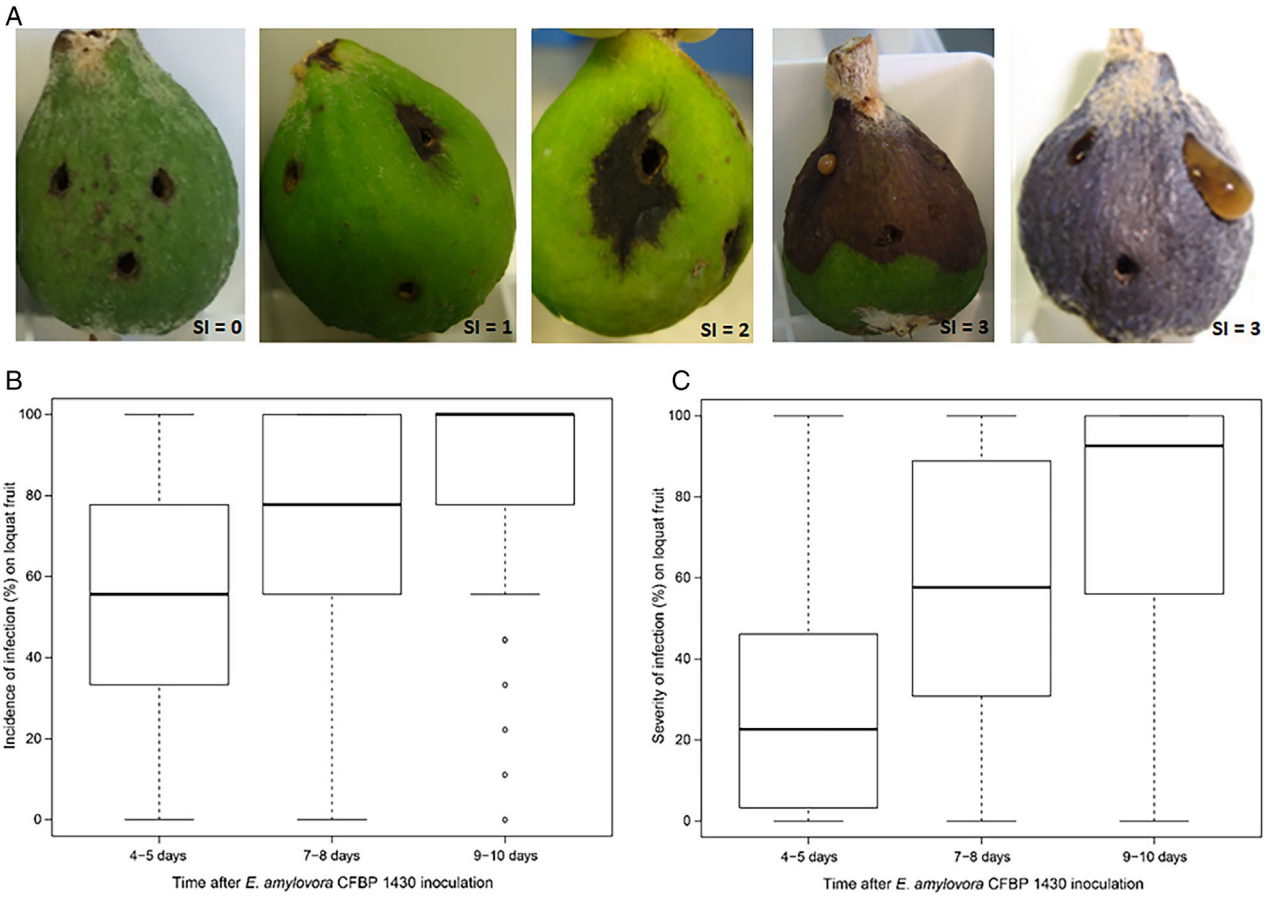

Fig. 1. Severity index (SI) (A), incidence (B) and severity (C) of infection in detached immature loquat fruits challenged with *Erwinia amylovora* CFBP 1430 strain after being sprayed with suspensions of a set of plant-associated bacterial isolates tested for biocontrol potential, 24 h before the pathogen inoculation. In A, SI = 0, no symptoms; SI = 1, exudates or necrosis located at the inoculation point; SI = 2, necrosis affecting area around the wound; SI = 3, necrosis expanding through the fruit. In B and C, middle line represents the median, box represents the upper and lower quartile, the dashed lines represent the greatest and lowest values excluding the outliers, and circles represent the outliers.

Further, moved to the plant environment, these results would mean that the potential biocontrol candidates would compete with the pathogen for space and nutrient resources before producing antibiotic- or siderophore-type substances (Santos Kron *et al*., 2020).

Selected plant bacterial isolates grew faster than E. amylovora

To comparatively evaluate the growth rate of the potential biological control agents and the pathogen as a trait that offers possible selective advantage to compete, the growth of the 10 isolates selected according to all previous trials and the strain 1430 GFP-1 of *E. amylovora* (Ordax *et al*., 2015) was monitored in diluted KB. As shown in Fig. 4A, all isolates grew faster than the *E. amylovora* strain. In general, the lag phase of the isolates was shorter than that of the pathogen. The generation time of *E. amylovora* strain was estimated to be about 9 h, compared to 8–6 h for candidate isolates for biocontrol, under the assayed conditions. This undoubtedly confers an advantage to its competitors, who would be able to colonize the same niche earlier. In fact, co-inoculation of strain 1430 GFP-1 with each one of the 10 isolates confirmed that indeed the population of the *E. amylovora* strain recovered after 48 h was lower in the presence of any of the antagonists than when the pathogen was alone (Fig. 4B). Thus, the faster growth of the isolates tested may limit the proliferation of the pathogen, since they would have more rapid access to the scarce resources present in the diluted medium. These results are consistent with the type of antagonistic mechanism observed in the *in vitro* tests.

Selected plant bacterial isolates exhibited growth promoting and/or biocontrol-related activities and did not trigger hypersensitivity response in tobacco

Selected isolates were able to produce one or more hydrolytic enzymes (Table S1). Thus, 20% of them were

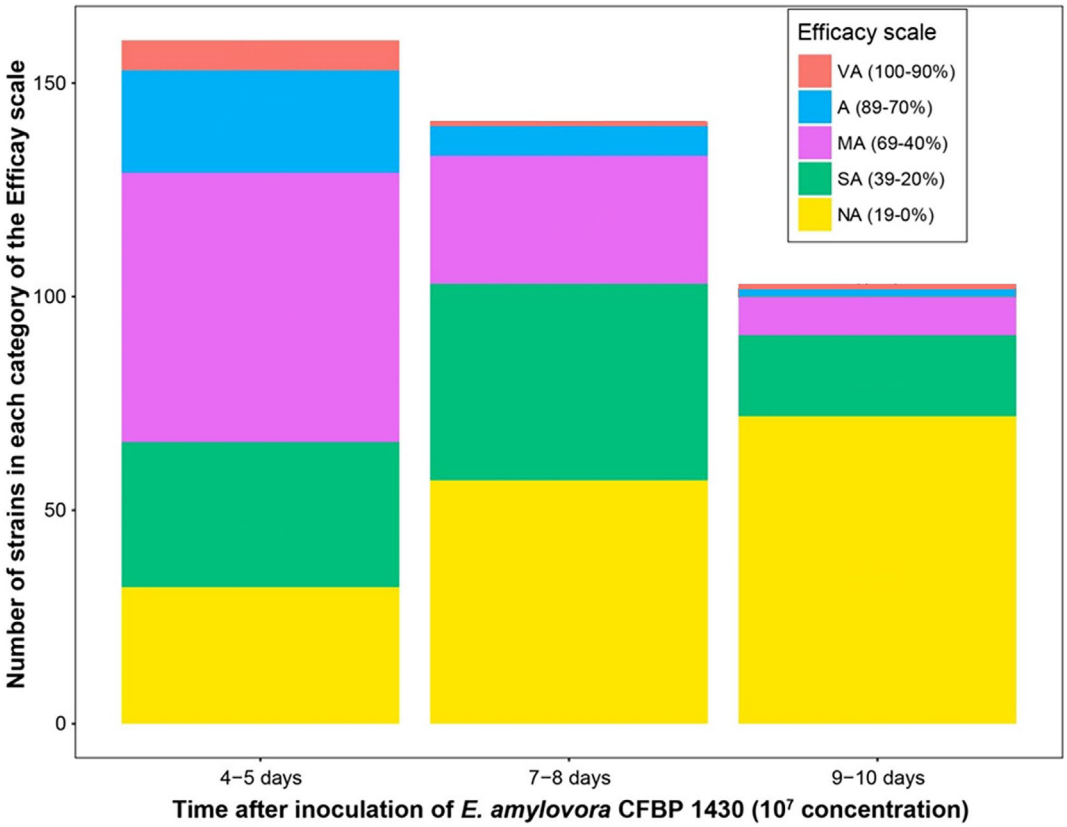

Fig. 2. Efficacy scale of a collection of plant-associated bacterial isolates for inhibiting or delaying fire blight symptoms in detached immature loquat fruits challenged with *Erwinia amylovora* strain CFBP 1430 at different days after pathogen inoculation. Fruits were pre-treated with the bacterial isolates 24 h before the inoculation of the pathogen. Degree of activity: non-active (NA) = 0%–19%, soft active (SA) = 20%–39%, moderately active (MA) = 40%–69%, active (A) = 70%–89% and very active (VA) = 90%–100%. The trial was done with the 196 selected isolates but only those that showed some efficacy after 4 days were represented ($n = 160$).

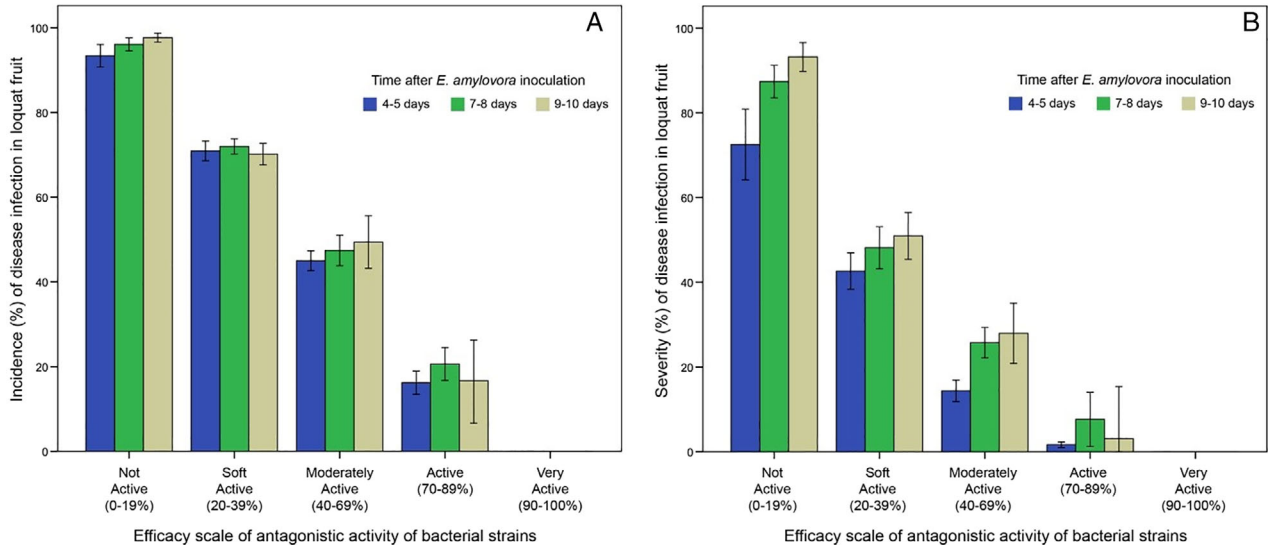

Fig. 3. Incidence (A) and severity (B) of infection by *Erwinia amylovora* strain CFBP 1430 in detached immature loquat fruits challenged with the pathogen after pre-treatment (24 h before) with a set of bacterial isolates with different efficacies for inhibiting or delaying fire blight symptoms, at different monitoring times. Error bars represent 95% confidence intervals.

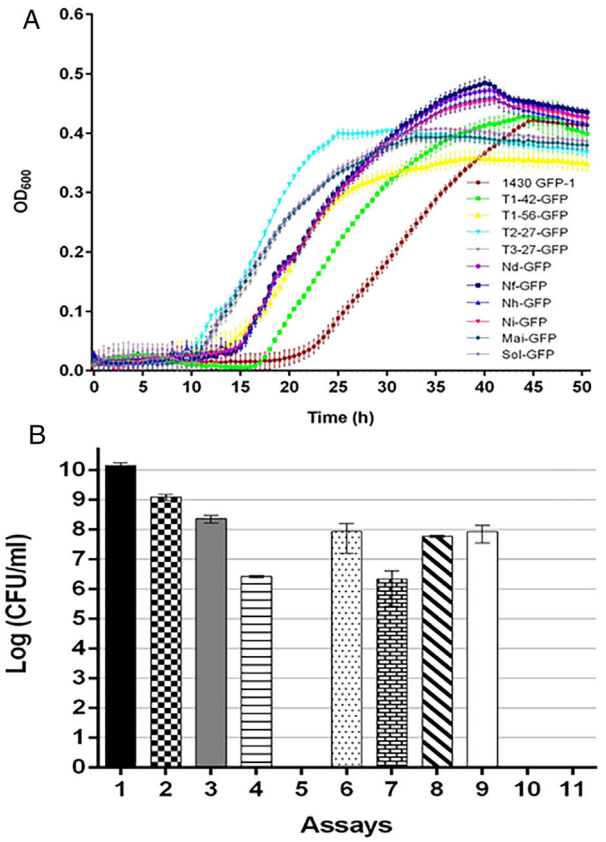

Fig. 4. Comparative growth of *Erwinia amylovora* 1430 GFP-1 strain and the plant-associated candidates to biocontrol agents against fire blight in KB 1/10 diluted liquid medium. Growth of all the strains separately, as measured by OD_{600} (A), and recovery of CFU ml^{-1} of 1430 GFP-1 strain (B) after co-inoculation with no strain (1), T1-42 (2), T1-56 (3), T2-27 (4), T3-27 (5), Nd (6), Nf (7), Nh (8), Ni (9), MAI (10), and SOL (11). All values represented are the average value obtained in at least four replicates of at least two independent experiments.

able to degrade starch, 50% gave positive results for lipase activity, 40% were able to degrade casein, and 30% to hydrolyse DNA. Interestingly, 90% of the isolates were able to produce siderophores, that limit iron availability to phytopathogens, indirectly protecting the plant (Rana *et al*., 2020), and participate in the initiation of induced systemic resistance in the host (Aznar and Dellagi, 2015). The ability to solubilize phosphate to a greater or lesser extent, producing organic acids (Ahmed and Shahab, 2011; Rana *et al*., 2020), was present in 100% of isolates. They exhibited very mucous colonies, and it is known that exopolysaccharides (EPS) can be involved in phosphorous solubilization (Yi *et al*., 2008). EPS production also provides protection to nitrogenase from inactivation by oxygen in aerobic bacteria able of fixing atmospheric nitrogen; and all selected isolates were able to grow on nitrogen-deprived culture medium, suggesting their ability to fix nitrogen. All these results indicate that the metabolic diversity and versatility of the selected isolates could contribute to their antagonism against *E. amylovora*, through competition for plant nutrients according to the tests performed, as proposed for antagonistic bacteria of other plant pathogens (Caulier *et al*., 2018).

The isolates T1-42, T1-56, T2-27, T3-27, Nd, MAI and SOL consistently did not trigger hypersensitivity response, that is considered a major element of plant disease resistance to show the potential of bacteria to be pathogenic, while isolates Nf, Nh and Ni gave variable results, causing necrosis only in 30% of the infiltrated leaf area in some of the replicates.

Five plant bacterial isolates were selected for their characteristics and consistent biocontrol activity

The 10 isolates selected according to the first screening in loquat fruits were then again challenged with loquat fruits of two different cultivars, Tanaka and Algerie, to check the robustness of the results. Most of these biocontrol candidates again delayed the onset of fire blight symptoms, and some of them effectively reduced the severity of the disease (Fig. S2A). Two isolates, SOL and MAI, were able to completely suppress fire blight symptoms until the end of the experiment. The five isolates SOL, MAI, Nd, T2-27 and T3-27 showed the highest activity, as had occurred in the *in vitro* antagonism assays. This correlation, in line with recent works (Bahadou *et al*., 2018; Llontop *et al*., 2020), is very interesting because it supports the value of *in vitro* assays as a first approach. Thus, fruits pre-treated with isolates SOL, MAI and Nd did not show any symptoms at any of the monitored times.

A third round of trials on loquat fruits of cultivars Algerie and Tanaka with a subset of five selected isolates (MAI, SOL, Nd, T2-27 and T3-27) showed that again MAI and SOL controlled the infection to a large extent, since no symptoms were observed throughout the experiment, regardless the assayed cultivar (Fig. S2B–E). The effect of the isolates T2-27, T3-27 and Nd was more pronounced in cv. Tanaka than Algerie, with an incidence and severity indexes of less than 15% for all isolates in cv. Tanaka. The biocontrol potential of these five isolates was also tested on immature pear fruits, and with two different *E. amylovora* strains, CFBP 1430 and IVIA 1892.1, the last one isolated from pear tree (Fig. 5). The isolates that best controlled the infection by CFBP 1430 were Nd and T2-27, with incidence rates of 11.1% and severity rates of 3.7%, at the end of the experiment, although the differences in both incidence and severity for all isolates in comparison to the positive control were significant ($P < 0.05$) (Fig. 5A and B). For the other three isolates, the incidence never reached 45% and the severity was less than 28%. Interestingly, the control of *E. amylovora* IVIA 1892.1 infection shown by the isolates was more pronounced than with

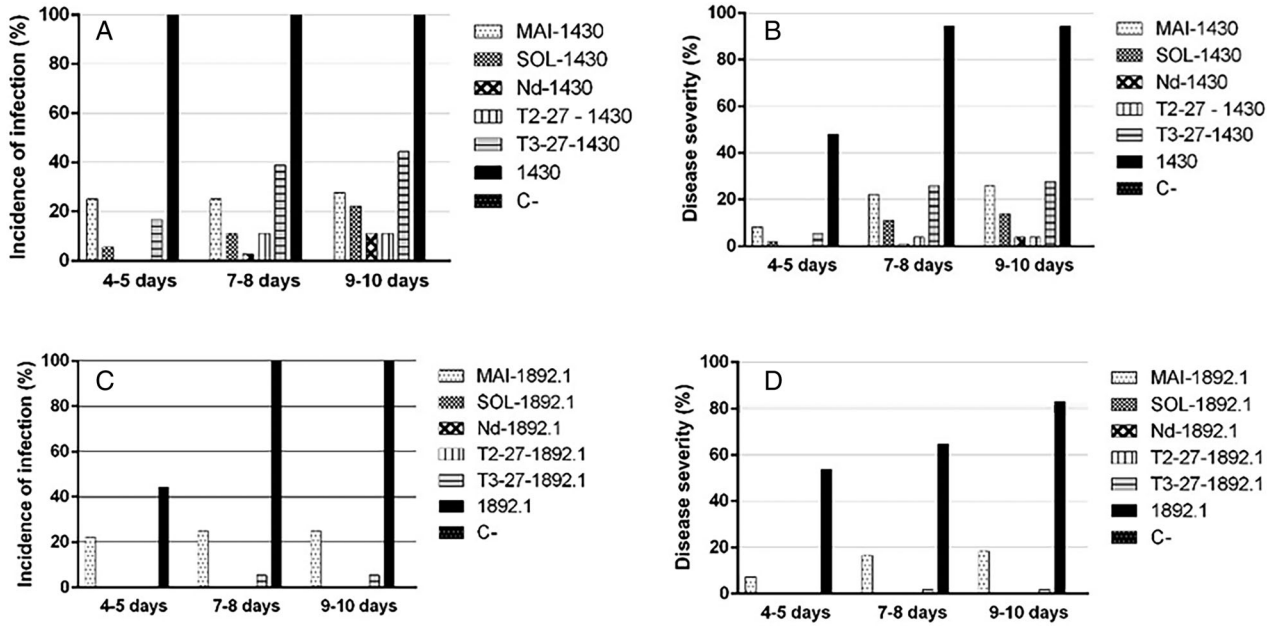

Fig. 5. Incidence (A, C) and severity (B, D) of infection by *Erwinia amylovora* strain CFBP 1430 (A, B) and strain IVIA 1892.1 (C, D) in detached immature pear fruits challenged with the pathogen after spraying with suspensions of selected plant-associated isolates tested for biocontrol potential.

E. amylovora CFBP 1430 strain (Fig. 5C and D). In fact, no symptoms developed when fruits were pre-treated with SOL, Nd and T2-27. The differences with the positive control in both incidence and severity were significant ($P < 0.05$) for the five isolates tested.

Finally, the biocontrol potential of these five isolates was challenged on pear shoots of cultivars differing in susceptibility with two different concentrations of *E. amylovora* strains. In all cases, regardless of the greater or lesser susceptibility of the cultivar, the five candidates delayed the onset of symptoms, more pronounced when the antagonist was inoculated at a concentration 10 times higher than the pathogen. The isolate that consistently, and after 8 days of incubation, completely suppressed fire blight symptoms on pear shoots was Nd. Re-isolation and real-time PCR (Gottsberger, 2010) of *E. amylovora* from symptomatic fruits and shoots of the several *ex vivo* trials confirmed the presence of the pathogen. However, re-isolation of *E. amylovora* from symptomless shoots was unsuccessful at 8 dpi, while antagonists were recovered in populations of approximately 10^5 CFU g^{-1} of tissue.

Molecular identification of selected bacterial isolates revealed the plant microbiota diversity as a source of potential biocontrol agents

The isolates showing the most suitable characteristics as potential biocontrol agents were then identified by sequencing the 16S rRNA gene (Table S2). Isolate T1-42 was identified as *Pseudomonas rhizosphaerae*, a species able to degrade nicotinic acid and nicotinamide (Paternoster *et al*., 2010), essential growth factors for *E. amylovora*, that colonizes the hypanthium of the apple blossom and has been more effective in controlling fire blight in greenhouse assays than the commercial agent *P. fluorescens* A506 (Paternoster *et al*., 2010). The isolate T1-56 was identified as *Rosenbergiella epipactidis*, one of the most abundant species in nectar (Bartlewicz *et al*., 2016; Lenaerts *et al*., 2014, 2017), a very limiting habitat in which only some highly specialized lineages are able to live (Álvarez-Pérez *et al*., 2013). Strains T1-42 and T1-56 belong to species able of inhabiting nectar, which ensures their survival in the flower, the main point-of-entry of *E. amylovora*, a favourable scenario to avoid the multiplication of the pathogen. The isolate T2-27 was identified as *Enterobacter cancerogenus*, a species widely distributed in nature and recovered from plant sources (Kazaks *et al*., 2012). In fact, numerous *Enterobacter* species are endophytic bacteria (Davin-Regli *et al*., 2019) reported as potential biocontrol agents (Kim *et al*., 2020). Competition can be established by the secretion of antibacterial proteins in the periplasm of the bacterial target, as this species contains loci for type VI secretion system (T6SS) (Navarro-Garcia *et al*., 2019). The isolate T3-27 was identified as *Curtobacterium flaccumfaciens*, a bacterial species able to inhibit the *in vitro* growth of other pathogens, such as *Xylella fastidiosa* or *Acidovorax citrulli* (Lacava *et al*., 2007; Horuz

and Ayssan, 2018), and to drastically reduce the incidence of crown gall caused by *Agrobacterium tumefaciens* (Tolba and Soliman, 2013).

The isolates Nd, Nf, Nh and Ni were identified as *Pseudomonas azotoformans*, a species isolated from diverse environmental sources, even from inside the plants, and reported as an effective biocontrol agent (Sang *et al*., 2014; Kiarood *et al*., 2020), producing secondary metabolites likely related to biocontrol (Fang *et al*., 2016). The abilities displayed by these isolates to solubilize phosphate, fix nitrogen, produce siderophores and, in general, all their metabolic potential make them particularly promising candidates to continue in the selection process. The isolates MAI and SOL were identified as *Serratia plymuthica*, a ubiquitous species recovered from rhizospheres worldwide. Its ability to control several bacterial phytopathogens of *Pectobacteriaceae* family has been reported (Czajkowski *et al*., 2012; Krzyzanowska *et al*., 2019). Moreover, it is known that this species produces several antimicrobial compounds and is considered to have great potential as a broad-spectrum biocontrol agent (Vleesschauwer and Höfte, 2007).

All characteristics above described could explain why the 10 isolates selected, and particularly five of them, have been effective in limiting the growth of *E. amylovora* and fire blight symptoms in *ex vivo* assays. We prefer to keep the 10 strains for the following stages of the selection process, in order to achieve a consortium of bacteria with complementary biocontrol traits (Santhanam *et al*., 2019) that may have a synergistic effect more effective than the use of single strains. The present work has allowed the screening and choice of strains in a first stage of the biological control agent selection process, and the selected strains will be further studied in terms of their survival and efficacy *in planta* against *E. amylovora*, their interaction with the rest of the host plant microbiota and their response to environmental conditions.

Experimental procedures

Plant material collection and sampling areas

A total of 223 samples of loquat (Algerie cultivar) blossoms or leaves were collected in November 2015 from two distinct orchards in the province of Alicante (Spain) and a greenhouse of Instituto Valenciano de Investigaciones Agrarias (IVIA) in Valencia (Spain), while 23 samples of loquat, pear trees and potato tubers and rhizosphere were collected in 2017–2018 in Algeria.

Sample processing and bacterial isolation

Sets of 8–11 detached loquat flowers, or approximately 1 g of leaves and petioles from each loquat or pear tree per sample were processed according to EPPO (2013). Growth media used for the isolation of bacterial epiphytic microbiota from pear and loquat samples were KB Levan and CCT media (EPPO, 2013). Samples of potato tubers and potato rhizosphere were processed according to Palacio-Bielsa *et al*. (2006). In all cases, colonies representing different morphological types were purified on KB and cryopreserved at −80°C with glycerol (25%–30%).

Bacterial strains and growth conditions

Strains of *E. amylovora* and those used as positive and negative controls in all the tests have been cited through the different sections.

. Unless otherwise stated, all isolates were grown in KB medium at 26°C for 24–48 h.

Ex vivo *antagonistic activity on detached immature fruits*

Immature Algerie loquat fruits (2–3 cm diameter) from greenhouses under controlled conditions (20 ± 2°C and 55%–60% HR) were collected around 5-weeks after fruit set and were used as a first approach for screening the bacterial collection. Following disinfection, each fruit was wounded in triplicate with a micropipette tip (Barbé *et al*., 2013). Then, loquats were sprayed with each suspension of biocontrol candidates (at 10^8 CFU ml^{-1}) and placed in plastic boxes at 26°C and 43% RH. After 24 h, each fruit wound was inoculated with 10 μl of a suspension of *E. amylovora* CFBP 1430 strain at 10^7 CFU ml^{-1} (Roselló *et al*., 2013). In a second approach, the previous trial was repeated using loquats from two cultivars, Algerie and Tanaka, and also immature pears cv. Ercolini of 2–3 cm diameter, collected at 6-weeks after fruit set. In addition to the reference strain CFBP 1430, the Spanish strain IVIA 1892.1 was also used separately in this trial to test the antagonistic spectrum on another strain of *E. amylovora*. Fruits were incubated at 26°C in a humid chamber and the appearance and severity of fire blight symptoms were monitored periodically. *B. subtilis* QST713 and *P. fluorescens* CHA0 were used as biocontrol reference strains. Negative control fruits were inoculated with 10 μl of PBS, and positive ones only with 10 μl of CFBP 1430 and/or IVIA 1892.1 strains.

The efficacy of *E. amylovora* infection inhibition by the tested bacterial strains, as well as the disease incidence and severity were evaluated and calculated 4–5, 7–8, and 9–10 days after pathogen inoculation (Cabrefiga and Montesinos, 2005). Wounds were considered infected when drops of bacterial exudates and/or necrosis were detected in and around them. The evaluation of severity index was made according to the necrosis extent/degree in a scale from 0 to 3 (Barbé *et al*., 2013) (Fig. 1A).

According to the efficacy value (*E*), the bacterial strains were categorized based on their degree of activity: non-active (NA) = 0%–19%, soft active (SA) = 20%–39%, moderately active (MA) = 40%–69%, active (A) = 70%–89% and very active (VA) = 90%–100%. Two independent experiments were performed for each loquat and pear cultivar in triplicate.

Re-isolations and molecular identification by Gottsberger (2010) qPCR from challenged loquat and pear fruits showing fire blight symptoms were performed to confirm that they were produced by *E. amylovora*.

Ex vivo *antagonistic activity on detached pear shoots*

Antagonistic activity was also evaluated on detached young pear shoots (cv. Blanquilla and cv. Passe Crassane). Shoots were immersed in a suspension of each antagonist at 10^8 CFU ml^{-1}, and after drying for 24 h a young leaf was cut down to the main vein with scissors dipped in suspensions of *E. amylovora* cells at 10^8 and 10^7 CFU ml^{-1} (EPPO, 2013). Shoots were maintained at 26°C and high RH with 16 h light. The development of symptoms was recorded periodically for up to 15 days.

In vitro *antagonistic activity on agar media*

Antagonistic activity against *E. amylovora* CFBP 1430 was tested by the double layer agar method (Iacobellis *et al*., 2005). Briefly, drops of 10 μl of suspensions (10^8 CFU ml^{-1}; OD_{600} of 0.20 ± 0.02) from 48 h grown cultures of 10 selected isolates (Table S1) were deposited on the bottom layer of KB 1:10 diluted medium with 1.5% agar. The strain CHA0 of *P. fluorescens* was included as positive control on separate plates. After 24 h at 26°C a second layer of 4.5 ml of melted KB 1:10 0.8% agar mixed with 0.5 ml of a 10^8 CFU ml^{-1} (OD_{600} 0.1 ± 0.02) suspension of *E. amylovora* in PBS was added. Plates were incubated at 26°C and growth inhibition halos recorded after 24 and 48 h. In order to determine the inhibition mechanism of the selected antagonists, they were also tested by inactivating them, after 24 h of growth on plates, by chloroform vapours exposure for 15 min; after chloroform evaporation, plates were overlaid with top agar mixed with *E. amylovora* and incubated 24 and 48 h at 26°C to detect growth inhibition halos.

Growth curves of selected bacterial isolates

The growth rates of the 10 selected isolates were determined and compared with the *E. amylovora* strain CFBP 1430, by measuring the OD_{600} for 48 h at 26°C, under gently shaking, in a spectrophotometer plate reader (Tecan Infinite). A volume of 180 μl per well of liquid KB 1:10 medium was inoculated with 20 μl of each suspension of the tested isolates in PBS at 10^4 CFU ml^{-1}. Moreover, co-inoculations assays (1:1) were also performed, challenging *E. amylovora* 1430 GFP-1 strain (Tet^R) (Ordax *et al*., 2009) against each one of the selected isolates, with CFU counts after incubation. Four replicates per strain were performed in two independent assays.

Characterization of selected isolates

Amylase, lipase, protease and DNase activities were assayed for selected isolates (Table S1), using the strains CECT 495 of *B. cereus* and CECT 204 of *Azotobacter vinelandii* as positive controls and CECT 101 of *Escherichia coli* as negative one. Nitrogen fixation, as the ability to grow on the minimal medium AB (Chilton *et al*., 1974), was also tested. Phosphate solubilization and siderophore production (Pikovskaya, 1948; Schwyn and Neilands, 1987) were assayed, including strains CECT 378 of *P. fluorescens* and CECT 495 of *B. cereus* as positive and negative controls, respectively. For all activities, plates were incubated at 26°C for up to 7 days.

Hypersensitivity response

Selected isolates were further evaluated for triggering the hypersensitivity response in tobacco plants cv. Xanthi. *P. syringae* pv. *tomato* DC3000 and *E. amylovora* CFBP 1430 were used as positive controls and PBS as negative one in all trials (Charkowski *et al*., 1998). These assays were repeated at least twice.

Molecular identification of selected isolates

Identification of selected isolates was performed by amplification of the 16S rRNA gene, using 16SBACF and 16SBACR primers, as described by Martínez-Murcia *et al*. (1999) and following Sanger sequencing procedures. Sequences were compared with available sequences in NCBI databases using BLASTn (Altschul *et al*., 1990) and their phylogenetic position was assessed.

Statistical analyses

ANOVA was performed to analyse differences between incidence of infection, severity and efficacy of antagonistic activity with significance at $P < 0.05$. HSD Tukey post hoc test (with 95% confidence intervals) was used to examine these differences at selected time points after *E. amylovora* inoculation. The number of bacterial isolates in each efficacy category was compared at the three time points following *E. amylovora* inoculation on

the fruits using a Chi-Square 2 × 2 contingency table test (http://www.socscistatistics.com/tests/chisquare/Default2.aspx). Statistical analyses were performed in R 3.3.1. and SPSS v15.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Effect of the antagonistic activity of plant-associated isolates MB-IVIA-Nd (A), MB-IVIA-SOL (B), and IVIA T3-27 (C), active (live cultures, pictures above) and inactivated (bacterial cells killed with chloroform vapours, pictures below), against *Erwinia amylovora* strain CFBP 1430 on KB 1:10 diluted medium after 48 h incubation at 26°C.

Figure S2. Severity of infection by *Erwinia amylovora* strain CFBP 1430 in detached immature loquat fruits cv. Algerie challenged with the pathogen after spraying (24 h before) with suspensions of a set of ten plant-associated bacterial isolates tested for biocontrol potential (A), of which 5 were selected for comparative evaluation of their effect in incidence (B, D) or severity (C, E) on cv. Algerie (B and C) or Tanaka (D and E).

Table S1. Biocontrol-related activities of selected plant-associated bacterial isolates (positive results are shading).

Table S2. Molecular identification by 16S rRNA sequencing of plant-associated bacterial isolates candidate to biocontrol agents against *Erwinia amylovora*.