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Antagonistic activity of olive endophytic bacteria and of *Bacillus* spp. strains against *Xylella fastidiosa*

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ABSTRACT

Strains of *Xylella fastidiosa* subsp. *pauca* characterized by a specific genotype, the so called sequence type "ST53", have been associated with a severe disease named Olive Quick Decline Syndrome (OQDS). Despite the relevant research efforts devoted to control the disease caused by *X. fastidiosa*, so far there are no therapeutic means able to cure the infected host plants. As such, the aim of this study was the identification of antagonistic bacteria potentially deployable as bio-control agents against *X. fastidiosa*. To this end, two approaches were used, *i.e.* the evaluation of the antagonistic activity of: i) endophytic bacteria isolated from olive trees located in an infected area but showing mild or no symptoms, and ii) *Bacillus* strains, as they are already known as bio-control agents. Characterization of endophytic bacterial isolates revealed that the majority belonged to different species of the genera *Sphingomonas, Methylobacterium, Micrococcus* and *Curtobacterium*. However, when they were tested *in vitro* against *X. fastidiosa* ST53 none of them showed antagonistic activity. On the contrary, when strains belonging to different species of the genus *Bacillus* were included in these tests, remarkable antagonistic activities were recorded. Some *B. velezensis* strains also produced culture filtrates with inhibitory activity against *X. fastidiosa* ST53. Taking also into account that two of these *B. velezensis* strains (namely strains D747 and QST713) are already registered and commercially available as bio-control agents, our results pave the way for further studies aimed at the development of a sustainable bio-control strategy of the OQDS.

1. Introduction

Xylella fastidiosa is one of the most important bacterial plant pathogen causing severe diseases of important crops with relevant economic damages (Almeida and Nunney, 2015; Rapicavoli et al., 2018). This pathogen has a very wide host range, including plants belonging to more than 500 species (EFSA (European Food Safety Authority), 2018), but it is particularly well known as the causal agent of Pierce's disease of grapevine and of Citrus Variegated Chlorosis in North and South America. At European level, *X. fastidiosa* is regulated as a quarantine pathogen and only sporadic detections were reported until 2013 when the bacterium was detected for the first time in Europe associated with the outbreak of a new severe disease (Saponari et al., 2013) which is devastating olive trees in the Southern part of the Apulia region (Southern Italy), one of the major Italian olive growing area. The disease, named Olive Quick Decline Syndrome (OQDS), has a highly destructive impact on the infected trees and is characterized by leaf scorching, desiccation of leaves, twigs and branches and leads the whole tree to death within few years. Genetic and genomic investigations revealed that infected trees harbored a previously undescribed genotype, categorized as sequence type ST53, related to strains of the subspecies *pauca* (Giampetruzzi et al., 2017). Conclusive evidence that isolates with this genotype are able to cause OQDS was achieved upon culturing the bacterium from infected olives and performing artificial inoculations on olive plants (Saponari et al., 2017). The bacterium is known to be naturally transmitted by insect vectors (xylem-sap feeders). While sharpshooters are the most common vectors in the American continent, spittlebugs appear to have a major role in the outbreaks so far discovered in Europe (Cornara et al., 2019), with *Philaenus spumarius* identified as the predominant species responsible for the epidemic spread of *X. fastidiosa* in Apulia (Cornara et al., 2017a, 2017b; Cavalieri et al., 2019. Unfortunately, the favorable epidemiological conditions in Apulia and the difficulties in applying phytosanitary control measures to restrain the spread of the bacterium to the initial

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area (approx. 8000 ha), concurred to the current alarming expansion of the infections, affecting about 715,000 ha, with millions of olive trees grown in the so called “demarcated area” (Saponari et al., 2019). Such rapid expansion of the front of the infections implied the adoption of containment measures, *i.e.* to put in place all available means to mitigate the impact of the bacterial infections entrenched in this territory and not eradicable. In this context, the containment of the bacterial disease is achieved mainly through actions against the insect vector populations and reduction of the inoculum sources (*i.e.* removal of the infected plants). Implementing vector control strategies is fundamental to stop the new infections and re-infections and several strategies can be used to lower vector populations (Dongiovanni et al., 2018), on the other hand, the control of the bacterium in the host plants still poses several challenges for the research. Attempts to develop therapeutic tools to cure infected plants have been and still are among the main drivers of the research programs. Previous efforts to mitigate the impact of OQDS using chemical treatments with an antimicrobial bio-complex (composed of zinc, copper and citric acid) (Scortichini et al., 2018), showed only a partial efficacy reducing the *X. fastidiosa* population in the infected olive plants; whereas the application of several inducers of plant resistance did not prove to alleviate the symptoms on susceptible infected olive trees (Dongiovanni et al., 2017). Thus, the lack of any therapeutic formulation for curing infected olives further emphasizes the need to develop effective and sustainable control strategies. In this regard, it is promising the identification of traits of resistance against *X. fastidiosa* in some olive cultivars (*i.e.* in “Leccino” and in the selection “FS17″) which, although infected, show mild or no symptoms and host a markedly lower population of the pathogen than the highly susceptible cultivars (*i.e.* “Ogliarola salentina” and “Cellina di Nardò”) (Giampetruzzi et al., 2016; Boscia et al., 2017). As the factors conferring resistance to *X. fastidiosa* are still unknown or not completely elucidated (Saponari et al., 2019), we undertaken a study to characterize the endophytic bacterial populations occurring in symptomless olive trees selected in the infected area, in the attempt to isolate and identify cultivable endophytic bacteria with antagonistic activity. It is known that endophytic bacteria may promote plant growth and improve plant health also by inhibiting phytopathogens (Afzal et al., 2019). In particular, it has been shown that the endophyte *Curtobacterium flaccumfaciens* inhibited the growth of *X. fastidiosa in vitro* and reduced the symptoms caused in *Catharanthus roseus* (Lacava et al., 2007). Moreover, Baccari et al. (2019) recently reported that the endophytic bacterium *Paraburkholderia phytofirmans* strain PsJN controls *X. fastidiosa* infections in grapevine.

In this work we describe the isolation, characterization and identification of endophytic bacterial strains isolated from selected olive trees and the evaluation of their antagonistic activity against *X. fastidiosa.* This study was also extended to strains belonging to different species of the *Bacillus* genus as they are, in general, already know as able to produce a number of antimicrobial substances (Caulier et al., 2019; Kaspar et al., 2019) and some of them are also used as bio-control agents against plant pathogens (Fira et al., 2018).

2. Materials and methods

2.1. Selection of olive trees

Olive trees used to isolate endophytic bacteria were selected in an orchard covering approximately 0.5 ha, located in Sannicola (40°07′13.77″N, 18°02′40.51″E, Lecce, Italy), within the heavily infected area of the Apulia region, where both resistant and susceptible cultivars co-existed under the same management practices (irrigation and yearly pruning) and trees were approximately of the same age (15 years old). More specifically, trees were selected on the basis of the following criteria: i) although grown in heavily infected olive groves, *i.e.* next to highly symptomatic olive trees, they showed mild or no symptoms of OQDS, and harbored a low population of *X. fastidiosa* which remained undetectable in some of the selected trees; ii) they belonged to different cultivars, either resistant like “Leccino” or “FS17” or susceptible like “Kalamata”. The presence and the bacterial population size of *X. fastidiosa* in the selected trees were indirectly determined by quantitative PCR (Harper et al., 2010).

2.2. Isolation of endophitic bacteria

Eight twigs of about 0.5 cm in diameter were sampled from each tree in the mid part of the canopy from the four cardinal directions, excluding those with superficial damages, according to sampling guidelines of EPPO (EPPO (European and Mediterranean Plant Protection Organization), 2019). Twigs were immediately stored in a refrigerated box prior to be transferred during the same day in the laboratory for processing. After washing with running tap water, surface disinfection of 10-cm-long twig sections was carried out by consecutive dipping in 70 % ethanol for 2 min, 2% (available Cl) sodium hypochlorite solution for 2 min, and 70 % ethanol for 30 s, followed by three rinses in sterile distilled water. The efficacy of the above-described disinfection procedure was ascertained by plating aliquots of the final rinse in sterile distilled water on Tryptic Soy Agar (TSA, Biofile) and incubation at 25 °C for 1 week. After surface disinfection, the end of each twig section and the bark were removed and scrapings of the external woody tissue were obtained by using a sterile scalpel. Tissue scrapings (500 mg) were homogenized in 5 ml of sterile phosphate-buffered saline (PBS, NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, pH 7.4) with a Homex homogenizer (Bioreba AG, CH) and serial dilutions of the obtained suspensions were seeded on plates of TSA and R2A (BD Dikinson) media. The plates were incubated at 25 °C for 3 weeks and when the growth was observed the single colonies were picked up and purified by repeated streaking on the same medium.

2.3. Molecular characterization and identification of endophytic bacteria

A molecular characterization of the isolates was performed by rep-PCR. Bacterial DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI USA) and amplified with primer pair REP-1R-Dt/REP-2R-Dt (5′-IIINCGNCGNCATCNGGC-3′; 5′-NCGNCTTATCNGGCCTAC-3′) (Hyytiä-Trees et al., 1999). The PCR reactions were carried out in a total volume of 25 µl containing 23 µl of Mega Mix (Microzone Ltd., United Kingdom), 2 µM of each primer and 1 µl of genomic DNA. Amplifications were conducted in a GeneAmp PCR system 9700 (Applied Bio-systems, Foster City, CA, USA) starting with 7 min at 95 °C, followed by 35 cycles of denaturation for 30 s at 90 °C, annealing for 1 min at 40 °C, elongation at 65 °C for 8 min and a final extension at 65 °C for 16 min. Amplicons were separated by microfluidic electrophoresis using the DNA7500 LabChip kit with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) according to the manufacturer's instructions. The rep-PCR profiles were analyzed and compared using the software provided by the same company.

Bacterial isolates were grouped on the basis of their rep-PCR profile and isolates representative of each different profile were identified by 16S rRNA gene sequencing. Almost all the gene (about 1400 bp) was amplified using the primer pair 27f-YM/1492 r (5′-AGAGTTTGATYM-TGGCTCAG-3′/5′-TACCTTGTTACGACTT-3′) (Frank et al., 2008). Each 50 µL reaction mixture contained 5 µL of 10x AccuPrime™ Pfx Reaction Mix, 1.25 U of AccuPrime™ Pfx DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 0.3 µM of each primer and 1 µL of genomic DNA. PCR amplifications were performed in a GeneAmp PCR system 9700. Reaction mixtures were first incubated for 2 min at 95 °C, and then cycled for 35 cycles according to the following temperature profiles: 30 s at 95 °C, 30 s at an annealing temperature of 60 °C for the first five cycles, 55 °C for the next five cycles and 48 °C for the last 25 cycles, then 3 min at 68 °C, followed by a final extension for 10 min at 68 °C.

PCR products were sequenced by using the BigDye™ Terminator cycle sequencing kit on an ABIPrism 3730xl DNA Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Partial sequences were assembled using the BioNumerics v. 5.1 software (Applied Maths, Inc., Austin, Texas, USA). The Blast N program, available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) was used to compare the 16S rRNA gene sequence of endophytic bacteria with sequences of type strains in database ("Sequences from type material" option). Endophytic bacterial strains were tentatively assigned to a bacterial species on the basis of the highest score of the alignment and the percentage of identity between their 16S rRNA gene sequences and those of type strains in database.

2.4. Bacillus strains

Most of the *Bacillus* strains tested in this study against *X. fastidiosa* are deposited in the Culture Collection of the Institute of Sciences of Food Production (ISPA Collection, CNR, Bari, Italy) and were previously isolated and identified (De Bellis et al., 2015; Valerio et al., 2012). In particular, those strains belong to the following species: *B. amyloliquefaciens* (strains N3.2 and S106.1b isolated from durum wheat semolina, S109.3 from durum wheat grain and S77.1 from bread), *B. pumilus* (strain S110.1 isolated from durum wheat grain and N60.2 from durum wheat semolina), *B. subtilis* (strain N67.A from durum wheat semolina), *B. licheniformis* (strain N13 from durum wheat semolina), *B. safensis* (strain S109.4, from durum wheat grain), *B. megaterium* (strain S108.3 from durum wheat grain), *B. simplex* (strain N58.2 from durum wheat semolina), *B. mojavensis* (strain N67.B2 from durum wheat semolina) and *B. oleronius* (strain S95 from brewer yeast). *B. clausii* strain DSM8716 (isolated from garden soil) and *B. firmus* strain DSM12 were obtained from DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. In addition, *B. amyloliquefaciens* subsp. *plantarum* strain D747 (isolated from atmosphere, Accession N. NRRL B-50405, NRRL Collection, Agricultural Research Service Culture Collection, Peoria, Illinois, USA) (SANCO - European Commission Health and Consumers - Directorate General, 2014) and *B. velezensis* strain QST713 (isolated from a peach orchard) (Pandin et al., 2018) were included in the study since they are already registered as biocontrol agents and commercially available.

2.5. Antagonistic activity against Xylella fastidiosa

The antagonistic activity of endophytic bacteria against *X. fastidiosa* ST53 (strain De Donno; CFBP 8402) was studied by the dual culture method on solid nutrient media. Preliminary experiments were carried out to set up and define the best conditions of co-cultivation of *X. fastidiosa* and the endophytic bacteria, using the four most commonly used media: PD3 (Davis et al., 1980), BCYE (Wells et al., 1981), PW (Davis et al., 1981) and PWG (modified after Hill and Purcell, 1995). PD3 and PWG media were then selected to perform in triplicates the experiments using the endophytic strains; while *Bacillus* strains were tested only on PD3 as, in this case, it was the only suitable for a dual-culture assay with *X. fastidiosa*. In detail, to estimate the antagonist activity, bacteria were co-plated as follow: three drops of 20 µl each of the suspension of *X. fastidiosa* (10^7 CFU/ml) were placed at the top of the petri dish, at 1 cm from each other, and slowly let to flow down to the opposite side of the plate, generating 3 parallel rows of *X. fastidiosa* cultures. After 24 h of incubation at 25 °C, an aliquot of 3 µl of a suspension in NaCl 0.85 % of the endophyte/*Bacillus* strain (10^9 CFU/ml) was placed on the top of the middle row of *X. fastidiosa* cultures. The antagonistic activity was detected after 7–10 days of further incubation at 25 °C as an area of *X. fastidiosa* growth inhibition and measured as the distance between the edges of *X. fastidiosa* growth and the growth of the tested strain.

2.6. Antimicrobial activity against X. fastidiosa in culture filtrates of Bacillus strains

All the *Bacillus* strains used in this study were evaluated for the production in liquid culture of antimicrobial compounds against *X. fastidiosa* ST53 (strain De Donno). *Bacillus* strains were 2% inoculated in 20 ml of PD3 broth and incubated under shaking (140 rpm) at 25 °C. The growth of each strain was monitored by measuring the optical density (OD) value at 600 nm. After 18, 42, 66, and 90 h of incubation, the cultures were sampled and cell-free culture filtrates were obtained by centrifugation (13,000 rpm, 4 °C, 10 min) and filtration through 0.22 µm filters. The antimicrobial activity against *X. fastidiosa* strain De Donno of the culture filtrates was evaluated by an agar well diffusion method. In particular, plates containing 20 ml of PD3 agar medium were seeded with a *X. fastidiosa* suspension to obtain three rows as described in the previous paragraph. Then, a well (8 mm diameter) was made at the end of the central row and filled with 150 µL of culture filtrate. Tests were performed in triplicate. The plates were incubated at 28 °C for 6 days and the antimicrobial activity was detected as an area of *X. fastidiosa* growth inhibition and measured as the distance between the well and the *X. fastidiosa* growth.

2.7. Statistical analysis

Statistical analysis was performed using SigmaPlot 12.0 software (ExactGraphs and Data Analysis, Systat Software Inc., USA). Data concerning antagonistic activity were compared by applying a one-way ANOVA followed by Tukey's test to determine significantly different values ($P < 0.05$).

3. Results

3.1. Selection of olive trees

To isolate endophytic bacteria, a total of 11 olive trees were selected: 4 of the cv. Leccino, 3 of cv. FS17 and 4 of the cv. Kalamata. OQDS symptoms were not observed on selected trees of cvs. Leccino and FS17 while those of the cv. Kalamata showed only few scattered desiccations that did not compromise the general architecture and vegetation of the plants. *X. fastidiosa* was undetectable by qPCR in some of the selected trees or present at low bacterial population sizes (in the range of 10^3 - 10^5 CFU/g) in the tissue used for the isolation of endophytic bacteria (Table 1).

3.2. Isolation and identification of endophytic bacteria

In general, a low number of cultivable endophytic bacteria was isolated from olive tissues, it was also highly variable depending on the plant (ranging from 6.6×10 to 7.1×10^2 CFU/g). Strains of the genus *Sphingomonas* (assigned to the species *aquatilis, yunnanensis, aerolata*, *insulae*, and *panacis*) were repeatedly isolated from plants of the cv. Kalamata. Other strains isolated from that cultivar mainly belonged to the genera *Curtobacterium* and *Methylobacterium* (Table 1). The genus *Sphingomonas* was also isolated from plants of the cv. FS17 and the strains were assigned to species *panacis, aerolata* and *aquatilis* (Table 1). This cultivar also hosted strains of the genus *Methylobacterium* assigned to species *phyllostachyos* and *pseudosasicola.* The genus *Agrococcus* was isolated from a single plant of the cv. FS17. On the contrary, the cv. Leccino seems to be mainly characterized by the presence of cultivable strains of the genus *Micrococcus* (assigned to the species *yunnanensis* and *aloeverae*) while strains of the genus *Sphingomonas* were rarely isolated from that cultivar. Strains belonging to different species of the genus *Methylobacterium* were also isolated from the cv. Leccino (Table 1) which also hosted strains belonging to the genus *Frondihabitans,* differently from cvs. Kalamata and FS17. A rep-PCR analysis was carried out to reveal clonal relationship between isolates. Results from these

Table 1
Endophytic bacteria isolated from the selected olive trees and their antagonistic activity against *Xylella fastidiosa* strain De Donno.

Cultivar	Olive tree	Level of *X. fastidiosa* population in olive tissues	Endophytic bacterial species	% of 16S rRNA gene identity	Number of isolates	N. of tested isolates and their antagonistic activity (+/-)**
KALAMATA	**FS53**	N.D.*	***Methylobacterium pseudosasicola***	99.7-99.5	2	2 (-)
	FS54	~10^5 CFU/g	***Curtobacterium ammoniigenes***	98.4-98.1	7	5 (-)
			Curtobacterium citreum	99.3	1	1 (-)
			Sphingomonas aerolata	99.2	1	1 (-)
			Novosphingobium soli	97.2	1	1 (-)
			Methylobacterium pseudosasicola	99.4	1	1 (-)
	FS55	~10^3 CFU/g	***Sphingomonas aquatilis***	99.4	2	2 (-)
			Sphingomonas insulae	98.6	1	1 (-)
	FS56	~10^5 CFU/g	***Sphingomonas aquatilis***	99.4-98.5	7	5 (-)
			Sphingomonas yunnanensis	99.5-99.0	3	3 (-)
			Sphingomonas panacis	98.2	1	1 (-)
			Sphingomonas aerolata	99.0	1	1 (-)
			Methylobacterium pseudosasicola	99.5	1	1 (-)
			Methylobacterium phyllostachyos	99.4	1	1 (-)
			Micrococcus yunnanensis	100.0	1	1 (-)
LECCINO	**FS114**	~10^4 CFU/g	***Methylobacterium pseudosasicola***	99.5-99.4	3	3 (-)
			Frondihabitans sucicola	98.7	2	2 (-)
			Amnibacterium soli	99.9	1	1 (-)
			Methylobacterium phyllostachyos	99.5	1	1 (-)
			Xanthomonas sp.	100.0	1	1 (-)
	FS118	N.D.	***Micrococcus yunnanensis***	99.9-99.4	37	15 (-)
			Micrococcus aloeverae	100.0	3	3 (-)
			Methylobacterium cerastii	98.7	1	1 (-)
			Frondihabitans sucicola	98.7	1	1 (-)
			Sphingomonas aquatilis	99.0	1	1 (-)
	L1	~10^4 CFU/g	***Micrococcus yunnanensis***	99.9-99.6	8	8 (-)
			Curtobacterium citreum	99.3	1	1 (-)
			Micrococcus aloeverae	100.0	1	1 (-)
			Pseudomonas lutea	100.0	1	1 (-)
	L3	N.D.	***Micrococcus yunnanensis***	99.9-99.8	7	7 (-)
			Curtobacterium citreum	99.3	5	5 (-)
			Methylobacterium pseudosasicola	99.5	1	1 (-)
FS17	**FS1**	N.D.	***Sphingomonas panacis***	98.6-97.6	6	6 (-)
			Sphingomonas aerolata	99.5-98.9	5	5 (-)
			Methylobacterium pseudosasicola	99.6-99.2	5	5 (-)
			Methylobacterium phyllostachyos	99.5-99.0	2	2 (-)
			Curtobacterium ammoniigenes	99.3	1	1 (-)
	FS3	~10^5 CFU/g	***Agrococcus jenensis***	99.7-99.6	4	4 (-)
			Sphingomonas aquatilis	99.3	1	1 (-)
	FS18	~10^4 CFU/g	***Methylobacterium phyllostachyos***	99.8-99.1	8	7 (-)
			Methylobacterium pseudosasicola	99.5-98.3	7	5 (-)
			Sphingomonas aquatilis	98.7-97.6	5	4 (-)

*: N.D., Not Detectable; **: The antagonistic activity was evaluated by dual-culture bioassays on PD3 and PWG media; (-): no inhibition of *X. fastidiosa* growth; (+): an halo of *X. fastidiosa* growth inhibition was detected.

assays highlighted diverse rep-PCR profiles indicating the genetic diversity of the bacterial isolates even if obtained from the same plant and belonging to the same species (Fig. 1).

3.3. Antagonistic activity against Xylella fastidiosa

A total of 120 endophytic bacteria isolated from the selected olive plants, representative of all the identified bacterial species (Table 1) and of the different rep-PCR profiles, were tested against *X. fastidiosa* strain De Donno; none of them showed antagonistic activity (Table 1) on both the used media (PD3 and PWG). On the contrary, when strains belonging to different species of the genus *Bacillus* were used in dual-culture bioassays, very strong activities were detected (Table 2). In particular, strains belonging to the species *B. velezensis* and *B. amyloliquefaciens* showed the highest antagonistic activity against *X. fastidiosa* strain De Donno, causing very large inhibition halos (Fig. 2). The activity of strains belonging to the species *B. subtilis, B. simplex, B. mojavensis, B. safensis* and *B. pumilus* was also remarkable, even if significantly lower than that showed by *B. velezensis* and *B. amyloliquefaciens* strains. The antagonistic activity of the above-mentioned *Bacillus* strains was proven to be bactericidal as *X. fastidiosa* cells sampled within the inhibition halos and transferred on a fresh PD3 agar plate were unable to grow. In contrast, the strains belonging to the other *Bacillus* species used in this study did not cause any inhibition of *X. fastidiosa* growth.

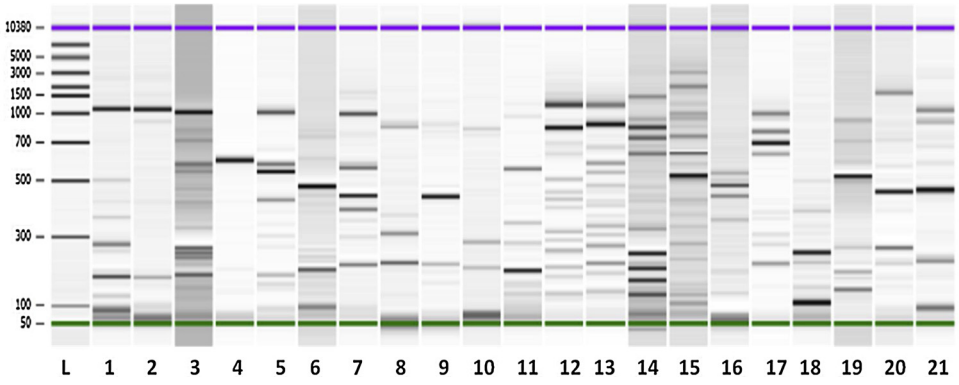

Fig. 1. Gel-like image of rep-PCR profiles of endophytic bacteria isolated from olive plants. L: DNA 7500 Ladder; 1-2: *Curtobacterium ammoniigenes*; 3-5: *Sphingomonas aquatilis*; 6-7: *Methylobacterium pseudosasicola*; 8-11: *Micrococcus yunnanensis*; 12-14: *S. panacis*; 15-17: *S. aerolata*; 18-19: *Methylobacterium phyllostachyos*; 20-21: *M. pseudosasicola*.

Table 2
Antagonistic activity of *Bacillus* strains against *Xylella fastidiosa* strain De Donno.

Bacterial species	Strain	Size (mm ± SD) of the growth inhibition halo
B. velezensis	QST713	29.0 ± 1.0[a]*
B. amyloliquefaciens	N3.2	26.6 ± 1.5[ab]
B. amyloliquefaciens	S109.3	26.3 ± 1.1[ab]
B. amyloliquefaciens	S77.1	26.0 ± 3.6[ab]
B. amyloliquefaciens	D747	25.3 ± 0.6[ab]
B. amyloliquefaciens	S106.1b	23.6 ± 0.6[b]
B. subtilis	N67.A	16.3 ± 0.6[c]
B. simplex	N58.2	16.3 ± 1.5[c]
B. mojavensis	N67.B2	15.6 ± 1.1[c]
B. safensis	S109.4	14.6 ± 1.1[c]
B. pumilus	S110.1	14.3 ± 1.1[c]
B. pumilus	N60.2	13.6 ± 1.5[c]
B. megaterium	S108.3	0
B. licheniformis	N13	0
B. oleronius	S95	0
B. firmus	DSM12	0
B. clausii	DSM8716	0

* Values with different letters are statistically different with $P < 0.05$ as determined by one-way analysis of variance (ANOVA) followed by the Tukey test.

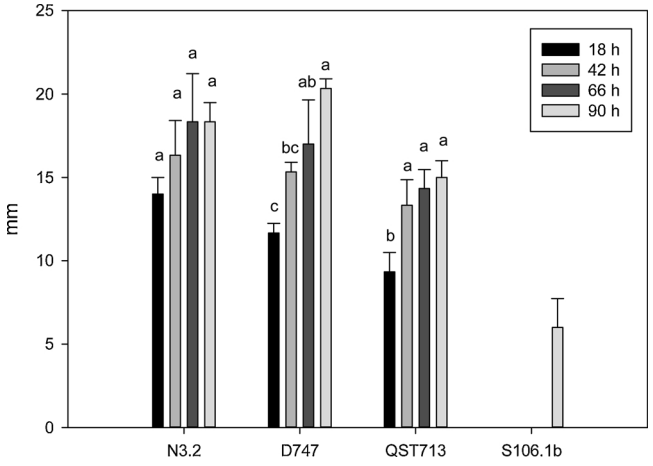

Fig. 3. Antimicrobial activity against *Xylella fastidiosa* strain De Donno of the culture filtrates obtained after 18, 42, 66 and 90 h of incubation of *Bacillus amyloliquefaciens* strains N3.2, D747, S106.1b and *B. velezensis* QST713. Different letters indicate statistically different antagonistic activities of the same strain at different times with $P < 0.05$ as determined one-way analysis of variance (ANOVA) followed by the Tukey test.

3.4. Antimicrobial activity against X. fastidiosa of culture filtrates of Bacillus strains

Regardless of the results of the dual-culture assay, all the *Bacillus* strains used in this study were also tested for the production in liquid culture of antimicrobial substances against *X. fastidiosa* strain De Donno, monitoring this activity for up to 90 h of growth. Results indicated that only the culture filtrates of three *Bacillus* strains, namely *B. amyloliquefaciens* strain N3.2, *B. amyloliquefaciens* strain D747 and *B. velezensis* strain QST713, showed a relevant antimicrobial activity. These activities, detected even after 18 h of incubation, increased with the time, although the increases were not always statistically significant (Fig. 3). With regard to the differences between strains, the antimicrobial activity of the culture filtrate of the strain QST713 was lower

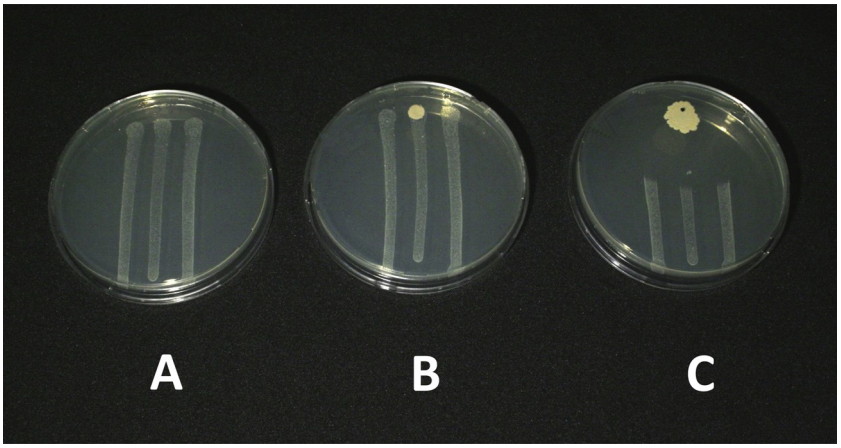

Fig. 2. Dual-culture assay of antagonistic activity of *Bacillus* strains against *Xylella fastidiosa* strain De Donno. **A)** Negative control, *X. fastidiosa*; **B)** *B. oleronius* strain S95 against *X. fastidiosa*; **C)** *B. amyloliquefaciens* strain N3.2 against *X. fastidiosa*.

than those of strains N3.2 and D747, and this difference was also statistically significant ($P < 0.05$) after 90 h of incubation. However, this could be related to the slightly lower ability of strain QST713 to grow (OD_{600} = 0.937 after 90 h) in comparison to the growth ability of strains N3.2 (OD_{600} = 1.146, after 90 h) and D747 (OD_{600} = 1.700 after 90 h). On the contrary, the very low activity in the culture filtrate of strain S106.1b (Fig. 3), as well as the complete lack of activity in the culture filtrates of all the other *Bacillus* strains, were certainly not related to a low ability to grow, as these strains reached very high values of OD_{600} (> 1.100) with the exceptions of *B. oleronius* strain S95 (OD_{600} = 0.684) and *B. firmus* strain DSM12 (OD_{600} = 0.529).

4. Discussion

There is an increasing evidence of the beneficial effects of bacterial endophytes on the host plants as they are considered able to promote plant growth and health also by indirect mechanisms such as inhibition of plant pathogens (Santoyo et al., 2016; Afzal et al., 2019). This last feature makes endophytic bacteria promising candidate bio-control agents, potentially able to compete with plant pathogens inhabiting the same niche. In the case of *X. fastidiosa*, selecting a potential bio-control agent among endophytic bacteria able to colonize the xylem should increase the possibility to achieve an effective control of that plant pathogen. To attain this goal we isolated endophytic bacterial strains from selected olive trees exposed to the natural inoculum pressure of *X. fastidiosa*, and tested their antagonistic activity against *X. fastidiosa* strain De Donno. Olive plants were selected among those testing negative to *X. fastidiosa* or infected but symptomless or with mild symptoms, hypothesizing that endophytic bacteria could be, at least in part, responsible for the status of these trees. However, our attempt to isolate an endophytic strain showing antagonistic activity against *X. fastidiosa* from those selected olive plants was unsuccessful. This result does not imply necessary that the involvement of endophytic bacteria in the resistance against *X. fastidiosa* is completely excluded. For example possible explanations of our failure are: i) the involvement of uncultivable endophytic bacteria, ii) an effect due to a complex community of endophytic bacteria, or iii) the triggering of Induced Systemic Resistance (ISR) by endophytic bacteria in the host plant (Afzal et al., 2019).

Indeed, there is a very limited knowledge on the presence of cultivable endophytic bacteria associated with the xylem tissues of olives. In general, it is known that the presence of endophytic bacteria varies depending on a number of factors including those related to the plant (such as the genotype, the age, the tissues) and environmental conditions (Hardoim et al., 2008). We found that the occurrence of cultivable endophyic bacteria was highly variable among the plants used in this study. The isolation and identification of endophytic bacteria was also carried out from some susceptible olive trees of the cv. Kalamata showing symptoms of OQDS and harboring high levels of *X. fastidiosa* populations (data not shown). However, on the basis of a comparison between endophytic bacteria isolated from resistant and susceptible plants, due to the observed variability, it was not possible identify bacterial species associated only to all the resistant plants. In fact, based on our results, it was not possible to ascertain any possible correlation between the occurrence of an endophytic bacterial species and the olive cultivar or between the occurrence of a bacterial species and the level of *X. fastidiosa* populations or the expression of symptoms. Nevertheless, rep-PCR characterization of the endophytic bacteria indicated the diversity of the endophytic isolates obtained from each plant; therefore, those isolated from the resistant ones, mainly belonging to different species of the genera *Sphingomonas, Methylobacterium, Micrococcus* and *Curtobacterium*, were selected to evaluate *in vitro* their potential antagonistic activity against *X. fastidiosa,* but none of them showed any activity. It is noteworthy that *Bacillus* strains were not found among the endophytic bacteria isolated in this study, even if endophytic strains identified as *B. amyloliquefacies* were isolated from olive trees originating from different locations by Müller et al. (2015). This difference could be also explained considering that we used different olive plants growing in a different soil and in a diverse geographic region; moreover, different tissues were used for the isolation. In particular, we isolated endophytic bacteria from the xylem tissues of olive twigs after carefully removing the bark; while Müller et al. (2015) used the whole terminal end of twigs, including leaves. Cheffi et al. (2019) also isolated an interesting endophytic *B. velezensis* strain, but from olive roots.

Nevertheless, our results clearly indicate a strong antagonistic activity, *in vitro*, of *Bacillus* strains belonging to different species against *X. fastidiosa* strain De Donno. In general, *Bacillus* strains are well known for their ability to produce a number of antimicrobial compounds, and some of them have been already considered and also registered as bio-control agents against different plant pathogens. In particular, *B. amyloliquefaciens* subsp. *plantarum* strain D747 and *B. velezensis* strain QST713 are the active ingredients of commercial formulations used on different crops for the bio-control of a number of plant pathogens including fungi and bacteria such as *Erwinia amylovora*, *Pseudomonas syringae* pv. *actinidiae* and *Xanthomonas arboricola* pv. *pruni*. Both the above-mentioned *Bacillus* strains produce lipopeptide biosurfactants with antimicrobial activity. In particular, strain QST713 produces lipopeptides belonging to the families of the surfactins, iturins and fengycins (Fiedler and Heerklotz, 2015). Moreover, the genes responsible for the production of these substances as well as of antibiotics, such as macrolactin, bacilysin and difficidin, have been identified in its genome (Pandin et al., 2018). It is significant that strains D747 and QST713 belong to the group of *Bacillus* strains tested in this study with the highest antagonistic activity against *X. fastidiosa* strain De Donno in dual-culture bioassay. Although it remains to be ascertained in further studies, the involvement of one or more of the above-mentioned antimicrobial substances in the antagonistic activity against *X. fastidiosa* is conceivable. Importantly, those strains, together with *B. amyloliquefaciens* strains N3.2 and S106.1b, and differently from the other *Bacillus* strains showing antagonistic activity against *X. fastidiosa*, also produce culture filtrates which inhibit the growth of the pathogen, likely secreting active substance/s in the liquid medium. This result suggests that the antagonistic mechanism of these strains is different from that of the other *Bacillus* strains used in this study. From the taxonomic point of view, it is interesting to note that the four strains producing active culture filtrates actually belong to the species *B. velezensis* (Rabbee et al., 2019). Indeed, *B. amyloliquefaciens* subsp. *plantarum* is a later heterotypic synonym of *B. velezensis* (Dunlap et al., 2016), and also strains N3.2 and S106.1b should be currently assigned to the species *B. velezensis* on the basis of a new data base search with their 16S rRNA gene sequences (unpublished results).

In conclusion, the endophytic bacteria isolated in this work from selected olive trees did not show any antagonistic activity against *X. fastidiosa* strain De Donno. On the contrary, we demonstrated that several *Bacillus* strains were able to inhibit *in vitro* the growth of *X. fastidiosa*. Moreover, some of these strains, belonging to the species *B. velezensis,* were also able to produce in liquid culture substances with inhibitory effect against *X. fastidiosa*. The evaluation of the capability of these *Bacillus* strains to inhibit *X. fastidiosa in vivo* and to control OQDS will require additional experiments on olive trees which will probably take years as the olive plants infected by *X. fastidiosa* may remain symptomless even for up to a year. Nevertheless, our results pave the way for further investigations aimed at the development of a sustainable strategy for the control of OQDS using bio-control agent(s) or their secreted metabolites.

Declaration of Competing Interest

All authors declare no conflict of interest.

CRediT authorship contribution statement

Stefania Zicca: Investigation, Methodology. **Palmira De Bellis:** Investigation, Formal analysis. **Mario Masiello:** Investigation. **Maria Saponari:** Conceptualization, Writing - review & editing. **Pasquale Saldarelli:** Conceptualization, Writing - review & editing. **Donato Boscia:** Writing - review & editing, Project administration, Funding acquisition. **Angelo Sisto:** Conceptualization, Writing - original draft, Supervision, Project administration, Funding acquisition.

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