Microbiological Research 219 (2019) 123–131

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Pseudomonas protegens MP12: A plant growth-promoting endophytic bacterium with broad-spectrum antifungal activity against grapevine phytopathogens

Marco Andreolli[a], Giacomo Zapparoli[a], Elisa Angelini[b], Gianluca Lucchetta[b], Silvia Lampis[a,*], Giovanni Vallini[a]

[a] Department of Biotechnology, University of Verona, 37134, Verona, Italy
[b] Research Centre for Viticulture and Enology, CREA-VE, 31015, Conegliano, TV, Italy

ARTICLE INFO

Keywords:
Biological control
Endophytic behavior
Esca disease
Plant growth-promoting bacteria
Pseudomonas protegens MP12
Vitis vinifera

ABSTRACT

Pseudomonas sp. MP12 was isolated from a soil sample collected in a typical warm-temperate deciduous forest near Brescia, Northern Italy. Phylogenetic analysis identified the species as *Pseudomonas protegens*. We evidenced in this strain the presence of the genes *phl*D, *plt*B and *prn*C responsible for the synthesis of the antifungal compounds 2,4-diacetylphloroglucinol (2,4-DAPG), pyoluteorin and pyrrolnitrin, respectively. *P. protegens* MP12 was also shown to produce siderophores and ammonia, yielded positive results with the indole-3-acetic acid test, and was capable of phosphate solubilization. Moreover, *P. protegens* MP12 exhibited inhibitory effects on *in vitro* mycelial growth of prominent grapevine (*Vitis vinifera*) phytopathogens such as *Botrytis cinerea*, *Alternaria alternata*, *Aspergillus niger*, *Penicillium expansum* and *Neofusicoccum parvum*. The strain showed activity even against *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum*, which cause the devastating tracheomycosis/esca disease of grapevine trunks for which no efficacious control methods have been demonstrated so far. Furthermore, the MP12 strain manifested *in vivo* antifungal activity against *B. cinerea* on grapevine leaves. Culture-dependent and culture-independent analysis revealed the ability of *P. protegens* MP12 to efficiently and permanently colonize inner grapevine tissues. These results suggest that *P. protegens* MP12 could be worth of exploitation as an antifungal biocontrol agent for applications in viticulture.

1. Introduction

The bacterial genus *Pseudomonas* includes a variety of species that occur in a broad range of ecological niches such as soils and water bodies as well as plant and animal tissues (Nikel et al., 2014). Soil-borne *Pseudomonas* species in particular show considerable metabolic versatility, which shapes and affects their interactions with other telluric biota (Raaijmakers and Mazzola, 2012; Silby et al., 2011). Moreover, a number of *Pseudomonas* strains have been isolated and characterized as plant growth-promoting (PGP) bacteria (Bensidhoum et al., 2016; Olorunleke et al., 2015). PGP bacteria confer beneficial effects on plant health and productivity by improving the availability and assimilation of nutrients, through phosphate solubilization and siderophore release, or by synthesizing specific active compounds (e.g. indole-3-acetic acid (IAA) and 1-aminocyclopropane-1-carboxylate (ACC)) that influence plant development. PGP bacteria can also antagonize phytopathogens and reduce the consequential loss of plant productivity via mechanisms such as competitive root colonization, the production of antimicrobial compounds, the release of hydrolytic enzymes, the production of HCN and ammonia, and the induction of systemic resistance (Lugtenberg and Kamilova, 2009; Hardoim et al., 2015).

Endophytes are defined as “all microorganisms which for all or part of their life time colonize internal plant tissues” (Hardoim et al., 2015). Endophytes colonize the majority of plant species including grapevine (Andreolli et al., 2017; Hardoim et al., 2015). Endophytic bacteria include species with different behavioral strategies in terms of plant–microbe interactions, such as latent pathogenicity, mutualism, commensalism and unilateral exploitation (Schulz and Boyle, 2006).

Pseudomonas spp. strains with PGP traits have been identified among the endophytes of different herbaceous and woody plants (Campisano et al., 2015; Wu et al., 2016). Indeed, the spontaneous invasion of internal plant tissues by non-deleterious PGP *Pseudomonas* strains often induces resistance against abiotic and biotic stress

* Corresponding author.
E-mail address: silvia.lampis@univr.it (S. Lampis).

https://doi.org/10.1016/j.micres.2018.11.003
Received 5 March 2018; Received in revised form 16 October 2018; Accepted 22 November 2018
Available online 23 November 2018

(Bensidhoum et al., 2016; Sandhya et al., 2010; Sang and Kim, 2014). Therefore, the use of beneficial endophytic bacteria to control plant pathogens offers an ecofriendly alternative to pesticides (Abdelwahab et al., 2016).

Grapevine (*Vitis* spp.) is the mostly widely-cultivated fruit crop in the world and economically the most important tree, mainly because the berries are processed to make wine (FAOSTAT, 2011). Therefore, opportunities to promote the healthy growth of grapevine crops by using biological control strategies to hinder the development of phytopathogens are of great interest. For instance, PGP rhizobacteria can stimulate grapevine vegetative development and mineral acquisition (Sabir et al., 2012) and increase chilling resistance (Ait Barka et al., 2006). Moreover, PGPR can retard water losses, induce accumulation of abscisic acid and the synthesis of defense-related terpenes in grapevine (Salomon et al., 2014). *Burkholderia phytofirmans* PsJN (now known as *Parabulkholderia phytofirmans*) is the most studied endophytic bacterial strain tested thus far that shows potential PGP activity in viticulture (Ait Braka et al., 2000; Ait Barka et al., 2002, 2006; Compant et al., 2005). Therefore, strains with PGP characteristics are highly desirable.

Here we report the taxonomic classification of the novel bacterial strain *Pseudomonas protegens* MP12, which was found in a woodland soil sample from the countryside near Brescia (Lombardia, Italy), and the characterization of its PGP traits. The ability of this strain to colonize the inner tissues of grapevine plants as an endophyte was also determined using culture-dependent and culture-independent methods.

2. Materials and methods

2.1. Strains and culture conditions

The *Pseudomonas* sp. strain named MP12 described herein was isolated from forest soil sampled in Brescia, northern Italy, a region dominated by hardwood forests predominantly containing the tree genera *Fagus*, *Castanea* and *Quercus*. Briefly, 5 g of soil samples were stirred in 45 ml of physiologic solution (0.9% NaCl) for 1 h. The samples were serially diluted, plated on R_2A-agar (Oxoid) and incubated at 27 °C for 5 days. Morphologically distinct bacterial colonies emerging on the plates were selected and streaked on fresh media to obtain pure cultures. Among these isolates, the strain MP12 was chosen for the characteristics described in Results.

The strain MP12 was grown and maintained in King B (King et al., 1954) broth or agar (King B broth containing 1.5% w/v bacteriological agar). Two reference strains obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) were also used in this study: *Pseudomonas protegens* DSM 19095^T as type strain of the *P. protegens* species and *Parabulkholderia phytofirmans* DSM 17436^T, previously known as *Bulkholderia phytofirmans* PsJN. This latter strain was chosen for comparison as it is a well-known bacterial strain tested so far for its PGP activity in viticulture (Ait Braka et al., 2000; Ait Barka et al., 2002, 2006; Compant et al., 2005).

2.2. Phylogenetic analysis

Total DNA was extracted from cultures grown in King B broth for 2 days at 30 °C as described by Andreolli et al. (2016a). PCR was carried out in a 25-µl reaction volume comprising ~100 ng of genomic DNA, 0.8 µM of each primer, 0.4 mM of the four dNTPs, 1 unit of GoTaq™ DNA Polymerase (Promega, Milan, Italy), and 5 µl of 5X PCR buffer. The 16*S* rRNA gene (1500 bp) was amplified using primers fD1 and rP2 (Weisburg et al., 1991). Each PCR involved an initial denaturation step at 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 2 min, and a final extension step at 72°C for 5 min. Gene fragments corresponding to RNA polymerase subunit D (*rpo*D, 693 bp), RNA polymerase subunit B (*rpo*B, 509 bp) and DNA gyrase subunit B (*gyr*B, 587 bp) were amplified as previously described (Frapolli et al., 2007). The PCR products were cloned in *Escherichia coli* strain XL1-Blue using the Promega pGem-T vector system according to the manufacturer's protocol, and both strands were sequenced by GATC Biotech (Cologne, Germany).

The phylogenetic tree was constructed after concatenating the 16*S* rRNA, *rpo*B, *rpo*D and *gyr*B sequences. Other related sequences used in the phylogenetic tree were selected by searching for homology using BlastN against the NCBI nucleotide database (Altschul et al., 1997) or were based on published data (Ramette et al., 2011). The sequences were assembled, aligned using ClustalX v1.83 (Thompson et al., 1997), and manually adjusted. The phylogenetic tree was assembled using the neighbor-joining method in MEGA v5.0 with 1000 bootstrap replications (Tamura et al., 2011). *Pseudomonas* sp. MP12 nucleotide sequences determined herein were deposited in NCBI GenBank under the following accession numbers: KJ467812 for the 16*S* rRNA gene, KX236070 for *rpo*B, KX236071 for *rpo*D, and KX236072 for *gyr*B.

2.3. Amplification and sequencing of genes required for the synthesis of antifungal metabolites

P. protegens can synthesize the antimicrobial secondary metabolites 2,4-diacetylphloroglucinol (2,4-DAPG) and pyoluteorin, which require the *phl*D and *plt*B genes, respectively (Ramette et al., 2011), and pyrrolnitrin, which requires the *prn*C gene (Hammer et al., 1995). We therefore amplified these genes to confirm the species identity. As above, PCR was carried out in a 25-µl total reaction volume comprising ~100 ng of genomic DNA, 0.8 µM of each primer, 0.4 mM of the four dNTPs, 1 unit of DNA Polymerase, and 5 µl of 5X PCR buffer. The sequences of *phl*D (746 bp), *plt*B (792 bp) and *prn*C (720 bp) were amplified as previously described (Mavrodi et al., 2001). The PCR products were isolated, cloned and sequenced as described above. Sequence identity was determined by using BlastN against the NCBI nucleotide database (Altschul et al., 1997). Nucleotide sequences were deposited in NCBI GenBank under the following accession numbers: KX236067 for *phl*D, KX236068 for *plt*B, and KX236069 for *prn*C.

2.4. Identification of plant growth-promoting traits

IAA was detected as described by Gordon and Weber (1951). The synthesis of siderophores was analyzed using the method provided by Schwyn and Neilands (1987). Phosphate solubilization tests were performed as reported by Andreolli et al. (2016a). The presence of 1-aminocyclopropane-1-carboxylate deaminase activity was detected as described by Penrose and Glick (2003). Cells were initially inoculated at OD_{600} nm = 0.05 and growth was monitored by UV/vis spectrophotometry (Eppendorf® BioPhotometer). The production of ammonia was tested in peptone water. Briefly, *Pseudomonas* sp. MP12 was inoculated in 10 ml of peptone water and incubated for 72 h at 27 °C. Thereafter, 1.5 ml of culture was centrifuged at 10,000 g for 5 min, and 1 ml of the supernatant was transferred to a new tube before adding 50 µl of Nessler's reagent. The development of a brownish color indicated the production of ammonia (Cappuccino and Sherman, 1992). All of the above-mentioned tests were performed in triplicate.

2.5. Screening for antifungal activity on agar plates

Visual agar plate assays were performed to test the ability of *Pseudomonas* sp. MP12 to inhibit the growth of 10 fungal phytopathogenic strains. Six strains (*Botrytis cinerea* BC, *Alternaria alternata* A41, *Aspergillus niger* AN, *Penicillium expansum* PE, *Fusarium verticillioides* F3 and *Neofusicoccum parvum* N12) were isolated from withered grapes (Lorenzini et al., 2013; Lorenzini and Zapparoli, 2015; Lorenzini et al., 2015).

Three strains (*Phaeomoniella chlamydospora* 13/13 and 22, and *Phaeoacremonium* sp. 20) came from the CREA-VE collection of grapevine pathogenic fungi, and were formerly isolated from Cabernet Sauvignon plants affected by esca disease at the CREA-VE experimental

vineyard in Spresiano (Treviso, Italy) (Andreolli et al., 2016a). These strains were selected among hundreds of other strains as representative of the respective species because of their high aggressiveness in grapevine *in vivo* (unpublished data). The last strain *Phaeoacremonium aleophilum* CBS 246.91^T was purchased from CBS-KNAW collection (Utrecht, NL), and it was chosen because it is a reference strain at international level.

Fungal strains were cultivated on malt extract agar (MEA, 20 g l^{-1} malt extract, 1 g l^{-1} yeast extract 1.5% w/v agar) at 25 °C for 4–10 days. We then removed 5-mm agar plugs from the mycelial fronts and transferred them to the center of Petri dishes containing R_2A (Oxoid)/ Malt-YE agar medium (R_2A supplied with 5 g l^{-1} malt extract, 1 g l^{-1} yeast extract and 15 g l^{-1} bacteriological agar). *Pseudomonas* sp. MP12 was streaked 3 cm from the fungal plugs. Each fungal strain was tested in triplicate. Antifungal activity was assessed by comparing the zones of mycelium growth inhibition with those in control plates without bacteria.

2.6. Antifungal activity assay on grapevine leaves

in vivo assay was carried out on young leaves excised from 15-year-old grapevines. Detached leaves were surface sterilized by 0.5% NaOCl, then rinsed with sterile water three times and placed on wet adsorbing paper on Petri dishes. Five mm plugs of *B. cinerea* BC, grown on MEA for 5 days at 25 °C 12 h light/12 h dark, were placed mycelium-side down onto surface sterilized plant tissues. A cell suspension of *Pseudomonas* sp. MP12 at 10^7 cell ml^{-1} (25 µl), obtained from 24h-old culture on King B medium washed with 0.9% NaCl, was deposited on each fungal plug. A sterile solution of 0.9% NaCl, instead of bacterial cell suspension, was used as negative control. Detached leaves were incubated at 25 °C 12 h light/12 h dark. Inoculation experiments were performed twice with ten replicates. Quantification of disease development was measured as the average diameter of necrotic area formed after seven days, and the average disease in the control was used to calculate the percentage of disease reduction.

2.7. Bacterial inoculation of grapevine plants

All experiments were performed using grapevine rootstock Kober 5BB clone ISV1 (*Vitis berlandieri* x *Vitis riparia*). *in vitro* stock cultures were used as the source and were cultivated on a modified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), with half-strength mineral salts, full-strength vitamins, 20 g l^{-1} sucrose and 0.8% agar, pH 5.8. The *in vitro* plants were maintained at 25 ± 1 °C with a 16-h photoperiod (60 µmol m^{-2} s^{-1} cool-white light) and were subcultured by node cutting every 3 months. Under sterile conditions, an aliquot (100 ml) of *Pseudomonas* sp. MP12 suspension with a concentration of 10^8 cells/ml was added to MS medium in which a node cutting was then placed. The inoculation was carried out on 50 new explants, while a further 10 non-inoculated explants were subcultured as controls.

2.8. Recovery and identification of inoculated bacteria

The recovery of inoculated *Pseudomonas* sp. strain MP12 was confirmed using culture-dependent plate counting in axenic culture, and culture-independent PCR-based denaturing gradient gel electrophoresis (PCR-DGGE).

Fifteen plantlets were randomly selected and sampled 25, 75 and 125 days after inoculation to evaluate the endophytic colonization of *Pseudomonas* sp. MP12 by plate counting. Therefore, the percentage of inoculated plants was calculated as follows: % of colonized plants = (n° of colonized plants x100)/ n° of total analyzed plants. Uninfected plantlets were sampled at the same time points as controls. The 25-day-old and 75-day-old plantlets were analyzed as entire plants, whereas each of the 125-day-old plantlets was dissected into three sections: roots, upper shoot and lower shoot. The plant tissues were carefully cleaned to remove agar substrate and washed in sterile water. The surface was then disinfected for 2 min with 70% ethanol and 5 min with 1% active chlorine before rinsing three times for 2 min with sterile physiological solution. To verify the efficacy of the disinfection protocol, 100 µl of physiological solution from the third rinse was plated on King B agar medium. The plant tissues were cut into small pieces under a sterile hood and placed on a rotary shaker for 1 h at 250 rpm. Thereafter, samples were serially diluted and plated on King B agar medium. The presence of *Pseudomonas* sp. MP12 was initially revealed due to its unique fluorescence. The abundance of the inoculated strain was determined by counting the colonies on King B agar plates incubated at 27 °C for 48 h. At each sampling, three colonies were randomly selected and the 16*S* rRNA gene was sequenced using the fD1 and rP2 primers to confirm the identity of *Pseudomonas* sp. strain MP12 (Weisburg et al., 1991).

PCR-DGGE analysis was carried out using five 125-day-old plantlets. Three leaves at different stem heights were harvested, sterilized and stored at −20 °C. The leaves were ground under liquid nitrogen and total DNA was extracted using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. Eubacterial 16*S* rRNA genes were amplified using primers fD1 and rP2 (Weisburg et al., 1991), then the hypervariable V3 region was amplified by nested PCR using primers p2 and p3 with a GC-clamp (Muyzer et al., 1993). The first PCR comprised an initial denaturation step at 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 45 °C for 30 s and 7 °C for 2 min, and a final extension step at 72 °C for 5 min. The nested PCR comprised an initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 45 s, and a final extension step at 72 °C for 5 min. DGGE analysis was applied to the V3 region amplicons as reported by Andreolli et al. (2016b). The 8% acrylamide/bisacrylamide (19:1) gel (BioRad, Hercules, CA, USA) was cast using a denaturing gradient of 30–60%, with 100% denaturant defined as 7 M urea and 40% (v/v) formamide.

The DGGE bands were excised and soaked in 50 µl sterile water at 37 °C for 4 h to allow diffusion, and 1 µl of the solution was used as the template for re-amplification. PCR was carried out as described above, albeit with non-GC-clamped primers. PCR products were introduced into *E. coli* Xl1blue cells using the pGEM-T vector system according to the manufacturer's instructions, sequenced on both strands, and finally searched for homology using BlastN against the NCBI nucleotide database (Altschul et al., 1997).

2.9. Statistical analysis

One-way analysis of variance (ANOVA) was used to compare specific pairs of means, with Tukey's test to determine statistical significance ($p < 0.05$). A chi-squared test (χ^2) was used to determine whether the distribution of categorical variables showed statistically significant differences ($p < 0.05$).

3. Results

3.1. Identification of strain MP12 by molecular analysis

Pseudomonas sp. MP12 evidenced fluorescent colonies when cultivated on King B. This medium is used primarily for the detection and differentiation of *Pseudomonas* species that produce the fluorescent metabolites fluorescein or pyoverdin (King et al., 1954; Ramette et al., 2011). The phylogenetic features of *Pseudomonas* sp. MP12 were identified by analyzing the 16*S* rRNA gene along with the sequences *rpo*B, *rpo*D and *gyr*B, encoding RNA polymerase subunits B and D and DNA gyrase subunit B, respectively. The sequences were used to construct a neighbor-joining tree (Fig. 1). The strain *Pseudomonas* sp. MP12 clustered within strains of *P. protegens* with a high bootstrap score (> 99%).

P. protegens carries the *phl*D and *plt*B genes, which are needed for the

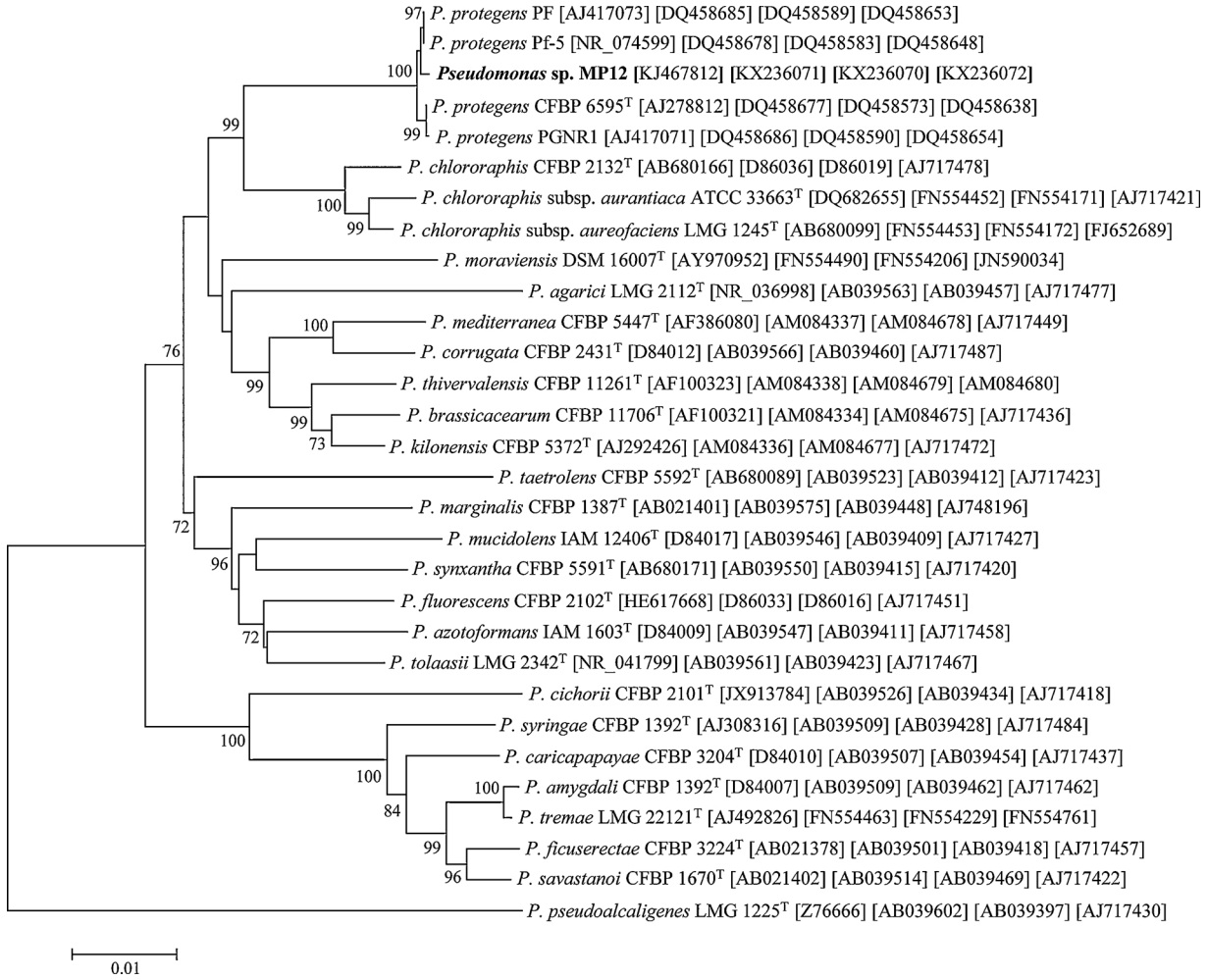

Fig. 1. Phylogenetic tree generated by the concatenation of the 16*S* rRNA, *rpo*D, *rpo*B and *gyr*B sequences retrieved from *Pseudomonas* sp. MP12 and related strains. Outgroup = *Pseudomonas pseudoalcaligenes* LMG 1225^T. Bootstrap values are shown at branch nodes and are based on 1000 replicates. The scale bar indicates 0.01 substitutions per nucleotide position.

synthesis of the antimicrobial secondary metabolites 2,4-DAPG and pyoluteorin, respectively (Ramette et al., 2011). PCR with *P. protegens* species-specific primers yielded amplicons of 745 bp (*phl*D) and 791 bp (*plt*B) from strain MP12 (data not shown). Furthermore, a partial *prn*C gene was also amplified, yielding a 719-bp product (data not shown), suggesting that *Pseudomonas* sp. MP12 is also able to synthesize the antifungal compound pyrrolnitrin (Hammer et al., 1995). These data provided convincing evidence that *Pseudomonas* sp. MP12 belongs to the species *P. protegens*.

3.2. Identification of bacterial PGP traits

In assays to determine whether bacteria can promote plant growth, we found that *P. protegens* MP12 was able to produce siderophores and ammonia, to yield positive results in a test for IAA, and to solubilize phosphate in all the media we tested except PVK/Ca medium (Table 1).

3.3. In vitro antifungal activity

The antifungal activity of *P. protegens* MP12, *P. phytofirmans* DSM 17436^T and *P. protegens* DSM 19095^T was tested against the phytopathogens *B. cinerea*, *N. parvum*, *A. alternata*, *A. niger*, *P. expansum*, *F. verticillioides*, *Phaeomoniella chlamydospora* 13/13 and 22, *Phaeoacremonium* sp. 20, and *Phaeoacremonium aleophilum* CBS 246.91^T (Table 2). *P. phytofirmans* DSM 17436^T inhibited the growth of *B. cinerea* but none of the other fungi. However, both *P. protegens* MP12 and

Table 1
Results of PGP traits tested on *P. protegens* MP12: phosphate solubilization by adopting NBRIP and PVK media supplemented with $Ca_3(HPO_4)_2$ or $CaHPO_4$; siderophores production (siderophores); 1-aminocyclopropane-1-carboxylate deaminase (ACC); indole-3-acetic acid (IAA); ammonia production (NH_4). (+) positive, (−) negative.

Siderophore	NH_4	IAA	ACC
+	+	+	−

Phosphate solubilization			
PVK/Ca	PVK/Ca_3	NBRIP/Ca	NBRIP/Ca_3
−	+	+	+

P. protegens DSM 19095^T significantly inhibited mycelial development in all of the phytopathogenic fungi tested except *F. verticillioides*. Both *P. protegens* strains also induced a significantly wider ($p < 0.01$) no-growth zone around *B. cinerea* colonies than *P. phytofirmans*. No significant differences in antifungal activity were observed between *P. protegens* MP12 and *P. protegens* DSM 19095^T.

3.4. In vivo antifungal activity

Detached leaves inoculated by *B. cinerea* BC and *Pseudomonas* sp. MP12 displayed a significantly ($p < 0.01$) lower necrotic area than the

Table 2
In vitro anti-microbial activities against grapevine pathogens by *P. protegens* MP12, *B. phytofirmans* DSM 17437^T and *P. protegens* DSM 19095^T. The antimicrobial activities were estimated by measuring the radius (mm) of the clear zone of growth inhibition. Results are expressed as the mean and standard deviation. Results of Tukey's test is also reported.

	(1) *B. phytofirmans* DSM 17436^T	(2) *P. protegens* DSM 19095^T	(3) *P. protegens* MP12	Tukey's test
***Botrytis cinerea* BC**	5.67 ± 2.52	10.67 ± 1.53	16.33 ± 3.21	1 *vs* 2, 3 $p < 0.01$ 2 *vs* 3 $p > 0.05$
***Neofusicoccum parvum* N12**	0	12.74 ± 1.33	14.67 ± 3.51	1 *vs* 2, 3 $p < 0.01$ 2 *vs* 3 $p > 0.05$
***Alternaria alternata* A41**	0	16.33 ± 1.53	17.00 ± 1	1 *vs* 2, 3 $p < 0.01$ 2 *vs* 3 $p > 0.05$
***Aspergillus niger* AN**	0	6.5 ± 0.71	6 ± 1	1 *vs* 2, 3 $p < 0.01$ 2 *vs* 3 $p > 0.05$
***Penicillum expansum* PE**	0	4.33 ± 2.52	3.67 ± 2.08	1 *vs* 2, 3 $p < 0.01$ 2 *vs* 3 $p > 0.05$
***Fusarium verticillioides* F3**	0	0	0	1 *vs* 2, 3 $p < 0.01$ 2 *vs* 3 $p > 0.05$
***Phaeomoniella chlamidospora* 13/13**	0	15.66 ± 0.57	16.00 ± 1.73	1 *vs* 2, 3 $p < 0.01$ 2 *vs* 3 $p > 0.05$
***Phaeomoniella chlamidospora* 22**	0	16.33 ± 1.53	17.30 ± 2.52	1 *vs* 2, 3 $p < 0.01$ 2 *vs* 3 $p > 0.05$
***Phaeoacremonium aleophilum* CBS 246.91**	0	12.33 ± 1.53	14 ± 1	1 *vs* 2, 3 $p < 0.01$ 2 *vs* 3 $p > 0.05$
***Phaeoacremonium* sp. 20**	0	17.33 ± 1.53	18.22 ± 1.52	1 *vs* 2, 3 $p < 0.01$ 2 *vs* 3 $p > 0.05$

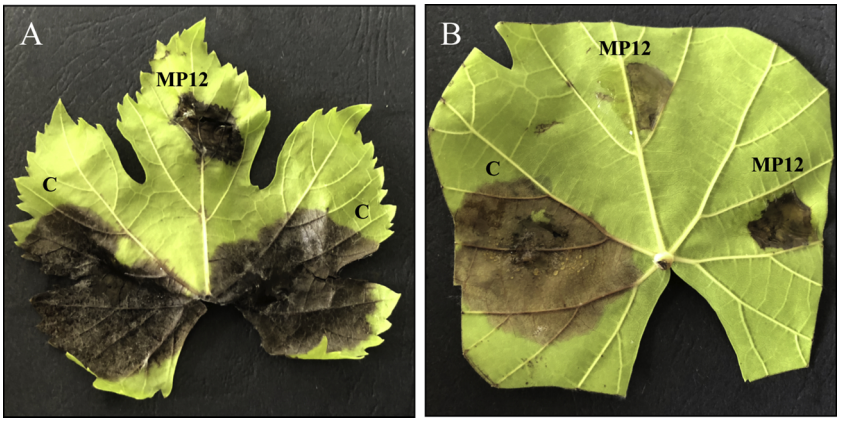

Fig. 2. Detached leaves at different development stages (A and B) infected by *Botrytis cinerea* BC and treated with *P. protegens* MP12 or 0.9% NaCl (C).

control. The reduction of symptomatic area calculated in the former with respect to the latter ranged from 50 to 88% (average 75 ± 10%) (Fig. 2).

3.5. Assessment of endophytic activity in grapevine tissues

Plate counts representing grapevine plants 25, 75 and 125 days after inoculation with *P. protegens* MP12 showed an infection percentage range of 42.8–63.6% (proportion of infected plants compared to all inoculated plants) without significant differences in the number of colony forming units (CFUs) throughout the experimental trial (Table 3). Bacterial cell concentrations were similar in three independently repeated tests (4.2–4.6 log CFUs g^{-1}) (Table 3). No bacterial cells were isolated from the control plantlets or from the rinsing

Table 3
A: Quantification of *P. protegens* MP12 within the whole plant. The percentage of colonized plant is also reported. **B**: Distribution of *P. protegens* MP12 within 3 plant sections after 125 days from inoculation. Different letters are statistically different ($p < 0.05$; Tukey's test).

A	log(CFUs g^{-1})	Infected plants (%)
After 25 days	4.22 ± 0.48	63.64
After 75 days	4.58 ± 0.25	42.85
Afer 125 days	4.65 ± 0.27	46.15

B	log(CFUs g^{-1})
Roots	3.57 ± 0.64[a]
Lower shoot	5.91 ± 0.30[b]
Upper shoot	4.36 ± 1.44[ab]

solution used to check disinfection efficiency. After 125 days, plants were dissected into three parts: roots, lower shoots and upper shoots. The concentration of *P. protegens* was 3.6 ± 0.6, 5.9 ± 0.3 and 4.4 ± 1.4 log CFU g^{-1} in the roots, lower shoots and upper shoots, respectively. There was a significant difference in bacterial abundance between the roots and lower shoots ($p < 0.05$).

The colonization of internal plant tissues was confirmed by the phenotypic and molecular analysis of colonies on plates streaked with samples from grapevine plantlets 25, 75 and 125 days after inoculation. The size of the colonies and their morphology (fluorescence, smoothness and convexity) were very similar to those of *P. protegens* MP12. Three representative isolates were randomly picked from plates for 16*S* rRNA gene sequence analysis, revealing an exact match with the *P. protegens* 16*S* rRNA gene (data not shown).

PCR-DGGE analysis was carried out on five randomly selected plants 125 days after inoculation, three from the infected group and two from the uninfected group (Fig. 3). Correctly band related to *P. protegens* MP12 was detected in the infected plants but not in the uninfected plants. This result confirmed the colonization of MP12 strain within the plants 125 days after inoculation. Additional bands, representing autochthonous bacteria, were also observed in the DGGE profiles (Fig. 3). Sequencing these bands revealed 97% identity to a non-cultivable cyanobacterium (band 1) and 96% identity to a member of the genus *Xanthomonas* (band 2). Therefore, the analysis revealed the presence of non-cultivable endophytes other than *P. protegens* MP12 in both the infected and uninfected grapevine plants.

4. Discussion

The bacterial strain *Pseudomonas* sp. MP12, isolated from a forest soil sample, was initially assigned to the species *P. protegens* based on the phylogenetic analysis of 16*S* rRNA, *rpo*B, *rpo*D and *gyr*B gene sequences according to Ramette et al. (2011). The MP12 strain was also shown to carry the *phl*D and *plt*B genes, which allow the synthesis of 2,4-DAPG and pyoluteorin, respectively, thus confirming *P. protegens* species designation. In fact, as demonstrated by Ramette et al. (2011), Phl^+ Plt^+ strains clustered independently from their nearest species *P. fluorescens* and *P. syringae*. Strains of *P. protegens* have frequently been isolated and identified from rhizosphere environments that suppress plant diseases (Keel et al., 1996; Ramette et al., 2011), but also from the phyllosphere (Müller et al., 2016), well water polluted with heavy metals (Bensidhoum et al., 2016) and hydroponic culture of lamb's lettuce (Polano et al., 2018). It was recently demonstrated that the inoculations of strains belonging to *P. protegens* improve the health and growth of annual crop plants (Bensidhoum et al., 2016; Fox et al., 2016). Moreover, previous studies reported that *P. protegens* strains such as *P. protegens* Pf-2 (Quecine et al., 2015) can synthesize antibiotics including pyrrolnitrin (Prn) (Howell and Stipanovic, 1979), pyoluteorin (Plt) (Howell and Stipanovic, 1979), 2,4-diacetylphloroglucinol (DAPG; Phl) (Nowak-Thompson et al., 1994), analogs of rhizoxin (Loper et al., 2008), hydrogen cyanide (Kraus and Loper, 1992), monoacetylphloroglucinol (MAPG) (Kidarsa et al., 2011), the lipopeptide orfamide A (Gross et al., 2007), and toxoflavin (Philmus et al., 2015). Therefore, the role of this species as a biocontrol agent has been well documented (Gross and Loper, 2009; Loper et al., 2007) and it is not surprising to find that *P. protegens* MP12 can also inhibit fungal growth. Previous investigations showed that different strains of *P. protegens* can inhibit the growth of a number of fungi, including *B. cinerea* and *A. niger*, both considered in this study, and various species in the genera *Fusarium*, *Alternaria* and *Penicillium* (Bensidhoum et al., 2016; Michelsen and Stougaard, 2012; Müller et al., 2016; Saini et al., 2016). On the other hand, the antifungal activity of *P. protegens* has not previously been tested against the genera *Neofusicoccum*, *Phaeomoniella*

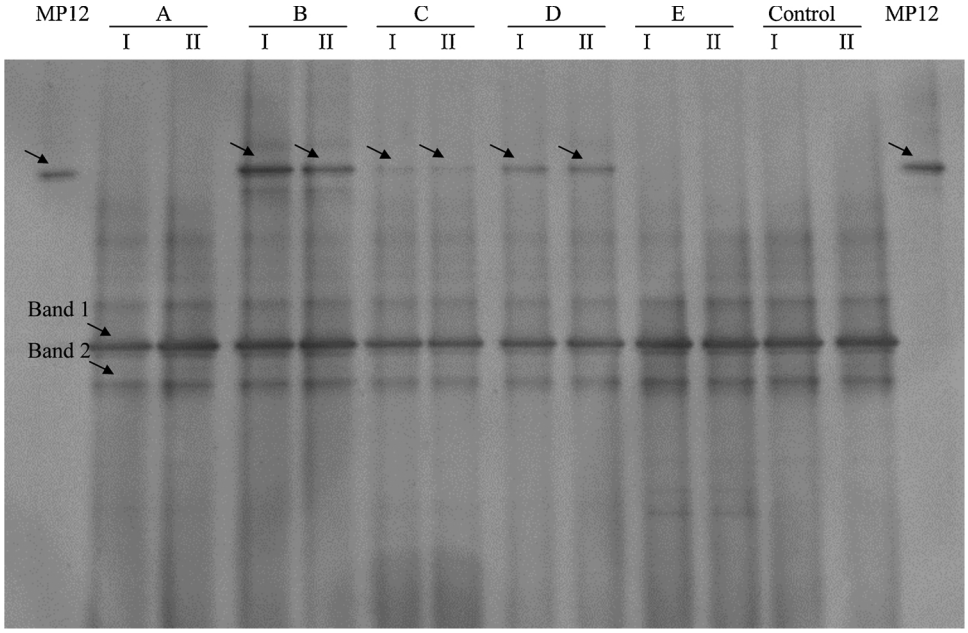

Fig. 3. DGGE analysis on five plants randomly selected among those either colonized (B, C and D) or not colonized (A and E) by *P. protegens* M12 125 days after inoculation. A non-inoculated plant is included as a control. The analysis was performed in duplicate. MP12 = positive control fragment amplified directly from *P. protegens* MP12. Bands 1 and 2 were excised from the gel for cloning and sequencing.

and *Phaeoacremonium*. To the best of our knowledge, the work described herein therefore provides the first evidence that *P. protegens* inhibits the phytopathogenic fungi *A. alternata*, *P. expansum*, *N. parvum*, *P. chlamydospora* and *P. aleophilum*. Previous studies reported the isolation of bacterial strains belonging to different genera with antifungal activity against *B. cinerea* (Ait Barka et al., 2002; Furuya et al., 2011), *A. niger* (Bensidhoum et al., 2016), *P. expansum* and *A. alternata* (Senthil et al., 2011; Lo Piccolo et al., 2016), *F. verticillioides* (Figueroa-López et al., 2014), *N. parvum* (Andreolli et al., 2016a; Wicaksono et al., 2017), *P. chlamydospora* and *P. aleophilum* (Álvarez-Pérez et al., 2017; Andreolli et al., 2016a; Lo Piccolo et al., 2016). However, to date only the here described *P. protegens* MP12 and *B. amyloliquefaciens* AG1 (Lo Piccolo et al., 2016) displayed noticeable growth inhibition against wide spectrum of grapevine phytopathogenic fungi.

All the fungi we tested herein are involved in important grapevine diseases: *B. cinerea* causes gray mold (Lorenzini et al., 2013; Masih et al., 2001), *N. parvum* is responsible for Botryosphaeria dieback (Masih et al., 2001; Úrbez-Torres et al., 2014), several strains of *A. alternata*, *A. niger* and *P. expansum* can release mycotoxins at concentrations exceeding regulatory limits in grapes and grape products such as wine (Logrieco et al., 2003; Serra et al., 2005), and *P. chlamydospora* and *P. aleophilum* are among the causative agents of tracheomycosis/esca syndrome, a devastating grapevine wood disease with no effective control measures (Larignon and Dubos, 1997).

The inability of *P. protegens* MP12 to inhibit *F. verticillioides* may reflect the production of mycetal metabolites such as fusaric acid, which limit the impact of bacterial antibiosis (Quecine et al., 2015).

Beside the antifungal activity, *P. protegens* MP12 also showed PGP traits. PGP bacteria can improve the growth of grapevine plants by: increasing nutrient availability and assimilation (e.g. phosphate solubilization, siderophore production), synthesizing specific compounds that usually plants require (e.g. synthesis of IAA), and/or protecting the plants from disease by competing with phytopathogens (e.g. antitiotics, siderophore production) (Quecine et al., 2012; Nabti et al., 2014; Andreolli et al., 2017). Therefore, antifungal activities jointly with PGP traits showed by *P. protegens* MP12 are encouraging features for the possible exploitation of this strain as endophytic inoculum in grapevine plants. Several studies have demonstrated the beneficial effects of inoculating plants with PGPB (Abbamondi et al., 2016; Subramanian et al., 2015). Endophytes with suitable fungal antagonistic proprieties have previously been isolated and exploited as biocontrol agents (Andreolli et al., 2017). For instance, two strains belonging to Curtobacterium fungal genus could reduce crown gall development in grapevine (Ferrigo et al., 2017), and endophytic Bacillus strains GLB191 and GLB197 effectively controlled grapevine downy mildew (Zhang et al., 2017). Moreover, it was demonstrated that a bacterial strain with suitable metabolic features, although originally isolated from a quite different environmental niche than internal plant tissues, might potentially be exploited as an endophytic inoculum in plants to improve growth, yield and health (Andreolli et al., 2013; Rolli et al., 2017; Wicaksono et al., 2017). The abundance of *P. protegens* MP12 in the internal tissues of infected grapevine plants was similar to or even greater than the levels previously reported for endophytes in grapevine and other plants (Andreolli et al., 2013; Compant et al., 2008; Germaine et al., 2004). The persistence of large bacterial populations starting from a small inoculum is one of the key requirements for the development of biocontrol agents based on PGP bacteria (Germaine et al., 2009). Endophytic colonization of the plants by *P. protegens* MP12 was confirmed by culture-independent technique (PCR-DGGE). The analysis also indicated that MP12 strain is able to migrate from the roots to the hypogenic upper part of the plants - the higher concentration of MP12 was found in lower shoots part - and can compete with autochthonous endophytes. The colonization and the spatial distribution of endophytes of grapevine differs in a strain-dependent manner (Andreolli et al., 2017). For instance, *Enterobacter ludwigii* EnVs6 and *Pantoea vagans* PaVv7 can colonize the root surfaces, the cortex and the central cylinder up to the xylem vessels, but cannot mount a systemic infection (Lòpez-Fernàndez et al., 2015). Moreover, Compant et al. (2011) evidenced that several isolates from different plant tissues correspond to identical phylogenetic bacterial groups: Firmicutes and Gammaproteobacteria were the predominant genera in epidermis of the flower and inside the xylem of ovaries, whereas high concentration of *Bacillus* spp. has been isolated in the berry pulp, flower ovules and inside the seeds (Comnpant et al., 2011).

We provide the first description of a *P. protegens* strain that can infect the inner tissues of multi-annual grapevine plants. On the other hand, Cyanobacteria and *Xanthomonas* spp. - here identified by PCR-DGGE - have already been found as grapevine endophytic genera (Bertani et al., 2016; Bell et al., 1995)

To date, *Paraburkholderia phytofirmans* DSM 17436^T, previously knows as *Burkholderia phytofirmans* PsJN (Sawana et al., 2014) is the only strain known causing grapevine exogenous infection and colonization jointly with fungal antagonistic effect in vitro against *B. cinerea* (Ait Barka et al., 2002; Compant et al., 2005, 2008). However, the present results showed a stronger antifungal activity of *P. protegens* MP12 against *B. cinerea* in comparison to *P. phytofirmans* DSM 17436 T. Also, MP12 was able to reduce the growth of additional grapevine fungal pathogens. Moreover, the concentration of *P. protegens* MP12 within the grapevine plants was found to be higher in comparison to the plants infected with *P. phytofirmans* DSM 17436^T (Compant et al., 2008). These results suggest that *P. protegens* MP12 strain could be developed as an antifungal biocontrol agent with applications in viticulture.

5. Conclusion

This investigation showed that *P. protegens* MP12 can efficiently colonize the internal tissues of grapevine plants. These characteristics suggest that *P. protegens* MP12 could be exploited as a biocontrol agent for grapevine crops. The prohibition in the use of chemical compounds like sodium arsenite has forced to take precautionary measures as the only control for esca disease. Therefore, the ability of *P. protegens* MP12 to inhibit *P. chlamydospora* and *P. aleophilum* should be investigated *in vivo*. However, a reliable protocol for the infection of grapevine plants with these phytopathogenic fungi must be developed before *P. protegens* MP12 can be tested in this manner.

Acknowledgements

This work was partially supported by the University of Verona through FUR2015 and FUR2017 Grants. The authors are grateful to Lorena Dalla Cia (CREA-VE) for excellent technical support.

References

Abbamondi, G.R., Tommonaro, G., Weyens, N., Thijs, S., Sillen, W., Gkorezis, P., Iodice, C., Rangel, Wd.M., Nicolaus, B., Vangronsveld, J., 2016. Plant growth-promoting effects of rhizospheric and endophytic bacteria associated with different tomato cultivars and new tomato hybrids. Chem. Biol. Technol. Agric. 3, 1.

Abdelwahab, R., Bensidhoum, L., Nacera, T., Yousra, B., Naili, F., Cruz, C., Nabti, E., 2016. A *Pseudomonas protegens* with high antifungal activity protects apple fruits against *Botrytis cinerea* Gray Mold. Int. J. Sci. Res. Sci. Technol. 6 (2), 227–237.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Álvarez-Pérez, J.M., González-García, S., Cobos, R., Olego, M.A., Ibañez, A., Díez-Galán, A., Garzón-Jimeno, E., Coque, J.H.R., 2017. Using endophytic and rhizospheric actinobacteria from grapevine plants to reduce fungal graft infections in nurseries that lead to young grapevine decline. Appl. Environ. Microbiol. 83 e01564-17.

Ait Barka, E., Belarbi, A., Hachet, C., Nowak, J., Audran, J.C., 2000. Enhancement of in vitro growth and resistance to gray mold of *Vitis vinifera* co-cultured with plant growth-promoting rhizobacteria. FEMS Microbiol. Lett. 186, 91–95.

Ait Barka, E., Gognies, S., Nowak, J., Audran, J.C., Belarbi, A., 2002. Inhibitory effect of bacteria on *Botrytis cinerea* and its influence to promote the grapevine growth. Biol. Control 24, 135–142.

Ait Barka, E., Nowak, J., Clément, C., 2006. Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth-promoting rhizobacterium

Burkholderia phytofirmans Strain PsJN. Appl. Environ. Microbiol. 72, 7246–7252.
Andreolli, M., Lampis, S., Poli, M., Gullner, G., Biró, B., Vallini, G., 2013. Endophytic *Burkholderia fungorum* DBT1 can improve phytoremediation efficiency of polycyclic aromatic hydrocarbons. Chemosphere 92, 688–694.
Andreolli, M., Lampis, S., Zapparoli, G., Angelini, E., Vallini, G., 2016a. Diversity of bacterial endophytes in 3 and 15 year-old grapevines of *Vitis vinifera* cv. Corvina and their potential for plant growth promotion and phytopathogen control. Microbiol. Res. 18, 42–52.
Andreolli, M., Albertarelli, N., Lampis, S., Brignoli, P., Khoei, N.S., Vallini, G., 2016b. Bioremediation of diesel contamination at an underground storage tank site: a spatial analysis of the microbial community. World J. Microbiol. Biotechnol. 32, 6.
Andreolli, M., Lampis, S., Vallini, G., 2017. Diversity, distribution, and functional role of bacterial endophytes in Vitis vinifera. In: In: Maheshwari, D.K. (Ed.), EndophYtes: Biology and Biotechnology Vol. 1. Springer, Switzerland, pp. 233–266.
Bensidhoum, L., Nabti, E., Tabli, N., Kupferschmied, P., Weiss, A., Rothballer, M., Schmid, M., Keel, C., Hartmann, A., 2016. Heavy metal tolerant *Pseudomonas protegens* isolates from agricultural well water in northeastern Algeria with plant growth promoting, insecticidal and antifungal activities. Eur. J. Soil Biol. 75, 38–46.
Bell, C.R., Dickie, G.A., Harvey, W.L.G., Chan, J.W.Y.F., 1995. Endophytic bacteria in grapevine. Can. J. Microbial. 41, 46–53.
Bertani, I., Abbruscato, P., Piffanelli, P., Subramoni, S., Venturi, V., 2016. Rice bacterial endophytes: isolation of a collection, identification of beneficial strains and microbiome analysis. Environ. Microbiol. Rep. 8, 388–398.
Campisano, A., Pancher, M., Puopolo, G., Puddu, A., Lòpez-Fernàndez, S., Biagini, B., Yousaf, S., Pertot, I., 2015. Diversity in endophyte populations reveals functional and taxonomic diversity between wild and domesticated grapevines. Am. J. Enol. Vitic. 66, 1.
Cappuccino, J.C., Sherman, N., 1992. Microbiology: A Laboratory Manual, 3th ed. Benjamin/Cummings Pub. Co, New York.
Compant, S., Reiter, B., Sessitsch, A., Nowak, J., Clément, C., Ait Barka, E., 2005. Endophytic colonization of *Vitis vinifera* L. by plant growth-promoting bacterium *Burkholderia* sp. Strain PsJN. Appl. Environ. Microbiol. 71, 1685–1693.
Compant, S., Kaplan, H., Sessitsch, A., Nowak, J., Ait Barka, E., Clément, C., 2008. Endophytic colonization of *Vitis vinifera* L. by *Burkholderia phytofirmans* strain PsJN: from the rhizosphere to inflorescence tissues. FEMS Microbiol. Ecol. 63, 84–93.
Compant, S., Mitter, B., Colli-Mull, J.G., Gangl, H., Sessitsch, A., 2011. Endophytes of grapevine flowers, berries, and seeds: identification of cultivable bacteria, comparison with other plant parts, and visualization of niches of colonization. Microb. Ecol. 62, 188–197.
FAOSTAT, 2011. available to http://faostat.fao.org.
Ferrigo, D., Causin, R., Raiola, A., 2017. Effect of potential biocontrol agents selected among grapevine endophytes and commercial products on crown gall disease. Biocontrol 62, 821–833.
Figueroa-López, A.M., Cordero-Ramírez, J.D., Quiroz-Figueroa, F.R., Maldonado-Mendoza, I.E., 2014. A high-throughput screening assay to identify bacterial antagonists against Fusarium verticillioides. J. Basic Microbiol. Suppl 1, S125–33.
Fox, A.R., Soto, G., Valverde, C., Russo, D., Lagares Jr, A., Zorreguieta, A., Alleva, K., Pascuan, C., Frare, R., Mercado-Blanco, J., Dixon, R., Ayub, N.D., 2016. Major cereal crops benefit from biological nitrogen fixation when inoculated with the nitrogen-fixing bacterium *Pseudomonas protegens* Pf-5 X940. Environ. Microbiol. 18, 3522–3534.
Frapolli, M., Défago, G., Moënne-Loccoz, Y., 2007. Multilocus sequence analysis of biocontrol fluorescent *Pseudomonas* spp. producing the antifungal compound 2,4-diacetylphloroglucinol. Environ. Microbiol. 9, 1939–1955.
Furuya, S., Mochizuki, M., Aoki, Y., Kobayashi, H., Takayanagi, T., Shimizu, M., Suzuki, S., 2011. Isolation and characterization of *Bacillus subtilis* KS1 for the biocontrol of grapevine fungal diseases. Biocontrol Sci. Technol. 21, 705–720.
Germaine, K., Keogh, E., Garcia-Cabellos, G., Borremans, B., van der Lelie, D., Barac, T., Oeyen, L., Vangronsveld, J., Moore, F.P., Moore, E.R.B., Campbell, C.D., Ryan, D., Dowling, D.N., 2004. Colonisation of poplar trees by gfp expressing bacterial endophytes. FEMS Microbiol. Ecol. 48, 109–118.
Germaine, K.J., Keogh, E., Ryan, D., Dowling, D.N., 2009. Bacterial endophyte mediated naphthalene phytoprotection and phytoremediation. FEMS Microbiol. Lett. 296, 226–234.
Gordon, S.-A., Weber, R.P., 1951. Colorimetric estimation of indoleacetic acid. Plant Physiol. 26, 192–195.
Gross, H., Stockwell, V.O., Henkels, M.D., Nowak-Thompson, B., Loper, J.E., Gerwick, W.H., 2007. The genomisotopic approach: a systematic method to isolate products of orphan biosynthetic gene clusters. Chem. Biol. 14, 53–63.
Gross, H., Loper, J.E., 2009. Genomics of secondary metabolite production by *Pseudomonas* spp. Nat. Prod. Rep. 26, 1408–1446.
Hammer, P.E., Hill, S., Ligon, J., 1995. Characterization of genes from *Pseudomonas fluorescens* involved in the synthesis of pyrrolnitrin. Phytopathology 69, 480–482.
Hardoim, P.R., van Overbeek, L.S., Berg, G., Pirttilä, A.M., Compant, S., Campisano, A., Döring, M., Sessitsch, A., 2015. The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiol. Mol. Biol. Rev. 79, 293–320.
Howell, C.R., Stipanovic, R.D., 1979. Control of *Rhizoctonia solani* in cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. Phytopathology 69, 480–482.
Keel, C., Weller, D.M., Natsch, A., Dé Fago, G., Cook, R.J., Thomashow, L.S., 1996. Conservation of the 2,4-diacetylphloroglucinol biosynthesis locus among fluorescent *Pseudomonas* strains from diverse geographic locations. Appl. Environ. Microbiol. 62, 552–563.
Kidarsa, T.A., Goebel, N.C., Zabriskie, T.M., Loper, J.E., 2011. Phloroglucinol mediates crosstalk between the pyoluteorin and 2,4-diacetylphloroglucinol biosynthetic pathways in *Pseudomonas fluorescens* Pf-5. Mol. Microbiol. 81, 395–414.
King, E.O., Ward, M.K., Raney, D.E., 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44, 301–307.
Kraus, J., Loper, J.E., 1992. Lack of evidence for a role of antifungal metabolite production by *Pseudomonas fluorescens* Pf-5 in biological control of Pythium damping-off of cucumber. Phytopathology 82, 264–271.
Larignon, P., Dubos, B., 1997. Fungi associated with esca disease in grapevine. Eur. J. Plant Pathol. 103, 147–157.
Logrieco, A., Bottalico, A., Mulé, G., Moretti, A., Perrone, G., 2003. Epidemiology of toxigenic fungi and their associated mycotoxins for some Mediterranean crops. Eur. J. Plant Pathol. 109, 645–667.
Loper, J.E., Kobayashi, D.Y., Paulsen, I.T., 2007. The genomic sequence of *Pseudomonas fluorescens* Pf-5: insights into biological control. Phytopathology 97, 233–238.
Loper, J.E., Henkels, M.D., Shaffer, B.T., Valeriote, F.A., Gross, H., 2008. Isolation and identification of rhizoxin analogs from *Pseudomonas fluorescens* Pf-5 by using a genomic mining strategy. Appl. Environ. Microbiol. 74, 3085–3093.
Lòpez-Fernàndez, S., Compant, S., Vrhovsek, U., Bianchedi, P.L., Sessitsch, A., Pertot, I., Campisano, A., 2015. Grapevine colonization by endophytic bacteria shifts secondary metabolism and suggests activation of defense pathways. Plant Soil 405, 155–175.
Lo Piccolo, S., Alfonzo, A., Burruano, S., Moschetti, G., 2016. Detection of bacterial endophytes in *Vitis vinifera* L. and antibiotic activity against grapevine fungal pathogens. In: Compant, S., Mathieu, F. (Eds.), Biocontrol of Major Grapevine Diseases: Leading Research. AIT Austrian Institute of Technology, Tulln an der Donau, Austria, pp. 182–190.
Lorenzini, M., Zapparoli, G., 2015. Occurrence and infection of *Cladosporium*, *Fusarium*, *Epicoccum* and *Aureobasidium* in withered rotten grapes during post-harvest dehydration. Anton. Leeuw. 108, 1171–1180.
Lorenzini, M., Cappello, M.S., Zapparoli, G., 2015. Isolation of *Neofusicoccum parvum* from withered grapes: strain characterization, pathogenicity and its detrimental effects on passito wine aroma. J. Appl. Microbiol. 119, 1335–1344.
Lorenzini, M., Azzolini, M., Tosi, E., Zapparoli, G., 2013. Postharvest grape infection of *Botrytis cinerea* and its interactions with other moulds under withering conditions to produce noble-rotten grapes. J. Appl. Microbiol. 114, 762–770.
Lugtenberg, B., Kamilova, F., 2009. Plant-growth-promoting rhizobacteria. Annu. Rev. Microbiol. 63, 541–556.
Masih, E.I., Deschaumes, S., Marmaras, I., Barka, E.A., Vernet, G., Charpentier, C., Adholeya, A., Paul, B., 2001. Characterization of the yeast *Pichia membranifaciens* and its possible use in the biological control of *Botrytis cinerea* causing the grey mould disease of grapevine. FEMS Microbiol. Lett. 202, 227–232.
Mavrodi, O.V., McSpadden, Gardener, B.B., Mavrodi, D.V., Bonsall, R.F., Weller, D.M., Thomashow, L.S., 2001. Genetic diversity of *phlD* from 2,4-diacetylphloroglucinol-producing fluorescent *Pseudomonas* spp. Biol. Control 91, 35–43.
Michelsen, C.F., Stougaard, P., 2012. Hydrogen cyanide synthesis and antifungal activity of the biocontrol strain *Pseudomonas fluorescens* In5 from Greenland is highly dependent on growth medium. Can. J. Microbiol. 58, 381–390.
Müller, T., Behrendt, U., Ruppel, S., von der Waydbrink, G., Müller, M.E.H., 2016. Fluorescent *Pseudomonads* in the phyllosphere of wheat: potential antagonists against fungal phytopathogens. Curr. Microbiol. 72, 383–389.
Murashige, T., Skoog, F., 1962. Revised medium for rapid growth and bioassays with tobacco cultures. Physiol. Plant. 15, 437–479.
Muyzer, G., De Waal, E.C., Uitierlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Am. Soc. Microbiol. 59, 695–700.
Nabti, E., Bensidhoum, L., Tabli, N., Dahel, D., Weiss, A., Rothballer, M., Schmid, M., Hartmann, A., 2014. Growth stimulation of barley and biocontrol effect onplant pathogenic fungi by a *Cellulosimicrobium* sp. strain isolated fromsalt-affected rhizosphere soil in northwestern Algeria. Eur. J. Soil Biol. 61, 20–26.
Nikel, P.I., Martínez-García, E., de Lorenzo, V., 2014. Biotechnological domestication of pseudomonads using synthetic biology. Nat. Rev. Microbiol. 12, 368–379.
Nowak-Thompson, B., Gould, S.J., Kraus, J., Loper, J.E., 1994. Production of 2,4-diacetylphloroglucinol by the biocontrol agent *Pseudomonas fluorescens* Pf-5. Can. J. Microbiol. 40, 1064–1066.
Olorunleke, F.E., Kieu, N.P., Höfte, M., 2015. Recent advances in *Pseudomonas* biocontrol. In: Murillo, J., Vinatzer, B.A., Jackson, R.W., Arnold, D.L. (Eds.), Bacterial-Plant Interactions: Advance Research and Future Trends. Caister Academic Press, Norfolk, pp. 167–198.
Penrose, D.M., Glick, B.R., 2003. Methods for isolating and characterizing ACC-deaminase-containing plant growth-promoting rhizobacteria. Physiol. Plantarum 118, 10.
Philmus, B.J., Shaffer, B.T., Kidarsa, T.A., Yan, Q., Raaijmakers, J.M., Begley, T.P., Loper, J.E., 2015. Investigations into the biosynthesis, regulation and self-resistance of toxoflavin in *Pseudomonas protegens* Pf-5. Chembiochem 16, 1782–1790.
Polano, C., Martini, M., Savian, F., Moruzzi, S., Ermacora, P., Firrao, G., 2018. Genome sequence and antifungal activity of two niche-sharing *Pseudomonas protegens* related strains isolated from hydroponics. Microbiol. Ecol. 76, 1–11.
Quecine, M.C., Araújo, W.L., Rossetto, P.B., Ferreira, A., Tsui, S., Lacava, P.T., Mondin, M., Azevedo, J.L., Pizzirani-Kleinera, A.A., 2012. Sugarcane growth promotionby the endophytic bacterium *Pantoea agglomerans* 33.1. Appl. Environ. Microbiol. 78, 7511–7518.
Quecine, M.C., Kidarsa, T.A., Goebel, N.C., Shaffer, B.T., Henkels, M.D., Zabriskie, T.M., Loper, J.E., 2015. An interspecies signaling system mediated by fusaric acid has parallel effects on antifungal metabolite production by *Pseudomonas protegens* strain Pf-5 and antibiosis of *Fusarium* spp. Appl. Environ. Microbiol. 82, 1372–1382.
Raaijmakers, J.M., Mazzola, M., 2012. Diversity and natural functions of antibiotics produced by beneficial and plant pathogenic bacteria. Annu. Rev. Phytopathol. 50, 403–424.
Ramette, A., Frapolli, M., Fischer-Le Saux, M., Gruffaz, C., Meyer, J.-M., Défago, G., Sutra,

L., Moënne-Loccoz, Y., 2011. *Pseudomonas protegens* sp. nov., widespread plant-protecting bacteria producing the biocontrol compounds 2,4-diacetylphloroglucinol and pyoluteorin. Syst. Appl. Microbiol. 34, 180–188.
Rolli, E., Marasco, R., Saderi, S., Corretto, E., Mapelli, F., Cherif, A., Borin, S., Valenti, L., Sorlini, C., Daffonchio, D., 2017. Root-associated bacteria promote grapevine growth: from the laboratory to the field. Plant Soil 410, 369–382.
Sabir, A., Yazici, M.A., Kara, Z., Sahin, F., 2012. Growth and mineral acquisition response of grapevine rootstocks (*Vitis* spp.) to inoculation with different strains of plant growth-promoting rhizobacteria (PGPR). J. Sci. Food Agric. 92, 2148–2153.
Saini, S., Verma, A., Kumar, A., Prakash, A., Sharma, S.K., Ramesh, A., Johri, B.N., 2016. Identification and characterization of antifungal metabolite producing *Pseudomonas protegens* strain BNJ-SS-45 isolated from rhizosphere of wheat crop (*Triticum aestivum* L.). Int. J. Appl. Pure Sci. Agric. 2, 69–76.
Salomon, M.V., Bottini, R., Apolinário de Souza Filho, G., Cohen, A.C., Moreno, D., Gil, M., Piccoli, P., 2014. Bacteria isolated from roots and rhizosphere of *Vitis vinifera* retard water losses, induce abscisic acid accumulation and synthesis of defense-related terpenes in in vitro cultured grapevine. Physiol. Plant. 151, 359–374.
Sandhya, V., Ali, Sk.Z., Grover, M., Reddy, G., Venkateswarlu, B., 2010. Effect of plant growth promoting *Pseudomonas* spp. on compatible solutes, antioxidant status and plant growth of maize under drought stress. Plant Growth Regul. 2010 (62), 21–30.
Sang, M.K., Kim, K.D., 2014. Biocontrol activity and root colonization by *Pseudomonas corrugata* strains CCR04 and CCR80 against Phytophthora blight of pepper. BioControl 59, 437–448.
Sawana, A., Adeolu, M., Gupta, R.S., 2014. Molecular signatures and phylogenomic analysis of the genus *Burkholderia*: proposal for division of this genus into the emended genus*Burkholderia* containing pathogenic organisms and a new genus *Paraburkholderia* gen. nov. harboring environmental species. Front. Genet. 5, 429.
Schwyn, B., Neilands, J.B., 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. 160, 47–56.
Schulz, B., Boyle, C., 2006. What are endophytes? In: Schulz, B., Boyle, C., Sieber, T.N. (Eds.), Microbial Root Endophytes. Springer, Berlin, Heidelberg, pp. 1–13.
Senthil, R., Prabakar, K., Rajendran, L., Karthikeyan, G., 2011. Efficacy of different biological control agents against major postharvest pathogens of grapes under room temperature storage conditions. Phytopathol. Mediterr. 50, 55–65.
Serra, R., Braga, A., Venancio, A., 2005. Mycotoxin-producing and other fungi isolated from grapes for wine production, with particular emphasis on ochratoxin A. Res. Microbiol. 156, 515–521.
Silby, M.W., Winstanley, C., Godfrey, S.A., Levy, S.B., Jackson, R.W., 2011. *Pseudomonas* genomes: diverse and adaptable. FEMS Microbiol. Rev. 35, 652–680.
Subramanian, P., Kim, K., Krishnamoorthy, R., Sundaram, S., Sa, T., 2015. Endophytic bacteria improve nodule function and plant nitrogen in soybean on co-inoculation with *Bradyrhizobium japonicum* MN110. Plant Growth Regul. 76, 327–332.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.
Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTALX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.
Úrbez-Torres, J.R., Haag, P., Bowen, P., O'Gorman, D.T., 2014. Grapevine trunk in British Columbia: incidence and characterization of the fungal pathogens associated with Esca and petri diseases of grapevine. Plant Dis. 98, 469–482.
Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697–703.
Wicaksono, W.D., Jones, E.E., Monk, J., Ridgway, H.J., 2017. Using bacterial endophytes from a New Zealand native medicinal plant for control of grapevine trunk diseases. Biol. Control 114, 65–72.
Wu, Y.-N., Feng, Y.-L., Paré, P.W., Chen, Y.-L., Xu, R., Wu, S., Wang, S.M., Zhao, Q., Li, H.-R., Wang, Y.-Q., Zhang, J.-L., 2016. Beneficial soil microbe promotes seed germination, plant growth and photosynthesis in herbal crop Codonopsis pilosula. Crop Pasture Sci. 67, 91–98.
Zhang, X., Zhou, Y., Li, Y., Fu, X., Wang, Q., 2017. Screening and characterization of endophytic Bacillus for biocontrol of grapevine downy mildew. Crop Prot. 96, 173–179.