



Competition assays revealed *Paenibacillus pasadenensis* strain R16 as a novel antifungal agent



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ABSTRACT

The development of new sustainable containment strategies of plant diseases is very important to guarantee food security while reducing the environmental impact of agriculture. Research of new biocontrol agents is a long and difficult process that involves several steps that start from the identification of possible candidates which, for example, show antibiotic activities, and ends with in field, large scale trials. In this study, the plant growth promoting potential and antifungal effect exerted by a novel, putative candidate biocontrol agent, strain R16, identified as *Paenibacillus pasadenensis* by sequence analysis of *16S rRNA* and *rpoB* genes, against three important plant pathogenic fungi (*Botrytis cinerea*, *Fusarium verticillioides*, and *Phomopsis viticola*), were assessed. Biochemical assays to determine plant growth promoting potential gave negative results for siderophore production and phosphate solubilization, and positive results for ACC-deamination and IAA production. Further biochemical assays for endophytic lifestyle and antifungal activity gave positive results for catalase and chitinase activity, respectively. *In vitro* antagonism assays showed that strain R16 is effective against *B. cinerea*, reducing mycelial growth both in dual-culture and through volatile substances, characterized to be mostly composed by farnesol, and inhibiting conidia germination. Good antagonistic potential was also observed *in vitro* towards *P. viticola*, but not towards *F. verticillioides*. Moreover, *in vivo* assays confirmed the strain R16 activity reduced the infection rate on *B. cinerea*-inoculated berries. The obtained results firstly proved that *P. pasadenensis* strain R16 is a putative plant growth promoter and effective against phytopathogenic fungi. Further studies will be needed to investigate the possible application of this strain as a biocontrol agent.

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1. INTRODUCTION

Plant diseases, due to their high impact on yield losses, cause severe economic damage and threaten both food safety and security. Containment of diseases is traditionally achieved through the use of chemical pesticides though, in recent years, the need to develop alternative strategies to control plant diseases has become evident (Siroli et al., 2015). This because of the growing concerns about the environmental problems related to these

Abbreviations: BC, *Botrytis cinerea* strain MG53; FV, *Fusarium verticillioides* strain GV2245; PV, *Phomopsis viticola* strain PV1; TGYA, Tryptone-Glucose-Yeast Extract-Agar medium; dpi, days post inoculation; GIP, Growth inhibition percentage; GIPv, Growth inhibition percentage determined by volatile compounds; GIPf, Growth inhibition percentage determined from the cultural filtrate; ISP, Inhibition of spore production; GR, Conidial germination rate; C%I, Percentage colonization index.

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molecules (Pimentel 2005) and the development of resistance to pesticides in pathogen populations (Sierotzki and Scalliet 2013). Among these, biological control is surely one of the most promising to either stimulate the plant's natural defenses or directly antagonize the pathogens (Lugtenberg and Kamilova 2009). While microbial biocontrol agents are commercially available and find use in agriculture (Lagerlof et al., 2015), the necessity of acting against a wide range of diseases on many different crops grown in variable environmental conditions urge researchers to continue looking for effective and novel biocontrol agents. Biocontrol agents must show a high ability in suppressing diseases of its host, while possessing some technical properties, such as ease of formulation, ability to effectively colonize the host, survival fitness in the agricultural environment, and not being pathogenic for non-target organisms (Bashan et al., 2013). In recent years, the study on the mechanisms through which bacteria interact with plant hosts and pathogens highlighted the great importance of volatile molecules, which can

have a role in signaling as well as a direct biological action against pathogens (Ping and Boland 2004; Sharifi and Ryu 2016).

Among non-pathogenic microorganisms associated with plants, particular attention should be paid to beneficial endophytic bacteria. These bacteria are stably associated with a host plant and can grant it various benefits, including a higher growth rate and, in many cases, an induction of systemic defense mechanisms (Hallmann et al., 1997; Kloepper et al., 2004).

In order to characterize biocontrol bacteria that can have an actual use on a specific crop, it is helpful to look at bacteria naturally present in association to that plant's organs, as those bacteria are already adapted to living and competing within the plant system of interest. Native endophytic bacteria are more likely to offer better control of some diseases and promotion of growth compared to organisms isolated from other plant species (Long et al., 2008).

It is important to note that a plant's ability to select microorganisms goes beyond a simple species level, and many different factors can influence the microbiome, such as the development stage (Andreote et al., 2010) and both biotic (Bulgari et al., 2014) and abiotic (Compant et al., 2010) stress conditions of the host plant. Relationships between pathogens and hosts have been recently proved to affect the whole microbiome of the infected plant. For example a loss of diversity and enrichment in some species associated with citrus roots after the infection with 'Candidatus Liberibacter asiaticus', a phloem-colonizing pathogen causing the Huanglongbing disease, have been reported (Trivedi et al., 2010). These results has led to the hypothesis that, as the presence of the pathogen can influence the microbiome, certain elements of the microbiome can be suppressive to the pathogen, being intrinsically related to the sanitary condition of the host plant. Such assumption is supported by evidence from other studies highlighting differences in microbiome of grapevine plants that were either healthy, infected, or underwent natural recovery from 'Candidatus Phytoplasma' strain (subgroup 16SrV-D) associated with Flavescence dorée disease. In particular, *Paenibacillus pasadenensis* and other endophytic bacteria have been detected exclusively in healthy and recovered plants, and not in diseased ones, hinting at a possible involvement of such microorganisms in the presence or absence of the pathogen (Bulgari et al., 2011, 2014).

P. pasadenensis as a species was first described in 2006, isolated from a spacecraft assembly facility near Pasadena, the place of isolation giving the species both its proper name, and the nickname of 'spacecraft bacterium' (Osman et al., 2006). Despite having been known for some years, data on this species are very few: *P. pasadenensis* is known as a Gram-positive, rod shaped, sporulating, slightly halophytic (3% salt concentration) soil bacteria. Interestingly, an alkaline chitinase purified from the *P. pasadenensis* strain NCIM 5434 showed efficient activity against pathogenic fungi such as *Aspergillus* spp. and *Penicillium* spp., reinforcing the idea that *P. pasadenensis* could act as a natural antifungal agent in plants (Loni et al., 2014), like other species of this genus (Grady et al., 2016).

In our study, we assessed the potential plant growth promoting effect and antifungal activity against three strains of important phytopathogens (*Botrytis cinerea*, *Fusarium verticillioides*, and *Phomopsis viticola*) exerted by *P. pasadenensis* strain R16 through *in vitro* and *in vivo* assays, constituting a first step in the assessment of this strain as a possible novel biocontrol candidate.

B. cinerea is a fungal pathogen that can be associated with several crops. Due to the broad host range on which *B. cinerea* can cause gray mold disease both pre- and post-harvest, this fungus is one of the most studied phytopathogenic fungi (Romanazzi et al., 2015). *P. viticola* is a fungal pathogen associated with diseases of grapevine, such as Phomopsis dieback and Phomopsis cane and leaf spot disease, the latter of which is reported to cause crop loss up to 30% (Urbez-Torres et al., 2013). *F. verticillioides* mainly colonizes maize and, in general, is associated with several diseases including stalk,

kernel, and ear rots known as fusarium rots (Leslie and Summerell 2006) but it may occur on maize as a kernel derived endophyte or infect the plant at various development stages (Venturini et al., 2011).

Based on the obtained results, we propose for the first time, to the best of our knowledge, *P. pasadenensis*, a putative endophytic bacterium, as possessing traits associated with plant growth promotion, as well as exerting an antifungal activity probably mediated by a mechanism that, if confirmed, would be firstly reported for a bacterial antifungal agent.

2. MATERIALS AND METHODS

2.1. Bacterial strain and cultivation

P. pasadenensis strain R16, previously described as 16R by Bulgari et al. (2011), was isolated from a leaf sample collected in July 2009 from a grapevine plant (*Vitis vinifera*, Barbera variety) in the 'Oltrepò Pavese' area of northern Italy. The plant was previously reported to be infected by the phytoplasma associated with Flavescence dorée disease (Quaglino et al., 2010) and a phenomenon of natural recovery was observed for several years before sampling. The strain was then cultivated on LB High Salt agar plates at 25 °C, and was stored in a 20% glycerol solution at –80 °C for long conservation periods.

To confirm the preliminary attribution of strain R16 to the *P. pasadenensis* species we sequenced a longer fragment of the 16S rRNA gene using the 27F/1495R primer pair (Lane, 1991), and a fragment of the highly conserved *rpoB* gene (encoding the β subunit of bacterial RNA polymerase) using the rpoB1206/rpoBR3202 primer pair (Ki et al., 2009). The reactions were carried out as previously described by Bulgari et al. (2009) and by Ki et al. (2009), respectively.

The DNA used for PCR assays was obtained from pure fresh culture of the strain R16 using the GenElute Bacterial Genomic DNA Kit (SIGMA), following the manufacturer's instructions adjusted for the extraction of Gram+ bacteria. The PCR products were sequenced by a commercial service (Eurofins, Italy), obtaining at least 5x coverage for each base. Nucleotide sequence data was assembled using the Bioedit software, version 7.2.5. (Hall, 1999). Sequences were compared with the GenBank database and search for the best identity was carried out using the software BlastN (<http://www.ncbi.nlm.nih.gov/BLAST/>).

2.2. Biochemical characterization of Plant Growth Promoting (PGP)-related traits of the strain R16

The following PGP-related biochemical characteristics of the strain R16 were assayed: (i) Phosphate solubilization, using Pikovskaya medium (Pikovskaya 1948) with insoluble Ca₃(PO₄)₂ added (5 g/liter); (ii) chitinase production, using the medium reported by Sridevi and Mallaiah (2008); (iii) siderophore production, using CAS agar medium as described by Alexander and Zuberer (1991); (iv) ACC-deaminase activity, using minimal DF medium (Dowrkin and Foster, 1958) prepared as described by Hunsen et al. (2009); (v) catalase activity, tested by dripping 3% hydrogen peroxide directly on the bacterial colonies; (vi) production of indole acetic acid, using the method described by Pilet and Chollet (1970).

2.3. Fungal strains and cultivation

Three fungal strains were used for the evaluation of antifungal activity of the bacterial strain R16 (i) *Botrytis cinerea* Pers. [MG53] isolated from wheat kernel in 2014, which will be referred to as BC in the rest of the paper; (ii) *Phomopsis viticola* (Sacc.) Sacc. [PV1] isolated from grapevine berry in 2012, which will be referred to

as PV in the rest of the paper; (iii) *Fusarium verticillioides* (Sacc.) Nirenberg [GV2245] isolated from maize ear in 2011, which will be referred to as FV in the rest of the paper. All the fungal strains were conserved in the fungal culture collection of the Mycology Laboratory at the Department of Agricultural and Environmental Sciences (DiSAA), University of Milan, Italy. The isolates were maintained on potato dextrose agar (PDA) at 4 °C.

2.4. Direct in vitro antagonism assays

Antagonism studies by dual-culture plate method were carried out on Tryptone Glucose Yeast Extract Agar (TGYA; containing 5 g/liter tryptone, 1 g/liter glucose, 3 g/liter yeast extract, 15 g/liter agar) plates, as the bacterial strain had difficulties growing on PDA medium, and the fungal strain had difficulties growing on LB medium. On each plate four 0.5 cm discs of sterilized filter paper were placed at approximately 4 cm from the center of the plate and evenly distributed on its surface. On each disc was placed a 20 µl drop of overnight strain R16 culture in LB broth. Liquid culture was obtained by adding 1 ml from a pre-culture of strain R16 (3 ml of LB broth inoculated with a single colony of strain R16 at 25 °C overnight) to 100 ml of LB broth in a 500 ml conical flask, incubated at 25 °C with constant shaking at 180 rpm. Based on growth kinetic of the strain in these conditions, we calculated that each 20 µl drop contain 2×10^5 CFUs. To test the difference in response over time, on separate plates the bacteria were inoculated 2 (T2), 1 (T1), and 0 (T0) days ahead of the fungal inoculation and incubated at 25 °C until the fungi were inoculated. The inoculum was a 0.5 cm in diameter mycelial plug excised with a sterilized cork borer from the edges of an actively growing fungal culture placed in the middle of the TGYA plate. As negative control, plates containing (i) the fungal strains alone, (ii) the fungi with blank sterilized filter paper discs, and (iii) the fungi and discs inoculated with 20 µl of sterilized LB broth were used.

Furthermore, to determine the presence or absence of an inhibition halo, we conducted an additional assay in which a single strain R16 colony was streaked across one half of a TGYA plate and, after 2 days of incubation at 25 °C, a fungal mycelial plug was inoculated in the other half of the plate. After fungal inoculations, all the plates were incubated at 25 °C in the dark. Fungal growth was measured 5, 7, and 14 days post inoculation (dpi) as mycelial growth diameter. Each test was carried out with plates in triplicate and three independent measures were made for each plate at each measuring time. Growth inhibition percentage (GIP) was calculated as $[1 - (D1/D2)] \times 100$, where D1 is the radial colony growth on bacteria-treated plate, D2 is the radial colony growth in the control plate.

2.5. Inhibition of fungal mycelial growth by volatile compounds

To evaluate strain R16's ability to inhibit fungal growth through the production of volatile compounds, a dual-plate assay was carried out (Chaurasia et al., 2004). Briefly, 100 µl of overnight strain R16 culture in LB broth (10^7 CFU/ml) was diffused on the surface of a TGYA plate and then, incubated overnight at 25 °C. After two days, a 0.5 cm in diameter, fungal mycelial plug was inoculated onto another TGYA plate, then the cover of the plate with the bacteria was replaced with the upturned plate containing the fungal cultures in sterile conditions, the plates were sealed together with Parafilm. After fungal inoculation, all the plates were kept at 25 °C in the dark and the fungal growth was measured 14 dpi. Each test was made with plates in triplicate and three independent measures were made for each plate. Growth inhibition percentage determined by volatile compounds (GIPv) was calculated as previously described.

2.6. Inhibition of fungal mycelial growth by cultural filtrate

To evaluate strain R16's ability to inhibit fungal growth through non-volatile compounds secreted in the growth medium, an assay was carried out by preparing TGYA plates added with different concentrations of strain R16 culture, previously purified from all cells, and inoculating the fungi on these plates. The liquid culture was obtained as previously described, however, TGY medium was used, in a final volume of 500 ml. After reaching late stationary phase (approximately 24 hours), the growth medium was centrifuged at 6800x g for 10 minutes to remove bacterial cells by pelleting. The supernatant was then vacuum filtered and the obtained suspension was used to prepare TGYA plates, with a final concentration of the strain R16 cultural filtrate being 25%, 50%, 75%, or 100% for each plate. To control the sterility of these plates, they were incubated at 25 °C overnight to check for bacterial growth. After the preparation of the cultural filtrate plates, the fungi were inoculated under sterile conditions. The inoculum was a 0.5 cm in diameter mycelial plug excised with a sterilized cork borer from the edges of an actively growing fungal culture, placed in the middle of the TGYA plate. As negative control, conventional TGYA plates containing the fungal inoculum were used. After fungal inoculations, all the plates were incubated at 25 °C in the dark. Fungal growth was measured 14 dpi as mycelial growth diameter. Each test was carried out with plates in triplicate and three independent measures were made for each plate. Growth inhibition percentage from cultural filtrate (GIPf) was calculated as described previously.

2.7. Characterization of Volatiles Organic Compounds (VOCs) produced by strain R16

Analysis of VOCs produced by strain R16 was carried out using divided Petri dishes with both halves filled with TGYA medium. In each half either strain R16, a pathogenic fungus (BC, FV, or PV) or nothing was inoculated, obtaining the following combinations: (i) plates in which only strain R16 was growing, (ii) plates in which only the fungus was growing, (iii) plates in which both strain R16 and the fungus were growing, and (iv) plates with only TGYA medium, to be used as negative controls. The plates, prepared in triplicates, were sealed with Parafilm® (Pechiney Plastic Packaging Co., Chicago, IL, USA) for minimizing volatile exchanges, and cultured for 14 days before VOCs analysis, using the method described by Lo Cantore et al. in 2015. In order to avoid the loss of volatiles and to keep the plates sealed for the full 14 days period, bacteria and fungi were inoculated on the plate at the same day in this assay, unlike what reported for the inhibition assay. VOCs from negative control plates were collected to provide a blank for the analysis. The analysis was performed using the Solid-Phase-Micro-Extraction technique followed by Gas Chromatography-Mass Spectrometry (SPME-GC-MS). VOCs were adsorbed on a Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) fiber and desorbed at 260 °C in the injection port of an Agilent Technologies 6890N/5973N gas chromatograph-mass spectrometer equipped with a 60 m × 0.25 mm × 0.25 µm 100% polyethylene glycol column (Zebtron ZB-WAX plus, Phenomenex). Helium was used as the carrier gas in constant pressure mode (150 kPa). The oven was programmed at 45 °C (5-min hold) and ramped up to 219 °C at 6 °C/min (16-min hold). The transfer line to the mass spectrometer was maintained at 280 °C, while the ion source was 230 °C, and the quadrupole was 150 °C. Acquisition was performed in electronic impact mode. VOCs were identified using the Wiley 7n-1 MS library on Agilent MSD ChemStation software (Agilent Technologies Inc.). Confirmation of the identity of the volatile compounds was achieved by comparing the GC retention indices and mass spectra of individual components with those of authentic reference compounds (Sigma-Aldrich Co.) under the

same operating conditions. The data collected refer to the peak area of the quant ion of each compound, and are expressed as a natural logarithm, for this reason the determination must be considered semi-quantitative. Compounds were considered “absent” (i.e. not produced) when the signal to noise ratio was below 2:1. To check the presence of carry-over effects, blank extractions were conducted regularly.

2.8. Production of spores in antagonized BC cultures

In order to evaluate the strain R16 ability to reduce the conidial formation in BC cultures, conidia were collected from one arbitrarily selected BC plates from each treatment (T2, T1, and T0) of the direct *in vitro* antagonism assays at 14 dpi. The plates were flooded with 10 ml of sterile 0.01% polysorbitan Tween® 20 aqueous solution, and the surface was gently scraped with a sterile loop in order to detach all the aerial mycelium and conidia. Mycelium was removed by filtering the suspension on a double layer of sterile gauze. The spore concentration in the suspensions was estimated through direct count of the conidia through an optical microscope (20X; EasyLab CX40, Olympus) using a Kova counting grid (Hycor Biomedical Inc., Garden Grove, California, USA) following appropriate dilutions. Spore production (SP) was calculated as presented by Fernandez-Ortuno et al. in 2013. The inhibition of spore production (ISP) was calculated according to the following formula SPo^*100 .

2.9. Inhibition of BC conidia germination

The strain R16's ability to reduce the germination of conidia produced by BC was evaluated. In this assay, solutions of freshly harvested conidia, with a final concentration of 10^4 conidia/ml, were tested for germination in TGY medium either alone, or in the presence of the strain. The bacterial strain R16 was added at a final concentration of approximately 10^5 CFU/ml, and the assay was carried out in 2 ml tubes, using a total volume of 1 ml. Both the treated samples and non-treated controls were set up in triplicates. The tubes were incubated for 72 hours on an orbital shaker, kept in the dark at a temperature of 20 °C, and germination rates were measured in triplicate after 6, 18, 24, 48, and 72 hours of co-culture. Germination was evaluated by direct observation under an optical microscope (20X; EasyLab CX40, Olympus) using a Kova counting grid, considering each spore to have germinated if the length of germination tube was twice as long as the conidium diameter (Chen et al., 2008). For each observation, 100 spores were visually analyzed and determined to be either germinated or non-germinated. Conidial germination rate (GR) was calculated as $(G/C) \times 100$, where G is the number of germinated conidia detected, and C is the total number of conidia counted. The visual examination was also confirmed with the spectrophotometry method reported by Raposo et al. in 1995 (data not shown).

2.10. In vivo BC antagonism assay

For the *in vivo* test of antifungal activity, ripe and healthy table grapes of Black Magic variety, obtained from organic farming and purchased in a local grocery, were used. Detached berries of uniform size and free from visible wounds or rots were surface sterilized in a solution containing 80 ml of NaOCl (7%), 100 ml of ethanol (96%) and 820 ml of sterile distilled water, for 5 minutes, rinsed in sterile water three times, and dried on sterile filter paper under sterile laminar flow hood. After drying, 4 wounds were made on the equatorial area of each berry using a sterile needle. Bacterial strain R16 were inoculated on the berry by soaking in a bacterial suspension (approximately 10^8 CFUs/ml) for 12 seconds and then left to dry on sterile filter paper under sterile laminar flow hood. BC conidia were inoculated by applying a 20 μ l drop of a conidial sus-

pension (5×10^5 conidia/ml) into each wound. For each treatment (non-treated, bacterial strain R16 only, BC only, bacterial strain R16 and BC) 10 berries were put on a sterile ceramic tray in a sterile glass chamber, containing a wet piece of sterile filter paper to maintain relative humidity of 95% inside the chamber, and incubated at 20 °C in the dark. Each treatment was carried out in triplicates. Fruits were visually evaluated to determine fungal colonization of the berries at 5 days after the inoculation. The results were expressed as visual classes as according to the scale presented in a previous work (Vercesi et al., 2014). Visual classes were transformed in a percentage colonization index (C%) according to the formula proposed by Townsend and Heuberger (1943).

2.11. Statistical analyses

The SPSS statistical package for Windows, v. 22.0 (SPSS Inc.), was used for all statistical analyses. Normal distribution and homogeneity of variances were verified using the Shapiro–Wilk test and the Levene's test, respectively. GIP, GIPv, GIPf, volatile production, ISP, GR, and C% did not meet the requirements for parametric tests (Conover and Iman, 1981), thus data were analyzed according to the Kruskal–Wallis non-parametric one-way analysis of variance (for GIP, GIPv, ISP, and some volatile compound abundance) and to Mann–Whitney test for the pairwise comparisons (for GR, C%, and some volatile compound abundance).

3. RESULTS

3.1. Species determination

BlastN analyses showed that nucleotide sequences of 16S rRNA and *rpoB* genes of the strain R16 shared 99% of identity (e-value=0.00) with *P. pasadenensis* strain NBRC 101214 [Accession number NR 113987 (16S rDNA) and HQ596205 (*rpoB*)]. The partial nucleotide sequences of 16S rRNA and *rpoB* genes were deposited in GenBank under the following Accession Numbers, respectively: KX161756 and KX161757.

3.2. Biochemical characterization of PGP-related traits of strain R16

Strain R16 gave negative results to the phosphate solubilization and siderophore production tests. The strain gave positive results in the tests for the production of chitinase, catalase, ACC-deaminase, and IAA.

3.3. Direct in vitro antagonism

The results, reported numerically in Table 1 and visually in Fig. 1, show that strain R16 effectively hindered the growth of BC and PV strains but was ineffective against FV, where the presence of the bacterium caused morphological differences when compared to the non-treated control, in particular mycelium becoming less compact and gaining a flaky appearance especially in the near proximity of bacterial colonies, but no actual growth inhibition (Fig. 1: I-L). The effect of inhibition, when present, was either stable or increasing with time in all cases, with the exception of the assay against BC with the T1 treatment. The assays that gave best results, such as BC with T2 treatment (GIP = 100) and PV with T2 treatment (GIP = 83), yielded these high results already from the first measurement at 5 dpi, and the effect persisted throughout the whole experiment. In general, the inhibitory effect of strain R16 was more effective against BC than against PV in all treatments, at each measurement time. Against FV only minimal inhibition effects were registered at 5 dpi and the fungus achieved normal growth at 7 and 14 dpi, show-

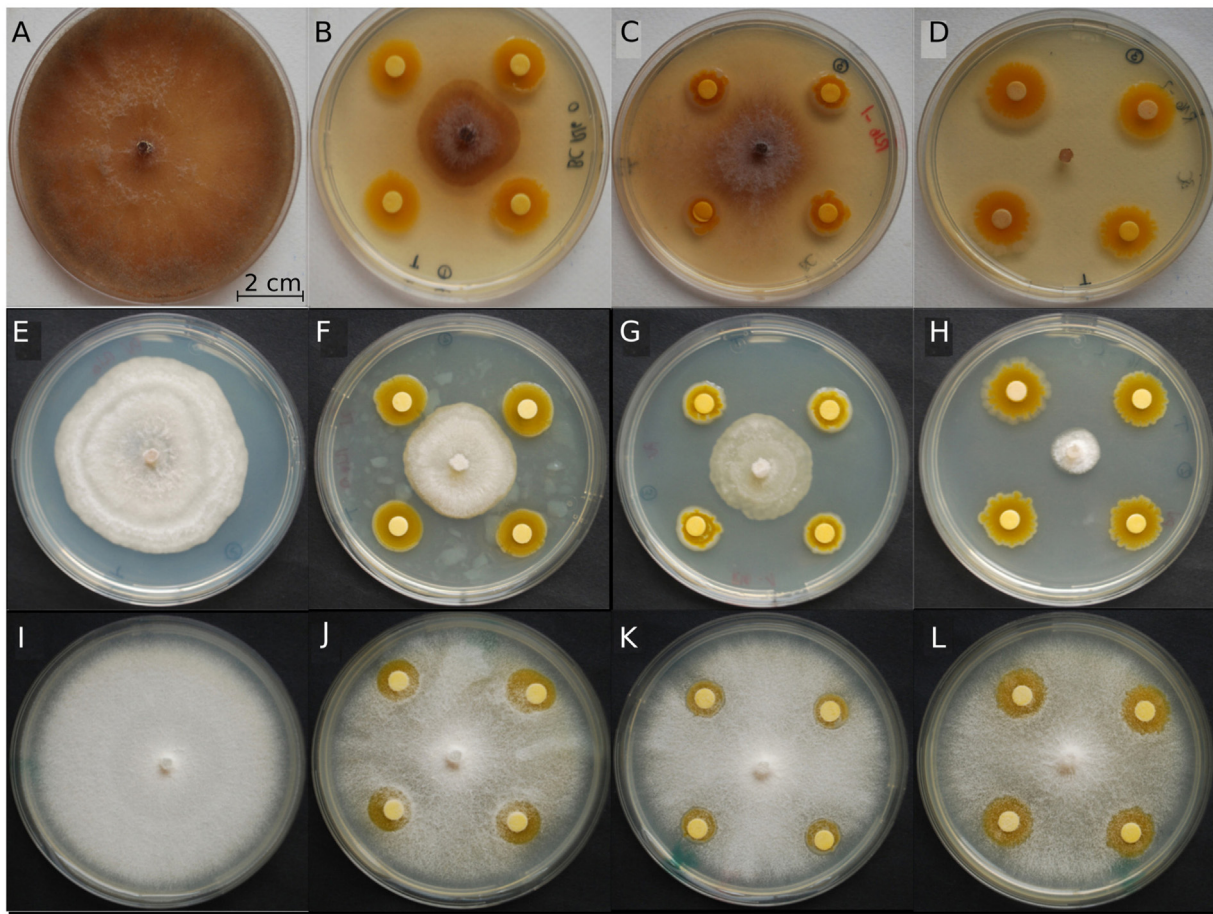


Fig. 1. Comparison between dual-culture treatments with strain R16 and a phytopathogenic fungus. Photographs of one representative plate per treatment, taken at 14 days post inoculation, are reported. From left to right are presented: non-treated control (A, E, I), T0 treatment (strain R16 inoculated the same day as the fungi) (B, F, J), T1 treatment (strain R16 inoculated 1 day ahead of the fungi) (C, G, K), and T2 treatment (strain R16 inoculated 2 days ahead of the fungi) (D, H, L). From top to bottom are presented: BC (A to D), PV (E to H), and FV (I to L).

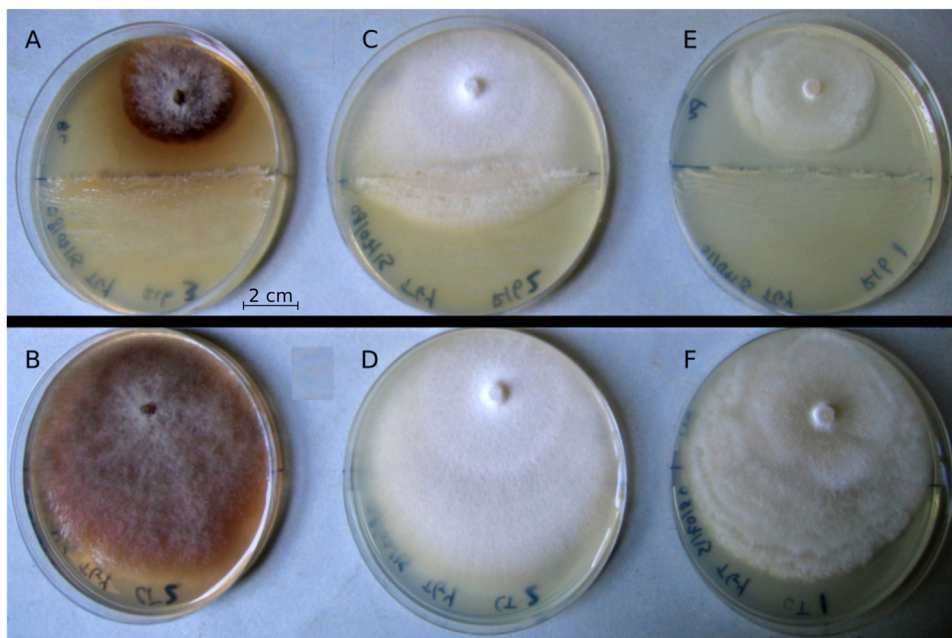


Fig. 2. Comparison between non-treated controls and strain R16 inoculated plates in inhibition halo dual-culture assays at 14 days post inoculation. The picture reports BC growing in the presence (A) or absence (B) of strain R16; FV growing in the presence (C) or absence (D) of strain R16; and PV growing in the presence (E) or absence (F) of strain R16.

TABLE 1

Results of dual-culture antagonism assays. For each of the pathogenic fungi are reported the results of mycelial growth inhibition percentage (GIP) measured at 5, 7, and 14 days post inoculation (d.p.i.). The results are organized in columns according to the treatment they refer to: T0 (strain R16 inoculated the same day as the fungi), T1 (strain R16 inoculated 1 day ahead of the fungi), or T2 (strain R16 inoculated 2 days ahead of the fungi). P values obtained with Kruskal-Wallis analysis are reported for the statistical comparison between the results obtained for each treatment against each fungus, at 5, 7, and 14 d.p.i. Significant difference is shown by different letters (a, b) beside significantly different results. In the column headed inhibition halo are reported the average values measured of minimum distance between strain R16 colonies and fungal mycelium in dual-culture assays.

Fungal strain	Measurement	T0	T1	T2	Inhibition Halo (cm)
<i>B. cinerea</i>	GIP 5 d.p.i.	74	68 a	100	1.3 ± 0.05
	MG53	77	64 a	100	
	BC	77	42 b	100	
	P value	0.793	0.033	–	
<i>F. verticillioides</i>	GIP 5 d.p.i.	10	7	17	0
	GV2245	0	0	0	
	FV	0	0	0	
	P value	–	–	–	
<i>P. viticola</i>	GIP 5 d.p.i.	35 a	34 a	78 a	0.5 ± 0.01
	PV1	47 b	48 b	82 b	
	PV	47 b	50 b	83 b	
	P value	≤0.001	0.003	≤0.001	

ing that strain R16 had no effect against this fungus. The assay to determine the formation of an inhibition halo gave positive results for BC and PV (Table 1) (Fig. 2: A, B, E, F). While no inhibition halo was registered for FV, it is noticeable that in these conditions the bacteria managed to reduce the growth of the fungus (Fig. 2: C, D).

3.4. Inhibition by volatile compounds

The results, reported in Fig. 3, show the effects of inhibition obtained in dual-plate assays. As with direct antagonism, strain R16 was effective against BC and PV, with a peak GIPv values of 71.3 and 39.3 respectively, while it was ineffective against FV. As with dual-culture assay, the effect against BC was higher than the inhibition against PV. It is interesting to notice that, while against BC the average inhibition caused by volatile molecules was of 61.5% compared to the 100% obtained in dual-culture, against PV the volatile substances were far less efficient, giving an average of 27.9% compared to the 83% registered in dual-culture. For FV the average GIPv value was equal to 4%, but showed great variability between the replicates including plates in which there was no inhibition, compatibly with the 0% of GIP seen in dual-culture assays.

3.5. Inhibition of fungal mycelial growth by cultural filtrate

The results, reported in Table 2, show that the cultural filtrate of strain R16 had little effect in inhibiting the growth of the tested fungi. The plates produced with the use of cultural filtrate showed no residual bacterial growth, indicating that the used protocol was efficient in removing bacterial cells from the medium (data not shown). Similarly to the dual-culture assay, the cultural filtrate from strain R16 did not manage to reduce the growth of FV (GIPf=0). Unlike in the dual-culture assay (GIP=83), the cultural filtrate from strain R16 had no effect on the growth of PV (GIPf=0). Instead, BC still had a reduced growth when confronted with a 100% cultural filtrate TGYA plate (GIPf=29), but the effect was greatly reduced when compared to that in dual-culture assay (GIP=100).

3.6. Characterization of volatiles produced by strain R16

The Total Ion Chromatograms of TGYA medium incubated alone (iv), the fungus incubated alone (i), strain R16 growing alone (ii), and both microorganisms incubated together (iii), were compared. TGYA medium alone (iv) gave production of isovaleraldehyde and benzaldehyde and these compounds are therefore considered to be part of the blank, rather than being produced by any of the microorganisms. The abundance of these two molecules is always

TABLE 2

Results of inhibition of fungal mycelial growth through cultural filtrate assays. For each of the pathogenic fungi are reported the results of mycelial growth inhibition percentage (GIPf) measured at 14 days post inoculation (d.p.i.). In the middle column, the concentration of strain R16 cultural filtrate used for the preparation of the medium is reported, being either 25, 50, or 100%. P values obtained with Kruskal-Wallis analysis are reported for the statistical comparison between the results obtained. Significant difference is shown by different letters (a, b, c) beside significantly different results.

Fungal strain	R16 Culture %	GIPf
<i>B. cinerea</i>	25	0.5 a
	50	14 b
	BC	29 c
	P value	≤0.001
<i>F. verticillioides</i>	25	0
	GV2245	0
	FV	0
	P value	–
<i>P. viticola</i>	25	0
	PV1	0
	PV	0
	P value	–

significantly lower when the medium is inoculated with a microorganism. BC inoculated alone (i) produced dimethyl disulfide and phenylethyl alcohol, the latter of which is produced at no significantly different level also by strain R16. The following compounds were found only when R16 was incubated alone (ii), or in presence of BC (iii), and are therefore considered to be produced by the bacterial strain: butanol, active amyl alcohol, isoamyl alcohol, DMNT, farnesol, isobutyl styryl ketone. These molecules were found to be produced at a not significantly different level between the (ii) and (iii) treatments. The abundance for each molecule is reported in table (Table 3).

3.7. Production of spores in antagonized BC

The spore production was reduced in antagonized *B. cinerea* cultures. In particular, we registered ISP = 40.3% with T0, and above 99% with T1, and T2, compared to a non-treated plate in which the production of conidia amounted to 2×10^6 spores/ml. It can be seen that strain R16 showed an antagonistic effect by reducing the production of conidia even at T1, when it had a lower GIP value. The results obtained with T1 and T2 were not statistically different, but were different from T0 and from the non-treated control ($P \leq 0.001$, according to Kruskal–Wallis test).

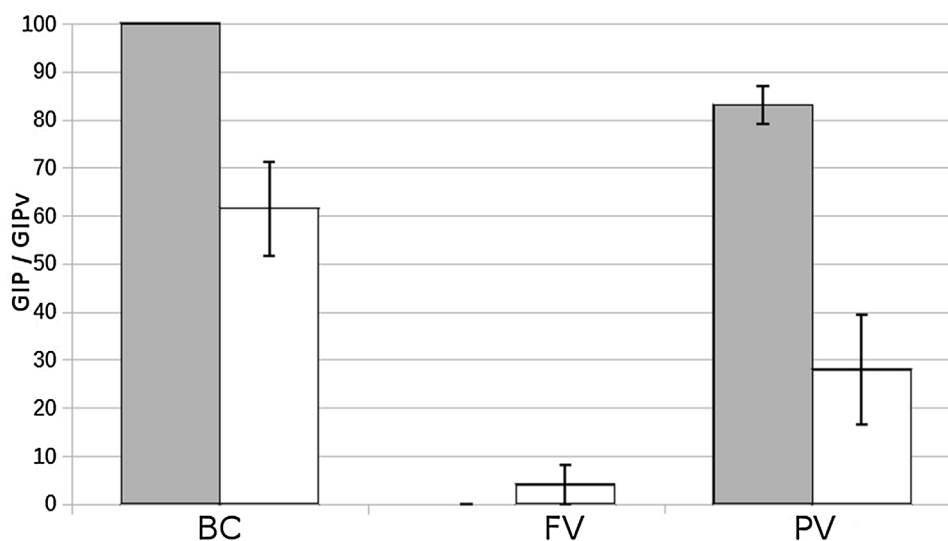


Fig. 3. Comparison between growth inhibition percentage (GIP) obtained in dual-culture assays at 14 days post inoculation (d.p.i.) with T2 treatment (bacteria inoculated two days ahead of the fungus) in gray, and growth inhibition percentage obtained only through volatile components (GIPv) at 14 d.p.i. and bacteria inoculated 2 days ahead of the fungus in white. Bars show the error, calculated as standard deviation from the mean.

TABLE 3

List of volatile molecules produced by the TGYA medium without inoculation (iv), BC (i), strain R16 (ii), and strain R16 in presence of BC (iii), as detected through SPME-GC-MS. In the first column the retention index is reported. In the second column, trivial name is reported, followed by IUPAC between brackets when the two are different. In the third column, quant ion specific for each compound is reported. In the fourth, fifth, sixth, and seventh column, the mean value of the natural logarithm of the peak area of each VOC is reported for TGYA, BC, R16, and BC+R16 treatments respectively. Significantly different values according to Kruskal-Wallis test (isovaleraldehyde, dymethyl disulfide, benzaldehyde, phenylethyl alcohol) or Mann-Whitney test (remaining compounds) in each row are identified with different letters.

Retention index	Compound name	Quant	TGYA	BC	R16	R16 + BC
731	Isovaleraldehyde [3-methylbutanal]	41	6.06 ^a	5.63 ^{ab}	5.48 ^b	4.53 ^b
979	Dymethyl disulfide [(methyldisulfanyl)methane]	43	–	6.21 ^a	4.84 ^b	4.96 ^b
1132	Butanol [Butan-1-ol]	56	–	–	5.81	5.59
1223	Active amyl alcohol [(2S)-2-methylbutan-1-ol]	57	–	–	5.88	5.50
1226	Isoamyl alcohol [3-methylbutan-1-ol]	55	–	–	5.79	5.73
1368	DMNT [(3E)-4,8-dimethylnona-1,3,7-triene]	69	–	–	6.37	5.97
1709	Benzaldehyde	69	6.71 ^a	4.31 ^b	5.27 ^b	4.32 ^b
2043	Farnesol [(2E,6E)-3,7,11-trimethyldodeca-2,6,10-trien-1-ol]	91	–	–	7.15	6.98
2177	Phenethyl alcohol [2-phenylethanol]	94	–	5.76 ^a	5.46 ^a	5.37 ^b
2642	Isobutyl styryl ketone [(1E)-5-methyl-1-phenyl-1-hexen-3-one]	105	–	–	6.18	6.34

3.8. Inhibition of BC conidia germination

The observation of BC conidia germination revealed that the bacterium is capable of inhibiting this process. The results, described in Fig. 4, show that the non-treated conidia had a high GR value, reaching a maximum of 88% after 18 h from the beginning of the assay. Instead, conidia treated with strain R16 showed a slight early GR, reaching 10% of germination 6 h post inoculation, but showed lower GRs until the end of the assay at 72 h post inoculation, determining a strong inhibitory effect. After the first observation at 6 hours post inoculation, in which the treated and untreated have similar GRs, there were statistically significant differences between GRs of the treated sample and untreated control all along the experiment (Mann-Whitney test; $P \leq 0.001$).

3.9. In vivo antagonism assays

The non-treated and bacteria-inoculated-only samples show the same values of fungal colonization, as only 1 berry out of 30 was rotted, probably due to earlier fungal contamination that was not removed through surface sterilization. All berries inoculated with BC conidia showed gray mold on the surface, with different colonization levels and an overall average C%I = 76.7%. With this treatment, most of the berries showed the maximum colonization rank (class 6: gray mold visible on 100% of the berry and presence of sporulation) and the minimum colonization rank observed was class 3 (gray mold visible on 50% to 75% of the berry). Berries that were both treated with strain R16 and inoculated with BC showed far more contained colonization (Fig. 5) and an overall average C%I = 51.9%. The reduction of the index was coupled with an overall reduction of the colonization severity, with most of the berries showing class 4 colonization (gray mold visible on more

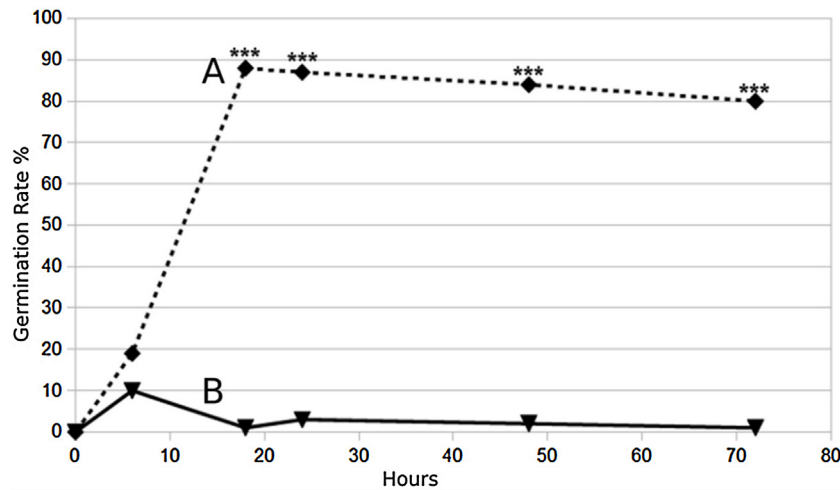


Fig. 4. Comparison between germination rate (GR) of BC conidia over time in the non-treated control (A) and strain R16 inoculated sample (B). Values marked with *** are statistically different for $P \leq 0.001$ according to Mann-Whitney test.



Fig. 5. Difference between berries inoculated only with BC conidia (A) and berries inoculated with both BC conidia and strain R16 liquid suspension (B). Difference in mycelial growth on berries at 5 days post inoculation can be observed.

than 75% of the berry) rather than class 6, and the less colonized berries being in class 1 (gray mold visible on 10% to 25% of the berry surface). These two results are significantly different between themselves, and from the non-inoculated control (Mann-Whitney test; $P = 0.048$).

4. DISCUSSION

While the genus *Paenibacillus*, most importantly *P. polymyxa*, has been known from a long time as a source of plant growth promoting (Holl et al., 1988) and biocontrol agents (Timmusk and Wagner 1999), data on other species of *Paenibacillus* in relation to biocontrol are few. There are reports of biocontrol activity for *P. illinoisensis* (Jung et al., 2005), *P. macerans* (Larsen et al., 2009), and for *P. lentimorbus* (Kumar et al., 2016), but many other reports are for *Paenibacillus* spp., without indication of species (Ghazalibiglar et al., 2016; Senthilkumar et al., 2009).

Obtained results proved, for the first time, plant growth promotion-related traits and antifungal activity by the use of whole cells of *P. pasadenensis*, strain R16. The biochemical *in vitro* assays carried out in this study show that *P. pasadenensis* strain R16 has several biochemical traits often reported as important for promotion of plant growth and adaptation to life inside of plant tissues. In particular, it did not show traits typical of bacterial

biofertilizers, as it lacks the ability to solubilize phosphorus and produce siderophores (Pii et al., 2015), and therefore seems unable to provide resources to a plant host. On the contrary, it could be able to act at hormonal level, using well-known mechanisms such as producing indole-acetic acid and deaminating the stress-regulating hormone 1-aminocyclopropane-1-carboxylate (ACC) (Sessitsch et al., 2005). Moreover, strain R16 produces catalase, an enzyme involved in detoxification of hydrogen peroxide. This enzyme is a key factor in allowing endophytic lifestyle of bacteria inside a plant host, since plants tend to produce hydrogen peroxide as a response to stresses (Joseph et al., 2007). Furthermore, it was reported by Musetti et al. (2007) that grapevine plants that underwent natural recovery from phytoplasmatic diseases, such as the ones from which strain R16 was isolated (Bulgari et al., 2011), have a much higher content of hydrogen peroxide compared to healthy plants. All these features confirmed that strain R16 showed a putative role as a PGP endophyte.

The results obtained from both *in vitro* and *in vivo* competition assays proved for the first time that a strain of the species *P. pasadenensis*, strain R16, has an antifungal activity against phytopathogenic fungi. Moreover, experimental evidences disclosed several interesting points about the strain R16's ability to influence fungal pathogens behavior.

- (i) Dual-culture assays highlighted that strain R16 demonstrated good inhibitory effect against both *Botrytis cinerea* MG53 (BC) and *Phomopsis viticola* PV1 (PV). Inhibition values against BC were higher than those presented for known biocontrol species such as *Bacillus subtilis* (Touré et al., 2004) and *B. amyloliquifaciens* (Ji et al., 2013). Comparison with effects of other biocontrol agents against PV is not possible because no numerical data are available prior to our work. However, it is reported by Kotze et al. (2011) that a strain of *B. subtilis* managed to express an inhibitory effect against the fungus. Furthermore, the presence of an inhibition halo, observed in dual-culture assays carried out with BC and PV in the present study, hints at the production of antibiotic molecules by strain R16 that diffuse in the agarized substrate. In contrast, strain R16 proved to have no particular effect against *Fusarium verticillioides* GV2245 (FV) in dual-culture assays when compared with previously reported biocontrol agents (e.g. *B. subtilis*) (Abiala et al., 2015; Cavaglieri et al., 2005). However, the reduction of FV mycelial growth observed in the inhibition halo assay proves that strain R16 is not completely ineffective against this pathogen. Further studies should be carried out to determine how and in which conditions the strain could have an antifungal effect against FV.
- (ii) We can generally observe that strain R16 inhibits the growth of phytopathogenic fungi with more efficacy when inoculated two days ahead of the fungi (Fig. 1: D, H, L). This result suggests that the antagonistic molecules are not produced by strain R16 as a response to the presence of fungal pathogens, but as part of its regular metabolism, at least in the analyzed experimental conditions. A similar phenomenon was already reported for *Burkholderia phytofirmans* strain PsJN which, when co-cultured with *B. cinerea*, could exhibit a great inhibitory effect when inoculated two days ahead of the pathogen, but had a reduced effect when inoculated at the same time as the pathogen (Barka et al., 2002).
- (iii) The inhibitory effect of strain R16, when present, was proved to remain consistent over a period of 2 weeks, longer than the 5 or 7 days period usually analyzed in previous studies (Essghaier et al., 2009; Todorova and Kozhuharova, 2009), showing a long-lasting efficacy of the antifungal activity.
- (iv) The volatile compounds produced by the strain R16 are very important for the antifungal activity, especially against BC, where most of the inhibition could be achieved through volatile compounds alone (Fig. 3). While the presence of an inhibition halo suggested the production of antibiotic molecules by strain R16, the cultural filtrate alone had little effect in inhibiting the fungal pathogens. It is of note that both diffusible (GIPf=0) and volatile (GIPv=39) molecules alone produced by strain R16 proved to be ineffective against PV, suggesting their synergistic effect in achieving growth inhibition (GIP=83). On the contrary, it is interesting to note that against BC, the GIPf (29) and GIPv (71) sum up perfectly to the observed GIP value (100). As these results suggest a major involvement of volatile molecules in the antifungal effects of strain R16, in the present study we focused on identifying the volatile molecule bouquet produced by strain R16 in order to investigate the putative mechanisms underlying its antifungal activity.
- (v) Many of the volatile compounds that strain R16 produces, in particular butanol, active amyl alcohol, and isoamyl alcohol, were already reported in literature as antifungal compounds (Batista et al., 2011; Chaves-Lopez et al., 2015). Dimethyl disulfide is known as an antifungal compound among its various biological functions (Weisskopf, 2013), still in this case the production for strain R16 is lower than that for BC alone, hinting both that this molecule is not related to the antifungal effect, and that the production detected when both microorganisms are incubated together is given by strain R16 alone, not from BC. Similar conclusions apply to phenylethyl alcohol, which is produced at comparable levels both BC and R16 alone, but the production is significantly reduced, albeit the numeric difference is slight, when both microorganisms are developing together. Moreover, isobutyl styryl ketone could be important for fungal growth inhibition. In fact, it is interesting to note that several styryl ketone molecules, namely the conjugated styryl ketones, have been reported as antifungal and antimicrobial compounds thanks to their effect as proton pumps inhibitors (Afeltra and Verweij 2003). Still, there is no evidence or previous report in literature about such effects of isobutyl styryl ketone. Therefore, while it is possible that this molecule could play a role in the antifungal activity of strain R16, its effect on fungal pathogens should be properly characterized.
- (vi) The most abundant volatile molecule produced by strain R16 is farnesol, which, if expressed in percentage rather than an absolute value, would amount in average to 70.43% of the total VOCs produced by strain R16 growing alone. Farnesol is a terpenic compound reported as a quorum-sensing molecule of fungi and, at high concentrations, as a powerful antifungal molecule (Krom et al., 2016). The possibility that farnesol could have great importance in the inhibitory mechanism of strain R16 is reinforced by the studies of Cotoras et al. (2013), since the inhibition obtained using purified farnesol against *B. cinerea* is perfectly comparable to that exerted by strain R16. Since the main mode of action for farnesol was proven to be induction of apoptosis in fungal cells (Shirtliff et al., 2009), the noticeable swelling of BC cells seen during competition assays (Fig. S1 A,B), which is compatible with apoptosis, seems to confirm this mechanism of antifungal activity. We therefore hypothesize that the reduced amount of farnesol detected when BC and R16 are growing together, which is not a statistically significant difference, could be caused by the uptake of this molecule from BC, rather than a less abundant production from strain R16. Farnesol was demonstrated to have antifungal effects also on *F. graminearum* (Semighini et al., 2008), but there are no studies carried out on *F. verticillioides* species. Likewise, no information is available about the interaction of farnesol with *P. viticola*.
- (vii) DMNT is a terpenic compound known to be involved in the defense mechanisms of plants against insects, known to be an attractive molecule for predators and parasitoids of herbivore insects (Tholl et al., 2011). As such, for its functions known by the scientific community at the present time, it seems unlikely that it is involved in direct fungal growth inhibition, though it could play a role in cross-talk between strain R16 and a plant host. The possible involvement of DMNT produced by strain R16 in its antifungal effect and induction of plant defenses should be further evaluated in later studies.
- (viii) Strain R16 had a strong effect against germination of BC conidia, suggesting that the strain could prevent germination of conidia if it were to be applied in postharvest conditions. The inhibition of germination was obtained at the concentration of 10^5 CFU/ml, which is three times lower than the 3×10^5 CFU/ml reported for *Brevibacillus brevis* strain Nagano, a strain known to produce fungicidal molecules (Edwards and Seddon, 2001).
- (ix) Strain R16 managed to sensibly reduce the rate of infection of grape berries by BC. In fact, strain R16 reduced the incidence of gray mold by 27.5%, which is a result comparable to that obtained by several *Paenibacillus* strains assayed by Haidar et al. (2016) in similar experimental conditions, interestingly giving a greater inhibition than the *P. polymyxa* strain tested in that study.
- (x) The strain R16 is capable of producing chitinase, and the chitinolytic enzymes synthesized by the *P. pasadenensis* species are already reported as effective for biocontrol of *Aspergillus* spp. and *Penicillium* spp. (Loni et al., 2014). In the present study the

chitinase did not seem to be involved in the antifungal activity since the assays were performed in a medium, TGYA, rich in other more easily available sources of carbon. However, preliminary observation at optical microscope suggested that a possible effect of strain R16 chitinase could be the degradation of fungal hyphae and spores of *F. verticillioides* (Fig. S1C,D).

In conclusion, this study proved for the first time that *Paenibacillus pasadenensis* strain R16 possesses traits related to plant growth promotion and exerts an antifungal activity against phytopathogenic fungi pathogens. The various assays performed hint towards production of farnesol being the main mechanism involved in this antifungal action which, to the best of our knowledge, was never before reported for a bacterial biocontrol agent. This hypothesis will need further validation through the use of knock-out mutants unable to produce the molecule.

These results open an interesting scenario for further studies investigating the possible application of this endophytic bacterium as a plant-growth-promoting and biocontrol agent through *in planta* studies. As the strain R16 was isolated from grapevines that recovered from phytoplasma infection, in future studies its biocontrol activity will be tested also against phytoplasmas.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.micres.2017.02.001>.

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