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Screening and modes of action of antagonistic bacteria to control the fungal pathogen *Phaeomoniella chlamydospora* involved in grapevine trunk diseases

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ABSTRACT

The antagonistic activity of 46 bacterial strains isolated from Bordeaux vineyards were evaluated against *Phaeomoniella chlamydospora*, a major grapevine pathogen involved in Esca. The reduction of the necrosis length of stem cuttings ranged between 31.4% and 38.7% for the 8 most efficient strains. Two *in planta* trials allowed the selection of the two best strains, *Bacillus pumilus* (S32) and *Paenibacillus* sp. (S19). Their efficacy was not dependent on application method; co-inoculation, prevention in the wood and soil inoculation were tested. The involvement of antibiosis by the secretion of diffusible and/or volatile compounds in the antagonistic capacity of these two strains was assessed *in vitro*. Volatile compounds secreted by *B. pumilus* (S32) and *Paenibacillus* sp. (S19) were identified by gas chromatography/mass spectroscopy (GC/MS). The volatile compounds 1-octen-3-ol and 2,5-dimethyl pyrazine were obtained commercially and tested, and they showed strong antifungal activity against *P. chlamydospora*, which suggested that these compounds may play an important role in the bacterial antagonistic activity *in planta*.

Furthermore, the expression of 10 major grapevine defense genes was quantified by real-time polymerase chain reaction, which demonstrated that the two strains significantly affected the grapevine transcripts four days after their application on the plants. High expression levels of different genes associated with *P. chlamydospora* infection in *B. pumilus* pre-treated plants suggests that this strain induces systemic resistance in grapevine. For the first time, we demonstrated the ability of two bacterial strains, *B. pumilus* and *Paenibacillus* sp., isolated from grapevine wood, to control *P. chlamydospora via* direct and/or indirect mechanisms.

1. Introduction

Grapevine trunk diseases (GTDs), such as Esca, Eutypiosis and Botryosphaeriae diebacks markedly impact the worldwide wine and grape industry. The heavy economic losses caused by these diseases, especially Esca, indicates that they are becoming a growing threat to grapevine and the production of quality wine wherever grapevines are cultivated (Lorrain et al., 2012). For example in France, approximately 13% of vineyards are unproductive because of GTDs (Bruez et al., 2013).

Symptoms of Esca are wood decay, symptoms on the leaves and brown spots on the berries. Foliar symptoms include leaf chlorosis and necrotic tiger stripes (Lecomte et al., 2012). Symptoms present on the wood are central necrosis, black punctuate necrosis, sectorial necrosis and a discolored xylem stripe and white rot, which is the most common and specific symptom of Esca (Lecomte et al., 2012; Maher et al., 2012). In severe cases, these symptoms result in the death of the plant.

Isolation and identification of the fungi associated with Esca have revealed several pathogenic species, including *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingfield & L. Mugnai) P.W. Crous & W. Gams (Pch), *Phaeoacremonium aleophilum* (*P. mini-*

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mum com.nov.) (Gramaje et al., 2015) and *Fomitiporia mediterranea* (Fom) M. Fischer (Ciancio and Mukerji, 2008; Surico, 2009). These three species are considered as the major pathogens associated with Esca (Bertsch et al., 2013; Larignon and Dubos, 1997). Nevertheless, other fungal species, *i.e.*, *Eutypa lata, Stereum hirsutum* and *Botryosphaeriaceae*, are also frequently isolated from infected plants and may be associated with Esca to some extent (Larignon and Dubos, 1997; Laveau et al., 2009). Because these pathogens have never been detected in the leaves of infected plants, the foliar symptoms of Esca have been hypothesized to originate from toxin transport from colonized wood to the leaves (Andolfi et al., 2011; Spagnolo et al., 2012) and/or from the disruption of the vessel sap flow (Lecomte et al., 2012). Environmental stress has also been reported as a major influence on the expression of Esca (Luini et al., 2010). Furthermore, wounds due to grapevine pruning are generally considered as the principal port of entry for the fungal pathogens associated with GTDs (Chapuis, 1998; Graniti et al., 2006; Niekerk et al., 2011). *P. chlamydospora* has been consistently isolated from grapevines showing symptoms of Esca. Pathogenicity tests have clearly demonstrated the presence of this pathogen at the origin of typical Esca necrosis lesions in the wood (Laveau et al., 2009). The susceptibility of fresh pruning wounds colonized by *P. chlamydospora* conidia has been reported (Eskalen et al., 2007; Larignon et al., 2000). The pycnidia produced by *P. chlamydospora* on the exposed vascular tissue on the cordons and spurs of grapevines could serve as an inoculum source in the vineyard (Eskalen et al., 2001; Eskalen and Gubler, 2002; Larignon et al., 2000). *P. chlamydospora* has been isolated from rootstock mother plants (Aroca et al., 2009) and from scion cuttings (Zanzotto et al., 2007). The presence of *P. chlamydospora* on nursery vine plants has also been reported by Vigues et al. (2009). In addition, Moyo et al. (2014) suggested that many different arthropods could carry *P. chlamydospora* spores and might serve as an inoculum source.

Sodium arsenate was banned in 2001 due to its human and environmental toxicity and was the only pesticide registered for the control of GTDs; since then, no treatment has been developed that efficiently controls Esca. Therefore, the development of an alternative method such as biocontrol is desirable. Thus, to prevent the spread of GTDs and to reduce the use of pesticides to control grapevine diseases, studies of complementary and/or alternative methods, especially biocontrol, have sparked great interest in viticulture.

As previously reviewed, various microorganisms have been tested to control the fungal pathogens associated with Esca (Bertsch et al., 2013; Compant et al., 2013). Most studies have focused on the biocontrol effect of *Trichoderma* spp. (incl. *T. harzianum, T.atroviride*) against several fungal pathogens related to GTDs (Di Marco et al., 2004; Fourie and Halleen, 2006; Halleen and Lombard, 2010; Kotze et al., 2011; Mounier et al., 2014). The antagonistic potential of various bacterial strains has been explored *in vitro* or on wood disks for the biological control of these fungi in grapevines (Alfonzo et al., 2009; Lebrihi et al., 2009). Furthermore, only a low number of antagonistic bacterial strains have been reported to suppress GTD agents *in planta*, including *P. chlamydospora*, *N. parvum*, *N. australe*, *Diplodia seriata*, *Lasiodiplodia theobromae*, *E. lata*, and *Phomopsis viticola* (Ferreira et al., 1991; Haidar et al., 2016; Kotze et al., 2011). *In vitro* assays have shown that the inhibitory effect of metabolites of *Bacillus subtilis* (AG1) on the growth of *L. theobromae*, *P. chlamydospora* and *P. aleophilum* is efficient (Alfonzo et al., 2009). Moreover, the biocontrol activity of the bacterial strain *B. subtilis* B1a, *Erwinia herbicola* (strains JII/E2 and JII/E4) and the A*ctinomycete* strain A123 was demonstrated *in vitro* and on autoclaved grapevine wood discs against *E. lata* (Schmidt et al., 2001). More specifically, the efficacy of *B. subtilis* for the protection of grapevine against *P. chlamydospora* by reducing pruning wound infections (Kotze et al., 2011) has been shown. In another study, a different strain of *B. subtilis* isolated from grape wood arm has been demonstrated to significantly reduce infection by *E. lata* in grapevine wood (Ferreira et al., 1991).

Lastly, despite several attempts, no commercial bacterial biocontrol products have been developed to control GTDs. For example, only one fungal product (Esquive®) based on *Trichoderma atroviride*, is currently registered in France. Thus, in order to find a suitable biological control agent (BCA) adapted to vineyard conditions, we evaluated 46 bacterial strains, all of which originated from vineyards, either from grapevine wood or grape berries, and all had already been tested against two other major grapevine fungal pathogen species, *Botrytis cinerea* and *N. parvum* (Haidar et al., 2016).

The objectives of this study were (1) to screen the 46 bacterial strains isolated from grapevine for significant antagonism against *P. chlamydospora*, and (2) to identify the mechanisms of action that potentially accounted for the inhibition of the pathogen by the most effective bacterial strains. In addition, we examined the disease control efficacy of selected bacteria by comparing three application methods (*i.e.*, co-inoculation, preventive inoculation in the hole and preventive inoculation by soil drenching).

2. Materials and methods

2.1. Microorganisms and cultural media

2.1.1. P. chlamydospora

P. chlamydospora strain (SO44) was selected from the INRA-UMR 1065 SAVE collection, Bordeaux and was used in all experiments. This strain was originally obtained in 1996 from a Cabernet Franc cultivar in Moncaup, France. It was characterized as highly aggressive in previous studies at INRA (Laveau et al., 2009). The strain was subcultured on Malt Agar (MA) medium and incubated at 22 °C (12 h light/12 h dark) for one month before being utilized for artificial inoculation in bioassays with cuttings and for two weeks for *in vitro* tests (confrontation and volatiles).

2.1.2. Bacterial strains

A total of 46 bacterial strains were tested which were derived from a previous study, *i.e.*, Haidar et al., 2016 (see Table S1 in Supplementary material in the online version at DOI: 10.1016/j.micres.2016.07.003). All the strains were isolated from grapevine, including 35 strains from wood tissue (Bruez et al., 2015) and 11 strains from the grape berry surface (Martins, 2012). In these two last articles, the bacterial strains were typed. The strains from the grape berry surface originated from the "Centre de Ressources Biologiques en Oenologie" (University of Bordeaux and Bordeaux INP "Institut National Polytechnique of technology"). For the *in vitro* trials, strains were grown beforehand on Trypto-Casein Soy Agar medium (TSA, Biokar diagnostics, France) for 24 h at 28 °C. In both 2013 and 2014 bioassays, the bacterial preparation was done as described in Haidar et al., 2016.

In the 2015 bioassay, the bacterial cell concentration of S19 and S32 was adjusted to 10^8 CFU/ml for co-inoculation. For preventive inoculation by soil drenching and in the hole, the bacterial cell concentration of *Paenibacillus* sp. (S19) and *B. pumilus* (S32) was adjusted to 10^7 and 10^6 CFU/ml, respectively.

2.2. Stem disease bioassays

2.2.1. Bacterial and fungal inoculation treatments

In all bioassays, each cutting stem was surface-sterilized by rubbing it with a paper towel soaked with 95% ethanol. Then an artificial wound was made by drilling a hole in the bark (4 mm in

diameter) in the central part of each cutting stem below the upper bud.

2.2.1.1. Bacterial treatments. For co-inoculation, a fresh wound was immediately drop-inoculated with 40 μL of bacterial suspension. Once the liquid had dried for 20–40 min at ambient temperature, the hole was inoculated with a mycelial plug of *P. chlamydospora*. In the preventive inoculation method in the hole, the fresh wound was drop-inoculated as described above, but four days were allowed to elapse before inoculation with the fungal pathogen. In the preventive soil inoculation method, rooted cuttings were inoculated by pouring 50 mL of bacterial suspension at the collar of each plant. For this last method, holes in the samples were made on the same day as the bacterial treatment. The treated and untreated wounds in both preventive inoculation methods (*i.e.*, in the hole and on the soil) were covered with Parafilm® (Scellofrais film) for protection before and after fungal inoculation.

2.2.1.2. Fungal inoculation. The hole in each cutting was filled with a *P. chlamydospora* mycelium plug cut off from the margin of a fresh mycelial MA culture, with the mycelium facing the inner part of the stem. The treated wounds were covered with Parafilm® (Scellofrais film) for protection during the incubation period.

2.2.2. Plant material and experimental design

Rooted cuttings of a Cabernet Sauvignon cultivar of grapevine were used in all of the bioassays and originated from the INRA experimental vineyards near Bordeaux. They were processed and prepared as described by Laveau et al. (2009). For incubation in the 2013 and 2014 bioassays, the plants were grown in an open greenhouse, but for the third bioassay in 2015, the plants were always grown and incubated in a closed greenhouse.

2.22.1. 2013 cutting bioassay. Two trials were conducted between April and October 2013. The first was conducted with 26 bacterial strains (S1–S26), and the second with 20 strains (S27–S46) as described by Haidar et al. (2016). The only bacterial application method used was co-inoculation. All treatments and the associated experimental design were as described by Haidar et al. (2016). Briefly, the principal treatments corresponded to the bacterial strains co-inoculated with the pathogen and the control treatments consisted of (i) an uninoculated, untreated control (UUC), in which the samples were not inoculated with the fungus and not treated with bacteria, (ii) an untreated control (UC), in which the samples were inoculated only with the fungus *P. chlamydospora*, and (iii) the fungicide controls (FC1 and FC2), in which the samples were treated with a Fluazinam (Sekoya, Syngenta France SAS, 50% *a.i.*, 250 g *a.i.* 100 L) fungicide solution and with a Thiophanate methyl (20 mg/L) fungicide solution, respectively. The UUC cuttings were treated with sterile bacterial culture medium and sterile MA plugs.

2.2.2.2. 2014 cutting bioassay. Nine bacterial strains were selected for the 2014 bioassay on the basis of their antagonistic capacity against *P. chlamydospora* and *N. parvum* (Haidar et al., 2016) as indicated by the results of the 2013 bioassay, which was conducted between May and September 2014 under open greenhouse conditions with three modes of bacterial application (Table 1).

The experimental design was a randomized complete block with 20 replications, *i.e.*, cuttings, for the untreated control (UC) and 15 replications per treatment for the other treatments. The experimental treatments consisted of cuttings (i) co-inoculated with the bacterial strains and *P. chlamydospora*; (ii) treated in the hole with the bacterial strains, and inoculated with *P. chlamydospora* in the holes four days later; (iii) treated on the roots with 50 mL of bacterial suspension by soil drenching, and inoculated with *P. chlamydospora* in the hole four days later. The control treatments consisted of (i) an uninoculated untreated control (UUC), in which the samples were not inoculated with the fungus and not treated with bacteria, (ii) untreated control in which the samples were inoculated with the fungus *P. chlamydospora* alone, either without or with sterile bacterial culture medium (UC or UCM, respectively), (iii) a BC (bacterial control) in which the samples were treated in the hole with the bacterial suspension alone iv) a fungicide control (FC), treated by co-inoculation with a solution of the fungicide Fluazinam (Sekoya, Syngenta France SAS, 50% *a.i.*, 250 g *a.i.* 100 L). The UUC cuttings were treated with sterile bacterial culture medium and sterile MA plugs. Two types of untreated control were tested with (UCM) or without bacterial medium (UC) for investigating the effect of the bacterial medium on the development of necrosis by the pathogen.

Table 1
Bacterial strains and methods of bacterial treatments performed in the three bioassays performed in 2013, 2014, 2015.

		2013 bioassay 46 strains	2014 bioassay 9 strains (S1, S3, S18, S19, S24, S27, S28, S41, S32)	2015 bioassay 2 strains (S19, S32)
	Bacterial strains tested			
Application methods	Co-inoculation	+	+	+
	4-day pre-inoculation at hole	–	+	+
	4-day pre-inoculation by soil drench	–	+	–

2.2.3. Evaluation of stem necrosis

The incubation period duration was of 122 ± 11 dpi (days post inoculation) and 127 ± 8 dpi in the 2013 and 2014 bioassays, respectively, after which the stem of each plant was cut longitudinally in the middle, and the length of the internal vascular lesions (or necroses) in the cutting wood just under the bark was visually evaluated by measuring the necrotic lesions upwards and downwards from the wound-inoculation hole. To better detect and expose the necrotic zones, the wood tissue around the top-margin of the necrotic zone was sometimes removed as wood shavings with a scalpel.

2.3. In vitro *assessments of antifungal effects*

2.3.1. Antagonism in dual culture

Dual cultures were used to test the effect of the 46 bacterial strains on the mycelial growth of *P. chlamydospora*. A mycelial plug of *P. chlamydospora* (4 mm of diameter) was inoculated at the center of a MA Petri dish. Then, 15 days later, a loop of bacterial cells from a 1-day-old culture grown on TSA plates was placed as a line on one side of the dish approximately 2.5 cm from the dish center. As extra controls, a set of dishes were inoculated similarly, but with the pathogen only. Three dishes were replicated per each bacterial strain X pathogen combination. The dishes were then incubated in the dark at 22 °C. After 16 days, the radial mycelial growth of the pathogen was assessed by measuring the colony radius in millimeter. The inhibition percentage was calculated using the following growth inhibition equation (GI%): $GI\% = 100 \times (R2 - R1)/R2$, where R1 is the minimal distance between the center of the mycelial plug and the fungal colony margin in the direction of the antagonistic bacteria. The control value for fungal colony radius was R2 and was assessed on the same plate as the distance between the center of

the mycelial plug and the fungal colony margin on the opposite side of the bacteria.

2.3.2. Effect and identification of antifungal volatile compounds

2.3.2.1. Production of antifungal volatile compounds by the bacterial strains. An *in vitro* trial was carried out to evaluate the potential secretion of antifungal volatile metabolites by the bacterial strains that affected the mycelial growth of *P. chlamydospora*. A one day-old bacterial culture was cultivated on TSA medium at 28 °C for 24 h. Cultures of *P. chlamydospora* (15-days) old were prepared on MA dishes. The cultures were incubated in the dark at 22 °C. The bottom parts of both dishes were then placed face-to-face with the TSA bacterial culture at the bottom and the MA culture of the pathogen at the top. This prevented physical contact between the two microorganisms. The dishes were sealed with transparent adhesive tape and Parafilm and incubated at 22 °C. As a control, a MA half bottom dish containing the pathogen alone was sealed with a half bottom dish containing TSA. Each bacterial strain and the control were represented by three double-dish replicates. After 14 days incubation, two perpendicular colony diameters were assessed to calculate the mycelial inhibition percentage.

2.3.2.2. Identification of the volatile compounds (VOCs) from bacterial strains pumilus *(S32) and* Paenibacillus *sp. (S19).* Two bacterial strains *B. pumilus* (S32) and *Paenibacillus* sp. (S19) were selected for this trial because of their high antagonistic activity toward *P. chlamydospora* as indicated in the 2013 and 2014 bioassays. The bacterial strains were streaked onto a TSA plate and incubated in the dark at 28 °C for 3 days. The volatile compounds produced by the bacteria were collected using solid-phase micro-extraction (SPME) as described by Zhou et al. (2014). The SPME fiber used was 65 μm polydimethylsiloxane/divinylbenzene (Supelco). The sampling of VOCs was performed at 30 °C for 30 min. Then, the fiber was thermally desorbed in the injector of an Agilent 7820A Series Gas Chromatograph system (HP-5 ms capillary chromatographic column, 30 m, 0.25 mm, 0.25 μm) connected for 5 min to an 5977 insert Mass Selective Detector (Agilent Technologies). The volatile compound profile of the tested bacteria was compared with an uninoculated TSA plate. The temperature program was as follows: an initial temperature of 45 °C for 1 min, followed by an increase to 300 °C at a rate of 10 °C min^{-1}. The temperatures of the injector and detector were 260 °C and 280 °C, respectively. High-purity helium was used as a carrier gas with a flow rate of 1 mL min^{-1}. Splitless injection was used, and the purge time was 0.6 min. The mass spectrogram of each peak in the total ion chromatography was compared in the National Institute of Standards and Technology (NIST) database to identify the corresponding compound.

2.3.2.3. Effect of pure 1-octen-3-ol and 2,5-dimethyl pyrazine on the mycelial growth of P. chlamydospora. The effect of 1-octen-3-ol and 2,5-dimethyl pyrazine on mycelial growth of *P. chlamydospora* was assessed as described by Zhao et al. (2011) with some modifications. Briefly, 15 day-old cultures on MA dishes (9 cm in diameter) of *P. chlamydospora* were used. Measured amounts of the two commercial volatile compounds 1-octen-3-ol and 2,5-dimethyl pyrazine (≥98% purity, Sigma-Aldrich, France) were added to a second dish, respectively. Two volumes of a pure solution of 1-octen-3-ol (50 and 500 μL) that corresponded to a concentration of 41 and 410 mg/ L(air) were tested. The same tests were conducted with three volumes of 2,5-dimethyl pyrazine (10, 100 and 200 μL) that corresponded to a concentration of 100, 1000 and 2000 mg/L(air). The two portions (105 mL) were rapidly sealed with transparent adhesive tape and Parafilm and incubated at 25 °C. Plates with the pathogen alone were used as controls. Four replicates were performed for each treatment and the diameter (mm) of the colony was measured after 4 days of incubation.

2.4. Assessments of induction of grapevine defense (2015 bioassay)

The 2015 bioassay investigated the hypothesis that resistance to the fungal pathogen could be induced by the bacterial strains *B. pumilus* (S32) and *Paenibacillus* sp. (S19). This mode of action was tested on leaf samples from the grapevine cuttings prepared in 2015, as described above (2.1).

2.4.1. Experimental design of the 2015 bioassay

This experiment was conducted between March and June 2015 with the 2 bacterial strains (*B. pumilus* (S32) and *Paenibacillus* sp. (S19)) that showed the highest antagonistic effect on *P. chlamydospora* in the 2013 and 2014 bioassays. The two strains were applied to grapevine cuttings (prepared as described in Section 2.1) by co-inoculation and 4d-preventive inoculation in the hole (Table 1) to assess their ability to induce a plant defense. The experimental design was a randomized complete block with 32 cuttings per treatment.

The experimental treatments consisted of cuttings (i) co-inoculated with one of the bacterial strains and *P. chlamydospora*; (ii) treated in the hole with one of the bacterial strains and then inoculated with *P. chlamydospora* in the hole four days later. The control treatments consisted of (i) an uninoculated, untreated control (UUC and UUCM) not inoculated with the fungus and not treated with bacteria, but UUCM was treated with sterile bacterial medium, (ii) an untreated control with samples inoculated with the fungus *P. chlamydospora* alone without sterile bacterial culture medium (UC), (iii) a bacterial control with samples treated in the hole with bacterial suspension alone (BC), and iv) a fungicide control (FC) treated with a Fluazinam (Sekoya, Syngenta France SAS, 50% *a.i.*, 250 g *a.i.* 100 L) fungicide solution. The UUC cuttings were treated with sterile bacterial culture medium and sterile MA plugs. The bacterial and fungal treatments were carried out as previously described (Section 2.2).

Leaves at the third or fourth foliar level were sampled at 0 dpi, (*i.e.*, 2 h after *P. chlamydospora* artificial infection), and at 15 dpi. Six plants per treatment (2 plants by 3 biological replicates) were collected each time. All samples were immediately frozen in liquid nitrogen, and stored at −80 °C for subsequent molecular analyses. Lastly, a stem necrosis assessment was carried out after an incubation period of 90 dpi and a visual evaluation of stem necrosis was conducted as described in Section 2.3.

2.4.2. Plant total RNA extraction and reverse transcription

The RNA extraction protocol was performed according to Reid et al. (2006) from frozen leaves of each of three biological replicates that had been stored at −80 °C. After grinding in liquid nitrogen, the leaf powder was added to extraction buffer (20 gmL^{-1}) preheated to 56 °C (300 mM Tris–HCl, pH 8.0, 25 mM EDTA, 2 mM NaCl 2% CTAB, 2% poly-vinyl poly-pyrrolidone (PVPP), 0.05% spermidine trihydrochloride and 2% β-mercaptoethanol added extemporaneously). The mixture was stirred vigorously and incubated in a water-bath at 56 °C for 10 min with regular stirring. An equal volume of chloroform: isoamyl alcohol (24:2, v/v) was added, and then, the mixture was centrifuged at 3500*g* for 15 min at 4 °C.

Using the manufacturer's protocol for the Spectrum™ Plant Total RNA Kit, the RNA extraction steps were as follows. The RNA was bound to a column using a unique binding solution, which effectively prevented polysaccharides as well as genomic DNA from clogging the column. Residual impurities and most residual genomic DNA were removed by DNase treatment and wash solutions according to the manufacturer's protocol. Purified RNAs

were eluted in RNase-free water. The amount of RNA obtained was determined by measurements at 260 nm and 280 nm (NanoDrop 1000 Spectrophotometer, France). The RNA integrity was assessed either by agarose gel electrophoresis or with a Bioanalyzer (Agilent technology, France). Lastly, RT-qPCR was conducted following the MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines (Bustin et al., 2009). Ten micrograms of RNA were reverse-transcribed using 2 μM oligo-d $(T)_{15}$, a ribonuclease inhibitor and M-MLV reverse transcriptase (Promega, France) according to the manufacturer's instructions in final volume of 900 μL at a final concentration between 70 and 150 ng μL^{-1}. The cDNAs obtained were then stored at −20 °C. Each data point is based on three independent biological replicates (biological and non-technical replicates).

2.4.3. Quantitative polymerase chain reaction

The expression level of the major 10 genes involved in the grapevine defense responses was assessed using real-time quantitative PCR (Dufour et al., 2013; Yacoub et al., 2016). The genes included four genes that code for PR protein: PR protein1 (*VvPR1*), PR protein10 (*VvPR10*), Chitinase class III (*VvCHIT3*) and ß-1,3 glucanase (*VvGLU*). Four other genes code for enzymes involved in phenylpropanoid and indole biosynthesis pathways: Phenylalanine ammonia lyase (*VvPAL*), Stilbene synthase (*VvSTS*), Chalcone synthase (*VvCHS*) and Antranilate synthase (*VvANTs*). The last two genes code for Glutathione *S*-transferase (*VvGST*) involved in redox status and callose synthase (*VvCALS*) involved in cell wall reinforcement. The γ-chain of Elongation Factor 1 (*VvEF1*γ, GenBank AF176496) (Dufour et al., 2013) and glyceraldehyde-3-phosphate dehydrogenase genes (*VvGAPDH*, GenBank CB973647) (Reid et al., 2006) were used as housekeeping genes.

Gene expression was assessed with a Stratagene Mx3005P qPCR system (Agilent technologies) using SYBR Green to detect dsDNA synthesis. For each reaction, 1 μL of each primer at 1 μM and 7 μL of 2× MESA BLUE qPCR MasterMix Plus for SYBR® Assay Low ROX (Eurogentec) including Hot start DNA polymerase, dNTP and $MgCl_2$ and 5 μL of cDNAs, were used according to the manufacturer's instructions with 350–750 ng of cDNA per well. Each PCR reaction was performed in duplicate. PCR was performed at 94 °C for 15 min, followed by 40 cycles at 95 °C for 10 s, 55 °C for 20 s and 72 °C for 20 s. The data were analyzed with MxPro QPCR Software (Agilent technologies) as the cycle of quantification (Cq), where the fluorescence signal of the amplified DNA intersected with the background noise. A mean Cq value was obtained for each gene and modality. A ΔCq value was obtained by subtracting Cq mean of a reference gene (EF1γ) from Cq value of the target gene. The Relative Expression (RE) was calculated with the $2^{-\Delta\Delta Cq}$ method for every sample, where ΔΔCq was the ΔCq between the two samples. The expression levels were calculated based on a multiple gene normalization method. The geometric mean of several carefully selected reference genes was used as an accurate normalization factor (Vandesompele et al., 2002).

2.5. Statistical analyses

The experimental data were compared using an analysis of variance (ANOVA) followed by Tukey's or Newman-Keuls' test ($P = 0.05$). The standard deviations were calculated for all mean values. Two statistical software packages were used: StatBox (Version 6.6, Grimmer© Logiciels, Paris) and XLSTAT (Version 2014.4.04, Addinsoft©, www.xlstat.com). A statistical analysis for the expression levels of the genes was carried out using the non-parametric Kruskal-Wallis test.

3. Results

3.1. Stem disease bioassays

In all biocontrol bioassays, all of the stem cuttings (cv. Cabernet Sauvignon) inoculated with *P. chlamydospora* alone and co-inoculated with a bacterial strain and the pathogen exhibited internal necrotic lesions. The lesions developed both above and below the inoculation point.

3.1.1. Reduction of internal necrotic lesion length by the bacterial strains in the 2013 bioassay

In the control cuttings inoculated with the pathogen alone (untreated controls, UC), the mean lengths of the necrotic lesions reached 90.9 mm and 90.6 mm after 122 ± 11 dpi incubation (data not shown). An evaluation of the protective effect of the bacterial strains against *P. chlamydospora* indicated that some strains were significantly different from the control (Fig. 1). The inhibition rates of the eight strains *Enterobacter* sp. (S24), *Paenibacillus* sp. (S18, S19), *B. pumilus* (S32), *Brevibacillus reuszeri* (S28, S31), *Bacillus* sp. (S34), *Paenibacillus illinoisensis* (S13) (most efficient to least efficient) were significantly different at $P = 0.05$ from the untreated

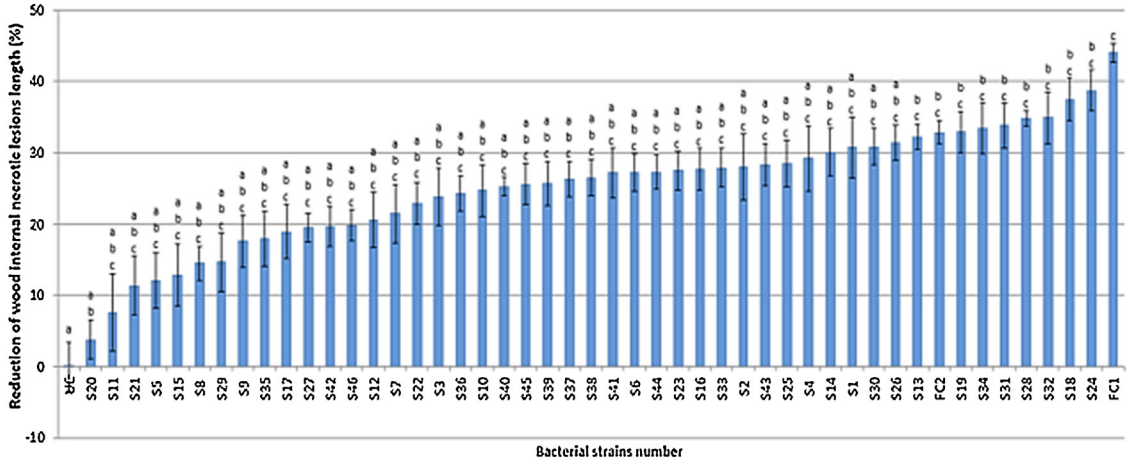

Fig. 1. Percentage of symptom inhibition displayed by 46 bacterial strains on the development of wood inner necrosis due to *P. chlamydospora* in the 2013 bioassay. Cutting stems (cv. Cabernet Sauvignon) were co-inoculated with bacteria and one mycelial plug of the pathogen mycelium before incubation in an open greenhouse for 122 ± 11 days. The untreated controls (UC) were inoculated with the pathogen only. The fungicide controls (FC1 and FC2) were treated with fungicide solutions of Fluazinam and Thiophanate methyl, respectively. Each value represents the mean of 16 or 11 cuttings depending on the test in the bioassay (see Section 2.1.1). Bars with the same letter are not significantly different at $P = 0.05$ according to Tukey's test after ANOVA. The error bar corresponds to the standard deviation of the mean.

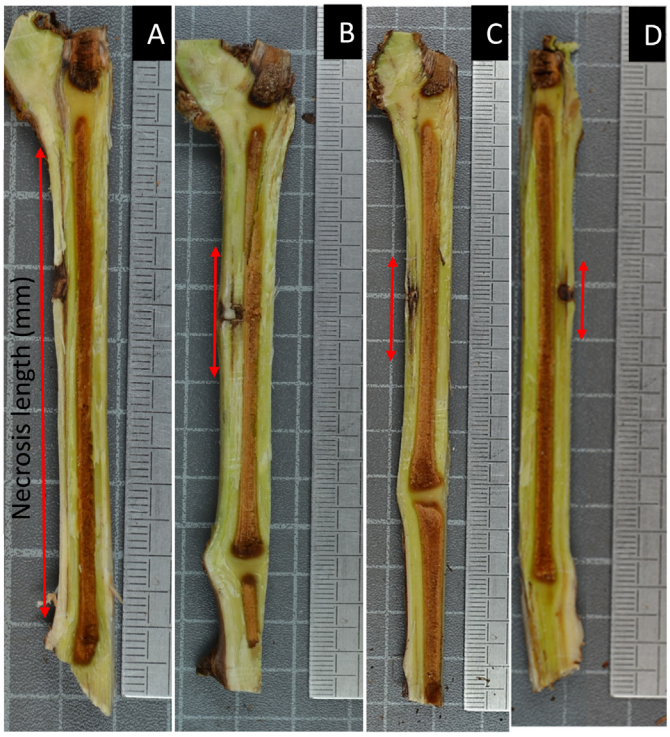

Fig. 2. Necrosis lesions observed on grapevine cuttings due to *P. chlamydospora* and/or drilling effect. (A) Inoculated with *P. chlamydospora* only; (B) Co-inoculated with *P. chlamydospora* and *B. pumilus* (S32); (C) Co-inoculated with *P. chlamydospora* and *Paenibacillus* sp (S19). (D) Necrosis lesions due only to the drilling effect.

control (UC), indicating marked antagonism (Fig. 2). The associated efficacy for lowering the internal necrotic lesion length varied between 31.4% and 38.7%. Interestingly, except for *P. illinoisensis* (S13) which was the last strain that showed a significant effect, all of the best strains originated from grapevine wood (SD 1). Moreover, the two fungicide treatments significantly reduced the lengths of the necrotic lesions with inhibition rates of 44% and 33% for Fluazinam and Thiophanate methyl, respectively. However, the efficacy of fungicide treatments was not much greater than that observed for the most efficient bacterial strains (Fig. 1).

3.1.2. Reduction of internal necrotic lesion length by the bacterial strains in the 2014 bioassay

Five of the eight bacterial strains that exhibited strong antagonistic activity against *P. chlamydospora* in the 2013 cutting bioassay *Enterobacter* sp. (S24), *Paenibacillus* sp. (S18, S19), *B. pumilus* (S32), *B. reuszeri* (S28) were chosen for testing in 2014. The purpose of this bioassay was to determine the most efficient method of bacterial application to the grapevine cuttings. In addition to these five strains, four other bacterial strains *Pantoea agglomerans* (S1, S3), *B. reuszeri* (S27) and *Bacillus firmus* (S41) were also tested in 2014 because they had been demonstrated to significantly reduce both necrosis and canker due to *N. parvum* in grapevine cuttings (Haidar et al., 2016). The nine strains were applied to the grapevine cuttings using three application methods; (i) co-inoculation, (ii) preventive inoculation in the hole, and (iii) preventive inoculation *via* soil drenching (Table 1).

The co-inoculation results showed that all of the bacterial strains tested significantly reduced the length of the internal necrosis after the artificial infection of the stem cuttings by *P. chlamydospora* compared to both the untreated control inoculated with the pathogen alone, with (UCM) or without (UC) sterile bacterial medium (Fig. 3). These two untreated controls showed a clear, significant difference in the length of pathogen necrosis (97 mm in UCM *vs* 83 mm in UC) and showed a nutritive stimulatory effect from the bacterial medium on pathogen development. However, an untreated control (UC) should be used in this bioassay to calculate the percentage of inhibition of the necrotic lesions (as in the 2013 bioassay, Fig. 1). Because two strains *B. reuszeri* (S27) and *Enterobacter* sp. (S24) showed a lower protective effect (Fig. 3), the comparison of the different application methods was focused on the seven most efficient strains. The inhibition levels of the seven strains ranged from 27.7% for S18 to 32.8% for *P. agglomerans* (S3). Similar to the bacterial treatment, the fungicide treatment also significantly reduced the length of pathogen necrosis (Fig. 3). The efficacy of Fluazinam in decreasing internal *P. chlamydospora* necrotic lesion length reached 34.5%.

The comparison of the three modes of bacterial application demonstrated that the mean wood necrosis lengths due to *P.*

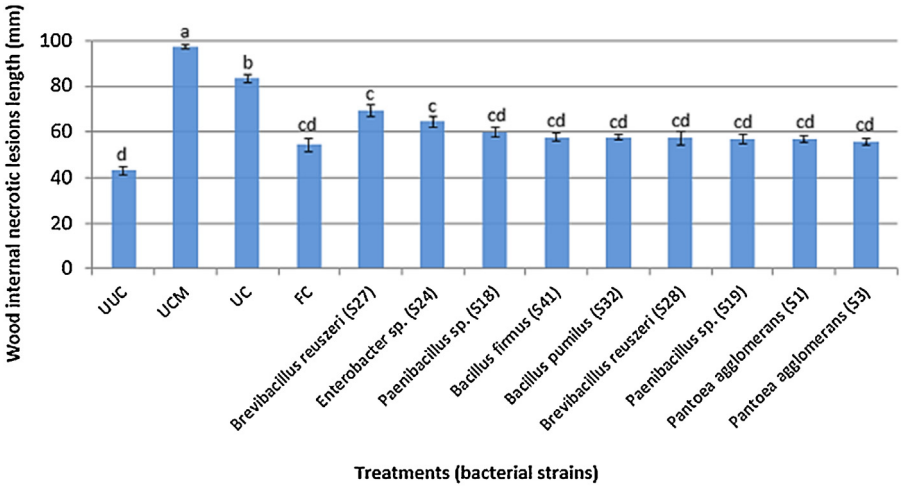

Fig. 3. Effect of selected bacterial strains on the development of wood inner necrosis following co-inoculation with *P. chlamydospora* in the 2014 bioassay. Cutting stems (cv. Cabernet Sauvignon) were co-inoculated with bacteria and one mycelial plug of the pathogen before incubation in an open greenhouse for 127 ± 8 days. The untreated controls, UC and UCM, were both artificially inoculated with the pathogen only, but the latter were treated with sterile bacterial medium before pathogen inoculation. The fungicide controls (FC) were treated with Fluazinam before pathogen inoculation. The un-inoculated untreated control (UUC) were not inoculated with the fungus and not treated with bacteria. Each value represents the mean of 15 cuttings (see Section 2.1.2). Bars with the same letter are not significantly different at $P = 0.05$ according to Newman and Keuls' test after ANOVA. The error bar corresponds to the standard deviation of the mean.

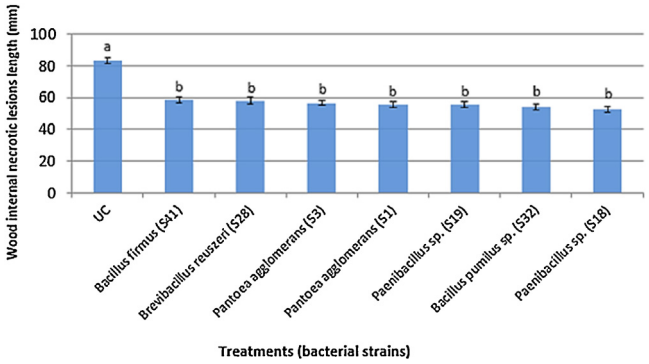

Fig. 4. Effect of selected bacterial strains on development of wood inner necrosis by *P. chlamydospora* on the basis of three methods of bacterial application in the 2014 bioassay. The untreated controls (UC) were inoculated with the pathogen only. Each value represents the mean of 15 cuttings (see Section 2.1.2). Bars with the same letter are not significantly different at $P = 0.05$ according to Newman and Keuls' test after ANOVA. The error bar corresponds to the standard deviation of the mean.

chlamydospora were 60.7, 57.9 and 59.0 mm for co-inoculation, preventive inoculation in the hole and application by soil drenching, respectively. The main effect of the application mode did not significantly affect the mean wood internal necrosis length (ANOVA, df = 2, *F*-value = 1.12, *P*-value = 0.33) but the main bacterial effect was highly significant (df = 7, *F*-value = 20.6, *P*-value < 0.001). These results showed that the bacterial treatment, regardless of the application method, resulted in significantly smaller lesions than in the control untreated cuttings (UC) inoculated with the pathogen alone (Fig. 4). The reduction of the internal necrotic lesion length by the seven selected bacterial strains ranged between 29.3% for *B. firmus* (S41) to 36.6% for *Paenibacillus* sp. (S18), confirming that these selected bacterial strains efficiently protected against *P. chlamydospora* in the grapevine wood and that the effect was irrespective of the bacterial inoculation method. Lastly, the interaction between the main effect of the bacterial strain and the main effect of the application method was not significant (ANOVA, df = 14, *F*-value = 0.92, *P*-value = 0.54).

3.1.3. *Reduction of internal necrotic lesion length by the bacterial strains in the 2015 bioassay*

To better understand the mode of action involved in the biocontrol of *P. chlamydospora* by two of the most efficient strains, a greenhouse experiment was performed. Strains S19 (*Paenibacillus* sp.) and S32 (*B. pumilus*) were applied to grapevine cuttings using two application methods (co and preventive inoculation). The purpose of this bioassay was to examine the resistance induced in the plants at the foliar level (*i.e.*, leaf tissues) after bacterial inoculation. The length of the necrosis in the grapevine wood was measured three months after inoculation by *P. chlamydospora,* (Fig. 5). Compared to control untreated cuttings (UC), plants treated with bacteria regardless of application method displayed significantly lower necrosis length. The inhibition percentages ranged from 43% (co-inoculation with S32) to 35% (co-inoculation with S19). When we drill the plant stem before inoculating the pathogen and/or the bacterial strain, we observed the formation of slight necrosis lesions only due to the drilling effect. The lesion in control plants treated only with bacteria was not significantly different in size from that in control plants without the pathogen nor bacteria.

3.2. In vitro *assessment of antagonism against* chlamydospora

The potential effect of the bacterial strains on fungal mycelial growth could be due to the action of diffusible metabolites or VOCs produced by the bacteria. The results from the two different *in vitro* experiments (Table 2) indicated that the bacterial inhibition of the fungi varied. In a dual culture assay, six bacterial strains *Bacillus ginsengihumi* (S38), *P. agglomerans* (S8), *B. reuszeri* (S30, S37), *Bacillus* sp. (S43, S46)) showed greater than 60% inhibition. Additionally, two strains *Bacillus licheniformis* (S44) and *Paenibacillus polymyx* (S15) also displayed a good level of inhibition of greater than 39%. Of these eight strains, four were in the genus *Bacillus: Bacillus ginsengihumi* (S38), *Bacillus* sp. (S43, S46), *Bacillus licheniformis* (S44) and two were *B. reuszeri* (S30 and S37), suggesting that bacterial strains related to the Bacillale order showed strong antifungal activity against this pathogen in dual culture. Furthermore, important antifungal activity was observed for most bacterial strains tested against *P. chlamydospora* due to VOC secretion. Of 46 strains, 44 exhibited more than 50% inhibition and all strains tested showed an inhibition rate of the radial mycelial growth of the pathogen greater than 34%. Fourteen strains had a biocontrol efficacy greater

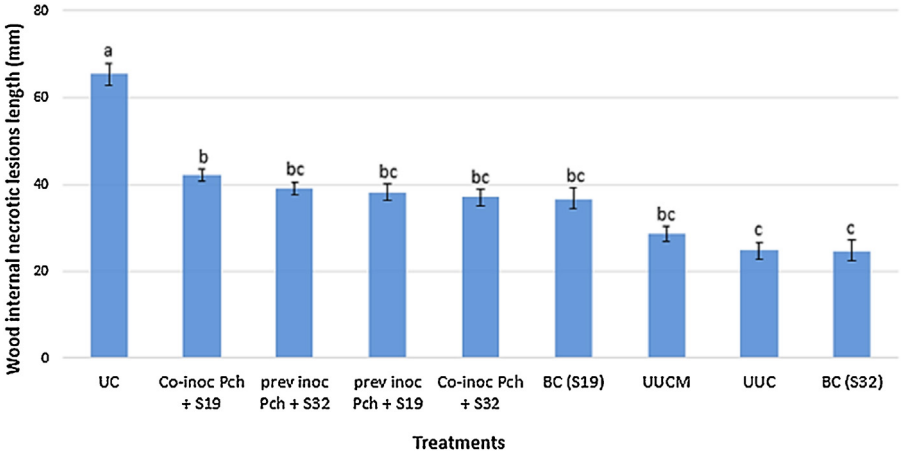

Fig. 5. Effect of the two selected bacterial strains (*Paenibacillus* sp. (S19) and *B. pumilus* (S32)) on the development of wood inner necrosis following either preventive or co-inoculation with *P. chlamydospora* in the 2015 bioassay. Cutting stems (cv. Cabernet Sauvignon) were co- or pre-inoculated (4 days) with bacteria and one mycelial plug of the pathogen before incubation in greenhouse for 90 days. The untreated controls (UC) were inoculated with the pathogen only. The bacterial controls (BC) were treated in the hole with bacterial suspension only. The un-inoculated untreated control (UUC and UUCM) were not both inoculated with the fungus and not treated with bacteria, but the latter were treated with sterile bacterial medium. Each value represents the mean of 14 cuttings (see Section 2.4.1). Bars with the same letter are not significantly different at $P = 0.05$ according to Newman and Keuls' test after ANOVA. The error bar corresponds to the standard deviation of the mean.

Table 2
In vitro mycelial growth inhibition activity of bacterial strains screened against the fungal pathogen *P. chlamydospora*.

Bacterial strains	Inhibition rate (%)/dual culture trial*	Inhibition rate (%)/VOCs emission trial*
S1	2.8 a	34 g
S11	2.8 a	58.5 de
S17	2.8 a	72.6 abcd
S2	2.8 a	69.4 abcde
S23	2.8 a	65.6 bcde
S28	2.8 a	68.8 bcde
S31	2.8 a	75.4 abc
S42	2.8 a	65.3 bcde
S9	2.8 a	69.7 abcde
S14	3.0 a	73.7 abcd
S16	5.3 a	60.1 cde
S39	5.6 a	63.9 bcde
S40	5.6 a	73.2 abcd
S29	5.6 a	72.4 abcde
S25	5.6 a	78.6 ab
S26	7.9 ab	67.5 bcde
S6	8.3 ab	68 bcde
S3	8.6 ab	64.5 bcde
S22	8.6 ab	59.3 cde
S27	8.8 ab	70.5 abcde
S13	11.1 ab	68.8 bcde
S4	12.5 ab	59 cde
S18	13 ab	59.2 cde
S36	16.7 ab	62 cde
S7	16.9 ab	67.2 bcde
S41	20.2 ab	84.7 a
S5	20.7 ab	65.8 bcde
S34	21.6 ab	61.2 cde
S45	23 ab	65 bcde
S32	23.2 ab	71 abcde
S33	23.7 ab	75.1 abcd
S12	24.2 ab	45.7 f
S19	24.9 b	74.3 abcd
S20	25.8 b	66.9 bcde
S24	26.3 b	66.4 bcde
S21	26.9 b	72.4 abcde
S44	38.9 c	66.4 bcde
S15	47.2 c	65.6 bcde
S38	63.4 d	74.3 abcd
S8	67.7 d	63.9 bcde
S30	73.1 de	58.5 de
S43	73.5 de	64.7 bcde
S37	82.8 e	62 cde
S46	86.1 e	57.1 e
S35	NA	65.6 bcde
S10	NA	78.6 ab

* Means in the same columns denoted by the same letter are not significantly different as determined by the test of Duncan ($P < 0.05$).

than 70% and S41 (*B. firmus*) achieved the highest control inhibition rate of 84.7%.

3.3. *VOC analysis of* Paenibacillus *sp. (S19) and* B. pumilus *(S32)*

GC–MS analyses of VOCs produced by *Paenibacillus* sp. (S19) and *B. pumilus* (S32) allowed us to identify 7 volatile compounds by their mass spectral properties in the NIST database (Table 3). The volatile secretion profile of these strains was compared with uninoculated medium control profiles. On the one hand, 2,5-dimethyl pyrazine was detected as the major volatile component from a 4 day-old S32 culture. Less abundant compounds in this strain were 3-octanone, trimethyl-pyrazine and 2-ethyl-3,5-dimethyl-pyrazine. On the other hand, the major volatile component from a 4 day-old S19 culture was of the pyrazine type, but it was not unequivocally identified. Other less-important volatile compounds that were identified were 2,6-Bis (2-methylpropyl) pyrazine and 1-Octen-3-ol. The NIST match percentage for all of the compounds detected

Table 3
Volatile organic compounds VOCs produced by the two antagonistic strains *Paenibacillus* sp. (S19) and *Bacillus pumilus* (S32) (GC/MS analysis).

Bacterial strain	Volatile compound	Retention time (minute)	Molecular weight (g/mol)
S19 (*Paenibacillus* sp.)	Compound of pyrazine type	12.8	–
	2,6-Bis (2-methylpropyl) pyrazine	12.4	192.3
	1-Octen-3-ol	6.9	128.22
S32 (*Bacillus pumilus*)	2,5-dimethyl Pyrazine	5.4	108.14
	3-octanone	6.6	128.21
	trimethyl-pyrazine	6.8	122.17
	2-ethyl-3,5-dimethyl pyrazine	8.1	136.19

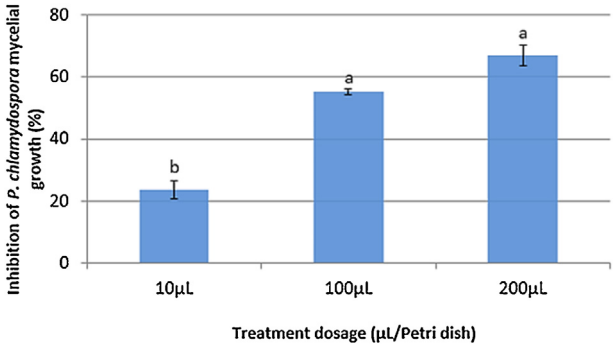

Fig. 6. *In vitro* inhibition (%) of *P. chlamydospora* by pure 2,5-dimethyl pyrazine after ten days of incubation at 25 °C. Different letters indicate mean values (three replicates) significantly different at $P = 0.05$ according to Newman and Keuls' test. The error bar corresponds to the standard deviation of the mean.

was equal or greater than 90%. These compounds most likely play an important role in the fungal antagonism shown by these strains.

3.4. *1-octen-3-ol and 2,5-dimethyl pyrazine inhibit the mycelial growth of* P. chlamydospora

As the antifungal activity of 1-octen-3-ol and 2,5-dimethyl pyrazine was reported in the literature, we further investigated to what extent these volatile compounds produced by *Paenibacillus* sp. (S19) and *B. pumilus* (S32), respectively, might inhibit the mycelial growth of *P. chlamydospora*. The two compounds inhibited pathogen development and the mycelial growth decreased at a high concentration, especially with 2,5-dimethyl pyrazine. When different quantities of this compound were added, dose-dependent mycelial inhibition was observed compared to the control. Significant inhibition of mycelial growth (55 and 67%) was observed with 100 and 200 µL/respectively (ANOVA, $P < 0{,}001$) (Fig. 6). Interestingly, the two levels of 1-octen-3-ol tested showed significant mycelial inhibition of greater than 96%.

3.5. *Effect of* Paenibacillus *sp. (S19) and* pumilus *(S32) on the grapevine defense response*

Plants defend against pathogens with three mechanisms of defense: reinforcement of plant cell walls, production of pathogenesis-related proteins (PR proteins) and the production of phytoalexins. To determine whether the necrosis reduction observed in stem cuttings following introduction of bacteria in the hole was associated with a differential induction of defense responses, the expression of ten defense genes, involved in these

	T0				T15			
	Pch	Co-inoc S19	prev-inoc S19	S19	Pch	Co-inoc S19	prev-inoc S19	S19
VvPR1	0,4	**0,1**	**0,5**	**8,9**	0,7	**5,2**	7,6	3,0
VvPR10	0,7	0,4	0,7	**3,0**	1,5	1,8	0,5	**0,2**
VvCHIT3	0,8	0,7	0,7	**5,4**	**2,9**	0,6	0,4	**0,2**
VvGLU	1,1	0,6	2,4	2,6	**0,1**	0,2	**0,2**	**0,1**
VvCALS	**0,4**	**0,4**	0,9	**6,1**	1,0	0,9	0,9	1,6
VvGST	0,5	2,2	1,9	**3,0**	0,9	0,9	0,9	1,9
VvANTS	0,8	**0,5**	1,0	**0,2**	1,0	1,2	1,2	1,3
VvSTS	1,0	0,9	0,8	1,3	2,0	0,9	**0,2**	0,4
VvCHS	1,0	**0,5**	0,8	0,6	**0,3**	0,4	0,9	**4,4**
VvPAL	1,3	0,8	1,2	1,4	**0,5**	**0,3**	**0,3**	0,8

Fig. 7. Relative expression of 10 major defence-related genes following inoculation by the bacterial strain *Paenibacillus* sp. (S19).
Genes are significantly at $P = 0.05$ induced (bold and underlined) or repressed (bold and underlined) in leaves of grapevine cuttings of all treatment (see 2.4.1) with the bacterial strain tested at 2 hpi (T0) and 15 dpi (T15) after pathogen inoculation compared to the control "UUCM" (Uninoculated Untreated Control with sterile bacterial Medium). Although not significant, genes over expressed appear surrounded in red, corresponding to an expression level higher than 2 times compared with the control, while those repressed appear surrounded in gray, with an expression level 1.5 times lower compared with the control. ***VvPR1***: PR protein 1, ***VvPR10***: PR protein 10, ***VvCHIT3***: chitinase class III, ***VvPAL***: phenylalanine ammonia lyase, ***VvSTS***: stilbene synthase, ***VvCHS***: chalcone synthase, ***VvANTS***: antranilate synthase, ***VvCALS***: callose synthase, ***VvGST***: Glutathione S-transferase, ***VvGLU***: b-1,3 glucanase.

	T0				T15			
	Pch	Co-inoc S32	prev-inoc S32	S32	Pch	Co-inoc S32	prev-inoc S32	S32
VvPR1	0,4	**0,1**	0,5	7,5	0,7	**30,4**	**13,8**	**2,2**
VvPR10	0,7	0,7	0,6	2,3	1,5	**1,8**	**3,6**	1,0
VvCHIT3	0,8	1,0	**0,4**	4,3	**2,9**	**1,8**	1,3	1,0
VvGLU	1,1	2,1	0,7	3,1	**0,1**	1,1	**1,5**	0,4
VvCALS	**0,4**	**0,4**	0,9	**4,8**	1,0	**6,3**	0,8	1,2
VvGST	0,5	1,0	1,8	0,8	0,9	**16,6**	1,2	0,4
VvANTS	0,8	0,8	0,8	**0,4**	1,0	**2,0**	**1,5**	**1,5**
VvSTS	1,0	1,9	0,6	1,8	2,0	**3,4**	1,1	**0,5**
VvCHS	1,0	0,9	**0,5**	0,8	**0,3**	2,7	**0,03**	0,4
VvPAL	1,3	1,0	0,8	**2,3**	**0,5**	**0,4**	**0,3**	**0,2**

Fig. 8. Relative expression of 10 major defence-related genes following inoculation by the bacterial strain *Bacillus pumilus* (S32).
Genes are significantly at $P = 0.05$ induced (bold and underlined) or repressed (bold and underlined) in leaves of grapevine cuttings of all treatment (see 2.4.1) with the bacterial strain tested at 2 hpi (T0) and 15 dpi (T15) after pathogen inoculation compared to the control "UUCM" (Uninoculated Untreated Control with sterile bacterial Medium). Although not significant, genes over expressed appear surrounded in red, corresponding to an expression level higher than 1.5 times compared with the control, while those repressed appear surrounded in gray, with an expression level 2 times lower compared with the control. ***VvPR1***: PR protein 1, ***VvPR10***: PR protein 10, ***VvCHIT3***: chitinase class III, ***VvPAL***: phenylalanine ammonia lyase, ***VvSTS***: stilbene synthase, ***VvCHS***: chalcone synthase, ***VvANTS***: antranilate synthase, ***VvCALS***: callose synthase, ***VvGST***: Glutathione S-transferase, ***VvGLU***: b-1,3 glucanase.

grapevine defense paths, was evaluated by quantitative RT-PCR in leaves 2 h and 15 days post inoculation of *P. chlamydospora*.

Our results showed that the response of plants was different between T0 and T15 depending on the treatment and the genes selected (Figs. 7 and 8).

In the *P. chlamydospora* inoculated treatments, all grapevine transcripts were not affected significantly at 2 hpi, except for *VvCALS*. In contrast, at 15 dpi two genes involved in the phenylpropanoid pathway (*VvPAL*, *VvCHS*) and a PR protein (*VvGLU*) were significantly repressed and only one gene (*VvCHIT3)* was upregulated in the same treatment.

In the plants treated with the bacterial suspension alone four days before sampling, the grapevine transcripts were different depending on the strain (*B. pumilus* (S32) or *Paenibacillus* sp. (S19)) and the sampling time (2 hpi or 15 dpi). For *Paenibacillus* sp. (S19), while the expression of five genes (*VvPR1*, *VvPR10*, *VvCHIT3*, *VvCALS* and *VvGST*) was upregulated at 2 hpi, only one *VvCHS* was upregulated at 15 dpi. Conversely, at 15 dpi, the majority of PR proteins (*VvPR10*, *VvCHIT3* and *VvGLU*) were down regulated (Fig. 7).

The transcripts of all PR proteins (*VvPR1*, *VvPR10*, *VvCHIT3* and *VvGLU*), *VvCALS*, *VvSTS* and *VvPAL* were overexpressed in the leaves of the S32-treated plants four days before sampling. However, two genes (*VvPAL* and *VvCALS)* were significantly up-regulated. At 15 dpi, two genes (*VvPR1*, *VvANTS*) were significantly over-expressed and the two genes coding for the two important enzymes involved in the stilbene biosynthesis pathway (*VvSTS* and *VvPAL*) were significantly down regulated. Interestingly, transcripts of *VvGST* which is involved in the oxidative stress response system were up regulated at 2 hpi in the preventive inoculation treatment for S19 (*Paenibacillus* sp.). In plants co-inoculated with S32 and *P. chlamydospora,* the response of genes was greatly different between 2 hpi and 15 dpi. While only two genes (*VvGLU* and *VvSTS*) were upregulated at 2 hpi, seven (*VvPR1*, *VvPR10*, *VvCHIT3*, *VvCALS, VvGST, VvANTS* and *VvSTS*) were overexpressed at 15 dpi. When the plants were treated with the S32 four days before inoculation with the pathogen (prev-inoc S32), the expression of 8 selected genes did not significantly change at 2 hpi (Fig. 8). Only two genes (*VvCHIT3* and *VvSTS*) were significantly down regulated at 2 hpi. In the prev-inoc S32 treatment, three PR protein transcripts (*VvPR1, VvPR10* and *VvGLU*) and *VvANTS* transcripts involved in indole biosynthesis, were up-regulated and two genes involved in the phenylpropanoid pathway (*VvCHS*, *VvPAL*) were significantly down regulated at 15 dpi.

In prev-inoculation *Paenibacillus* sp. (S19) treatment, only *VvPR1* was overexpressed at 2 hpi, whereas at 15 dpi, *VvGLU*, *VvSTS* and *VvPAL* were significantly repressed (Fig. 7).

4. Discussion

In this study, we analyzed the antagonistic activity of 46 bacterial strains against *P. chlamydospora,* a major fungus involved in Esca disease, under different *in vitro* and *in planta* conditions. A previous publication (Haidar et al., 2016) reported the screening of the same strains isolated from vineyards against the two other major grapevine pathogens *Botrytis cinerea* and *Neofusicoccum parvum*.

Of the 46 bacterial strains, eight significantly reduced the length of the necrosis lesions due to *P. chlamydospora* in grapevine cuttings under greenhouse conditions:

Enterobacter sp. (S24), *Paenibacillus* sp. (S18, S19), *B. pumilus* (S32), *B. reuszeri* (S28, S31), *Bacillus* sp. (S34), *P. illinoisensis* (S13) These strains exhibited good inhibition ranging from 31.4% to 38.7%. Similar results were obtained with the oomycete *Pythium oligandrum*, which colonized the plant root system, and significantly reduced (40–50%) necrosis due to *P. chlamydospora* in grapevine cuttings under greenhouse conditions (Yacoub et al., 2016). The inhibitory efficacy of these 8 strains (except for strain *P. illinoisensis* (S13)) was greater than the efficacy of the fungicide Thiophanate methyl, which was an active ingredient reported as one of the most efficient of four fungicides tested for the protection of pruning wounds for controlling nine GTD pathogens, including *P. chlamydospora* (Rolshausen et al., 2010). Moreover, *P. chlamydospora* infections of grapevine pruning wounds were reduced by a single paste or a single spray application of thiophanate-methyl, and pre-infection paste application provided the best control (Díaz and Latorre, 2013). Little information exists in the literature about the efficiency *in vivo* of Fluazinam and Thiophanate-methyl for the control of *P. chlamydospora*. However, Fluazinam was the most efficient treatment in our study, inhibiting necrosis by 44%. Similarly, Fluazinam treatment of pruning wounds inhibited *E. lata* in field trials (Halleen and Lombard, 2010; Pitt et al., 2011). Interestingly, except for one strain of *Enterobacter* sp. (S24), all of the strains that efficiently inhibited *P. chlamydospora* in our *in-vivo* screening were in the bacterial order Bacillales, in the genera of

Paenibacillus, *Bacillus* and *Brevibacillus*. This result was contrary to our previous findings with *N. parvum*, under the same experimental conditions, which showed that the four most efficient strains (*P. agglomerans*, *Enterobacter* sp.) were Enterobacteriaceae (Haidar et al., 2016). Few reports in the literature indicate that Bacillales strains may be promising BCA candidates against *P. chlamydospora*. For example, *B. subtilis* was applied at the surface of fresh pruning wounds in the field, and decreased the incidence of various GTD pathogens including *P. chlamydospora* eight months after inoculation (Kotze et al., 2011). The same authors also reported a 77% reduction in the incidence of *P. chlamydospora* after treatment with *Trichoderma* sp. USPP-T1. However, it should be noted that many reports of biocontrol and/or prevention of *P. chlamydospora* infection concern *Trichoderma*. Many isolates and species of *Trichoderma*, including *T. harzianumt*, *T. asperellum*, *T. gamsii*, *T. atroviride* have been demonstrated to colonize grapevine wounds or prevent and reduce vascular streaking by *P. chlamydospora* in grapevine under greenhouse, field and nursery conditions (Aloi et al., 2015; Di Marco et al., 2004; Di Marco and Osti, 2007; Pertot et al., 2016; Prodorutti et al., 2012).

In our experiments, seven of the eight most effective bacterial strains against *P. chlamydospora* were originally isolated from grapevine wood. This observation should be further investigated and confirmed with more strains. However, it is possible that these strains are better adapted to the grapevine wood environment than others, especially strains originating from the fruit surface. These results agree with those reported by Pancher et al. (2013), who showed that bacterial strains isolated from domestic grapevine (*V. vinifera* subsp. *vinifera*) provided better *in vitro* biocontrol of *P. chlamydospora* than strains isolated from wild grapevine (*V. vinifera* subsp. *sylvestris*).

Because the best performance of a BCA for disease prevention depends on an appropriate application method (Whipps and McQuilken, 2009), we used three different methods to apply the best bacterial strains to the grapevine cuttings (*i.e.*, co-inoculation, preventive inoculation in the hole or soil drenching). Preventive application at the soil surface or in the hole in the wood cutting in which the fungal pathogen was inoculated did not significantly improve the bacterial inhibitory efficacy for *P. chlamydospora* lesions. In fact, the bacterial efficiency was more strain dependent than on the inoculation method. Interestingly, we observed that the biocontrol efficacy of the same bacterial strains against another major GTD pathogen *(N. parvum)* varied with the application method. Drenching the plant soil with the same bacterial strains was less efficient for inhibiting *N. parvum* symptoms than the application in the hole (unpublished data). Similarly, in other studies, the mode of application of bacterial BCAs affected the level of disease control, for example, for the severity of grey mold in grapevine (Magnin-Robert et al., 2007).

We carried out other experiments to study the biocontrol mechanisms of the most antagonistic strains. While *P. agglomerans* strains showed the greatest antagonism against *N. parvum* (Haidar et al., 2016), of the eight most efficient strains against *P. chlamydospora* under the same conditions, *Enterobacter* sp. (S24) significantly reduced the necrosis produced by *N. parvum*. Similarly, *B. pumilus* (S32) also exhibited high antagonism against both *P. chlamydospora* and *N. parvum*. The efficacy of these two strains for decreasing the internal necrotic lesion length caused by the two pathogens was greater than 30%. Further investigations should be conducted for studying the efficacy of these bacterial strains to control other pathogens involved in GTDs.

In vitro and *in planta* experiments were carried out to understand the mechanism(s) by which the microbial BCAs exerted an effect on the pathogen to facilitate successful future development and application. The *in vitro* assays showed the inhibitory effects of the bacterial strains against the mycelial growth of *P. chlamydospora* in both dual culture and assays of volatile compounds, but a relatively lower efficacy was observed in the dual culture assay. Thus, the production of various extracellular antifungal compounds as diffusible and/or volatile compounds by most of the bacteria tested may partially explain their mechanisms of action against the pathogen.

Bacterial volatile organic compounds (VOCs) can reduce fungal growth, impair fungal spores and hyphae, and/or promote plant growth (Effmert et al., 2012; Kai et al., 2007, 2009; Weisskopf, 2013). Several VOCs produced by *Bacillus* and *Paenibacillus* species have been shown to exhibit antibacterial and/or antifungal activity (Berrada et al., 2012; Cernava, 2012; Liu et al., 2008; Rybakova et al., 2015; Ryu et al., 2003, 2004; Zhang et al., 2013). In fact, solid phase micro extraction (SPME) coupled with gas chromatography–mass spectrometry (GC–MS) allows the analysis of a wealth of bacterial VOCs. Compared with diffusible compounds, volatile metabolites could facilitate the interactions between physically separated microorganisms. A potential antagonistic interaction could take place easily between VOCs produced by endophytic bacteria and pathogens present in the same wood environment. All of the tested strains produced VOCs with antifungal activities that ranged from 34% to 74.3%. Two of the most effective strains *in planta*, *Paenibacillus* sp. (S19) and *B. pumilus* (S32) were tested for the production of VOCs by SPME headspace analysis. Four VOCs were produced by *B. pumilus* (S32), while three were detected from *Paenibacillus* sp. (S19), which could account for the antagonistic effects. Interestingly, these two strains emitted distinct pyrazine derivatives into the headspace. It is likely that pyrazine is the predominant molecule produced by these bacteria. Similarly, *B. polymyxa* has been shown to produce different volatile pyrazine compounds (Cernava, 2012). Many studies have reported the production of pyrazine derivatives from several species of *Bacillus*, such as *B. subtilis* and *B. cereus* (Adams and De Kimpe, 2007; Owens et al., 1997). The antifungal activity of some of the compounds we detected has been previously reported, such as 1-octen-3-ol and 2,5-dimethyl-pyrazine produced by *Paenibacillus* sp. (S19) and *B. pumilus* (S32), respectively (Chuankun et al., 2004; Munjal et al., 2016; Zhao et al., 2011; Zheng et al., 2013). It has also been previously reported that 1-octen-3-ol produced by *P. polymyxa* strain BMP-1 effectively inhibited the mycelial growth of eight fungal pathogens, including *B. cinerea* (Zhao et al., 2011). This compound also induced defensive responses in *Arabidopsis thaliana* and enhanced the resistance of the plant against *B. cinerea* (Kishimoto et al., 2007).

In this study we demonstrated for the first time the suppression by 1-octen-3-ol and 2,5-dimethyl pyrazine produced by *Paenibacillus* sp (S19) and *B. pumilus* (S32), respectively, of *P. chlamydospora*.

The production of diffusible compounds or antibiotics is another important inhibition mechanism that explains the efficacy of biocontrol bacteria. Because the mycelial growth of *P. chlamydospora* is slow, the bacteria in dual culture assays were inoculated when the *P. chlamydospora* culture was 15 days old. Six strains: *Bacillus ginsengihumi*, (S38), *P. agglomerans* (S8), *B. reuszeri* (S30, S37), *Bacillus* sp. (S43, S46) potently inhibited (>60%) fungal growth as indicated by the formation of an inhibition zone due to diffusible bacterial metabolites. Of these six, only one (S30) showed an inhibition level greater than 30% in *in planta* bioassays (Fig. 1). However, this result confirmed that a positive correlation does not always exist between *in vitro* inhibition and *in planta* control of infection and/or the development of symptoms of infection (Donmez et al., 2011; Duffy et al., 2003; Köhl, 2009; Köhl et al., 2011; Pliego et al., 2011). The inhibition of *P. chlamydospora* by *Bacillus subtilis* (AG1) has been shown *in vitro* (Alfonzo et al., 2009). Similarly, two grapevine endophytic strains of *Bacillus* sp. (3R1 and 3R4) inhibited both *P. chlamydospora* and *P. aleophilum in vitro* (Andreolli et al., 2016).

The induction of grapevine resistance by beneficial bacteria has been considered as a major mode of action and has been often

reported against several pathogens including *B. cinerea*, *Plasmopara viticola* and *Erysiphe necator* following the application of various biotic or abiotic treatments, for example, by *Pseudomonas fluorescens*, *P. putida* or a grapevine elicitor such as Benzothiadiazole "BTH" (Dufour et al., 2013; Verhagen et al., 2010). To our knowledge, no investigation of resistance induced by bacteria against GTD fungi in grapevine has been reported. Recently, the oomycete *Pythium oligandrum* has been shown to reduce *P. chlamydospora* necrosis by inducing grapevine defense systems (Yacoub et al., 2016). We explored this facet of the bacterial induction of resistance by focusing on 10 major grapevine genes involved in defense mechanisms, and demonstrated the potential of the most efficient strains (*B. pumilus* (S32) and *Paenibacillus* sp (S19)) to induce plant defense systems. *P. chlamydospora* infection has been shown to induce grapevine defense mechanisms in young plants (Martin et al., 2009) and in *V. vinifera* cell cultures (Lima et al., 2011). In our experiments, plants inoculated with *P. chlamydospora* alone did not show significant up regulation of transcript expression at 2 hpi and 15 dpi, except for *VvCHIT3,* which was over-expressed at 15 dpi. This PR-protein gene could result in chitin lysis in the fungal cell wall. This difference in specific grapevine responses to *P. chlamydospora* infection may result from the different plant tissues used and/or to the time before the assessment of gene expression (Martin et al., 2009; Lima et al., 2011). Infection by the pathogen mostly led to the significant repression of defence-related genes, confirming that pathogens affect the metabolism of the growing host plant, and exploit plant cellular resources and/or suppress defense mechanisms *via* effectors (Dufour et al., 2013; Polesani et al., 2008).

In plants treated with bacteria alone (*Paenibacillus* sp. (S19) or *B. pumilus* (S32)), the expression of various genes including PR transcripts and the callose synthase gene (*VvCALS*), was induced four days after bacterial treatment, suggesting the direct activation of the grapevine defense processes by these bacteria.

It was noteworthy in this study that prior treatment of the plants with bacteria (20–40 min or 4 days before inoculation) did not induce a plant response at 2 h after *P. chlamydospora* inoculation. The absence of gene overexpression was in accordance with other results showing down regulation of many defence-related genes in *Pseudomonas fluorescens* PTA-CT2–infected plants after challenge with *B. cinerea* (Gruau et al., 2015). Furthermore, the antagonistic bacterium *Pseudomonas fluorescens* (PTA-CT2) was shown to stimulate grapevine plants by inducing an early oxidative burst and produce phytoalexins that protected the grapevine against gray mold disease (Gruau et al., 2015; Verhagen et al., 2010, 2011).

However, an intense up-regulation of grapevine transcripts was observed 15 days after pathogen inoculation in plants treated with *P. chlamydospora* and *B. pumilus* (S32), especially when co-inoculated (bacterial inoculation 20–40 mn before pathogen inoculation). These results are in accord with the increase seen in the *VvPR*-protein *(VvPR10* and *VvCHIT3)* and the *VvGST* (oxidative stress response system) transcript levels observed after inoculation by *P. chlamydospora* in plants pretreated with the biocontrol agent *P. oligandrum* (Yacoub et al., 2016). In contrast, Yacoub et al. (2016) showed an up regulation of *VvPAL*, a key enzyme in the phenylpropanoid pathway, which was not confirmed by our findings 15 days after pathogen inoculation. Furthermore, the case of *VvANTS* was interesting because this gene is involved in the secondary salicylic pathway (isochorismate pathway) according to Dufour et al. (2013). The transcripts of *VvANTS* were up-regulated 15 days after pathogen inoculation in plants leaves treated with *B. pumilus* (S32). As for the preventive inoculated plants with *B. pumilus* (S32) (four days before pathogen inoculation), there was a relative decrease in expression of some genes which could be due to the longer bacterial stay, during 19 days, within the plant allowing us to detect a lowering in plant defense reaction. Lastly, all these results suggest that *B. pumilus* (S32) may trigger a systemic immune response in grapevine and this response could decrease over time after 15 days of bacterial presence. Regarding *Paenibacillus* sp. (S19), except for *VvPR1*, the genes in plants inoculated with the bacterium alone tended to be more induced compared with plants inoculated by both microorganisms. Our results suggest that the inhibition by *Paenibacillus* sp. strain S19 could mostly be a result of other modes of action, such as the production of VOCs. Overall, this study showed the activation of grapevine defense mechanisms by both bacterial strains (*Paenibacillus* sp. (S19) and *B. pumilus* (S32)) tested four days after their introduction in the grapevine cutting wood. At 15 days after inoculation by the pathogen, this effect was only maintained in the leaves of plants treated with *B. pumilus* (S32) and *P. chlamydospora*. This suggests that the induction of systemic resistance in the grapevine is a possible important mechanism of action for *B. pumilus* (S32) against *P. chlamydospora* because it is initiated early and more long-lasting. Further experiments at different times are needed in order to confirm these first results. Systemic resistance is consistent with studies on other *B. pumilus* strains that elicit plant defenses against various diseases in different host plants (Choudhary and Johri, 2009; Lanna-Filho et al., 2013). Furthermore, *B. pumilus* may be considered as endophytic bacteria in grapevine because it was isolated from grapevine wood (Bruez et al., 2015) as well as *in vitro* from grapes (Thomas, 2004).

To conclude, two of our most efficient bacterial strains originating from vineyards, *B. pumilus* (S32) and *Paenibacillus* sp. (S19) are potent biological agents against *P. chlamydospora* infection in grapevine to prevent Esca symptoms. The production of volatile compounds was identified as a major mode of action of these two bacterial strains. Moreover, specific grapevine defense responses were induced following treatment with each of these bacteria alone, particularly in plants pretreated with *B. pumilus*, in which the induction of resistance in the grapevine was even more longterm.

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