Biocontrol of grapevine aerial and root pathogens by *Paenibacillus* sp. strain B2 and paenimyxin *in vitro* and *in planta*

Zhipeng Hao^{a,b}, Diederik Van Tuinen^b, Daniel Wipf^b, Léon Fayolle^b, Odile Chataignier^b, Xiaolin Li^c, Baodong Chen^a, Silvio Gianinazzi^b, Vivienne Gianinazzi-Pearson^b, Marielle Adrian^{b,*}

^a State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, 100085 Beijing, China

^b Agroécologie, AgroSup Dijon, CNRS, INRA, Univ. Bourgogne Franche-Comté, 21065 Dijon cedex, France

^c College of Resources and Environmental Sciences, China Agricultural University, 100193 Beijing, China

HIGHLIGHTS

- *Paenibacillus* sp. strain B2 and its antagonistic factor paenimyxin control grapevine pathogens.
- The bio-control effects are active against aerial or root pathogens *in vitro* or *in planta*.
- Paenimyxin acts as a biopesticide against the oomycete but does not trigger plant defense.
- *Paenibacillus* sp. strain B2 can induce plant defense gene expression.

ARTICLE INFO

Article history:

Received 31 October 2016

Revised 22 February 2017

Accepted 6 March 2017

Available online 12 March 2017

Keywords:

Paenibacillus

Paenimyxin

Grapevine pathogens

Biocontrol

Defence related genes

ABSTRACT

Paenibacillus sp. strain B2, isolated from the mycorrhizosphere of *Sorghum bicolor*, was previously shown to possess antagonistic activity against fungi and bacteria due to the secretion of the peptide paenimyxin. The effects of strain B2 and paenimyxin were assessed against grapevine pathogens as they potentially represent a non-polluting complement or an alternative biocontrol agent to existing disease management strategies. In this study, the effects of *Paenibacillus* sp. strain B2 and paenimyxin were analyzed *in vitro* or *in planta* on the aerial or root pathogens of grapevine *Botrytis cinerea*, *Plasmopara viticola* and *Xiphinema index*. The bacterium significantly reduced *B. cinerea* development *in vitro*, and substantially decreased *X. index* numbers in soil and gall formation in the root system of grapevine plants. *In vitro*, paenimyxin inhibited the mycelial growth of *B. cinerea*, the mobility of the zoospores of *P. viticola* and *X. index* activity. Supplying paenimyxin at the time of inoculation of leaf tissue with *P. viticola* decreased infection sites, compared to application prior to fungal inoculation. Using the *P. viticola*/grapevine pathosystem, we showed that paenimyxin acts as a biopesticide against the oomycete but does not trigger plant defense. Conversely, *Paenibacillus* sp. strain B2 can induce the expression of the defense genes *CHI* (chitinase), *PR-3* (proteinase inhibitor) and *LOX* (lipoxygenase). Altogether, the results obtained highlight the interest of *Paenibacillus* sp. strain B2 and paenimyxin for the biocontrol of different grapevine diseases.

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1. Introduction

Grapevine (*Vitis* spp.) is susceptible to several pathogenic microorganisms including bacteria, viruses, oomycetes, fungi and nematodes. *Botrytis cinerea* is the fungus responsible for grey mold, a disease known to cause severe qualitative and quantitative damage of the grape harvest. Chemical fungicides are problematic because of the risks of non-target impacts appearing and the establishment of resistant *Botrytis* sp. strains (Leroch et al., 2011), as well

as the presence of residues in wines (Edder et al., 2009). Biological control strategies against *B. cinerea* have been developed but remain poorly used as their efficacy under field conditions is often limited (Elmer and Reglinski, 2006; Hahn, 2014). Another serious grapevine disease, downy mildew, is caused by the oomycete *Plasmopara viticola*. This pathogen enters the plant via stomata and colonizes inner leaf tissues by developing an intercellular mycelium before emerging through other stomata for sporulation. As the life cycle of this oomycete is reduced to one week in optimal temperature and humidity conditions, the control of the disease requires numerous treatments, especially in vineyards subjected to rainy springs. Numerous chemical fungicides have been developed, but some of them lead to the development of resistant strains (Nanni

* Corresponding author at: UMR Agroécologie, INRA, 7 rue Sully, BP 86510, 21065 Dijon cedex, France.

E-mail address: marielle.adrian@u-bourgogne.fr (M. Adrian).

et al., 2016). Copper is practically the unique solution in organic farming as all other alternatives to chemicals are not efficient enough in situations of middle to high disease pressure. However, the secondary effect of copper accumulation in soils is problematic (Brunetto et al., 2014). The *Grapevine Fanleaf Virus* (GFLV) is responsible for fanleaf disease, one of the most detrimental grapevine diseases worldwide, with yield decreases up to 80% and an alteration in berry quality (Andret-Link et al., 2004; Martelli, 2012). The ectoparasitic root-feeding nematode *Xiphinema index*, which also has direct pathogenicity, is the vector for the transmission of GFLV. *Xiphinema* sp. can be difficult to control using chemical nematocides, which may have a poor penetration potential in some soils, a high acute toxicity and a detrimental effect on beneficial non-target soil organisms (Barker and Koenning, 1998; Abawi and Widmer, 2000). In addition, their use is now forbidden in some countries. Nematicidal plants have been identified but are often not efficient enough (Aballay et al., 2001), and the recommended 10-year fallow therefore remains the most efficient strategy (Demangeat et al., 2005), although highly impractical.

Research over the past decades has led to a significant improvement in knowledge about the beneficial effects of antagonistic microorganisms (Cook, 2000), which can offer protection against insect herbivores and phytopathogens, including bacteria, fungi, nematodes, and viruses (Gu et al., 2010; Han et al., 2017; Saophuong et al., 2014). These act as antagonists against pathogens through competition (Bloemberg and Lugtenberg, 2001), production of antibiotics, extracellular enzymes or metabolites (Ongena and Jacques, 2008; Degenkolb and Vilcinskis, 2016a,b), and induced systemic resistance (ISR) (Shoresh et al., 2010; Schouteden et al., 2015). In a previous study the gram-positive bacterium *Paenibacillus* sp. strain B2, isolated from the mycorrhizosphere of *Sorghum bicolor* inoculated with *Glomus mosseae* (Budi et al., 1999), was shown to possess antagonistic activity towards soil-borne fungal pathogens partly due to the secretion of hydrolytic enzymes (Budi et al., 2000). In addition, *Paenibacillus* sp. strain B2 secretes the peptide paenimycin, an antagonistic factor active against fungi as well as gram-positive and gram-negative bacteria (Selim et al., 2005). When applied to soil, paenimycin transiently impacts the genetic structure and density of soil bacterial communities (Selim et al., 2007). Paenimycin also induces defense reactions in *Medicago truncatula*, especially the production of hydrogen peroxide and the expression of defense-related genes (Selim et al., 2010). Previous papers reported that the mode of action of *Paenibacillus* species against microorganisms involves the induction of plant defense. For example, *Paenibacillus alvei* K165 protects *Arabidopsis* plantlets against *Verticillium dahliae* through both salicylate and jasmonate-dependent pathways (Gkizi et al., 2016; Sang et al., 2014). We therefore wanted to know if *Paenibacillus* strain B2 and paenimycin were able to trigger grapevine defense mechanisms.

The present study was conducted to assess the efficacy of *Paenibacillus* sp. strain B2 and paenimycin against the problematic grapevine pathogens *B. cinerea* (fungus), *P. viticola* (oomycete), and *X. index* (nematode). The potential of *Paenibacillus* sp. strain B2 and paenimycin in biocontrol strategies against grapevine pathogens offers a non-polluting complement or alternative to existing strategies of grapevine disease management in order to reduce the use of chemical pesticides.

2. Material and methods

2.1. Biological material

Cuttings of grapevine cv. Marselan (Cabernet sauvignon × Grenache) plants and of the rootstock SO4 (*V. berlandieri* × *V. riparia*) were used for shoot and root pathogen tests, respectively. Marse-

lan plants were obtained from herbaceous cuttings placed in individual pots (10 × 10 cm) containing a mixture of blond peat, sand, and perlite (4:1:1, vol/vol/vol). They were grown in a glasshouse at a temperature of 24 and 18 °C (day and night, respectively) with a 16 h light photoperiod at a relative humidity (RH) of 70 ± 10% until they developed six leaves, as previously described (Allège et al., 2007). Plants were watered daily and a fertilizing solution (Plant-Prod, Puteaux SA, France) was supplemented once a week. SO4 plants were grown *in vitro* in Murashige and Skoog medium (Murashige and Skoog, 1962) in a growth cabinet at 24 °C with a 16 h light photoperiod.

B. cinerea isolate BO5.10 (Bessire et al., 2007) was grown on PDA (potato dextrose agar; Difco). Conidia were harvested in water, filtered through glass wool and suspended in 1/2 PDB (potato dextrose broth; Difco). The suspension was adjusted to a concentration of 5.10⁴ spores per milliliter for bioassays.

P. viticola was isolated from a Burgundy vineyard and maintained on *V. vinifera* L. cv. Marselan herbaceous cuttings in the glasshouse as previously described (Trouvelot et al., 2008). The plants were placed in the dark at 100% RH overnight in order to induce sporulation. Sporangia were collected from the abaxial leaf side using a brush and suspended in distilled water. The concentration was adjusted to 10⁴ sporangia per milliliter using a Malassez hemacytometer before inoculation.

X. index Thorne and Allen (1950) was collected in a Burgundy vineyard and reared under greenhouse conditions on *Ficus carica* L. to provide a permanent source of virus-free nematodes (Coiro and Brown, 1984). Nematodes were extracted from soil using the Baermann funnel and sieving procedure as previously described (Hao et al., 2012). Active nematodes were recovered in the bottom of the Petri dish after 48 h; adults and juveniles were counted in the final suspension with an etched grid. Nematode inoculation consisted of dispensing a water suspension of 100 nematodes (10 nematodes mL⁻¹) into evenly spaced 6–8 cm deep holes around plants. Non-inoculated plants received an equivalent volume of water.

Paenibacillus sp. strain B2 (Budi et al., 1999) was grown in liquid Luria-Bertani (LB) culture medium at 28 °C, agitated at 125 rpm for 20 h to reach a stationary phase. *Escherichia coli* strain MRAP2 was cultured at 37 °C on LB culture medium.

The antagonistic peptide paenimycin was purified from the bacterial culture medium by cation-exchange, reverse-phase, and size exclusion chromatography as previously described (Selim et al., 2005).

2.2. Analysis of antagonistic activities of *Paenibacillus* sp. strain B2

To test the antagonistic activity of *Paenibacillus* sp. strain B2 against *B. cinerea* BO5.10, a single drop of *Paenibacillus* sp. strain B2 culture, or of *E. coli* as negative control, was inoculated on each side of a 9 mm petri dish containing PDA medium, and incubated 3 days at 20 °C prior to inoculation in the center of a disk (~2 mm in diameter) of *B. cinerea* from a PDA culture. Mycelial growth of the pathogen was observed after 1 week. The experiment was repeated 3 times.

The effect of *Paenibacillus* sp. strain B2 on *X. index* mortality was tested *in vitro* in microtitration plates (Sigma-Aldrich, Milan, Italy). A volume (0.5 ml) of a *Paenibacillus* sp. strain B2 suspension (0, 10⁶, 10⁷, 10⁸, 10⁹ CFU mL⁻¹) was added to 15 adult females. Nematodes were then observed under a light microscope at 1, 2, 4, 8, 24 and every 8 or 16 h intervals for 120 h. The active/inactive nematodes were recognized by the reaction of their body to pricking with soft fibers. The experimental design was randomized, with each concentration replicated four times, and each experiment was repeated at least twice with separate controls.

The effect of *Paenibacillus* sp. strain B2 on *X. index* development *in planta* was evaluated on SO4 grapevine plants in a two factorial experiment, with a bacterial treatment (inoculation with *Paenibacillus* sp. strain B2) and a pathogen treatment (inoculation with *X. index*). Four conditions were used as follows: 1, control (no *Paenibacillus* sp. strain B2 and no *X. index*) (CK); 2, inoculation with *Paenibacillus* sp. strain B2 at repotting time (B2); 3, inoculation with *X. index* (Xi) 1 week after repotting; 4, inoculation with *Paenibacillus* sp. strain B2 at repotting time and *X. index* 1 week later (B2 + Xi). Glass jars (750 ml) were filled with 240 ml of a 1:1 (v/v) mix of sterilized (180 °C, 6 h) terragreen® (OilDri-US special, Mettman, Germany) and a γ -irradiated clay-loam soil (pH 7.96 (soil:water, 1:2.5), 14.4 g kg⁻¹ organic matter, 67 mg kg⁻¹ Olsen-P, 1.46 mg kg⁻¹ NaOH-extractable N, 0.241 g kg⁻¹ NH₄Ac-exchangeable K) and autoclaved (120 °C, 30 min) twice at 24 h interval. Ten microliters of a bacterial suspension (10⁹ cfu ml⁻¹ in 0.1 mM MgSO₄) were added to 65 ml sterile Long-Ashton nutrient solution, and the mixture supplied to each jar to obtain 60% of water holding capacity of the growth medium. Ten microliters 0.1 mM MgSO₄ without bacteria served as control. After each treatment, one *in vitro*-produced SO4 plant (with two leaves and about 2 cm long roots without agar) was transplanted into each jar, and allowed to establish for 1 week in an illumination incubator at 24 °C with a 16 h light photoperiod. Nematodes were then inoculated at the root level as indicated above (100 nematodes/pot). Five replicates per treatment were harvested at 35 days after inoculation with *X. index*. Shoot fresh weight was recorded. The number of nematodes in the soil and the number of galls on roots were determined as previously described (Xu et al., 2008). Root samples were weighed, ground in liquid nitrogen and stored at –80 °C until gene expression analysis.

2.3. Analysis of antagonistic activities of paenimyxin

2.3.1. Effect on *Botrytis cinerea*

The suppressive effect of paenimyxin on mycelial growth of *B. cinerea* was investigated *in vitro* according to Bessire et al. (2007). *B. cinerea* conidia (10⁴ spores ml⁻¹) were mixed with different concentrations of paenimyxin (0, 5, 10, and 50 μ g ml⁻¹), deposited on a glass slide at 100% relative humidity and were incubated for 12 h at 20 °C. Conidia germination and mycelial growth were evaluated by microscopy (Leica DFC 490, Leica Microsystems) and following image analyses using the Leica Application Suite 3.1 (Leica Microsystems). Experiments were performed with five replicates per condition and were independently repeated at least 3 times.

2.3.2. Effect on *Plasmopara viticola*

The effects of paenimyxin on the mobility of *P. viticola* zoospores were assessed *in vitro* as described by Pezet et al. (2004). Briefly, sporangia of *P. viticola* were collected and suspended in distilled water at a concentration of 10⁵.ml⁻¹. After 1 h of gentle agi-

tation at room temperature, zoospores were released from about 50% of the sporangia, leading to a suspension of 0.5 10⁵ mL⁻¹ mobile zoospores. Paenimyxin was added to the zoospore suspension at final concentrations of 0, 5, 10 and 50 μ g ml⁻¹, respectively. Five minutes after the addition of paenimyxin, the number of mobile zoospores was counted in an area of 0.11 mm² during a viewing period of 30 s under a light microscope (Leica-Leitz, magnification 60 $\times$). For each paenimyxin concentration, the measurement was repeated 5 times and the bioassays were repeated 3 times independently.

The effects of paenimyxin on the infection of grapevine leaves by *P. viticola* were investigated using three different experimental procedures. Discs (10 mm diameter) were punched out from mature leaves and placed in a Petri dish on wet filter paper. For the first set of experiments (called “PPv”), one droplet (30 μ l) of a mixture of a 10⁴.mL⁻¹ *P. viticola* sporangia suspension and a paenimyxin solution (see below) were deposited on the abaxial side of the leaf discs, and then droplets removed using laboratory tissues 24 h later. For the second set of experiments (called “Pd-Pv”), droplets of paenimyxin (30 μ l) were deposited on the abaxial side of leaf discs and left for 48 h to allow paenimyxin penetration into leaf tissues. Discs were then rinsed three times in 50 mM phosphate buffer (pH 7.0) to remove the residual paenimyxin and inoculated with a 30 μ l droplet of a 10⁴.mL⁻¹ *P. viticola* sporangia suspension for 24 h. For the third experiment set (called “Pf-Pv”), leaf discs were incubated in the paenimyxin solution (flotation) in Petri dishes for 48 h, rinsed three times in phosphate buffer (pH 7.0 50 mM) to remove the residual of paenimyxin, and inoculated with 1 droplet of a 10⁴.mL⁻¹ *P. viticola* sporangia suspension for 24 h. Paenimyxin was applied at final concentrations of 0, 0.25, 0.4, 2.5, 4 or 25 μ g ml⁻¹. The level of *P. viticola* sporulation was determined at 6 days post inoculation (dpi) by determination of the percentage of sporulating leaf area. Experiments were repeated three times.

2.3.3. Effect on *Xiphinema index*

The *in vitro* tests of the effects of paenimyxin on *X. index* mortality were carried out in microtitration plates (Sigma-Aldrich, Milan, Italy). 0.5 ml of paenimyxin (final concentration of 0.4, 2, 10 or 50 μ g ml⁻¹) or osmosed water as control was added to 15 adult females. Nematodes were then observed under a light microscope at every 8 or 16 h intervals for 120 h. The active/inactive nematodes were recognized by their response to pricking of their body with soft fibers. The experimental design was randomized, each concentration was replicated four times, and each experiment was repeated at least twice with separate controls.

2.4. Analysis of defense gene expression

The expression of the defense-related genes chitinase 1b (*CHI*, Busam et al., 1997), phenylalanine ammonia lyase (*PAL*), stilbene synthase 1 (*STS*, Sparvoli et al., 1994), glutathione-S-transferase

Table 1
Defense-related gene characteristics for expression analysis. *CHI*: chitinase 1b gene, *GST*: glutathione-S-transferase gene, *LOX*: lipoxygenase gene, *PAL*: phenylalanine ammonia lyase gene, *PIN*: proteinase inhibitor gene, and *STS*: stilbene synthase 1 gene. *Hero* and *HsI^{pro-1}* heterologous sequences of the tomato genes *Hero* and *HsI^{pro-1}* for resistance to nematodes (XM_002265532.1 and XM_002268530.1).

Gene	Accession number	Forward primer	Reverse primer	Annealing °C	Amplicon size (bp)	r ² of calibration curve	PCR efficiency
<i>CHI</i>	Z54234	CCCAAGCCTTCTGCCATA	TGTGATAACACAAAACCGGG	60	96	0.997	0.998
<i>GST</i>	AY156048	TGCATGGAGGAGGAGTTCGT	CAAGGCTATATCCCATTTCTTC	60	98	0.996	0.995
<i>LOX</i>	AY159556	CCCTTCTTGGCATCTCCCTTA	TGTTGTGTCCAGGGTCCATTC	56	101	0.994	0.933
<i>PAL</i>	X75967	TCCTCCCGGAAAACAGCTG	TCCTCCAAATGCCTCAAATCA	56	101	0.997	0.897
<i>PIN</i>	AY156047	AGTTCAGGGAGAGGTTGCTG	CACCAACCCAATGAGTCTATCC	59	185	0.996	0.974
<i>STS</i>	X76892	AGGAAGCAGCATTTGAAGGCTC	TGCACCGGCAATTTCTACACC	55	101	0.997	0.920
<i>HERO</i>	XM_002265532.1	CGGAAGAAAATGGGATGGAAGAG	ATGAGCAATAAGTCGGCGAGGG	59	188	0.993	0.950
<i>HS</i>	XM_002268530.1	GCTGTACCGCGAAAAGGTAG	AGATTGTAGACAACGAGTCC	57	188	0.997	0.932

(GST), lipoxygenase (LOX) and pathogenesis-related protein PR-6 (PIN) (Aziz et al., 2003), of the heterologous sequences of the tomato genes *Hero* and *Hs1^{pro-1}* for resistance to nematodes (XM_002265532.1 and XM_002268530.1), and of the constitutively-expressed reference gene encoding GAPDH (Reid et al., 2006) was studied in root samples (Table 1). Three biological repetitions were used for each treatment, and PCRs were carried out in triplicate per sample. RNA extraction and precipitation were performed as previously described (Hao et al., 2012). RNA quantity and quality were estimated by 260/280 nm absorbance and 1% denaturing gel electrophoresis. cDNA synthesis was performed with the M-MLV reverse transcriptase kit (Invitrogen) following the manufacturer's recommendations. Primers were defined for transcript quantification of selected genes and PCR conditions according to Hao et al. (2012). Transcripts were quantified using the Absolute™ QPCR SYBR green ROX mix (ABgene, Epsom, UK) and an ABI PRISM 7900 apparatus (Applied Biosystems, Foster City, CA, USA). Real time PCR was carried out in 1 µl of 100× diluted cDNA as template in a final volume of 20 µl containing 1× SYBR green mix, and 20 nM of each gene-specific primer, as recommended by the manufacturer. PCR cycling consisted of an initial denaturation step at 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s (Applied Biosystems). Standard amplification curves were determined from duplicate samples of plasmid DNA [TOPO plasmids containing each amplicon were quantified by UV absorbance spectroscopy (Eppendorf BioPhotometer) and linearized by *NotI* (Promega) digestion] at 10², 10³, 10⁴, 10⁵, 10⁶, and 10⁷ copies. A melting curve analysis was included at the end of each PCR run in order to verify amplification of each target cDNA. Quantification cycle (C_q) were analyzed by SDS 2.2 software (Applied Biosystems, Foster City, CA, USA) and target-gene expression data were plotted as $2^{(40-C_q)}/10$, as explained in Czechowski et al. (2004), and normalized using the internal reference *GADPH* gene. PCR amplification efficiency ($10^{-1/\text{slope}} - 1$) were determined from the slope of the log-linear portion of the calibration curve, when the logarithm of the initial template concentration is plotted on the x axis and C_q is plotted on the y axis (Table 1).

Fig. 1. Antagonistic activity of *Paenibacillus* sp. strain B2 (B2) against *Botrytis cinerea* in vitro, compared with the reference bacterial strain *E. coli* MRAP2. Single colonies of both bacteria were inoculated on opposite sides of a Petri dish three days before *B. cinerea* inoculation. The photograph was taken one week after *B. cinerea* inoculation.

2.5. Data analysis

Statistical analysis of the data was carried out by analysis of variance (ANOVA) using the SPSS package (version 13.0, SPSS Inc., USA). Significant differences between means were established by calculation of LSD at the 5% level. Furthermore, two-way ANOVA was performed to compare the effects of *Paenibacillus* sp. strain B2 and *X. index* inoculation on plant growth, nematode development and gene expression using SPSS (version 13.0, SPSS Inc., USA).

3. Results

3.1. Inhibitory effect of *Paenibacillus* sp. strain B2 and paenimyxin on *Botrytis cinerea* growth

Paenibacillus sp. strain B2 inhibited the growth of *B. cinerea*, as indicated by the formation of clear zones surrounding the bacterial colony in plate assays (Fig. 1). Conversely, no effect was observed for *E. coli* which was included as negative control.

The peptide paenimyxin significantly reduced the mycelium growth of *B. cinerea*. At the concentrations of 5 and 10 µg ml⁻¹, the percentages of inhibition were 54% and 81%, respectively, when

Fig. 2. Effect of paenimyxin on *Botrytis cinerea* development in vitro. Different concentrations of paenimyxin (0 to 50 µg ml⁻¹) were added to a conidial suspension (10⁴ spores.ml⁻¹) of the fungus on slides. Observations were made using a microscope equipped with a camera and mycelium length was determined using the Leica Application Suite 3.1 (Leica Microsystems).

Fig. 3. Effect of paenimyxin on *Plasmopara viticola* zoospore mobility in vitro. Zoospores were incubated with different paenimyxin concentrations (0 to 50 µg ml⁻¹) and observed by microscopy. The number of active zoospores was counted on a surface of 0.11 mm² during 30 s under a light microscope (Leica-Leitz, magnification 60×).

compared to water (Fig. 2). At concentrations higher than $20 \mu\text{g ml}^{-1}$, paenimyxin completely inhibited the mycelial growth.

3.2. Antagonistic effect of paenimyxin on *Plasmopara viticola* in vitro and in planta

Paenimyxin significantly reduced the mobility of *P. viticola* zoospores. At 5, 10 and $50 \mu\text{g ml}^{-1}$, the number of mobile zoospores was reduced by about 30%, 64% and 74%, respectively (Fig. 3). The effect of paenimyxin on *P. viticola* infection of leaf tissue was highly dependent on the way of application (Fig. 4). When applied together with the sporangia suspension (PPv), paenimyxin strongly inhibited *P. viticola* development and sporulation; sporulation was scarce at the concentration of $0.25 \mu\text{g ml}^{-1}$ and absent at $2.5 \mu\text{g ml}^{-1}$ and higher concentrations. When applied by flotation (Pf-Pv) before inoculation, paenimyxin was less active, although sporulation was reduced by >60% at 4 and $25 \mu\text{g ml}^{-1}$. Finally, when applied as droplets (Pd-Pv) before inoculation, paenimyxin was no longer active, even at the concentration of $25 \mu\text{g ml}^{-1}$.

3.3. Effects of *Paenibacillus* sp. strain B2 and paenimyxin on *X. index* activity

Inoculation of SO4 plants with *Paenibacillus* sp. strain B2 promoted root biomass, while nematode inoculation had no effect at

Fig. 4. Effect of paenimyxin on *Plasmopara viticola* development in planta. The abaxial side of foliar discs of Marselan grapevine was treated with a droplet of a mixture of paenimyxin and *P. viticola* sporangia suspension (PPv), a droplet of paenimyxin for 24 h prior to inoculation with a *P. viticola* sporangia suspension (Pd-Pv), or paenimyxin by flotation for 48 h before inoculation with a *P. viticola* sporangia suspension (Pf-Pv). Paenimyxin was added at concentrations of 0, 0.25, 0.4, 2.5, 4 or $25 \mu\text{g ml}^{-1}$. Values correspond to the percentage area of leaf discs showing *P. viticola* sporulation.

Table 2

Effect of *Paenibacillus* sp. strain B2 on the development of *Xiphinema index* on grapevine roots and in soil. Two-way ANOVA was used to compare the influence of the factors *Paenibacillus* sp. strain B2 (Bac), *X. index* (Nem), and the interaction between both factors (Bac $\times$ Nem) on plant growth and nematode development. Significant *P* values ($P < 0.05$) are indicated in bold. CK: control (no *Paenibacillus* sp. strain B2 and no *X. index*); B2: inoculation with *Paenibacillus* sp. strain B2 only at repotting time; Xi: inoculation with *X. index* only; and B2 + Xi: inoculation with *Paenibacillus* sp. strain B2 at repotting time and with *X. index* one week later.

Treatments	Shoot fresh weight (g)	Root fresh weight (g)	Gall number/plant	Nematode number/jar
CK	1.24 b	0.42 b	0.0 c	0.0 c
B2	1.41 a	0.54 a	0.0 c	0.0 c
Xi	1.31 ab	0.40 b	14.0 a	101.2 a
B2 + Xi	1.34 ab	0.55 a	7.2 b	73.7 b
Factors				
Bac	0.012	<0.001	0.001	0.010
Nem	0.932	0.613	<0.001	<0.001
Bac $\times$ Nem	0.055	0.943	0.001	0.010

35 dpi (Table 2). This growth promoting effect was not affected by inoculation with *X. index*. Shoot biomass showed a similar trend, and inoculation or co-inoculation with *X. index* had no significant effect, compared with the control treatment. No nematode infection was observed in the non-inoculated control treatment whilst in the nematode-inoculated control plants, *X. index* successfully developed (about 100 nematodes/jar) and infected roots, as indicated by the occurrence of galls (about 14 galls/plant). Application of *Paenibacillus* sp. strain B2 significantly decreased *X. index* development in planta, as indicated by a significant reduction in the number of galls per plant (twice lower than inoculated control) and nematodes in soil (Table 2).

The activity of nematodes decreased slightly in vitro during the time course of the experiment (Fig. 5A). Application of *Paenibacillus*

Fig. 5. Effects of *Paenibacillus* sp. strain B2 (A) and paenimyxin (B) on *Xiphinema index* activity. A bacterial suspension ($0, 10^6, 10^7, 10^8$ or 10^9 CFU ml^{-1}) or paenimyxin ($0, 0.4, 2, 10$ or $50 \mu\text{g ml}^{-1}$) were added to 15 *X. index* adult females. The active/inactive nematodes were recognized by the response of their body to pricking with soft fibers from 0 to 120 h incubation.

sp. strain B2 induced a decrease in the proportion of active nematodes from 24, 48, and 72 h post treatment for concentrations of 10^9 , 10^8 , and 10^7 – 10^6 bacteria ml^{-1} , respectively (Fig. 5A). At the end of the experiment, the percentage of active nematodes was reduced to about 30%, whatever the bacterial concentration. The activity of nematodes was also decreased in the presence of paenimycin (Fig. 5B). From 48 h, there was a dose- and time-dependent inhibition of the nematode activity. At 120 h, the activity of nematodes was practically zero in presence of paenimycin at $50 \mu\text{g ml}^{-1}$ and strongly decreased at 2 and $10 \mu\text{g ml}^{-1}$.

3.4. Expression of defense-related grapevine genes

The expression of genes encoding phenylalanine ammonia lyase (PAL), proteinase inhibitor (PIN), and glutathione-S-transferase (GST) was unaffected by the presence of *X. index* and/or *Paenibacillus*

lus sp. strain B2 (Fig. 6). The expression of the stilbene synthase 1 (STS) gene was slightly induced in the presence of *X. index* whereas it was not modified in presence of the bacteria (Fig. 6, Table 3). Interestingly, expression of the gene encoding chitinase 1b (CHI) was significantly induced in response to co-inoculation by *X. index* and *Paenibacillus* sp. strain B2, but not for inoculation with the bacterium or *X. index* alone. The response was similar for the expression of the lipoxygenase (LOX) gene, except that inoculation with *X. index* alone slightly enhanced the gene expression (Table 3).

The expression of the nematode “resistant” genes *Hero* and *Hs1^{pro-1}* is presented in Fig. 7. *Hero* expression was activated in *X. index*-inoculated plants, although the expression was slightly reduced in the presence of *Paenibacillus* sp. strain B2. Conversely, *Hs1^{pro-1}* was slightly induced in response to co-inoculation with *X. index* and the bacteria.

Fig. 6. Relative expression of grapevine defense-related genes. The expression of chitinase 1b (CHI), phenylalanine ammonia lyase (PAL), glutathione-S-transferase (GST), pathogenesis-related protein PR-6 (PIN), lipoxygenase (LOX), stilbene synthase 1 (STS) encoding genes was studied in roots of SO4 grapevine plantlets in response to different treatments. CK: control (no *Paenibacillus* sp. strain B2 and no *X. index*); B2: inoculation with *Paenibacillus* sp. strain B2 only at repotting time; Xi: inoculation with *X. index* only; and B2 + Xi: inoculation with *Paenibacillus* sp. strain B2 at repotting time and with *X. index* one week later. Three biological repetitions of each treatment were analyzed at 35 days after inoculation with *X. index*.

Table 3

Two-way ANOVA for influence of the factors *Paenibacillus* sp. strain B2 (Bac), *Xiphinema index* (Nem), and the interaction between both factors (Bac × Nem) on relative expression of grapevine defense-related and nematode-resistant genes. Significant *P* values (*P* < 0.05) are indicated in bold. CK: control (no *Paenibacillus* sp. strain B2 and no *X. index*); B2: inoculation with *Paenibacillus* sp. strain B2 only at repotting time; Xi: inoculation with *X. index* only; and B2 + Xi: inoculation with *Paenibacillus* sp. strain B2 at repotting time and with *X. index* one week later. CHI: chitinase 1b gene, GST: glutathione-S-transferase gene, LOX: lipoxygenase gene, PAL: phenylalanine ammonia lyase gene, PIN: proteinase inhibitor gene, and STS: stilbene synthase 1 gene. *Hero* and *Hs1^{pro-1}* heterologous sequences of the tomato genes *Hero* and *Hs1^{pro-1}* for resistance to nematodes (XM_002265532.1 and XM_002268530.1).

Factors	CHI	GST	LOX	PAL	PIN	STS	HERO	Hs1 ^{pro-1}
Bac	0.003	0.774	0.162	0.958	0.076	0.377	0.852	0.020
Nem	0.006	0.738	0.014	0.337	0.450	0.004	0.028	0.001
Bac × Nem	<0.001	0.987	0.029	0.392	0.220	0.369	0.080	0.360

Fig. 7. Relative expression of genes resistant to nematodes. The expression of *Hero* and *Hs1^{pro-1}* genes (heterologous sequences of the tomato nematode resistance-related genes XM_002265532.1 and XM_002268530.1) in roots of SO4 grapevine plantlets was determined in response to different treatments. CK: control (no *Paenibacillus* sp. strain B2 and no *X. index*); B2: inoculation with *Paenibacillus* sp. strain B2 only at repotting time; Xi: inoculation with *X. index* only; and B2 + Xi: inoculation with *Paenibacillus* sp. strain B2 at repotting time and with *X. index* one week later. Three repeats of each treatment were analyzed at 35 days after inoculation with *X. index*.

4. Discussion

The results obtained here clearly show that the bacterium *Paenibacillus* sp. strain B2 can inhibit development of the fungal pathogen *B. cinerea* *in vitro*, and affect activity of the ectoparasitic nematode *X. index* *in vitro* as well as *in planta*. Previous *in vitro* studies have reported inhibition of mycelial growth also of *Phytophthora parasitica* and *Fusarium oxysporum* by *Paenibacillus* sp. strain B2 (Budi et al., 1999). The genus *Paenibacillus* includes several species producing active compounds able to inhibit plant pathogens (Ongena and Jacques, 2008). For example, *Paenibacillus polymyxa* strain E681 has been used against a complex of soil-borne sesame diseases (Ryu et al., 2006) and, more recently, Kim et al. (2016) reported the antagonistic activity of *P. polymyxa* APEC128 against two *Colletotrichum* species responsible for apple anthracnose. Antagonistic activity of *P. polymyxa* has also been demonstrated against hatching and penetration of the root-knot nematode *Meloidogyne javanica* (Siddiqui et al., 2007). Treatment of *Arabidopsis thaliana* with *Paenibacillus alvei* K165 was shown to protect against *Verticillium dahliae* by triggering ISR (Gkizi et al., 2016). All these observations suggest that the genus *Paenibacillus* has a broad spectrum of antagonistic activity, and therefore could be of potential interest for the biological control of different phytopathogens.

The antagonistic activity of *Paenibacillus* sp. strain B2 towards phytopathogens involves extracellular chitinolytic, cellulolytic, proteolytic and pectinolytic enzyme activities (Budi et al., 2000), and other substances (Budi et al., 1999) like paenimycin (Selim et al., 2005, 2010). Paenimycin, which consists of a mixture of polymyxins or polymyxin-related peptides (Selim et al., 2005), may act by binding to microbial lipopolysaccharides with an irreversible obstruction of membrane permeability, as do other antagonistic factors (Ongena and Jacques, 2008; Budi et al., 2000). Purified paenimycin has inhibiting activity against bacteria, fungi and oomycetes (Selim et al., 2005). The present study clearly shows that it also directly inhibits the growth of *B. cinerea*, as well as the zoospore mobility and development of *P. viticola*.

Some biocontrol agents or their metabolites reduce disease incidence by activation of defense systems in the host plant (Ongena et al., 2005; Jourdan et al., 2009), and some rhizobacteria or their metabolites can induce systemic resistance (ISR, van Loon et al., 1998; Ongena et al., 2007). In the present study, no defense gene activation was detected in the roots of grapevine plants treated by *Paenibacillus* sp. strain B2 alone. However, *CHI* and *LOX* genes were activated with decreased *X. index* activity in response to co-inoculation of the bacterium and the nematode, although the low expression of the resistance genes *Hs1^{pro-1}* and *Hero* (Sobczak et al., 2005) suggest that the enhanced grapevine resistance was not related to these specific resistance genes. It would be interest-

ing to analyse the kinetics of defense gene expression in leaves and roots treated with *Paenibacillus* sp. strain B2 to gain insight into the molecular mechanisms of the antagonistic activity of the bacterium in grapevine. Selim et al. (2010) have reported that paenimycin elicits the production of hydrogen peroxide (H_2O_2) and upregulates several defense-related genes in cell suspensions of the model legume *Medicago truncatula*, and Lee et al. (2013) observed that fusaricidin, a cyclic depsipeptide from *P. polymyxa* E681, significantly decreased the severity of red-pepper *Phytophthora* blight by the induction of systemic resistance. However, in the present bioassays with *P. viticola*, paenimycin application by droplets onto leaf discs prior to oomycete inoculation (to detect defense elicitor activity) did not induce resistance against downy mildew of grapevine tissues. Conversely, addition of paenimycin directly to *P. viticola* zoospores significantly decreased disease development, suggesting a direct action of this antagonistic peptide. Application of paenimycin by flotation prior to pathogen inoculation had a slight effect on disease development, compared to application by droplets, but this might be explained by a direct effect of paenimycin which had accumulated within leaf discs against *P. viticola* during the first stages of pathogen development.

Previously researches showed that *Paenibacillus* can promote plant development via the production of the phytohormone indole-3-acetic acid (IAA), biological nitrogen fixation, phosphate solubilization, and siderophore secretion which enables iron acquisition (Fernandes et al., 2014; Zhou et al., 2016). Inoculation of grapevine plants with *Paenibacillus* sp. strain B2 promoted root and shoot biomass, while reducing nematode root infection and decreasing nematode numbers in soil. Budi et al. (1999) have previously shown that *Paenibacillus* sp. strain B2 promotes tomato root growth, but has no effect on shoot growth, while inhibiting development of *P. parasitica*. These observations are consistent with a role of *Paenibacillus* as a PGPR (Venkadasamy et al., 2010).

Altogether, the present and previous data demonstrate that *Paenibacillus* sp. strain B2 and paenimycin could be effective in strategies to control grapevine pathogens.

Author contribution

M.A., V.G.-P. Z.H., S.G., X.L., and D.v.T. planned and designed the research. Z.H., L.F., and O.C. performed experiments, Z.H., A.D., D.W. and B.C. analyzed data; Z.H., M.A., and V.G.-P. wrote the manuscript.

Acknowledgments

The authors are grateful to A. Manga for assistance in producing purified paenimycin. This research was supported by the Conseil

Régional de Bourgogne, France (Grant number 079201A AO4053619), National Natural Science Foundation of China (Grant number 41571250) and the China Scholarship Council (Grant to ZH).

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