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Bacteria isolated from roots and rhizosphere of *Vitis vinifera* retard water losses, induce abscisic acid accumulation and synthesis of defense-related terpenes in in vitro cultured grapevine

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Eleven bacterial strains were isolated at different soil depths from roots and rhizosphere of grapevines from a commercial vineyard. By 16S rRNA gene sequencing 10 different genera and 8 possible at species level were identified. From them, *Bacillus licheniformis* Rt4M10 and *Pseudomonas fluorescens* Rt6M10 were selected according to their characteristics as plant growth promoting rhizobacteria (PGPR). Both produced abscisic acid (ABA), indole-3-acetic acid (IAA) and the gibberellins A_1 and A_3 in chemically-defined medium. They also colonized roots of in vitro grown *Vitis vinifera* cv. Malbec plants. As result of bacterization ABA levels in 45 days-old in vitro plants were increased 76-fold by *B. licheniformis* and 40-fold by *P. fluorescens* as compared to controls. Both bacteria diminished plant water loss rate in correlation with increments of ABA. Twenty and 30 days post bacterization the plants incremented terpenes. The monoterpenes α-pinene, terpinolene, 4-carene, limonene, eucalyptol and lilac aldehyde A, and the sesquiterpenes α-bergamotene, α-farnesene, nerolidol and farnesol were assessed by gas chromatography-electron impact mass spectrometry analysis. α-Pinene and nerolidol were the most abundant (μg per g of tissue in plants bacterized with *P. fluorescens*). Only α-pinene, eucalyptol and farnesol were identified at low concentration in non-bacterized plants treated with ABA, while no terpenes were detected in controls. The results obtained along with others from literature suggest that *B. licheniformis* and *P. fluorescens* act as stress alleviators by inducing ABA synthesis so diminishing water losses. These bacteria also elicit synthesis of compounds of plant defense via an ABA independent mechanism.

Abbreviations – +ABA, treated with abscisic acid; +*B. licheniformis*, bacterized with *Bacillus licheniformis*; +*P. fluorescens*, bacterized with *Pseudomonas fluorescens*; ABA, abscisic acid; API, analytical profile index; CFU, colony forming units; dpb, days post bacterization; FW, fresh weight; GA, gibberellin; GC-EIMS, gas chromatograph-electron impact mass spectrometer; IAA, indole-3-acetic acid; ISR, induced systemic resistance; JA, jasmonates; LA, leaf area; LB, Luria Broth medium; MS, Murashige and Skoog; Nd, not detected; OD, optical density; PBS, phosphate buffer; PGPR, plant growth promoting rhizobacteria; RL, root length; ROS, reactive oxygen species; SL, shoot length; Tr, traces.

Introduction

In nature, rhizobacteria play a fundamental role in plant adaptation to the environment (Hallman et al. 1997). Plant growth promoting rhizobacteria (PGPR) represent a wide variety of rhizospheric and endophytic bacteria that when growth associated with plants stimulate growth of their hosts (Kloepper and Schroth 1978, Bashan and Holguin 1998). The interaction between plants and PGPR has profound effects on plant growth, development and health (Fulchieri et al. 1993, Compant et al. 2005). These microorganisms can induce plants to synthesize metabolites, so that upon exposure to stress primed plants are able to respond more efficiently than non-primed individuals (Compant et al. 2005). Moreover, microorganisms from the rhizosphere of a specific plant may be better adapted to that plant and its environment and they may therefore provide better control of diseases than organisms originally coming from another rhizosphere (Cook 1993).

PGPR stimulate plant growth through several mechanisms, mainly by producing the phytohormones such as abscisic acid (ABA), gibberellins (GAs), indole-3-acetic acid (IAA), jasmonates (JA) and cytokinins (Bottini et al. 1989, Bastián et al. 1998, Arkhipova et al. 2005, Cohen et al. 2008, Piccoli et al. 2011), which stimulate different aspects of metabolism and growth (Bastián et al. 1998, Bottini et al. 2004). Different groups have focused in other PGPR abilities, like the capability to induce the plant synthesis of different defense compounds that help in pathogen control (Liu et al. 1995, Lugtenberg and Kamilova 2009), such as phytoalexins and proteins of resistance (Van Loon and Glick 2004, Van Loon 2007). In this respect, production of terpenes in plant tissues have been associated with defense-responses to pathogen (Neri et al. 2006, Escoriaza et al. 2013) and herbivore attack, as well as to abiotic stresses (Grassmann et al. 2002, Beckett et al. 2012, Gil et al. 2012, Piccoli and Bottini 2013). However, the induction of terpenes synthesis by PGPR as a mechanism of plant protection has been scarcely studied.

Since PGPR were characterized as beneficial for plants, different genera have been tested to improve growth and yield in different crops. Strains of *Pseudomonas* and *Bacillus* had been used to increase the yield in tomato, pepper and apple (Raupach et al. 1996, Szczech and Shoda 2004, Aslantaş et al. 2007). It was also found that *Azospirillum* sp. help maize plants to tolerate water stress probably through production of the stress-related hormone ABA (Cohen et al. 2009). In this respect, although grapevine is the most important economic fruit worldwide, reports about plant growth promotion and/or elicitation of defense mechanisms using beneficial microorganisms are scarce. *Burkholderia phytofirmans* PsJN is able to colonize several parts of grapevines (Compant et al. 2005), to increase root and shoot dry weight, to induce growth of secondary roots (Ait Barka et al. 2000, Compant et al. 2005) and to enhance resistance to low temperatures (Ait Barka et al. 2002, 2006). Likewise, *Pseudomonas* sp., *Pantoea* sp. and *Acinetobacter* sp. elicit defense-responses by stimulation of chitinases and β-1,3-glucanases activities and accumulation of phytoalexins so improving resistance against *Botrytis cinerea* (Magnin-Robert et al. 2007, 2013, Verhagen et al. 2011).

In Argentina, the *Vitis vinifera* cv. Malbec has found propitious ecological features for its development, mainly in the province of Mendoza, thus becoming the emblematic cultivar of the country's wine industry (Stajner et al. 2009). In this study 11 bacteria strains were isolated and characterized from roots and rhizosphere in commercial grapevines of this cv. The hypothesis was that rhizospheric microorganisms help the grapevine to cope more efficiently with the arid environment. In fact, even though grapevines in Mendoza's region are cultivated under irrigation, the environmental conditions are profoundly arid so the plants are submitted to extreme abiotic stresses (Berli et al. 2010). Thus, the aim of this work was to study the effect of inoculation of PGPR native from the grapevine environment in order to shed light on the mechanisms involved in the plant–PGPR relationship. Two of the isolates, *Pseudomonas fluorescens* Rt6M10 and *Bacillus licheniformis* Rt4M10, were studied in their capability to produce phytohormones in chemically-defined medium, to colonize in vitro grown grapevine cv. Malbec, where bacterization induces control of water loss rates and elicits production of defense-related terpenes.

Materials and methods

Bacteria isolation

Bacteria associated with grapevines were isolated from root-adjacent soil (rhizosphere) and roots of *V. vinifera* cv. Malbec plants from a commercial vineyard (Catena Zapata, Bodegas Esmeralda S.A.) located in Gualtallary at 1450 m a.s.l. (69°15′ W and 33°23′ S; Tupungato, Mendoza, Argentina). Roots and soil samples were collected every 10 cm to a depth of 80 cm and were placed in sterile recipients to be taken to the lab. Roots were washed in 50 ml of sterile phosphate buffer (PBS) and the supernatants were serially diluted from 10^{-1} to 10^{-5}. Then root's surfaces were disinfected with 70%

ethanol for 1 min and 1% commercial bleach (55 g l^{-1} active chlorine) for 5 min, and rinsed several times with sterile distilled water; then 1 g of each sample was macerated in PBS and diluted from 10^{-1} to 10^{-6}. Aliquots of 0.1 ml of each dilution were plated in Petri dishes with bacterial LB medium (Luria Broth, Sigma Chem. Co, St Louis, MO). Three days after incubation at 30°C colonies were grouped according to phenotypic characteristics.

Phenotypic characterization of bacterial isolates

The bacteria isolated were characterized according to their color, form, elevation, margin, diameter, surface, opacity and texture. Also, morphology, motility and size were evaluated by phase-contrast microscopy. The Gram reaction was performed using a 3% KOH solution followed by staining with crystal violet (Gram Britania, Buenos Aires, Argentina) on glass slides. The presence of cytochrome oxidase was measured with discs impregnated with dimethyl *p*-phenyl-enediamine (Sigma-Aldrich). The catalase activity was determined by O_2 production after adding a drop of 1.5% H_2O_2 to a young colony growing on LB agar. Then, all strains were characterized by the analytical profile index (API) micro-methods standardized for a rapid identification of non-fastidious Gram-negative and Gram-positive bacteria according to the recommendations of API, bioMérieux (Marcy l'Etoile, France). The production of acid metabolites from 49 carbohydrates was tested with API 50CHB strips for Gram-positive bacteria. Proteolysis of gelatin, activities of nitrate reductase, galactosidase, urease and tryptophanase, H_2S formation, production of acetoin, and citrate utilization were carried out with API 20E strips for Gram-negative bacteria. The API 20NE test kit was used to identify glucose-non-fermenting Gram-negative non-Enterobacteriaceae. Finally, the results were interpreted with the API database of the API-LAB PLUS software (version 3.3.3; bioMérieux).

DNA extraction

Bacteria preliminary characterized with the API database of the Api-Lab Plus software were grown at 30°C in LB liquid medium, and then harvested at the exponential phase as assessed as optical density (OD) and colony forming unit (CFU) for genomic DNA extraction. The extraction was performed with the commercial kit QIAamp, DNA mini kit (Qiagen, Hilden, Germany) following the provider instructions and then total DNA was quantified in agarose gel (0.8%) using a standard concentration (KS 50 ng μl^{-1}).

PCR amplification and sequencing of 16S rRNA gene

For phylogenetic identification the 16S rRNA gene was amplified using the primers FD1 Eubacteria (5′-AGATTTGATCCTGGCTCAG-3′) and RD1 (5′-AGGAGG TGATCCAGCC-3′). In order to precipitate the amplified products, polymerase chain reaction (PCR) products were transferred to micro-tubes and added with 40 µl of 65% isopropanol. After homogenization, micro-tubes were incubated at room temperature for 30 min and centrifuged 25 min at 14 000 *g*. The supernatants were discarded, added with 200 µl of 60% ethanol, centrifuged 10 min at 14 000 *g*, the pellets were incubated 10 min at 60°C and re-suspended in water.

Purified PCR products were sequenced in both directions by using FD1 and RD1 primers with the ABI PRISM™ BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Bases were read in an *ABI3130* sequence analyzer (Applied Biosystems). The obtained sequences were edited using Phred, Phrap and Consed programs (Linux Operating System). Then, the sequences were aligned with the obtained data deposited in GenBank by BLASTn at the NCBI database (National Center for Biotechnology Information) and five of the match sequences were selected (97–100% identity). Phylogenetic tree was generated by maximum likelihood using PHYLOGENETIC ANALYSIS USING PARSIMONY software 4.0b1 version (PAUP). Tree was drawn using FIGTREE v.1.4.0 and robustness was tested by bootstrap analysis with 1000 replicates.

Characterization and quantification of phytohormones in bacterial cultures

Bacillus licheniformis and *P. fluorescens* were grown to stationary phase (10^9 CFU; 1.5–2 OD_{530}) in 50 ml of NFb chemically-defined medium containing: 5 g l^{-1} malic acid, 0.5 g l^{-1} K_2HPO_4, 0.2 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.1 g l^{-1} NaCl, 0.2 g l^{-1} $CaCl_2$, 2 ml of micronutrient solution (0.1 g 100 ml^{-1} $Na_2MoO_2 \cdot 2H_2O$, 0.117 g 100 ml^{-1} $MnSO_4$, 0.14 g 100 ml^{-1} H_3BO_3, 0.004 g 100 ml^{-1} $CuSO_4 \cdot 5H_2O$, 0.012 g 100 ml^{-1} $ZnSO_4 \cdot 7H_2O$), 4 ml Fe-EDTA (1,64% w/v, aqueous), 4.5 g l^{-1} KOH, 1 ml vitamin solution (Biotin 10 mg 100 ml^{-1}, Piridoxal-HCl 20 mg 100 ml^{-1}) and 1.25 g l^{-1} NH_4Cl. As control sterile NFb medium was used. After incubation the cultures were acidified to pH 2.5 with phosphoric acid, sonicated three times for 5 min and centrifuged 10 min at 10 000 *g* and 4°C. The supernatants were collected, added with 100 ng of [$^2H_6$]-ABA (gift from Prof. R. P. Pharis, University of Calgary, Canada), 50 ng of [$^{13}C_6$]-IAA (gift from

Prof. J. D. Cohen, University of Minnesota, USA) and 50 ng each of [$^2H_2$]-$GA_{1/3}$ (Prof. l. Mander, University of Canberra, Australia), and left 3 h at 4°C in darkness to allow the equilibration of the isotopes. Hormone extraction was carried out by partition with equal volumes of water-saturated ethyl acetate with 1% acetic acid (pH 2.5). Ethyl acetate fractions were evaporated in vacuum at room temperature and the dried extracts suspended in 3 ml of distilled water pH 3 (1% phosphoric acid), transferred to C18 reverse phase cartridges (Water Corporation, Milford, MA) and eluted with 2 ml of hexane and 2 ml of 80% methanol (methanol:distilled water:phosphoric acid, 80:19:1, v/v/v). The methanol fraction was collected and evaporated in vacuum. After evaporation the extracts were dissolved in 2 ml of distilled water pH 7 (1% NH_4OH), transferred to Oasis cartridges (Water Corporation) and eluted with 2 ml ammonium hydroxide (methanol:ammonium hydroxide, 95:5, v/v), 2 ml of methanol, 2 ml of 2% formic acid (methanol:formic acid, 98:2, v/v) and 3 ml of 5% formic acid (methanol:formic acid, 95:5, v/v). Both formic acid fractions were collected, grouped and evaporated in vacuum. Dry extracts were dissolved in 10 μl methanol and sequentially derivatized by adding 50 μl of fresh CH_2N_2 (30 min at room temperature) and then 40 μl of *N,O*-bis(trimethylsilyl)trifluoreacetamide with trimethylchlorosilane (BSTFA) in an equal amount of dry pyridine (40 min at 70°C). After drying in vacuum, the extracts were suspended in 50 μl of hexane and 1 μl was injected split–splitless in a Perkin Elmer Elite-5MS, cross-linked methyl silicone capillary column (30 m length, 0.25 mm inner diameter and 0.25 μm film thickness) fitted in a capillary gas chromatograph-electron impact mass spectrometer (GC-EIMS; Clarus 500, Perkin Elmer, Shelton, CT). The GC column was eluted with He (0.7 ml min^{-1}). The GC temperature program was from 100 to 200°C at 20°C min^{-1}, then augmented to 280°C at 4°C min^{-1} and held for 15 min. The mass spectrometer was operated with electron impact ionization energy of 70 eV. The injector temperature was 230°C, ion source temperature was 120°C and the interface temperature was 150°C. After performing selected ion monitoring (SIM) the amount of each phytohormone was calculated by comparison of the peak areas (at their corresponding retention times) of the major ions relative to its non-labeled counterpart; i.e. [$^2H_6$]-ABAMe (194/166) vs [^{1}H]-ABAMe (190/162), [$^{13}C_6$]-IAAMe (195/136) vs [^{12}C]-IAAMe (189/130), [$^2H_2$]-GA_1Me-TMSi (508/493) vs [^{1}H]-GA_1Me-TMSi (506/491) and [$^2H_2$]-GA_3Me-TMSi (506/491) vs [^{1}H]-GA_3Me-TMSi (504/489); each bacterial culture was performed by triplicate.

In vitro plant material

Grape plants grown in vitro were obtained from a virus-free vineyard of *V. vinifera* cv. Malbec as previously described Escoriaza et al. (2013). Explants of virus-free grape plant were treated with indole-3 butyric acid to promote rooting and maintained in darkness at 4°C; after 21 days they were placed in pots (2.5 l) filled with pet-moss under greenhouse conditions. In order to obtain in vitro plants, one-nodal explants were taken from a young shoot and surface sterilized with 70% ethanol for 3 min and 15% commercial bleach (55 g l^{-1} active chlorine) for 10 min. After rinsed several times with sterile distilled water they were placed in 50 ml tubes with solid MS (Murashige and Skoog) medium (Murashige and Skoog 1962), supplemented with 30 g l^{-1} sucrose, 1 mg l^{-1} 6-benzylaminopurine and 7.5 g l^{-1} agar. For the assays, in vitro plants were subcultured on 350 ml flasks with MS medium containing half-concentration of macro- and micronutrients (except for Fe-EDTA) and supplemented with 30 g l^{-1} sucrose, 0.5 μM 1-naphthaleneacetic acid and 7.5 g l^{-1} agar. Plants were cultured in a growth chamber with 25 ± 2°C controlled temperature, under cool-white fluorescents tubes with photosynthetic photon flux density of 80 μmol m^{-2} s^{-1} and 16/8 h photoperiod.

Plant bacterization

Bacillus licheniformis and *P. fluorescens* were selected in order to test their effects in grape plants. Bacteria were grown in MS medium and used to inoculate in vitro cultured plants. Emerging roots of in vitro plants (15 days-old) grown on flasks were bacterized with an aliquot of 0.25 ml bacterial culture, previously grown in liquid MS medium to 0.7 OD_{530} (10^6 CFU ml^{-1}). Control plants were incubated with sterile MS medium. After, 10, 20 and 30 days post bacterization (dpb) or 25, 35 and 45 days-old in vitro grown plants were removed from agar. Leaves and roots samples and the agar adjacent to roots were collected to determine bacterial survival and to analyze metabolites. All morphologic parameters were measured at the end of the assay (45 days-old) and data collection to evaluate morphological aspects included: stem and root lengths (SL and RL, respectively), aerial (leaves and stems) and radical fresh weight (FW), leaf number and number and length of internodes. Also leaf area (LA) was measured using the IMAGE TOOL PROGRAM (version 3.00). Leaves and roots of 25, 35 and 45 days-old plants were stored at −80°C until processing.

Endophytic and root surface colonization

To determine bacterial survival and to have an indication of the endophytic aptitude samples of in vitro roots, agar

adjacent to roots and aerial parts, were taken at 10, 20 and 30 dpb and following the standard plate-counting method (Bastián et al. 1999). The tissue's surfaces (root and aerial parts) were disinfected with 70% ethanol for 1 min and 1% commercial bleach (55 g l^{-1} active chlorine) for 5 min, and rinsed several times with sterile distilled water; then 0.1 g of root and aerial tissues were soaked with PBS and ground to powder in a sterile mortar and pestle. In parallels 1 g of root-adjacent agar and sterile MS agar were soaked in PBS and finally all of them re-suspended in PBS to give a serial dilution of 10^{-1} to 10^{-6}. The number of typical colonies was counted after 2–5 days of incubation at 30°C by plating each dilution in LB medium. Three replicates were performed for each treatment and the CFU determined.

Characterization and quantification of phytohormones in plants

One hundred milligram of stored leaves and roots collected at 30 dpb (45 days-old plant bacterized or not with *B. licheniformis* and *P. fluorescens*) were homogenized in a mortar and pestle with liquid N_2 and then with 3 ml of 80% methanol (methanol:distilled water:phosphoric acid, 80:19:1, v/v/v). In order to extract the hormones, each sample of leaves and roots was macerated and maintained overnight at 4°C and then centrifuged at 10 000 *g*. The supernatant was collected and added with 100 ng of [$^2H_6$]-ABA, 50 ng of [$^{13}C_6$]-IAA and 50 ng each of [$^2H_2$]-$GA_{1/3}$ and left 3 h at 4°C in darkness. Methanol fraction was evaporated in vacuum at room temperature and sequentially purified with C18 reverse phase and Oasis cartridges, suspended in 5 μl of methanol and derivatized as described above. After dried in vacuum, samples were dissolved in 50 μl of hexane and 1 μl injected in GC-EIMS using the same column, program and analytical determination described above. The experiments were done by triplicate.

ABA treatment of in vitro plants

One hundred micromolar solution of *cis-trans* ABA (90%, Kelinon Agrochemical Co., Beijing, China) containing 0.1% Triton X-100 and a minimum amount of 96% aqueous ethanol (to initially dissolve the ABA) was sprayed in one application onto in vitro plants (23, 33 and 43 days-old) grown in flasks. The spray was accomplished at the beginning of the dark period to assure uptake and to minimize photo-degradation. This ABA dose was chosen according to previous work with a range of species. In parallel, a control treatment was conducted with a solution containing distilled water plus 0.1% TritonX-100 and a minimum amount of ethanol and sprayed once as described above. Forty-eight hours after the ABA application, 100 mg of leaves and roots from each plant were collected and processed for terpene assessment.

Water loss rate assay

Aerial parts of 45 days-old in vitro plants bacterized or treated with ABA were severed and their FW immediately measured along four consecutive intervals of time: from 0 to 50 min every 3 min, from 51 to 86 min every 6 min, from 87 to 138 min every 12 min and from 139 to 210 min every 24 min. Once the experiment was done, water loss rate was calculated multiplying the weight at each interval by 100, then divided by the initial weight. Ten replicates were done for each treatment.

Quantification of terpenes in leaves and roots

In order to quantify monoterpenes and sesquiterpenes at three different times of plant bacterization (10, 20 and 30 dpb), and after 48 h application of ABA in in vitro plants, 100 mg of leaves and roots (i.e. from 25, 35 and 45 days-old in vitro plants) were macerated with 1 ml of methanol:distilled water:formic acid (85:14:1, v/v/v) and 2 ml of dichloromethane. Then, the samples were transferred to glass vials (Perkin Elmer) and left overnight at 4°C. The mixtures were shaken and centrifuged 15 min at 10 000 *g*. From each dichloromethane phase, an aliquot of 100 μl was put into inserts with 1 ng μl^{-1} of *n*-hexadecane as internal standard, and 2 μl were injected in the GC-EIMS. The oven temperature program was: initial temperature at 45°C for 1 min, followed by an increase of 2°C min^{-1} to 130°C, then from 130°C to 250°C at a rate of 20°C min^{-1} and held for 10 min at 250°C. The ionization potential was 70 eV and a range of 40–500 atomic mass units was scanned. Compounds were identified by comparison of GC retention times and full mass spectra of the corresponding standards previously injected and/or data of the NIST library (Gil et al. 2012). Quantification of each compound was performed on the basis of the peak area as compared to the peak area of a known amount of *n*-hexadecane co-injected with the sample.

Statistical analysis

The statistical analysis was performed by ANOVA and comparisons were done with LSD of Fisher test, using STATGRAPHICS CENTURION XVI version 16.1.03 (Statpoint Technologies Inc., Warrenton, VA). Significant differences were considered at probability of $P \leq 0.05$. The replica numbers of reported data are specified in each figure legend.

Table 1. Bacteria isolated from rhizosphere of *Vitis vinifera* cv. Malbec of a commercial vineyard. 10, year of the isolation; M, Mendoza; number, isolation number; Rz, rhizosphere; Rt, root.

Designation	Gram	Soil deep (cm)	Source
Rz7M10	+	10	Rhizosphere
Rz1M10	+	20	
Rz19M10	+	40	
Rz2M10	+	40	
Rz3M10	+	40	
Rz8M10	+	50	
Rt5M10	+	10	Roots
Rt6M10	−	10	
Rt9M10	+	10	
Rt17M10	+	30	
Rt4M10	+	80	

Results

Isolation and phylogenetic identification of bacterial strains

From roots and rhizosphere of grapevine cv. Malbec eleven bacterial strains were isolated at different soil depths (10–80 cm), where only Rt6M10 was Gram (−) and the rest Gram (+) (Table 1). Comparison with GenBank database (NCBI) of the 16S rRNA gene sequences indicated 99% homology of seven isolates at genus level with *Arthrobacter* sp. (Rz7M10), *Bacillus* sp. (Rz1M10 and Rt4M10), *Brachybacterium* sp. (Rz8M10), *Micrococcus* sp. (Rz2M10), *Paenibacillus* sp. (Rz3M10) and *Pseudomonas* sp. (Rt6M10). One hundred percent homology was found between four isolates with *Kocuria* sp. (Rt5M10), *Microbacterium* sp. (Rz19M10), *Planococcus* sp.(Rt9M10) and *Terribacillus* sp. (Rt17M10) (see Fig. 1 and Appendix S1 in Supporting Information for identification of accession number from NCBI database). Even more, among the first hits the BLASTn analysis showed that eight of the isolates could be possible identified at species levels with *Arthrobacter parietis*, *Bacillus pumilus*, *B. licheniformis*, *Brachybacterium faecium*, *Micrococcus* sp., *Paenibacillus* sp. and *P. fluorescens* (Appendix S1).

On the basis of the literature indicating that *Bacillus* and *Pseudomonas* are well characterized genera of bacteria as PGPR (Gutiérrez-Mañero et al. 2001, Haas and Keel 2003, Suzuki et al. 2003, Trotel-Aziz et al. 2008, Verhagen et al. 2010), *B. licheniformis* Rt4M10 and *P. fluorescens* Rt6M10 isolated in this work were precisely selected to further study their capability to produce phytohormones in chemically-defined medium, to colonize in vitro grown grapevine plants, to induce control of water losses and to elicit production of defense-related terpenes.

Bacillus licheniformis and *P. fluorescens* produce phytohormones in chemically-defined medium

The GC-EIMS results of Table 2 show for the first time that *B. licheniformis* and *P. fluorescens* produce ABA in chemically-defined NFb medium, and they also confirm that IAA and GAs are produced by the bacteria in the same conditions. In *P. fluorescens* cultures more ABA and IAA were found while *B. licheniformis* produced more GAs (including GA_3 and GA_1) (see Appendix S2 in Supporting Information for spectra). In sterile NFb control medium no phytohormones were detected. Either pH or growth rate (OD and CFU) were similar in both bacterial liquid cultures (Table 2).

In vitro plant root colonization by *B. licheniformis* and *P. fluorescens*

Bacterial counting suggested that at 10, 20 and 30 dpb *B. licheniformis* and *P. fluorescens* had entered and colonized in vitro plants only at root levels, since no bacteria were found in aerial parts (stem and leaves). Table 3 shows that *B. licheniformis* has the capability to colonize roots faster than *P. fluorescens*, since number of colonies at 10 dpb reached up 1.1 10^3 CFU g^{-1} in comparison with 2.8 10^2 CFU g^{-1} reached up by *P. fluorescens*. Then, at 20 and 30 dpb the number of CFU was similar in both bacterized plants. The bacterial number of the adjacent zone bacterized with *P. fluorescens* (+*P. fluorescens*) was higher than *B. licheniformis* in all sampled times, even at 30 dpb where no colonies of *B. licheniformis* were found in the adjacent agar. No colonies were found in the control agar.

Bacillus licheniformis enhances in vitro plant growth

At the end of the assay, 45 days-old in vitro plants (30 dpb) bacterized with *B. licheniformis* (+*B. licheniformis*) showed enhancement of SL and RL (10.83 ± 1.8 and 13.9 ± 1.2 cm, respectively) in comparison with control plants (8.35 ± 1.8 and 9.23 ± 2.7 cm) (Table 4). As well, bacterized plants showed LA values higher (7.72 ± 1.85 cm^2) than controls (5.22 ± 0.88 cm^2). However, *P. fluorescens* did not modify significantly any growth parameters (Table 4). Both microorganisms affected slightly total FW (shoot and roots), but did not show any difference in leaves and internode number (data not shown). Nevertheless these results indicated that both bacteria behave as non-pathogenic, since colonized plants looked healthy without any growth inhibition symptoms during the whole period of assay as compared with controls.

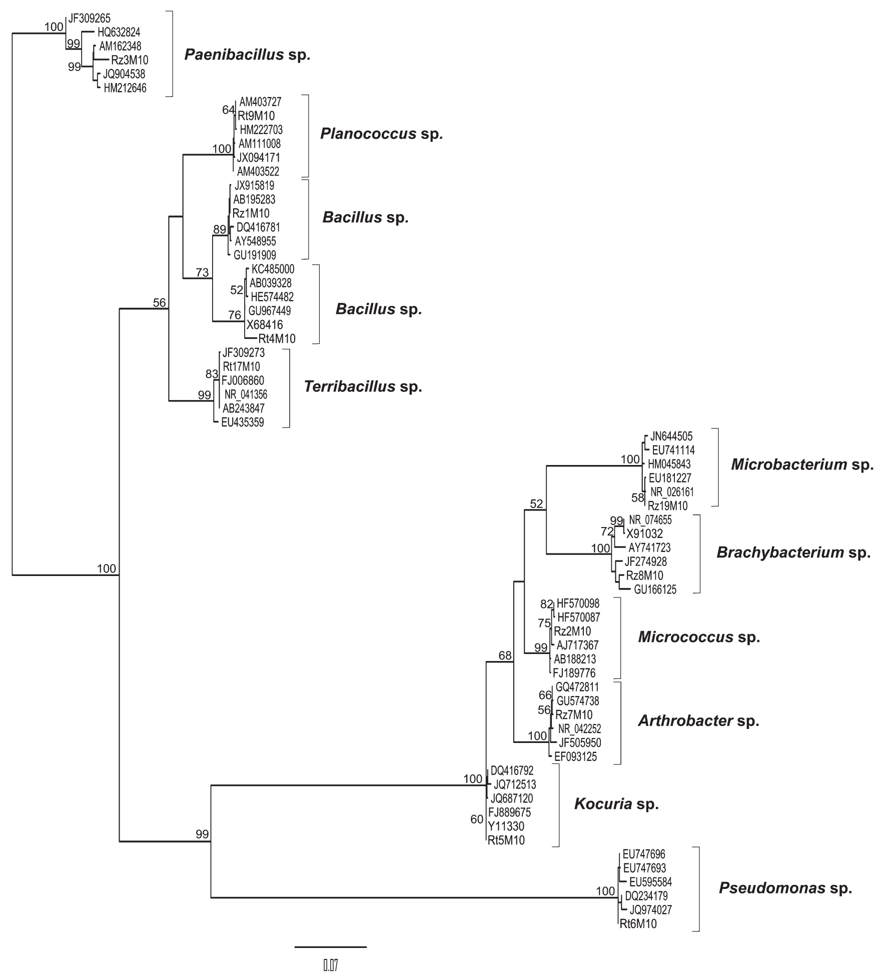

Fig. 1. Phylogenetic tree showing the relationship of isolates from grapevine with bacteria of the GenBank database (NCBI) based on the alignment of the 16S rRNA gene sequences. The tree was constructed by Maximun likelihood using Phylogenetic Analysis Using Parsimony (PAUP) and robustness was tested by bootstrap analysis with 1000 replicates. Bacteria isolated from grapevine: Rz7M10, Rz1M10, Rz19M10, Rz2M10, Rz3M10, Rz8M10, Rt4M10, Rt5M10, Rt6M10, Rt9M10 and Rt17M10; the other are database reference strains (see Appendix S1, Supporting Information for identification of accession number NCBI database).

Table 2. pH, OD_{530}, CFU (per ml) and ABA, IAA and gibberellin levels (ng ml^{-1}) produced by *Bacillus licheniformis* and *Pseudomonas fluorescens* isolated from *Vitis vinifera* cv. Malbec, grown in NFb chemically-defined medium to stationary phase and assessed by GC-EIMS. Values are means ± SD of n = 3 and the experiments were conducted twice; see Materials and methods for technical details and Appendix S2 in supporting information for spectra. Nd, not detected; Tr, traces.

	pH	OD_{530}	CFU 10^9	ABA	IAA	GA_1	GA_3
Pseudomonas fluorescens	7.7 ± 0.08	1.6 ± 0.007	5.8 ± 0.9	7.1 ± 3.8	28.5 ± 4.94	0.61 ± 0.04	Tr
Bacillus licheniformis	7.6 ± 0.12	1.5 ± 0.004	2.3 ± 0.6	0.43 ± 0.04	14.2 ± 1.34	0.86 ± 0.06	1.1 ± 0.05
Control NFb	6.5	<0.01	Nd	Nd	Nd	Nd	Nd

Bacteria increase phytohormone content and delay water loss in in vitro plants

At the end of the assay, 45 days-old in vitro plants bacterized with *B. licheniformis* and *P. fluorescens* showed a significant increase in ABA concentration measured in leaves but not at root level, where the hormone could not be detected by GC-MS analysis (Table 5). Grape plants bacterized with *B. licheniformis* showed the highest ABA concentration (3.93 ± 0.75 ng mg^{-1} leaf FW) which means an increase of 76-fold as compared with the control (0.052 ± 0.01 ng mg^{-1} leaf FW), whereas ABA levels of plants bacterized with *P. fluorescens*

Table 3. Bacterial counting ($CFU\,g^{-1}$) in agar adjacent to roots, roots, shoots and leaves of in vitro *Vitis vinifera* cv. Malbec plants bacterized with *Bacillus licheniformis* (+*Bacillus licheniformis*) and *Pseudomonas fluorescens* (+*Pseudomonas fluorescens)* assessed at 10, 20 and 30 dpb. Different letters indicate significant differences ($P \leq 0.05$); Values are means ± SD of n = 3 and the experiments were conducted twice. See section Materials and methods for details. Nd, not detected.

	+*Bacillus licheniformis*			+*Pseudomonas fluorescens*		
	Adjacent agar	Roots	Shoots and leaves	Adjacent agar	Roots	Shoots and leaves
10 dpb	$8.2\ 10^2$ a	$1.1\ 10^3$ a	Nd	$3.3\ 10^3$ a	$2.8\ 10^2$ b	Nd
20 dpb	$3.0\ 10^1$ b	$1.3\ 10^3$ a	Nd	$3.1\ 10^3$ a	$1.1\ 10^3$ a	Nd
30 dpb	Nd	$8.4\ 10^2$ a	Nd	$7.8\ 10^2$ b	$7.5\ 10^2$ a	Nd

Table 4. Shoot length (SL), root length (RL) and leaf area (LA) measured in 45 day-old in vitro *Vitis vinifera* cv. Malbec plants bacterized (or not, control) 30 dpb with *Bacillus licheniformis* and *Pseudomonas fluorescens*. Different letters indicate significant differences ($P \leq 0.05$). Values are means ± SD of n = 10 and the experiments were conducted twice.

Treatment	SL (cm)	RL (cm)	LA (cm^2)
+*Bacillus licheniformis*	10.83 ± 1.8 a	13.9 ± 1.2 a	7.72 ± 1.85 a
+*Pseudomonas fluorescens*	9.23 ± 1.8 ab	9.34 ± 2.7 b	6.68 ± 1.89 ab
Control	8.35 ± 1.8 b	9.23 ± 2.7 b	5.22 ± 0.88 b

Table 5. ABA and IAA concentration assessed by GC-EIMS ($ng\,mg^{-1}$ FW) in leaves and roots of 45 days-old *Vitis vinifera* cv. Malbec plants bacterized (30 dpb) with *Bacillus licheniformis* and *Pseudomonas fluorescens* and controls. Different letters indicate significant differences ($P \leq 0.05$). Values are means ± SD of n = 3 and the experiments were conducted twice. Nd, not detected.

	ABA ($ng\,mg^{-1}$ FW)		IAA ($ng\,mg^{-1}$ FW)	
Treatment	Leaf	Root	Leaf	Root
+*Bacillus licheniformis*	3.93 ± 0.75 a	Nd	0.43 ± 0.06 a	2.17 ± 0.27 a
+*Pseudomonas fluorescens*	2.1 ± 0.26 b	Nd	0.47 ± 0.08 a	2.31 ± 0.24 a
Control	0.052 ± 0.01 c	Nd	0.42 ± 0.07 a	1.02 ± 0.09 b

increased 40-fold (2.1 ± 0.26 $ng\,mg^{-1}$ leaf FW). Also, was found that IAA concentration was significantly increased in roots of bacterized plant (+*B. licheniformis* 2.17 ± 0.27 $ng\,mg^{-1}$ root FW and +*P. fluorescens* 2.31 ± 0.24 $ng\,mg^{-1}$ root FW) respect to controls (1.02 ± 0.09 $ng\,mg^{-1}$ root FW) (Table 5). No differences were observed in GAs levels (data not shown).

Fig. 2 shows that plants bacterized with *B. licheniformis* and with *P. fluorescens* lost 10% and 4% less water respectively than controls (between 38 and 170 min; indicated with rows in Fig. 2) and the loss observed in plants bacterized with *B. licheniformis* was similar to that observed in ABA-treated plants.

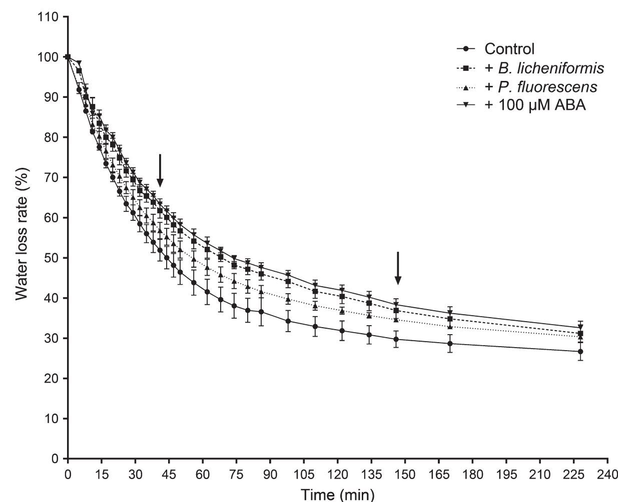

Fig. 2. Water loss rate of 45 days-old in vitro grown on MS agar *Vitis vinifera* cv. Malbec plants bacterized or not (controls) with *Bacillus licheniformis* and *Pseudomonas fluorescens*. Thirty days dpb aerial parts were cut off and weighed at different intervals of time. Arrows indicate the period of time in which plants bacterized with *B. licheniformis* lost 10% less water than controls. The data reported are means ± SD of n = 10 and the experiments were conducted twice.

Bacterial colonization induces synthesis of monoterpenes and sesquiterpenes

Fig. 3 shows the chemical structures of terpenes characterized by GC-EIMS from leaves and roots extract of plants bacterized with *B. licheniformis* or *P. fluorescens*. Six monoterpenes, including four hydrocarbons (α-pinene, terpinolene, 4-carene and limonene) and two oxygenated (eucalyptol and lilac aldehyde A) were identified. Four sesquiterpenes, two hydrocarbons (α-farnesene and α-bergamotene) and two oxygenated (nerolidol and farnesol) were characterized. The relative (%) and absolute ($\mu g\,g^{-1}$ FW) amounts of terpenes found in leaves and roots of bacterized plants at 20 and 30 dpb are shown in Tables 6 and 7 (at 10 dpb and in controls plants they were not detected or only traces were found).

Table 6 shows the mono and sesquiterpenes detected in leaves at 20 and 30 dpb. With *P. fluorescens* the

Table 6. Monoterpenes and sesquiterpenes assessed by GC-EIMS (in $\mu g\,g^{-1}$ FW and in % of leaves) from in vitro grape plants 20 and 30 dpb with *Bacillus licheniformis* (+*Bacillus licheniformis*) and with *Pseudomonas fluorescens* (+*Pseudomonas fluorescens*). At 10 dpb traces and in controls terpenes were not detected. Different letters indicate significant differences ($P \leq 0.05$). The data reported are means ± SD of n = 10 and the experiments were conducted twice. Nd, not detected.

	+*Bacillus licheniformis*				+*Pseudomonas fluorescens*			
	20 dpb		30 dpb		20 dpb		30 dpb	
	%	$\mu g\,g^{-1}$ FW	%	$\mu g\,g^{-1}$ FW	%	$\mu g\,g^{-1}$ FW	%	$\mu g\,g^{-1}$ FW
Monoterpenes								
α-Pinene	79.68	170.84	62.2	104.93	79.52	453.85	50.1	658.21
4-Carene	1.67	3.59	3.39	5.73	0.88	5.05	0.66	8.74
Limonene	Nd	Nd	Nd	Nd	Nd	Nd	0.076	0.98
Terpinolene	16.42	35.2	28.36	47.84	17.44	99.5	16.66	218.98
Eucalyptol	Nd	Nd	Nd	Nd	Nd	Nd	0.035	0.47
Lilac aldehyde A	2.23	4.78	6.05	10.19	2.16	12.35	32.47	426.54
Total	100	214.41	100	168.7	100	570	100	1314
Sesquiterpenes								
α-Bergamotene	0.007	0.04	0.07	0.65	0.075	0.84	0.16	3.4
α-Farnesene	0.022	0.12	0.2	1.88	0.12	1.32	0.7	14.28
Farnesol	0.11	0.62	0.47	4.6	0.09	0.97	0.52	10.63
Nerolidol	99.86	562.26	99.26	954.5	99.71	1109.77	98.62	1918.85
Total	100	563.04	100	961.63	100	1112.9	100	1947.16

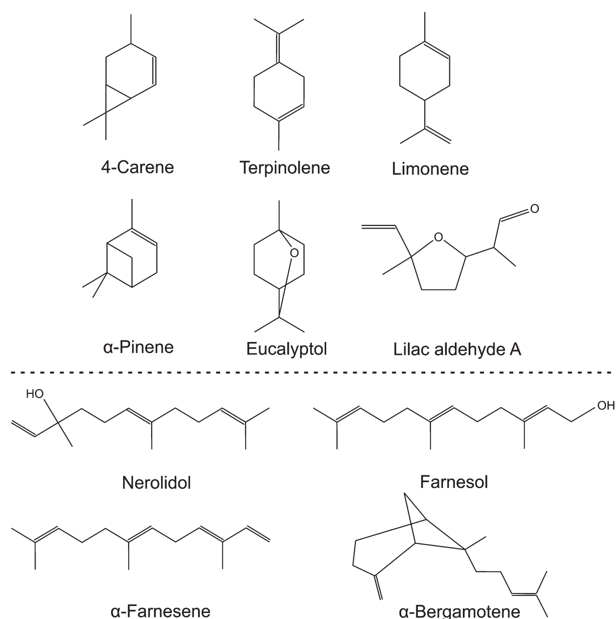

Fig. 3. Chemical structures of monoterpenes and sesquiterpenes identified by GC-EIMS in leaf extracts of in vitro grown grape plants.

total concentration of monoterpenes increased from 570 $\mu g\,g^{-1}$ FW to 1314 $\mu g\,g^{-1}$ FW in the period between 20 and 30 dpb, while with *B. licheniformis* decreased from 214.41 $\mu g\,g^{-1}$ to 168.7 $\mu g\,g^{-1}$ FW. Among the monoterpenes identified, the hydrocarbon compound α-pinene was the most abundant followed by terpinolene. At 20 dpb α-pinene represented 79.7% (*B. licheniformis*)

Table 7. Terpenes assessed by GC-EIMS ($\mu g\,g^{-1}$ FW of roots) from in vitro grape plants at 10, 20 and 30 dpb with *Bacillus licheniformis* (+*Bacillus licheniformis*) and *Pseudomonas fluorescens* (+*Pseudomonas fluorescens*). In the controls not detected. Different letters indicate significant differences ($P \leq 0.05$). The data reported are means ± SD of n = 10 and the experiments were conducted twice. Nd, not detected.

	+*Bacillus licheniformis*			+*Pseudomonas fluorescens*		
	10 dpb	20 dpb	30 dpb	10 dpb	20 dpb	30 dpb
Eucalyptol	Nd	0.0024 a	0.009 a	Nd	0.0028 a	0.0071a
Farnesol	Nd	0.0031 a	0.0026 a	Nd	0.0065 a	0.0096 a
Total		0.0055	0.0031		0.0133	0.0237

and 79.5% (*P. fluorescens*) of the total monoterpenes in leaves and 62.2 and 50.1% after 30 dpb. Terpinolene, 4-carene and lilac aldehyde A were also identified in lower concentration in both bacterized plants. It is noticeable that the oxygenated compound lilac aldehyde A was the less abundant in all the treatments with the exception of 30 dpb with *P. fluorescens* where the values reached 426.54 $\mu g\,g^{-1}$ FW, meaning 32.4%. Also the oxygenated compounds limonene and eucalyptol were detected, but only in plant bacterized with *P. fluorescens* at 30 dpb. In Fig. 4, the evolution in concentration along the time of the monoterpenes α-pinene (A), terpinolene (B), 4-carene (C) and lilac aldehyde A (D) is shown. In plants bacterized with *P. fluorescens* all compounds tended to augment, with the highest concentration at 30 dpb. On the other hand, in *B. licheniformis*-bacterized plants the compounds also augmented, except for α-pinene that diminished at 30 dpb. It is noticeable that the

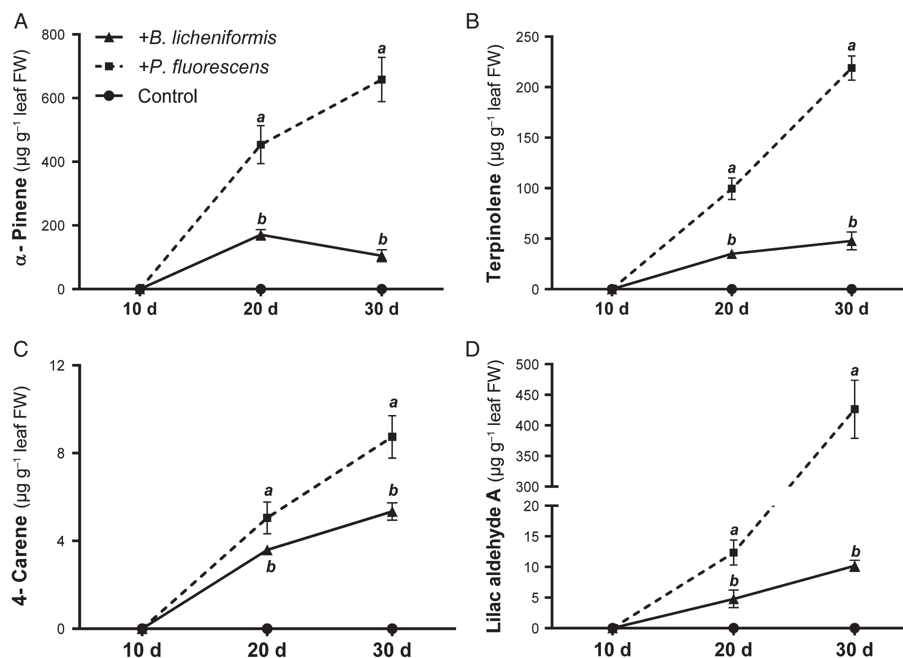

Fig. 4. Levels of monoterpenes assessed by GC-EIMS in leaves of in vitro grown *Vitis vinifera* cv. Malbec plants bacterized or not (controls) on roots with *Bacillus licheniformis* and *Pseudomonas fluorescens*, expressed in $\mu g\ g^{-1}$ leaf FW. Leaf samples were collected at 10, 20 and 30 dpb. Different letters indicate significant differences ($P \leq 0.05$). The data reported are means ± SD of n = 10 and the experiments were conducted twice. α-Pinene (A); Terpinolene (B); 4-Carene (C); Lilac aldehyde A (D).

concentration of all compounds was markedly higher in plants bacterized with *P. fluorescens* as compared with *B. licheniformis*-bacterized plants at 20 and 30 dpb. No monoterpenes were detected in control plants (Fig. 4).

The total concentration of sesquiterpenes in leaves augmented with bacterization (Table 6). With *P. fluorescens* the concentration of sesquiterpenes increased from $1112.9\ \mu g\ g^{-1}$ FW to $1947.16\ \mu g\ g^{-1}$ FW in the period between 20 and 30 dpb, while with *B. licheniformis* increased from $563.04\ \mu g\ g^{-1}$ FW to $962.63\ \mu g\ g^{-1}$ FW. The most abundant sesquiterpene identified was the oxygenated compound nerolidol the levels of which increased between 20 and 30 dpb from $562.26\ \mu g\ g^{-1}$ FW to $954.5\ \mu g\ g^{-1}$ FW in plants bacterized with *B. licheniformis* and from $1109.7\ \mu g\ g^{-1}$ FW to $1918.85\ \mu g\ g^{-1}$ FW with *P. fluorescens*. Nerolidol is the major sesquiterpene detected in *B. licheniformis* (between 99.86 and 99.26% at 20 and 30 dpb, respectively) and *P. fluorescens* (99.74 and 98.62%, respectively) treated plants. Plants bacterized with *P. fluorescens* showed the highest absolute nerolidol contents. The sesquiterpenes farnesol, α-bergamotene and α-farnesene were also detected but in lower concentrations in plants treated with these bacteria. Fig. 5 shows the evolution in concentration of the sesquiterpenes α-farnesene (A), α-bergamotene (B), farnesol (C) and nerolidol (D) with time. In both bacterized plants all compounds augmented. As it occurred with monoterpenes, the concentration of all sesquiterpenes was markedly higher in plants bacterized with *P. fluorescens* as compared to *B. licheniformis* at 20 and 30 dpb. Sesquiterpenes were found only as traces in control plants.

In roots (Table 7), terpenes were detected at very low concentration as compared with leaves, and they were represented only by the oxygenated monoterpene eucalyptol and the oxygenated sesquiterpene farnesol. Also, the concentrations did not change from 20 to 30 dpb and were similar in both bacterized plants.

Application of ABA to in vitro plants does not induce synthesis of terpenes

The terpenes analyzed in leaves and roots 48 h after the application of ABA to 25, 35 and 45 days-old plants (in accordance with the bacterization treatment, that is, 10, 20 and 30 dpb) were pinene, eucalyptol and farnesol (Table 8). These terpenes were identified at low concentration and only in leaves of 25 days-old plants. Both in roots of all treatments and in control plants, terpenes were not detected or were in low concentrations.

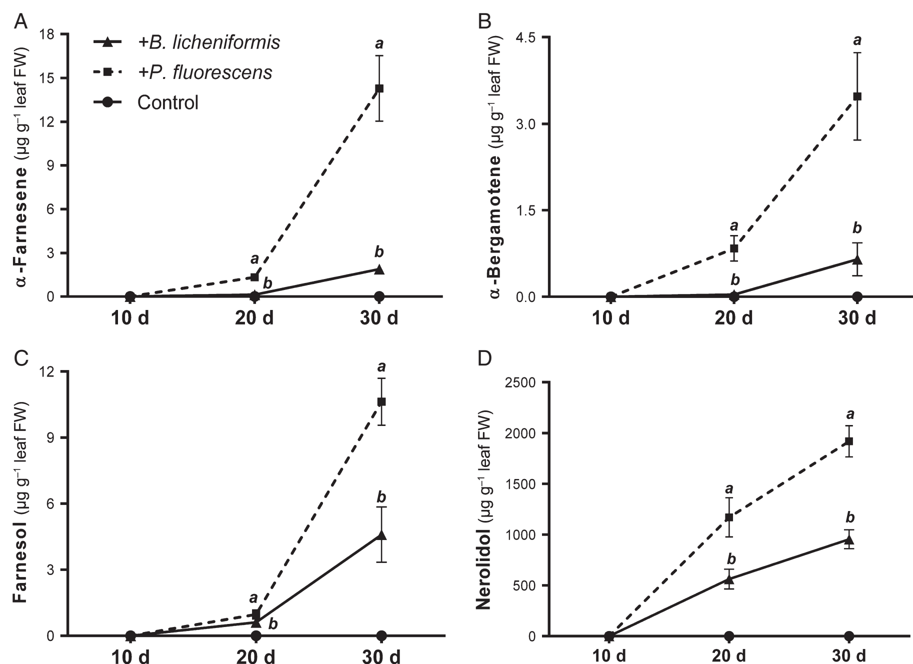

Fig. 5. Levels of sesquiterpenes assessed by GC-EIMS in leaves of in vitro grown *Vitis vinifera* cv. Malbec plants bacterized or not (controls) on roots with *Bacillus licheniformis* and *Pseudomonas fluorescens*, expressed in μg g^{-1} leaf FW. Leaf samples were collected at 10, 20 and 30 dpb. Different letters indicate significant differences ($P \leq 0.05$). The data reported are means ± SD of n = 10 and the experiments were conducted twice. α-Farnesene (A); α-Bergamotene (B); Farnesol (C); Nerolidol (D).

Table 8. Terpenes assessed by GC-EIMS (μg g^{-1}FW of leaves) from 25, 35 and 45 days-old in vitro grape plants after 48 h of treatment with ABA. Neither was detected in roots nor in controls. See Materials and methods for details. Different letters indicate significant differences ($P \leq 0.05$). The data reported are means ± SD of n = 10 and the experiments were conducted twice. Nd, not detected.

	+ABA		
	25 day-old	35 day-old	45 day-old
Pinene	0.0012 a	Nd	Nd
Eucalyptol	0.008 a	Nd	Nd
Farnesol	0.006 a	Nd	Nd
Total	0.152		

Discussion

Eleven strains were isolated at different soil depths from roots and rhizosphere of vineyard cv. Malbec and identified by comparison with GenBank database of the 16S rRNA gene sequences. Two of them, *B. licheniformis* and *P. fluorescens*, were selected for further studies. These bacteria belong to the genera well characterized as PGPR (Haas and Keel 2003, Kloepper et al. 2004), and they are widely distributed in nature since they have been isolated from different environments and associated to a variety of plants (Podile and Kishore 2006). It is important to note that although all the strains were isolated and more numerously in the range of 10–40 cm of soil depths, the total population varied according to the depth (data not shown). That is, each depth does not configure a niche to which each strain may be better adapted, but influences the size of the population. It is difficult to identify the specific factors that drive the distribution because multiple environmental factors change with soil depth (Eilers et al. 2012), including root exudates, nutrient accessibility, oxygen availability and humidity. These factors that were suitable to the microorganisms may account for the different density although most of them behave as endophytic.

On the basis of the antecedents in literature *B. licheniformis* and *P. fluorescens* were used in this study for further assays. Inoculation to grapevine showed that both bacteria behave as non-pathogenic, since bacterized plants looked healthy without any growth inhibition symptoms as compared with controls. *Bacillus licheniformis* increased shoot length (SL) and LA, as well as RL of in vitro grown plants (Table 4), which may be correlated with its capability to early colonize roots compared to *P. fluorescens* (10 dpb). It was demonstrated that both bacteria have the capability to produce phytohormones in chemically-defined medium, ability extensively reported in other PGPR. It has been previously reported that these bacteria synthesize IAA

(Gutiérrez-Mañero et al. 1996, Suzuki et al. 2003), and particularly *B. licheniformis* has the ability to produce different GAs (Gutiérrez-Mañero et al. 2001). GAs produced by *B. licheniformis* may be implicated in the extension of stems and roots (Bottini et al. 2004). These results agree with Probanza et al. (2002) who reported that inoculation with *B. licheniformis* enhanced growth of *Pinus pinea*, especially increasing RL so resulting in greater root area that enabled the plant to take up more nutrients collaborating to the plant growth. In the case of *P. fluorescens* there was also an increase in the growth values although they were not significant with respect to controls. Also, this is the first report that *P. fluorescens* and *B. licheniformis* produce ABA in chemically-defined medium.

ABA plays a fundamental role in plant responses to environmental conditions as stress signal. It is known that ABA is directly involved in regulation of stomata aperture-closure and it increases as a result of water stresses (Davies and Zhang 1991, Jiang and Zhang 2002). In this work it was found that bacterized plants with both studied bacteria increased ABA content (70-fold *B. licheniformis* and 40-fold *P. fluorescens*) in leaf tissues with respect to controls. The high amount of ABA found strongly suggests that the increase is due to production of the own bacteria (already proved in chemically-defined medium) and as a plant response to the interaction with the microorganisms. This was correlated with the results of water loss rate assay, where the plants bacterized with *P. fluorescens* and *B. licheniformis* lost 4% and 10% less water than controls, respectively. The enhancement of ABA content in plants that interacted with *B. licheniformis* hence delayed water losses so making these plants more prepared to cope with water scarcity in soils. In fact, Cohen et al. (2009) demonstrated that the endophytic PGPR *Azospirillum* sp. alleviated the plant water stress restoring growth parameters at control levels in plants of maize treated with fluridone (inhibitor of ABA biosynthesis), suggesting that the bacterium may supply the plant with ABA as to cover the hormonal deficit produced by the inhibitor. It is worth to note that plants may respond to bacteria by closing stomata through an ABA-independent mechanism in which oxylipins are involved (Montillet et al. 2013), which remain to debate here since bacterization was done via roots and these microorganisms were not detected at leaf and shoot level along the plant ontogeny. Another possible effect of the ABA-increased levels by bacteria may be the augmentation of carbohydrate transport. In fact, it has been previously shown that ABA application to pot-grown grapevines (Moreno et al. 2011) and field-grown wheat (Travaglia et al. 2007) increased carbohydrate allocation in grapes and grains, respectively.

Induced systemic resistance (ISR) has been reported as one of the mechanisms by which PGPR help the plant to reduce the incidence of diseases through the modification of physical and biochemical properties of the host that enhances the plants defensive capacity (Kloepper et al. 2004). In this work the induction of synthesis of terpenes by bacteria defined as PGPR was studied as a new possible mechanism of defense against pathogens in grapevine, based on the idea that their antimicrobial activities may inhibit the spread of the pathogen into plant tissues (Maffei 2010). As well, terpenes have been suggested to participate in the plant response before abiotic stresses (Beckett et al. 2012, Gil et al. 2012, 2013, Piccoli and Bottini 2013). In plants inoculated with *P. fluorescens* and *B. licheniformis* α-pinene, terpinolene, 4-carene, limonene, eucalyptol and lilac aldehyde A (monoterpenes) and α-bergamotene, α-farnesene, nerolidol and farnesol (sesquiterpenes) were identified in leaves. However in roots the terpenes found were reduced to eucalyptol and farnesol and in lower concentrations. This variety of compounds was detected only in bacterized plants, suggesting that they are synthesized *de novo* in response to bacterial elicitation. Because bacteria were inoculated on roots and the aerial parts were not colonized, the results indicate that *B. licheniformis* and mainly *P. fluorescens* have the ability to elicit an ISR in *V. vinifera* (cv. Malbec) plants because the synthesis of terpenes were detected mainly in leaves. Various inducible defense responses, similar to that characterized during interaction with pathogens, have been activated by biocontrol agents or biotic elicitors in grapevine (Elmer and Reglinski 2006, Magnin-Robert et al. 2007, 2013). Other works on biocontrol reported that grape inoculation with a soil bacteria increase the synthesis of the defense-compound resveratrol, even more than the pathogen *B. cinerea* (Paul et al. 1998). Such phytoalexins contribute to grapevine resistance since through the inhibition of mycelium growth of a wide range of fungi. Verhagen et al. (2010, 2011) also underline that *P. fluorescens* is able to induce early oxidative burst and phytoalexins (*trans*-resveratrol and *trans*-ϵ-viniferin) production in grapevine leaf cells that protect the plant against gray mold disease. Considering these antecedents, the induction of defense terpenic compound may be a new possible mechanism by with PGPR protects grape plant against pathogens.

The monoterpenes α-pinene and the sesquiterpene nerolidol were the most abundant terpenes identified mainly in plants inoculated with *P. fluorescens*. The oxygenated sesquiterpene nerolidol represented the most abundant terpene compound both at 20 and at 30 dpb, signifying 98–99% of the total sesquiterpenes identified in leaves. This compound is present in

essential oils of several plant species (Alissandrakis et al. 2005, Vila et al. 2010) and its concentration has been shown to increase in response to fungal pathogen elicitors (Leitner et al. 2008). In cell suspension cultures of grapevine Escoriaza et al. (2013) reported synthesis of nerolidol in response to infection with *Phaeoacremonium parasiticum*, one of the causal fungi of 'hoja de malvon' disease that affects grapevines. It has been previously reported (Brehm-Stecher and Johnson 2003) that nerolidol has antimicrobial properties that enhance the bacterial permeability and susceptibility to exogenous compounds by disruption of the cytoplasmic membrane. More recently it was reported that in fungi nerolidol provokes distortion and collapse of the hyphae as result of destruction of organelles in cytoplasm (Park et al. 2009). On the other hand, α-pinene was the most abundant monoterpene in leaves, representing almost 79% of the total at 20 dpb and 50–60% at 30 dpb, in plants bacterized with *B. licheniformis* and *P. fluorescens*. It has been previously reported that pinene is present in essential oils and increases in response to fungal and insect attack in cotton and *Picea abies* (Paré and Tumlinson 1997, Zhao et al. 2010).

It was also detected in both leaves and roots of bacterized plants low concentrations of the oxygenated sesquiterpene farnesol, compound with antimicrobial properties. Machida et al. (1998) showed that farnesol induces generation of reactive oxygen species (ROS) by indirect inhibition of the mitochondrial transport chain in *Saccharomyces cerevisiae*. Semighini et al. (2006) reported that this compound showed antifungal properties by triggering morphological features characteristic of apoptosis in *Aspergillus nidulans* suggesting that mitochondria and ROS participate in farnesol-induced apoptosis. The synthesis of these compounds was induced by the bacteria since they were found exclusively in bacterized plants and not detected in controls.

Terpenes may also have a plant protective function towards abiotic stresses due to their antioxidant activity that sequestrate free radicals so reducing oxidative damage (Aharoni et al. 2003, Wei and Shibamoto 2007) and inducing integrity and stability of membranes (Beckett et al. 2012).

Our experiment shows that exogenous ABA is not able to induce the synthesis of terpenes although it helps the plant to alleviate water losses.

In conclusion, the results suggest that *B. licheniformis* and *P. fluorescens* operate as PGPR in the interaction with the grapevines at least by two different mechanisms, one by inducing synthesis of terpenes as defense compounds, and another by producing and inducing synthesis of ABA that may alleviate plants from drought conditions. It is also clear that *P. fluorescens* induce most strongly the synthesis of terpenes since the plants bacterized with this bacterium showed significantly highest concentrations.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Definition of accession number (NCBI) of the sequences in phylogenetic tree.

Appendix S2. Spectra of identified phytohormones in bacteria cultures.

Appendix S3. GenBank accession numbers.

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