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Research article

Bacteria and smoke-water extract improve growth and induce the synthesis of volatile defense mechanisms in *Vitis vinifera* L.

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A B S T R A C T

Sustainable agricultural practices have been developed as alternative to the use of agrochemicals, and viticulture is not exempt of that. Plant growth promoting rhizobacteria (PGPR) and smoke water extracts (SW) are environmentally-friendly alternative to those agrochemicals. The aim of this study was to investigate the single or combined effects of SW and the PGPR *Pseudomonas fluorescens* (*Pf*) and *Bacillus licheniformis* (*Bl*) on the physiology and biochemistry of grapevines plants. After 38 days, single applications of SW solutions and bacterial suspensions increase rooting and root length. Combined treatments had a slight positive effect compared to the water control. At the end of 60-days pot trial, grapevine treated with 1:1000 SW and *Pf* applied alone showed increases in stem length, leaf area and fresh weight of the roots, shoot and leaves, although not significantly differences from the water control were found. In addition, *Pf* augmented chlorophyll relative content, all treatments decreased the stomatal conductance (mainly 1:500 SW, *Pf* and 1:1000 SW + *Bl*), as well as lipid peroxidation in roots (mainly in bacterial treatments), and induced the synthesis of mono and sesquiterpenes in leaves, where the effect was enhanced in combined treatments. In conclusion, PGPR and SW are effective to improve growth of *V. vinifera* cuttings as well as to increase the plants defense mechanisms that may help them to cope with biotic and abiotic stresses.

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1. Introduction

The continuous use of fertilizers and agrochemicals over decades has led to land becoming less productive (Kulkarni et al., 2011). Consequently, there is a trend towards replacing chemicals with more ecologically sustainable agricultural practices, which include application of different biological strategies (Loureiro et al., 2001). Furthermore, when consumers choose a food, they aim to ecologically-friendly products, not just for environmental concerns but also for health aspects (Torjusen et al., 2001; Brugarolas Mollá-Bauzá et al., 2005). Viticulture is not exempt of the changing habits of consumers and it is undergoing a gradual shift towards more sustainable production patterns (Gabzdylova et al., 2009; Villanueva-Rey et al., 2014). Producers have begun to apply field practices such as organic viticulture as a novel and attractive technique (Villanueva-Rey et al., 2014). Studies in the consumer's preferences when purchasing a wine found that there is a segment of the population willing to pay a higher price for organic wine (Brugarolas Mollá-Bauzá et al., 2005; Bernabéu et al., 2008).

The use of plant growth promoting rhizobacteria (PGPR) is an environmentally-friendly alternative that offers an attractive replacement to synthetic fertilizers and agrochemicals. PGPR improve plant growth by increasing the supply or availability of primary nutrients to the host (Vessey, 2003; Lugtenberg and Kamilova, 2009), producing plant growth regulators (PGR) such as gibberellins, abscisic acid and auxins (GAs, ABA and Aux; Piccoli and Bottini, 2013; Spaepen et al., 2007) and protecting plants from

Abbreviation: ABA, abscisic acid; *Bl*, *Bacillus licheniformis*; CFU, colony forming units; CRC, chlorophyll relative content; FW, fresh weight; GAs, gibberellins; IBA, indole-butyric acid; MDA, malondialdehyde; LA, leaf area; LB, Luria Broth medium; *Pf*, *Pseudomonas fluorescens*; PBS, phosphate buffer Saline; PGR, plant growth regulators; PGPR, plant growth promoting rhizobacteria; RL, root length; sg, stomatal conductance; SL, stem length; SW, smoke water extract.

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biotic and abiotic stresses due to production of antibiotics and/or inducing a systemic defensive response in the plant (Kloepper et al., 2004; Yang et al., 2009). Some of these responses are related to an increase in the antioxidant capacity of plant tissues via synthesis of different compounds and/or enzymatic activities (Paul et al., 1998; Kohler et al., 2009). PGPR have been successful in promoting growth of different crops, including grapevines (Compant et al., 2005; Fernandez et al., 2012; Salomon et al., 2014). In a previous study, we demonstrated that the PGPRs *Pseudomonas fluorescens* (*Pf*) and *Bacillus licheniformis* (*Bl*) isolated from a commercial Malbec vineyard, are able to enhance growth parameters, retard water loss rate by increasing ABA concentrations in leaves, and to induce a systemic response by eliciting synthesis of volatile terpenes of *in vitro* grown grapevines (Salomon et al., 2014). These compounds are derived from isopentenyl diphosphate (IPP, C5) and its isomer dimethylallyl diphosphate (DMAPP), and are related to defense against herbivory and pathogens, as well as abiotic stress (Dudareva et al., 2004; Gil et al., 2012; Nagegowda, 2010). In another study, the *Bl* strain was also able to significantly improved shoot biomass of okra plants (Papenfus et al., 2015).

Smoke is an alternative biotechnology for the cultivation of crops that has been explored in the last decade. The application of smoke-water solutions (SW, obtained from burning plant material and bubbling the plant-derived smoke through water) on different species has demonstrated that it significantly increases seed germination (Brown and Botha, 2004; Light and van Staden, 2004), enhances seedling vigor (Kulkarni et al., 2006; van Staden et al., 2006) and improves both growth and yield of crops (Kulkarni et al., 2008, 2010). Additionally, SW has antimicrobial properties (Kulkarni et al., 2011). These promotive effects of smoke and SW are due in part, to the biologically active butenolide compound karrikinolide (3-methyl-2Hfuro[2,3-c]pyran-2-one) which is thermostable, long-lasting in solution and highly active at very low concentrations (10^{-9} M; van Staden et al., 2004). Thus, SW has great potential for use in horticulture and agriculture (Light and van Staden, 2004).

The aim of this study was to investigate the single or combined effects of two environmentally-friendly products, SW and the PGPR *Pf* and *Bl* on the physiology and biochemistry of grapevines. Due to the characteristics of both technologies, we hypothesized that the inoculation of PGPR together with SW solutions would further increase growth compared to single application of either SW or PGPR, and increase antioxidant properties of the tissues by inducing terpenes synthesis.

2. Material and methods

2.1. Bacterial cultures

Pseudomonas fluorescens and Bl were previously isolated from the roots and rhizosphere of *Vitis vinifera* L. cv. Malbec growing in a commercial vineyard in Mendoza, Argentina (Salomon et al., 2014). Each bacterium was grown in 1 L of liquid Luria Broth medium (LB, Sigma Chem. Co, St Louis, MO) to the late exponential phase (OD_{530}; 10^9 CFU mL^{-1}). Cultures were centrifuged at 3000 *g* for 15 min at 4° C. The resulting pellets were rinsed twice with distilled water and then re-suspended in water at a final concentration of 10^6 CFU mL^{-1}.

2.2. Preparation of smoke-water extract

The SW was prepared as described by Baxter et al. (1994) by burning 5 kg dry leaf material of the grass *Cynodon dactylon* L. (Poaceae) and bubbling the smoke through 500 mL distilled water for 45 min. This grass was selected since it naturally grows between files of grapevine in the main grape region of Argentina. The obtained smoke extract was filtered through filter paper (No. 1; Whatman) and was used as stock solution. The concentrations used in the trials described below were prepared by diluting 1 mL of the stock solution with the corresponding volume of tap water.

2.3. Plant material and experimental design

In order to evaluate the effects of treatments on rooting, cuttings of *V. vinifera* cv. Malbec from a virus-free experimental vineyard of INTA-Mendoza (Argentina) were used. Cuttings were submerged (n = 8) in different SW concentrations (1:250, 1:500, 1:1000, 1:1500 and 1:2000 v/v), bacterial suspension (10^6 CFU mL^{-1} *Pf* and *Bl*) and combined treatments (*Pf* + 1:500 v/v SW, *Pf* + 1:1000 v/v SW, *Bl* + 1:500 v/v SW, *Bl* + 1:1000 v/v SW). Indole-butyric acid (IBA, Sigma Chem. Co, St Louis, MO) 0.6 μM and water were used as controls. After 24 h the cuttings were placed in perlite at 22 ±2° C and daily watered with tap water for 38 days. The rooting % and root length (RL) were evaluated in order to select the most effective treatments to continue the pot trials. After the treatment selection, *V. vinifera* cv. Malbec hardwood cuttings were placed in pots containing marc:perlite (2:1; v/v) and maintained under greenhouse conditions at Instituto de Biología Agrícola de Mendoza (33° 0′ S, 68° 52' W, 940 m a.s.l.) during one growing season (2013–2014). The following treatments (n = 5) were weekly applied as soil drench: water (control); 1:500 v/v SW; 1:1000 v/v SW; *Pf* + 1:500 v/v SW; *Bl* + 1:1000 v/v SW; *Pf*; and *Bl*. After 60 days, physiological and growth parameters were evaluated and samples of different tissues taken and stored at −80 °C until further analysis.

2.4. Growth and physiological parameters

At the end of the pot trial, the following growth parameters were measured: stem and root length (SL and RL, respectively); aerial (leaves + stems) and root fresh weight (aFW and rFW respectively); leaf area (LA); leaf number; node number; internode length. The following physiological parameters were also measured: chlorophyll relative content (CRC) using a portable chlorophyll meter (SPAD-502, Konica Minolta Sensing, Osaka, Japan); photosynthetic efficiency with a Fluorometer (Hansatech Instruments LTD, King's Lynn, Norfolk, UK); stomatal conductance (*sg*) with a steady-state diffusion leaf porometer (conductimeter SC-1, Decagon Devices, Inc, Pullman, WA, USA).

2.5. Lipid peroxidation

To evaluate oxidative damage as lipid peroxidation, malondialdehyde (MDA) content was measured in root and three leaf samples (collected from various positions on the stem - high, mid and low) following the procedure previously described by Berli et al. (2010). Briefly, 60 mg tissue (FW) was suspended in 1.5 mL stock solution comprising of 15% (w/v) trichloroacetic acid (0.5% (w/v) thiobarbituric acid and 0.25% (w/v) hydrochloric acid). The mixture was vigorously stirred and incubated at 70 °C for 45 min. After centrifugation at 9300*g* for 10 min, the supernatant was collected and the absorbance measured at 535 nm with 1 mm optical path cell. The concentration was calculated considering MDA molar extinction coefficient = 1.56 × 105 M^{-1} cm^{-1}.

2.6. Terpene quantification

Monoterpenes, sesquiterpenes and diterpenes were quantified by GC-MS as previously described in Salomon et al. (2014). Briefly, 0.1 g FW leaves were macerated with 1 mL of methanol: distilled water: formic acid (79: 20: 1, v/v/v) and 2 mL CH_3Cl_2. The extract

was left overnight at 4° C and then shaken and centrifuged for 15 min at 10000 × g. From each CH_3Cl_2 phase, a 100 μL aliquot was taken and mixed with 1 ng μL^{-1} *n*-hexadecane as internal standard. Then, 2 μL were injected in split–splitless mode into a capillary gas chromatograph-electron impact mass spectrometer (GC-EIMS; Clarus 500, Perkin Elmer, Shelton, CT) fitted with 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). 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 scanned. Compounds were identified by comparison of GC retention times and full mass spectra of the corresponding standards previously injected and/or data from the NIST library. 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.

2.7. Bacterial counting

In order to evaluate bacterial survival, samples of substrate and roots were taken at the end of the pot trial by the standard plate-counting method. The root sample (1 g FW) was disinfected with 70% ethanol for 1 min and 10% commercial bleach (55 g L^{-1} active chlorine) for 10 min and then rinsed several times with sterile distilled water. The root was then soaked with Phosphate Buffer Saline (PBS; 100 mM potassium phosphate buffer, pH 7.5) and ground to powder in a sterile mortar and pestle. A 200 μL aliquot of the supernatant was plated in duplicate in agar LB medium. In addition, 0.5 g substrate (taken close to the root) of each pot was soaked in 50 mL PBS and shaken for 2 h. Then, a serial dilution of 10^{-1} to 10^{-6} was performed and the extract plated (in duplicate) on LB medium. The number of typical colonies was counted after 2–5 days of incubation at 30 ± 2 °C.

2.8. Statistical analysis

Statistical analysis was performed by ANOVA and comparisons were done with LSD of Fisher test, using InfoStat version 2013 (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina). Significant differences were considered at $p \leq 0.05$.

3. Results

3.1. Smoke-water extract and selected bacteria improve rooting of hardwood cuttings

Fig. 1 (a) shows that all treatments increased rooting % from 0.2-fold up to 1.7-fold; being *Bl* alone and IBA 0.6 μM the most effective. SW in single application promoted rooting in a dose-responsive manner with a maximum effect at 1:1000 dilution, where the positive effect diminished at lower or higher concentrations. When SW dilutions were combined with bacteria also showed less promotive effects, nullifying the bacteria promotion and showing negative interactions. Similar results were found in RL (Fig. 1 b), although with exception of IBA treatment, the differences were not statistically significant. .

3.2. Pf and 1:1000 v/v SW concentration increase growth parameters

At the end of the 60 days pot trial the best growth response was observed in the 1:1000 SW and *Pf* treated plants, although not significantly different from the water control; that is, there were increases in SL (166.2 ± 7.4 and 166.3 ± 12.4 cm respectively vs.134.8 cm in the control; Fig. 2a) and LA (2658 ± 87 and 2502 ± 98 cm^2 respectively, vs. 2129 cm^2 in the control; Fig. 2b). The combination of 1:500 SW + *Pf* reduced the positive effect of these treatments applied separately (113.4 cm SL and 1889 cm^2 LA), especially in comparison with *Pf* in which the difference respect to SL was statistically significant. On the other hand, *Bl* and the combination 1:1000 SW + *Bl* had no effects on these growth parameters.

Likewise, Fig. 2 (c) shows that FW was significantly increased in 1:1000 SW (223.3 ± 9.4 g) as compared to control (180.8 ± 15.4 g), mainly due to an increment in root and stem FW. Regarding *Pf* alone, even it was not statistically significant a difference was observed (205.7 ± 3.9 g).

The combined treatments 1:500 SW + *Pf* and 1:1000 SW + *Bl* reduced the positive effect compared to single applications. No differences were found in the other growth parameters (RL, leaf number, node number and internode length; data not shown).

3.3. Pf increases CRC and decreases sg

As it is shown in Fig. 3 (a), *Pf* alone was the only treatment that significantly augmented CRC (27.2 ± 1.1) compared to control (24.5 ± 0.5), while in 1:1000 SW was slightly decreased and with no differences respect to control (23.0 ± 0.7). Nevertheless, photosynthetic efficiency was similar between all the treatments (data not shown). All treatments decreased **sg** with 1:500 SW, *Pf* and 1:1000 SW + *Bl* having a significant effect (264.4 ± 15.1, 284.3 ± 15.9 and 250.3 ± 63.6 M mol m^2 s^{-1} respectively; Fig. 3b) compared to control (341.8 ± 18.6 M mol m^2 s^{-1}).

3.4. Bacteria and SW decrease lipid peroxidation

All treatments significantly diminished lipid peroxidation in the roots as assessed by MDA content. The lowest MDA content was in all the bacterial treatments, irrespective of whether SW was included in the treatment (Fig. 4). No differences were found in leaf MDA content, although bacteria application tended to diminish it (data not shown).

3.5. Smoke-water extract and selected bacteria induce synthesis of mono and sesquiterpenes related to defense

At the end of the 60 days pot trial, terpenes were assessed in leaves of all plants treated with SW, bacterial suspensions and their combinations, while no terpenes were detected in the control plants (Table 1). The monoterpenes α-pinene, 4-carene and ocimene presented the highest concentrations in the leaves of the 1:500 SW treatment, being 4-carene and ocimene concentrations remarkably higher respect to the other treatments. Also, terpinolene was found in leaves, being its concentration significantly higher in 1:1000 SW, *Pf* + 1:500 SW and *Bl* + 1:1000 SW treatments. It is important to highlight that these compounds increased in the combined treatments compared to when the bacteria were applied alone, mainly regarding 4-carene and terpinolene concentrations. Futhermore, ocimene was only detected in plants treated with SW, irrespective of whether it was applied alone or in combination (Table 1). Amongst treatments, 1:500 SW and *Pf* + 1:500 SW increased the amount of monoterpenes more than the other treatments.

Even though the sesquiterpene nerolidol was detected in all treatments (Table 1), the highest concentrations were found in those with bacteria, especially with *Pf* in which concentrations were significantly higher as compared to SW alone. The combination of bacteria and SW increased nerolidol content from

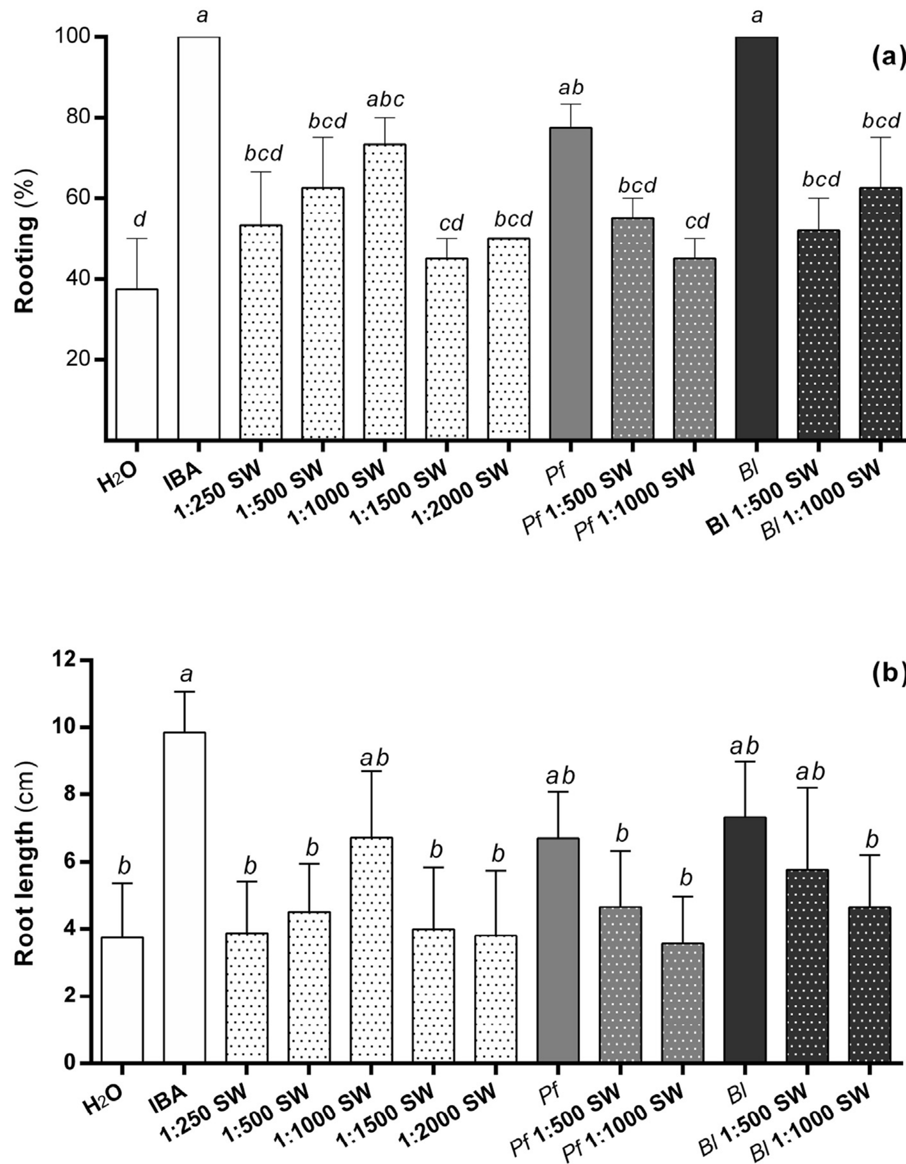

Fig. 1. Rooting % (a) and RL (b) of *V. vinifera* L. cv. Malbec cuttings (38 days post-treatment), treated with different SW concentrations, bacterial suspensions (*Pf* and *Bl*) and combined SW + bacteria treatments. Values are mean (n = 8) and different letters indicate significant differences ($P \leq 0.05$).

520,9 ± 171.3 in *Pf* to 662.7 ± 54.9 ng mg^{-1} Leaf FW in *Pf* +1:500 SW, and from 206.4 ± 52.4 in *Bl* to 329.0 ± 25.2 ng mg^{-1} Leaf FW in *Bl* +1:1000 SW. On the other hand, the diterpene beyerene was only found in leaves of plants treated with SW alone.

3.6. Root colonization by Bl and Pf in interaction with SW

After 60 days, CFU countings in pot substrates were similar in all treatments (approximately 2×10^7 CFU g^{-1} substrate) and did not differ from control (Table 2). In *Pf* treatments combined or not, between 40 and 45% of the total CFU were identified as *P. fluorescens*, while in *Bl* treatments only 11–22% of the total CFU corresponded to this bacterium. In roots, bacteria abundance was lower than in substrate and the highest CFU counting was found in water controls, being significantly different from *Pf*, *Pf* + 1:500 SW and 1:500 SW applied alone. Nevertheless the proportion of *Pf* increased almost three-fold when SW was included in the *Pf* treatment. In comparison, only 1.6% of the total CFU in roots were identified as *Bl*, and it was not found when SW was included (Table 2).

4. Discussion

Bacteria applied alone and SW (in dose-responsive manner) similarly increased rooting of *V. vinifera* cv. Malbec cuttings compared to water control, suggesting that both are effective although the differences were not always statistically significant. The results achieved with the application of bacterial suspensions may be explained in part because the ability of these bacteria to produce IAA (Salomon et al., 2014). Auxin and/or hormone-like substances produced by bacterial strains has been linked to an increase of graft union and callusing formation in different rootstock-scion combinations of grapevines treated with *Pseudomonas* and *Bacillus* (Köse et al., 2005). In the present study, 1:1000 SW and, to a lesser extent 1:500 SW treatments, increased rooting by 0.97 and 0.67-fold respectively compare to water control. Similar results were found in mung beans and rice by application of SW that improved rooting and number of lateral roots (Taylor and van

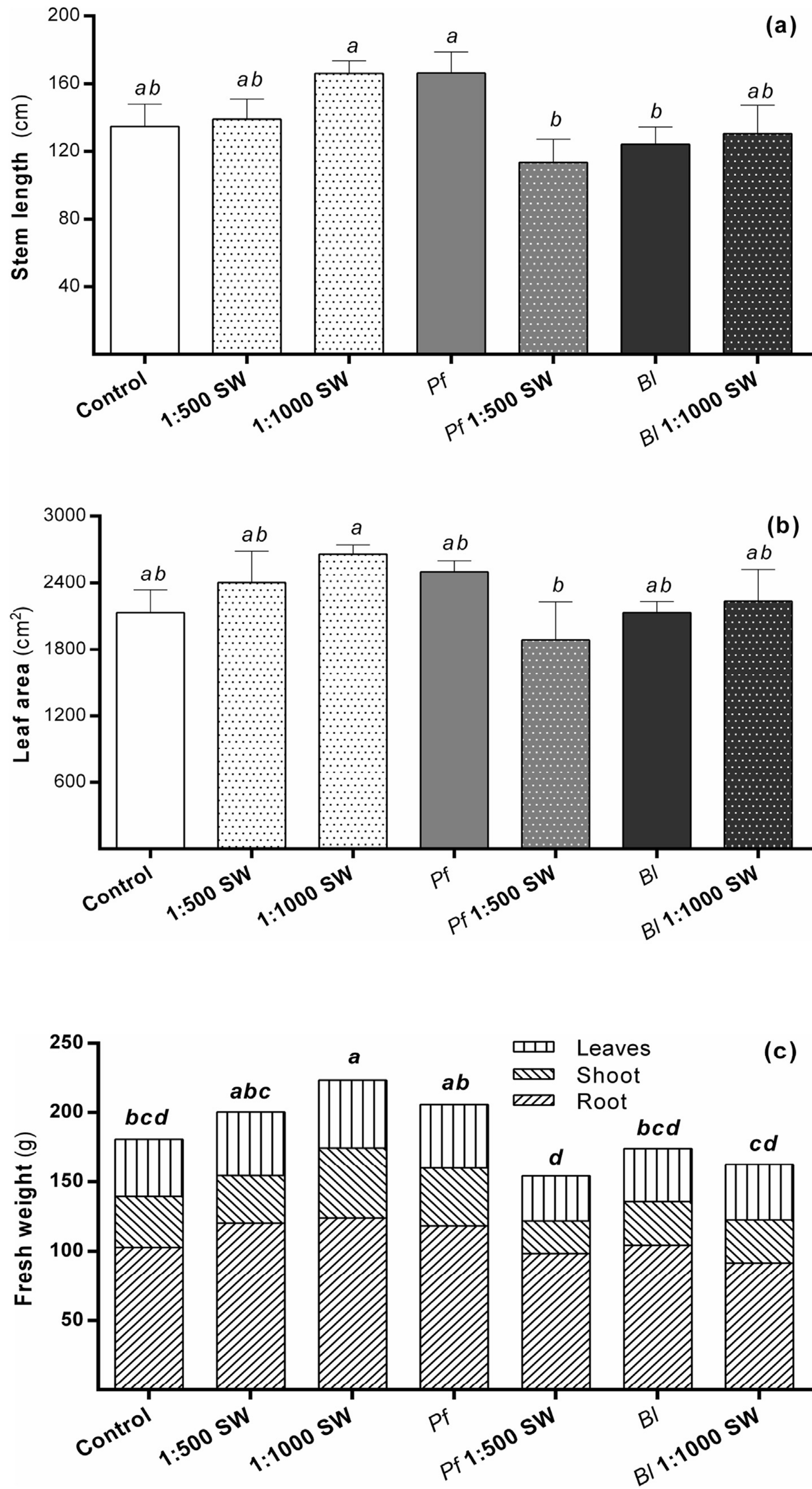

Fig. 2. SL (a), LA (b) and FW (c) of *V. vinifera* L. cv. Malbec plants, treated during a 60 days pot trial with SW, bacterial suspension cultures and combined SW and bacterial treatments. Values are mean ± SE (n = 5) and different letters indicate significant differences ($P \leq 0.05$).

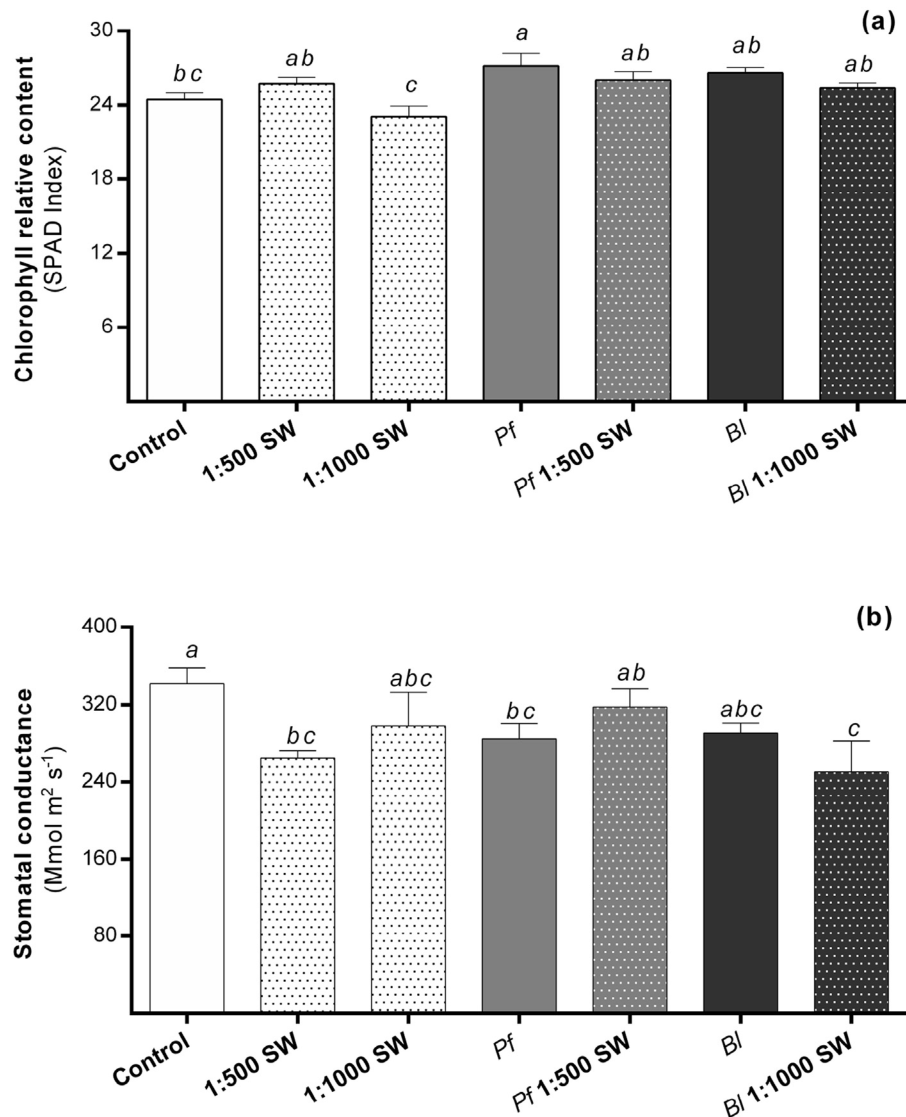

Fig. 3. CRC (a), photosynthetic efficiency (b) and 4 (c) of *V. vinifera* L. cv. Malbec plants treated during a 60 days pot trial with SW, bacterial suspension cultures and combined SW and bacterial treatments. Values are mean ± SE (n = 5) and different letters indicate significant differences ($P \leq 0.05$).

Staden, 1996; Kulkarni et al., 2006). These positive rooting results are probably due to the presence of karrikinolide in the SW that also enhanced lateral roots in rice seedlings (Kulkarni et al., 2006). Studies with different plant species have shown that application of both PGPR and SW can modify endogenous levels of phytohormones (e.g. cytokinin, GAs and IAA) and/or increase tissue sensitivity to them, thereby increasing plant growth (van Staden et al., 2004; Bottini et al., 2004; Salomon et al., 2014). In the present study, single application of 1:1000 SW and *Pf* suspension tended to increase SL, LA, and plant FW, indicating that when applied at optimum doses, these biotechnologies could be satisfactorily implemented in commercial vineyards.

At the end of the pot trial, the bacterial population in the substrate was similar in all treatments, including the water-irrigated control. In contrast, rhizospheric bacterial populations decreased with SW application in a pot trial using *B. licheniformis* and okra (Papenfus et al., 2015). The present results indicate that *Pf* has a greater survival capability (40% of the total CFU counting) than *Bl* (11% of the total CFU), which is consistent with previous studies when both bacteria were inoculated in *in vitro* grapevines (Salomon et al., 2014). In addition, when bacteria were applied in combination with SW, the survival of *Pf* did not change, while in *Bl* 22% of increase was observed as compared to the single application treatment. This indicates that SW did not affect *Pf* survival but improves *Bl* growth in the soil. On the other hand, *Pf* population in the roots increased almost three-fold (from 27% to 72% of the total CFU) in the combined treatment, while *Bl* was not detected, implying that *Pf* had greater ability to colonize the roots. These results showed that, although SW has antimicrobial properties (revised in Kulkarni et al., 2011), growth and survival of both PGPR were not negatively affected in the studied conditions.

The province of Mendoza is a semiarid region where the average annual rainfall is approximately 200 mm (Guevara et al., 1997), therefore vineyards are cultivated in oasis under irrigation. In such environment the sustainable use of water resources is critical for vine cultivation, being water availability the main factor limiting growth and yield (Chaves et al., 2008; Dayer et al., 2016). In the pot trial, a decrease of sg was observed in all the treated plants, with significant differences for *Pf*, 1:500 SW and *Bl* +1:1000 SW treatments compared to the control. This is an important trait to

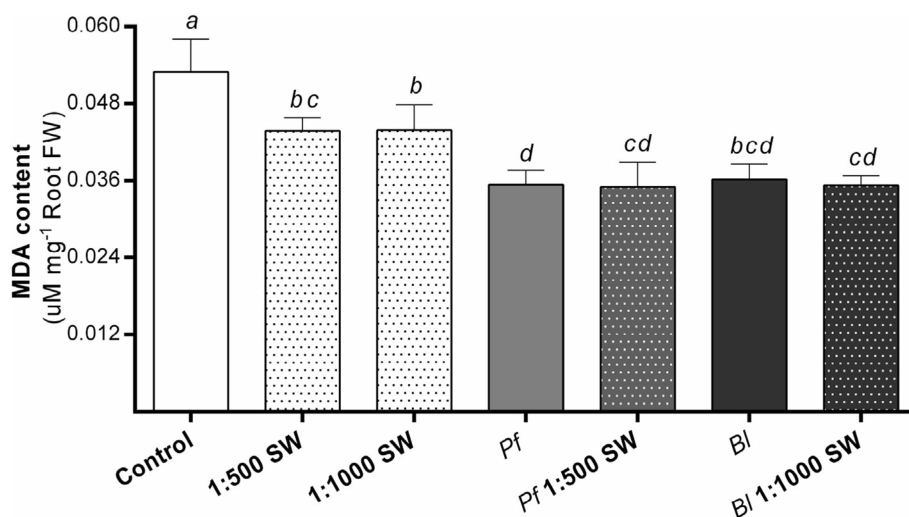

Fig. 4. Root MDA content of *V. vinifera* L. cv. Malbec plants treated during a 60 days pot trial with SW, bacterial suspension cultures and combined SW and bacterial treatments. Values are mean ± SE (n = 5) and different letters indicate significant differences ($P \leq 0.05$).

Table 1
Terpenes detected in leaves of *V. vinifera* L. cv. Malbec plants treated over 60 days with SW, bacteria culture suspensions and combined treatments of SW and bacteria. Values are means ± SE (n = 5) and different letters indicate significant differences ($P \leq 0.05$). Nd = not detected.

	Monoterpenes (ng mg^{-1} Leaf FW)				Sesquiterpene (ng mg^{-1} Leaf FW)	Diterpene (ng mg^{-1} Leaf FW)
	α-Pinene	4-Carene	Terpinolene	Ocimene	Nerolidol	(+)-Beyerene
1:500 SW	377.16 ± 181.26 ***a***	31.16 ± 21.24 ***a***	143,33 ± 20.1 ***ab***	21.27 ± 15.14 **a**	132.41 ± 15.63 **b**	3.28 ± 1.66 **a**
1:1000 SW	307.83 ± 26.16 ***a***	9.72 ± 0.78 ***b***	342.79 ± 29.49 ***a***	6.44 ± 1.11 **b**	122.74 ± 14.84 **b**	1.77 ± 0.60 **a**
Pf	241.93 ± 40.91 ***a***	0.38 ± 0.20 ***c***	86.05 ± 9.67 ***ab***	*Nd*	520,87 ± 171.26 **a**	*Nd*
Pf + 1:500 SW	316.42 ± 64.15 ***a***	4.17 ± 0.39 ***b***	313.30 ± 36.16 ***a***	0.32 ± 0.05 **b**	662.65 ± 54.92 **a**	*Nd*
Bl	120.26 ± 30.55 ***b***	0.68 ± 0.17 ***c***	61.56 ± 16.84 ***b***	*Nd*	206.38 ± 52.44 **ab**	*Nd*
Bl + 1:1000 SW	211.53 ± 33.39 ***ab***	7.02 ± 0.41 ***b***	377.02 ± 22.65 ***a***	2.22 ± 0.32 **b**	329.04 ± 25.18 **ab**	*Nd*
Control	*Nd*	*Nd*	*Nd*	*Nd*	*Nd*	*Nd*

Table 2
Total bacterial counting (CFU g^{-1}) in the substrate and root of *Vitis vinifera* cv. Malbec plants treated during 60 days with SW, bacteria culture suspensions and combined SW and bacterial treatments. Values are means ± SE (n = 3).

	Substrate (CFU g^{-1})	Substrate *Pf* or *Bl* %	Root (CFU g^{-1} FW)	Substrate *Pf* or *Bl* %
1:500 SW	2.00 ± 3.10 × 10^7 ***a***		1.23 ± 1.01 × 10^4 ***bcd***	
1:1000 SW	1.90 ± 1.00 × 10^7 ***a***		1.59 ± 0.87 × 10^4 ***abc***	
Pf	2.01 ± 1.00 × 10^7 ***a***	40.4	4.34 ± 1.60 × 10^3 ***cd***	27.5
Pf + 1:500 SW	2.46 ± 2.90 × 10^7 ***a***	45	4.97 ± 1.35 × 10^3 ***cd***	72
Bl	1.91 ± 1.53 × 10^7 ***a***	11	2.77 ± 1.75 × 10^4 ***a***	1.6
Bl + 1:1000 SW	2.90 ± 4.20 × 10^7 ***a***	22.2	2.57 ± 2.19 × 10^4 ***ab***	–
Control	2.03 ± 0.78 × 10^7 ***a***		2.86 ± 2.56 × 10^4 ***a***	

improve the water status of the vines, hence decreasing both the negative effect of limited water on plant growth and yield, and improving the effective use of the resource. These results are in agreement with a previous study where *Pf* and *Bl* increased ABA concentration in leaves of *in vitro*-grown Malbec plants which in turn was correlated with a decrease in rate of water loss (Salomon et al., 2014). SW significantly diminished *sg* when applied at 1:500 concentrations while 1:1000 SW was only effective when applied in combination with *Bl*. A possible explanation for this effect of SW on *sg* could be the acidity of the SW solution (pH 4.5) that incorporated by the plant may lead to the acidification of the leaf apoplast so altering membrane permeability to ABA and so affecting stomata closure (Anderson et al., 1994; Keeley and Fotheringham, 1997). Similarly, exposing *Chrysanthemoides monilifera* to aerosol smoke for 1 min significantly reduced *sg*, CO_2 assimilation rate and intercellular CO_2 in the short-term (Gilbert and Ripley, 2002). In plants treated with bacteria and SW, there were no changes in photosynthetic efficiency and CRC indicating that carbon fixation was not altered although gas exchange may be temporarily reduced by stomatal closure. This suggests that under the experimental conditions, plant water status was improved without affecting other physiological processes, mainly in those treatments where the *sg* was significantly decreased.

Soil salinization is a consequence of intensive irrigation (Chaves et al., 2008), and salt stress results in oxidative stress by generating ROS (superoxide ion, hydrogen peroxide and hydroxyl radicals) which negatively affects plant survival (Kohler et al., 2009). Both, salt and water hindrance stresses promote oxidative stress in plants (Cramer et al., 2007). In the present study, all treatments significantly diminished lipid peroxidation in the roots as assessed by MDA content. The antioxidant effect of PGPR may be due to the ability of bacteria to increase the activity of antioxidant enzymes

such as peroxidase (POX) and catalase (CAT) (Kohler et al., 2009; Heidari and Golpayegani, 2012). In this respect, Funes Pinter et al. (2017) showed that the same strains of *Pf* and *Bl* were able to increase CAT, POX and ascorbate peroxidase in leaves of grapevines subjected to stress, so the decreased MDA in roots may be consequence of the applied bacteria.

Additionally, application of PGPR and SW induced the synthesis of monoterpenes and sesquiterpenes. These compounds are related to plant defense mechanisms against both biotic and abiotic stress (Nagegowda, 2010; Gil et al., 2012). Some volatiles terpenes such as pinene and nerolidol have antioxidant properties, being able to reduce photooxidation of the amino acid tryptophan caused by ROS as well as to diminish the lesion diameter caused by the pathogen *Botrytis cinerea* (Salomon et al., 2016). All together, these results indicate that application of PGPR and SW not only improve water status but also increase defense protection in leaves and decrease lipid peroxidation in roots, hence help vines to cope with both water and salt stresses.

In conclusion, PGPR and SW biotechnologies are effective in improving growth of *V. vinifera* cuttings as well as improving the plant defense mechanisms to water and salt stresses. This makes these technologies an ecologically friendly alternative that may replace the use of PGR as a management practice in commercial vineyards and used in organic viticulture where agrochemicals are not permitted (Lotter, 2003). Other important benefits include reducing costs and avoiding burdensome processes due to management procedures required when using PGRs. Nevertheless, further investigations are required in order to evaluate the effect of these biotechnological strategies on the environment (Villanueva-Rey et al., 2014) as well as on the quality of the wine.

Contributions

MVS produced bacterial cultures and smoke water extract (following MK, WS and JvS indication), conducted the rooting and inoculation experiments together with IFP, carried out GC-EIMS analysis as well as interpreted all experiments. RB and PP designed and interpreted all experiments, and wrote the manuscript together with MVS. MK, WS and JvS help in the interpretation of the experiments and revised the manuscript in general.

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