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Research article

Rhizosphere associated bacteria trigger accumulation of terpenes in leaves of *Vitis vinifera* L. cv. Malbec that protect cells against reactive oxygen species

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ABSTRACT

It has been proposed that plant growth promoting rhizobacteria (PGPR) stimulate plant growth and development by inducing the biosynthesis of secondary metabolites, like terpenes, which reduce stress incidence. Three bacteria previously isolated from grapevine roots and adjacent soil (*Microbacterium imperiale* Rz19M10, *Kocuria erythromyxa* Rt5M10 and *Terribacillus saccharophilus* Rt17M10) were tested as PGPR. After 30 days since root inoculation of in vitro grown *Vitis vinifera* cv. Malbec plants, the monoterpenes α-pinene, terpinolene and 4-carene, and the sesquiterpene nerolidol were detected only in bacterized-plant leaves. Also, the concentrations of the diterpenes α and γ-tocopherol, and the sterols sitosterol and lupeol were significantly enhanced compared to controls. The leaf extracts of bacterized plants showed photoprotective properties since they decreased the oxygen consumption (that is photo-oxidation) of the amino acid tryptophan in a sensitized solution, thus indicating an increment of the antioxidant capacity of the tissues. In addition, experiments with α-pinene and nerolidol standards showed the capability to intercept reactive oxygen species in the sensitized solution. Moreover, bacterized plants infected with the pathogen *Botrytis cinerea* showed a reduction in the lesion diameter compared with non-bacterized plants. The results suggest that *M. imperiale, K. erythromyxa* and mainly *T. saccharophilus* are able to induce a systemic response that trigger increases on monoterpenes, sesquiterpenes, tocopherols and membrane sterols. These compounds enhance the antioxidant capacity in leaf tissues that may help grapevine to cope with stresses.

1. Introduction

Plant growth promoting rhizobacteria (PGPR) play an important role in the establishment and adaptation of plants to the environment, they have been used in different crops to enhance growth and yield (Hardoim et al., 2008), and in grapevine they have been reported to improve resistance towards biotic and abiotic menaces (Ait Barka et al., 2006; Trotel-Aziz et al., 2008). PGPR stimulate plant growth and yield through different mechanisms, including the ability to elicit plant defense responses that alleviate biotic and abiotic stresses (Salomon et al., 2014). The induced systemic resistance (ISR) against pathogens reduces the incidence of diseases by modifying physical and biochemical properties of the tissues that enhance plant resistance (Van Loon et al., 1998). When bacteria interact with roots, plants perceive them like elicitors, so transduction signals activate synthesis of secondary metabolites and pathogen related proteins (Heil and Bostock, 2002). The induced systemic tolerance (IST), acts similar to ISR but helping plants against abiotic stresses (Yang et al., 2009; Salomon et al., 2014). In both biotic and abiotic stresses terpene biosynthesis is involved.

Abbreviations: + *K. erythromyxa*, bacterized with *Kocuria erythromyxa*; + *M. imperiale*, bacterized with *Microbacterium imperiale*; + *T. saccharophilus*, bacterized with *Terribacillus saccharophilus*; ABA, abscisic acid; CFU, colony forming units; dpb, days post bacterization; DMAPP, dimethylallyl diphosphate; GC-EIMS, gas chromatograph-electron impact mass spectrometer; IPP, isopentenyl diphosphate; ISR, induced systemic resistance; IST, induced systemic tolerance; MEP, 2-C-methyl-d-erythritol-4-phosphate pathway; MS, Murashige and Skoog; MVA, mevalonic acid pathway; Nd, not detected; OD, optical density; PGPR, plant growth promoting rhizobacteria; Rf, riboflavin; ROS, reactive oxygen species.

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The terpenoids (or isoprenoids), derived from isopentenyl diphosphate (IPP, C5) and its isomer dimethylallyl diphosphate (DMAPP), constitute one of the most structurally diverse groups of natural compounds, and over 55,000 different terpenoids have been isolated (Gershenzon and Dudareva, 2007). In plants, there are two known biosynthetic pathways, the cytoplasmic mevalonic acid (MVA) pathway that generally provides IPP to form sesquiterpenes (C_{15}), sterols (C_{30}), and polyterpenes; and the plastidic 2-C-methyl-d-erythritol-4-phosphate (MEP) pathway (Lichtenthaler et al., 1997), in which the IPP and DMAPP are produced as precursors for monoterpenes (C_{10}), diterpenes (C_{20}) and tetraterpenes (C_{40}) (Gershenzon and Dudareva, 2007). All these metabolites have an important role in plant defense. Some monoterpenes and sesquiterpenes are associated with responses against pathogens (Pontin et al., 2015), and they could also protect plants against herbivory (De Moraes et al., 1998). Diterpenes such as tocopherols have antioxidant activity that protects membranes against lipid peroxidation (DellaPenna and Pogson, 2006), and triterpenes like sterols regulate membrane fluidity and permeability in plants under environmental stress (Gil et al., 2012). Recently, 11 bacteria were isolated and identified from roots and rhizosphere of *Vitis vinifera* L. in vineyards from where the most reputed wines of Argentina are produced. Two of them, *Pseudomonas fluorescens* Rt6M10 and *Bacillus licheniformis* Rt4M10, promoted growth of in vitro cultured grapevines by enhancing ABA levels in leaves so diminishing the water loss rate, and by inducing synthesis of terpenes purportedly related to cell protection (Salomon et al., 2014). In the present work, other three indigenous rhizobacteria, *Microbacterium imperiale* Rz19M10, *Kocuria erythromyxa* Rt5M10 and *Terribacillus saccharophilus* Rt17M10 were analyzed in the interaction with grapevine. These bacteria, scarcely studied as PGPR, were evaluated regarding their ability to stimulate synthesis of terpenes in leaves of in vitro grapevine cv. Malbec. As well, the enhancement of the photoprotective properties (antioxidant capacity) in leaves of bacterized plants, and particularly of the most abundant terpenes, α-pinene and nerolidol, was evaluated by sensitized photooxidation of the amino acid tryptophan compared with the antioxidant properties of trolox (well known antioxidant compound).

2. Materials and methods

2.1. Bacteria and culture condition

Microbacterium imperiale Rz19M10, *Kocuria erythromyxa* Rt5M10 and *Terribacillus saccharophilus* Rt17M10 were previously isolated from roots and rhizosphere of *Vitis vinifera* L. cv. Malbec (Mendoza, Argentina), and identified by 16S RNAr sequences (Salomon et al., 2014). In order to be inoculated in grapevine, they were grown in liquid MS medium to 0.7 OD_{530} (10^6 CFU mL^{-1}).

2.2. In vitro plant material and plant bacterization

In vitro grown grapevines were obtained from a virus-free vineyard of *Vitis vinifera* L. cv. Malbec as previously described (Salomon et al., 2014). Emerging roots of 15 days-old in vitro plants growing on flasks with modified MS-agar medium (Murashige and Skoog, 1962) were inoculated with an aliquot of 0.25 mL bacterial culture (previously grown in liquid MS medium). In control plants sterile MS-liquid medium was added. After 30 days post-bacterization (dpb; 45 days-old) plants were removed from agar and leaf samples were collected and stored at −80° C until metabolite analysis and evaluation of antioxidant capacity. For biocontrol assay leaves were immediately transferred to Petri dishes.

2.3. Terpenes quantification in leaves

Terpenes were extracted and quantified according to Salomon et al. (2014). For diterpenes and triterpenes analysis the GC oven temperature program was: initial temperature 60° C for 1 min, followed by an increase of 10° C min^{-1} to 280° C and held for 37 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. 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.

2.4. Photolysis and substrate conversion

The conversion of α-pinene (1) and nerolidol (4) (standards) was measured in absolute ethanol/distilled water solution (80/20; v/v), sensitized with riboflavin (Rf; 0.04 mM). This sensitizer is a natural pigment found in living organisms able to absorb visible light; thus the irradiation of riboflavin solution (in ground state) forms the electronically excited singlet and triplet states ($^1Rf^*$ and $^3Rf^*$ respectively). Then, the $^3Rf^*$ can react with dissolved oxygen and forms reactive oxygen species (ROS) (Edwards and Silva, 2001). The aerobic photolysis of the solutions was performed in constant stirring with a blue light (445 nm) emitting photolyzer (diodes, LEDs), thus ensuring that the incident light was only absorbed by Rf. In order to evaluate changes in substrates (absorbance at 201–206), the measurements were done at the beginning and at the end of the photolysis (considering the end when 10% of the substrate was converted) using a 10 mm optical path cells in a Cary-50 UV–Vis spectrophotometer (Varian Inc., Palo Alto, CA, USA). α-pinene (1) was used at 0.2 mM concentration and nerolidol (4) at 0.04 mM in a 50 mL final volume of sensitized-ethanol solution.

The reagents (terpenes standards and Rf) were purchased from Sigma-Aldrich (Steinheim, Switzerland), and absolute ethanol was Pro-analysis quality (99.99% purity; J.T. Baker, Center Valley, USA). All measurements were carried out at room temperature and with ethanol/water solution freshly prepared.

2.5. Oxygen consumption rate of tryptophan as indication of antioxidant capacity

In order to evaluate the antioxidant capacity of the terpenes α-pinene (1) and nerolidol (4), as well as leaf extract of bacterized plants, comparative experiments of oxygen uptake were performed. The oxygen consumption in absolute ethanol/distilled water solution with addition of each terpene (standards) or leaf extract and sensitized with Rf, was measured along the time by using a specific oxygen electrode (edgeTM, HANNA, Rumania).

The oxygen consumption in presence of trolox was used as reference. The aerobic photolysis of the solutions was performed as it is mentioned in section 2.4 and in the same conditions. The procedure was done for each terpene and trolox (at 0.2 mM concentration) and leaf extracts (100 μL; prepared as it is mentioned in section 2.3 according to Salomon et al., 2014), and then in presence of the amino acid tryptophan (photo-oxidizable with Rf through a well-known mechanism; Garcia and Silva, 1997) in a 50 mL final volume of Rf ethanol solution. The photo-oxygenation rates of substrates were calculated for each compound or extract and in combination with tryptophan by the slopes of oxygen consumption versus irradiation time in the solution. Trolox and tryptophan were purchased from Sigma-Aldrich (Steinheim, Switzerland).

2.6. Biocontrol assay

The assay was carried out with a virulent strain of *Botrytis cinerea*, isolated from a commercial vineyard in Mendoza (Argentina), gift of Dr. P. Pizzuolo (Lab. of Phytopathology, Facultad de Ciencias Agrarias, UNCuyo, Argentina). The pathogen was grown in Petri dishes on potato dextrose agar (PDA, Oxoid, Basingstoke, England) at 25° C for 15 days. Conidial suspension was obtained by adding sterile distilled water to the dishes and gently rubbing the mycelium, after counting the suspension was adjusted to 1.10^6 conidia mL^{-1}.

Grapevine leaves were cut from 45 days-old plants after 30 dpb (plants had been inoculated with each bacterium as it is mentioned in 2.2 section) and placed on wet absorbing paper in Petri dishes (5 leaves per dishes). Then, one needle-prick wound was done to each leaf and covered with 10 μL drops of the *B. cinerea* conidial suspension. The disease incidence was measured as the average diameter of the lesions registered 5 d post-infection with the fungus. The percentage (%) of disease reduction was calculated considering the average diameter in positive control as 100% (Trotel-Aziz et al., 2008).

2.7. Statistical analysis

The 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 probability of $p \leq 0.05$ and replica numbers of reported data are specified in each figure or table legend.

3. Results

3.1. Bacteria increase monoterpenes and sesquiterpenes content in leaves of grapevine

In leaves of plants bacterized via roots with *K. erythromyxa*, *M. imperiale* and *T. saccharophilus*, the monoterpenes α-pinene (1), terpinolene (2) and 4-carene (3), and the sesquiterpene nerolidol (4) were identified (see Fig. 1 for structures). The absolute (μg g^{-1} FW) and relative (%) amounts of each compound after 30 dpb are shown in Table 1. Plants bacterized with *T. saccharophilus* showed the highest total terpenes content (1803.35 μg g^{-1} FW) in respect to *M. imperiale* (1189.61 μg g^{-1} FW) and *K. erythromyxa* (695.97 μg g^{-1} FW). Nerolidol (4) was the major compound in these plants (836.75 ± 310.06 μg g^{-1} FW) representing 46.4% of the total content, and this concentration was higher as compared with the other bacteria treatments (+*K. erythromyxa* 148.96 ± 43.44 μg g^{-1} FW and +*M. imperiale* 154.18 ± 41.81 μg g^{-1} FW). In plants inoculated with *M. imperiale*, α-pinene (1) was the most abundant terpene representing 44.7% of the total, similar to inoculation with *T. saccharophilus* (530.37 ± 256.85 μg g^{-1} FW and 535.00 ± 136.26 μg g^{-1} FW, respectively). These concentrations were higher respect to+ *K. erythromyxa* (188.70 ± 28.55 μg g^{-1} FW). On the other hand, in the latter treatment, terpinolene (2) was the major terpene representing 51.48%, although its amount was

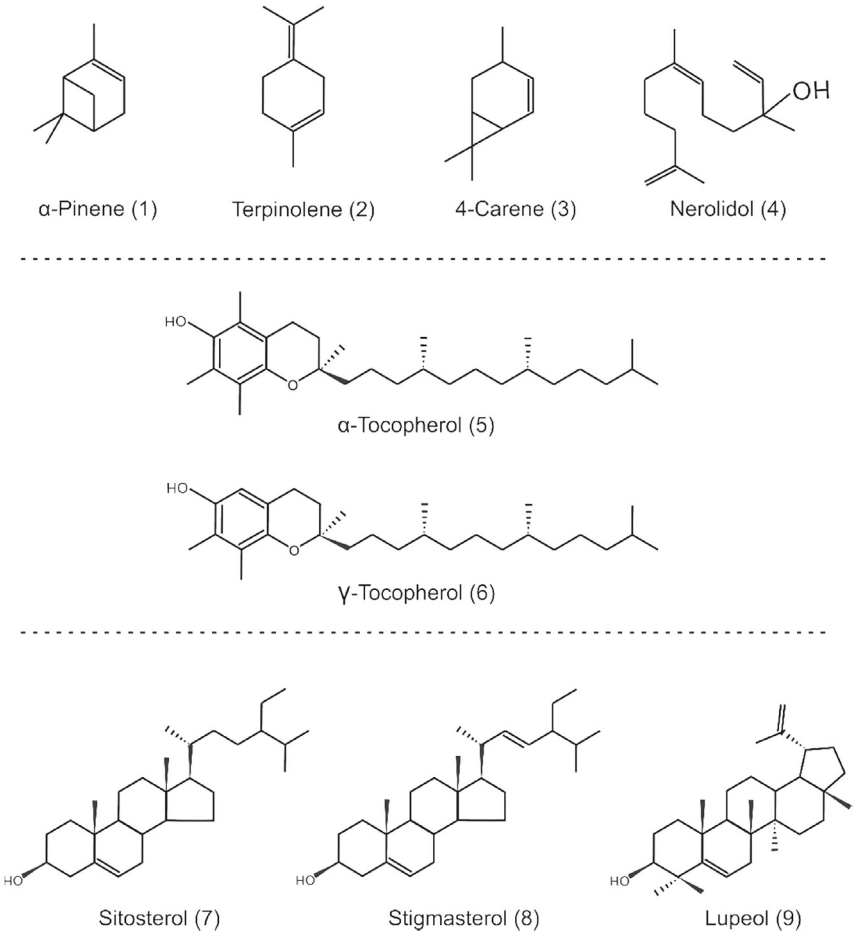

Fig. 1. Chemical structures of monoterpenes, sesquiterpene, tocopherols and sterols identified by GC–EIMS in leaves of grapevine bacterized with bacteria.

Table 1
Monoterpenes and nerolidol (sesquiterpene) assessed by GC-EIMS (in μg g^{-1} FW and in % of leaves) from leaves of in vitro grape plants collected 30 days post-bacterization with *Kocuria erythromyxa*, *Microbacterium imperiale* and *Terribacillus saccharophilus*. Different letters indicate significant differences ($p \leq 0.05$). Values are means ± SE of n = 3 and the experiments were conducted twice. *Nd*, not detected.

	+ *K. erythromyxa*		+ *M. imperiale*		+ *T. saccharophilus*		Control μg g^{-1}
	μg g^{-1}	%	μg g^{-1}	%	μg g^{-1}	%	
Monoterpenes							
α-Pinene (1)	188.70 ± 28.55 ***b***	27.11	530.37 ± 256.85 ***a***	44.7	535.00 ± 136.26 ***a***	29.67	*Nd*
Terpinolene (2)	358.31 ± 52.91 ***a***	51.48	495.90 ± 196.89 ***a***	41.79	422.15 ± 85.27 ***a***	23.41	*Nd*
4-Carene (3)	*Nd*	–	6.16 ± 3.4 ***a***	0.52	9.45 ± 2.53 ***a***	0.52	*Nd*
Sesquiterpene							
Nerolidol (4)	148.96 ± 43.44 ***b***	21.4	154.18 ± 41.81 ***b***	12.99	836.75 ± 310.06 ***a***	46.4	*Nd*
Total	**695.97**	**100**	**1189.61**	**100**	**1803.35**	**100**	–

slightly lower (358.31 ± 52.91 μg g^{-1} FW) compared with +*M. imperiale* and +*T. saccharophilus* (495.90 ± 196.89 and 422.15 ± 85.27 μg g^{-1} FW respectively). The monoterpene 4-carene (3) was only detected in plants bacterized either with *M. imperiale* or with *T. saccharophilus,* and in low content (6.16 ± 3.4 and 9.45 ± 2.53 μg g^{-1} FW, respectively). It is important to note that none of these compounds were detected in control non-bacterized plants. The results suggest that, although the same compounds were detected in all bacterized plants (similar qualitative profile), the content of each compound differed amongst bacteria (different quantitative profile), and the synthesis was presumably de novo since such compounds were not present in non-bacterized plants.

3.2. Bacteria modify di and triterpenes concentration in leaves of grapevine

As Table 2 shows, concentration of the diterpenes α-tocopherol (5) and γ-tocopherol (6) were significantly increased 30 dpb in leaves, compared to controls (see Fig. 1 for structures). Level of α-tocopherol (5) detected in controls (16.76 ± 10.71 μg g^{-1} FW) increased 8- to 9-fold in bacterized plants (132.78 ± 53.6, 134.86 ± 44.47 and 162.68 ± 30.26 μg g^{-1} FW in +*K. erythromyxa,* +*M. imperiale* and *T. saccharophilus* respectively). Although, α-tocopherol (5) was the least abundant tocopherol in controls, became the most abundant in bacterized plants. Also, the content of γ-tocopherol (6) augmented 2.5-fold in +*K. erythromyxa* and +*T. saccharophilus* (70.3 ± 9.18 and 68.62 ± 13.7 μg g^{-1} FW in respectively) and 1.7-fold in +*M. imperiale* (46.58 ± 19.81 μg g^{-1} FW), in respect to non-bacterized plants (27.39 ± 13.38 μg g^{-1} FW). There were no significant differences between treatments in phytol (7) content (Table 2).

Sitosterol (8) was increased from 100.27 ± 25.35 μg g^{-1} FW in controls plants to 203.51 ± 44.11 and 262.26 ± 94.47 μg g^{-1} FW in +*K. erythromyxa* and +*T. saccharophilus* treatments (2 and 2.5-fold respectively) (Fig. 1; Table 2). Also lupeol (9) increased more than 15-fold in bacterized plants (215.37 ± 138.41, 249.57 ± 89.16 and 211.29 ± 68.56 μg g^{-1} FW) compared to controls (13.8 ± 6.91 μg g^{-1} FW). A pronounced increment in the sterols precursor squalene was detected in bacteria-treated plants (Table 2). For stigmasterol (10) concentration no changes were detected.

3.3. The photolysis of riboflavin-ethanol solution induces changes in the absorption spectra of α-pinene (1) and nerolidol (4)

The reaction of α-pinene (1) and nerolidol (4) (substrates) with photolysis-generated ROS can be monitored as a change in their absorption spectrum after Rf photolysis. The visible-light irradiation of the ethanol solution sensitized with Rf increased the absorption spectra of α-pinene (1) and nerolidol (4) after 360 s of irradiation (Fig. 2 a and b). The absorbance of α-pinene (1) (206 nm) was increased from 1.16 to 1.21 at the end of the photolysis, which represent 3.27% of substrate conversion (Table 3 and Fig. 2a); while the absorbance of nerolidol (4) (200 nm) was increased from 1.09 to 1.22, representing 11.3% of conversion for the same irradiation time (Table 3 and Fig. 2b). Similar results were observed in the spectra when leaf extracts were added to the ethanol solution (data not shown).

3.4. α-pinene (1) and nerolidol (4) consume oxygen in Rf-sensitized solution

Fig. 3 shows the oxygen consumption rate of the different terpenes compared with the known antioxidant trolox, in ethanol solution sensitized with irradiated-Rf. α-pinene (1) and mainly nerolidol (4) showed oxygen consumption along the irradiation time (−0.15 and −0.27 ΔO_2/mg L^{-1} respectively at the end of the assay), although the rate was lower than that of trolox (−0.84 ΔO_2/mg L^{-1}). The oxygen consumption of nerolidol (4) was higher than that of α-pinene (1), showing a relative rate of 0.30 ± 0.01 and 0.18 ± 0.05 respectively (considering the oxygen consumption rate

Table 2
Diterpenes and triterpenes assessed by GC-EIMS (in μg g^{-1} FW) from leaves of in vitro grape plants collected 30 days pos-bacterization with *Kocuria erythromyxa*, *Microbacterium imperiale* and *Terribacillus saccharophilus*. Different letters indicate significant differences ($p \leq 0.05$). Values are means ± SE of n = 3 and the experiments were conducted twice.

	+ *K. erythromyxa*	+ *M. imperiale*	+ *T. saccharophilus*	Control
Diterpenes				
α-tocopherol (5)	132.78 ± 53.6 ***ab***	134.86 ± 44.47 ***ab***	162.68 ± 30.26 ***a***	16.76 ± 10.71 ***b***
γ-tocopherol (6)	70.3 ± 9.18 ***a***	46.58 ± 19.81 ***a***	68.62 ± 13.7 ***a***	27.39 ± 13.38 ***a***
Phytol (7)	81.24 ± 22.28 ***a***	41.55 ± 5.43 ***a***	81.43 ± 20.42 ***a***	58.63 ± 27.51 ***a***
Triterpenes				
γ-sitosterol (8)	203.51 ± 44.11 ***a***	5.38 ± 5.38 ***b***	262.26 ± 94.47 ***a***	100.27 ± 25.35 ***ab***
Lupeol (9)	215.37 ± 138.41 ***a***	249.57 ± 89.16 ***a***	211.29 ± 68.56 ***a***	13.8 ± 6.91 ***b***
Stigmasterol (10)	13.34 ± 3.23 ***a***	12.23 ± 1.59 ***a***	10.48 ± 1.3 ***a***	10.12 ± 3.47 ***a***
Squalene (11)	77,042.41 ± 15,723.13	49,074.92 ± 12,871.46	49,252.2 ± 11,862.65	78.74 ± 21.47

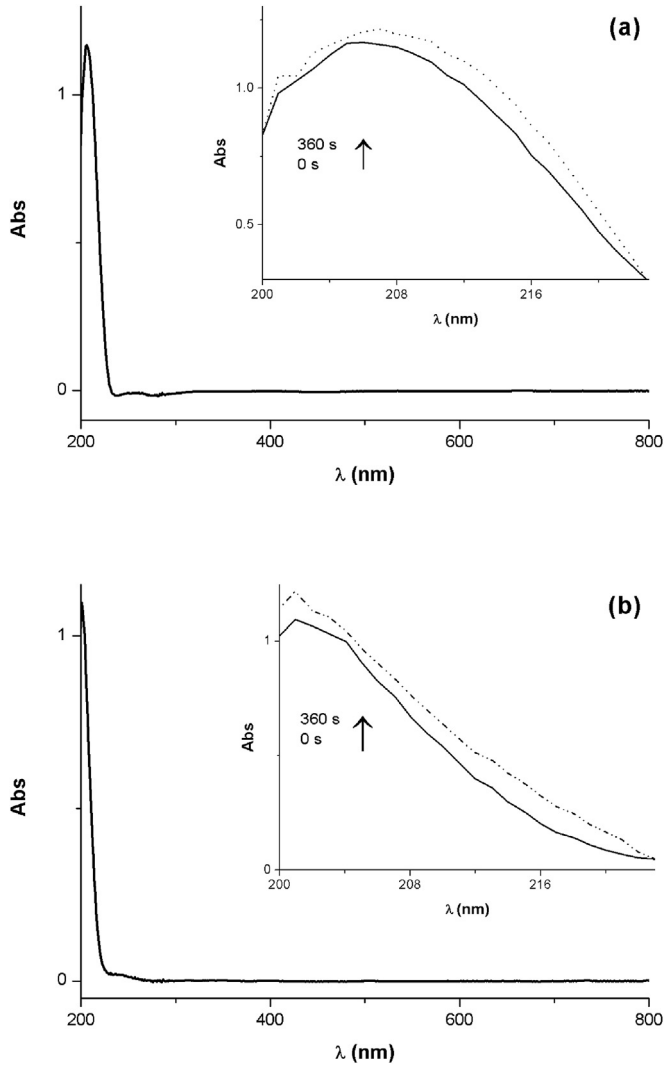

Fig. 2. UV–Vis absorption spectra of α-pinene (a) and nerolidol (b) in ethanol/water (80/20; v/v) solution sensitized with riboflavin and irradiated with a blue light (445 nm) emitting photolyzer. Measures assessed with UV–Vis spectrophotometer were done at the beginning and after 360 s of irradiation. The insets show the section of the spectrum where the absorbance of each terpene was measured. Measurements were conducted three times and the showed spectrum is representative of them.

Table 3
Changes in absorbance spectra of α-pinene (1) and nerolidol (4) (at 206 and 200 nm, respectively), and percentage of substrate conversion, in ethanol/water solution (80/20; v/v) sensitized with Rf (0.04 mM concentration) and irradiated with a blue light (445 nm) emitting photolyzer during 360 s. Measurements were conducted three times.

Compound	Abs (time 0)	Abs (360 s)	Conversion (%)
α-pinene (206 nm)	1.16	1.20	3.27
Nerolidol (200 nm)	1.09	1.22	11.31

of trolox as 1; Fig. 4). In Rf solution (ethanol solution plus Rf) the oxygen consumption was close to zero ($-0.04\ \Delta O_2/mg\ L^{-1}$).

3.5. α-pinene (1) and nerolidol (4) decrease the oxidation velocity of tryptophan in Rf-sensitized solution

Fig. 5 shows the oxidation velocity of tryptophan (assessed as oxygen consumption rate) in ethanol solution sensitized with

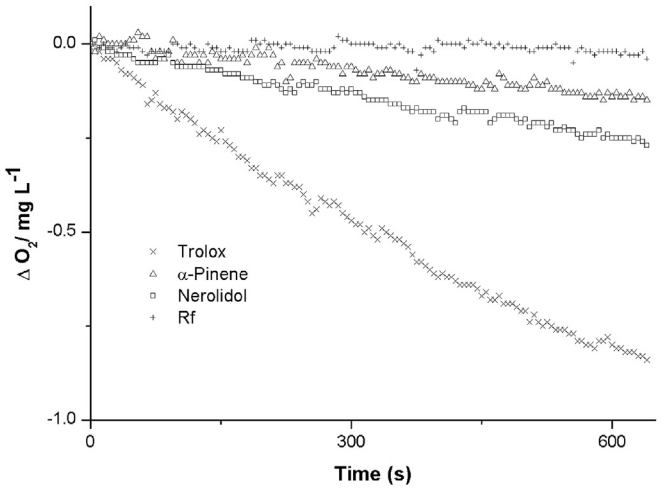

Fig. 3. Oxygen consumption rate of α-pinene (1), nerolidol (4) and trolox. Measures were assessed in ethanol/water solution (80/20; v/v) sensitized with Rf (0.04 mM concentration) and irradiated with a blue light (445 nm) emitting photolyzer, with a specific oxygen electrode. Terpenes and trolox were used at 0.2 mM concentration final volume. The measurements were conducted three times and values are means of them.

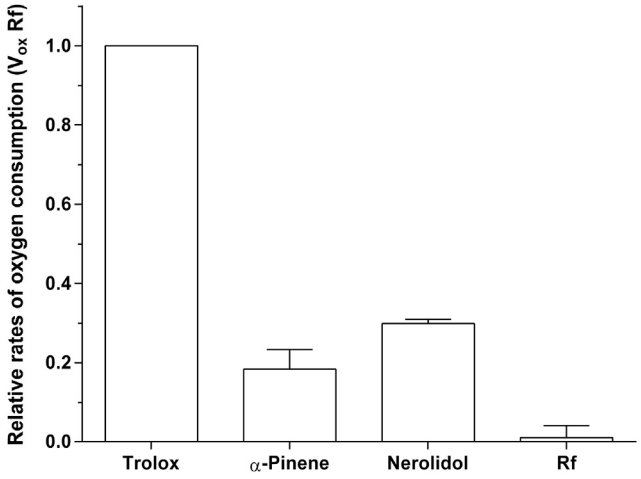

Fig. 4. Relative rates of oxygen consumption (V_{ox} Rf) of α-pinene (1) and nerolidol (4) compared with trolox (oxygen consumption rate of trolox was considered as 1). Measures were assessed by a specific oxygen electrode, in ethanol/water solution (80/20; v/v) sensitized with Rf (0.04 mM concentration) and irradiated with a blue light (445 nm) emitting photolyzer. All compounds were used at 0.2 mM concentration. Values are means of three measurements ±SE.

irradiated-Rf and with addition of terpenes. α-pinene (1) and mainly nerolidol (4) showed protective properties, since they were able to decrease the oxidation rate of tryptophan 7 and 10% respectively at the end of the assay (0.93 ± 0.06 and 0.90 ± 0.07 relative oxidation rate considering oxidation of tryptophan as 1; Fig. 6). However, the oxidation velocity of tryptophan in presence of terpenes was higher than that of trolox, where the velocity was reduced 15% (0.85 ± 0.08 relative oxidation rate; Fig. 6).

3.6. Extracts of bacterized plants consume oxygen in Rf-sensitized solution and protect tryptophan from oxidation

Fig. 7 shows that all bacterized and control leaf tissues consumed oxygen in the ethanol solution sensitized with irradiated-Rf along the irradiation time. At the end of the assay the

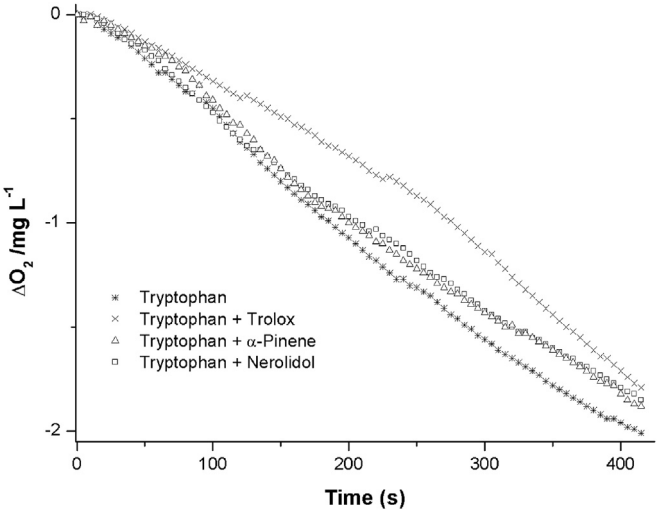

Fig. 5. Oxidation rate of tryptophan in ethanol/water solution (80/20; v/v) added with α-pinene (1), nerolidol (4) and trolox, assessed with a specific oxygen electrode. The solutions were sensitized with Rf (0.04 mM concentration) and irradiated with a blue light (445 nm) emitting photolyzer. Tryptophan, terpenes and trolox were used at 0.2 mM concentration final volume. The measurements were conducted three times and values are means of them.

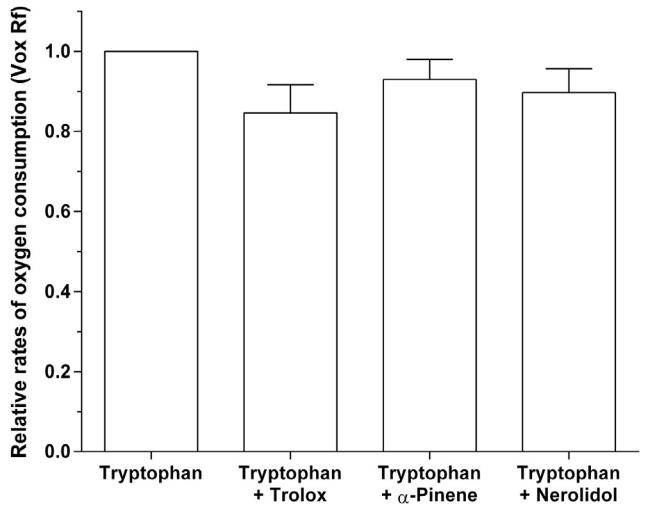

Fig. 6. Relative rates of tryptophan oxidation (V_{ox} Rf) in presence of α-pinene (1), nerolidol (4) and trolox (oxygen consumption rate of tryptophan alone was considered as 1), Measures were assessed by a specific oxygen electrode, in ethanol/water solution (80/20; v/v) sensitized with Rf (0.04 mM concentration) and irradiated with a blue light (445 nm) emitting photolyzer. All compounds were used at 0.2 mM concentration. Values are means of three measurements ±SE.

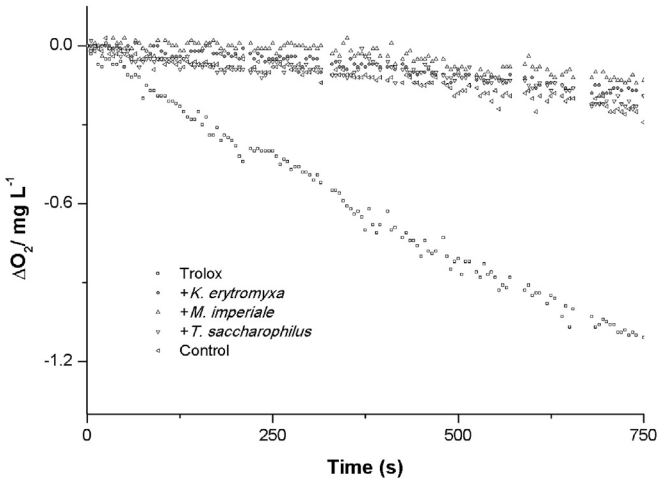

Fig. 7. Oxygen consumption rate of leaf extracts of bacterized plants (100 μL) and trolox. (at 0.2 mM concentration). Measures were assessed in ethanol/water solution (80/20; v/v) sensitized with Rf (0.04 mM concentration) and irradiated with a blue light (445 nm) emitting photolyzer, with a specific oxygen electrode. Values are mean of n = 3 (three leaf extract per treatment) and the experiments were conducted twice.

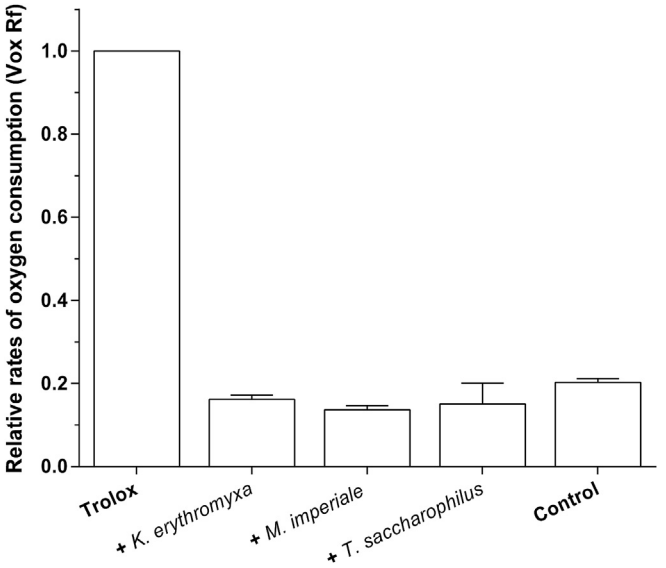

Fig. 8. Relative rates of oxygen consumption (V_{ox} Rf) of leaf extracts of bacterized and control plants compared with trolox (oxygen consumption rate of trolox was considered as 1). Measures were assessed by a specific oxygen electrode, in ethanol/water solution (80/20; v/v) sensitized with Rf (0.04 mM concentration) and irradiated with a blue light (445 nm) emitting photolyzer. Values are means of n = 3 ± SE.

rate was similar in bacterized plants (between −0.13 and −0.19 ΔO_2/mg L^{-1}). In comparison with trolox (1.11 ΔO_2/mg L^{-1}), the relative oxygen consumption rate of the extracts was 0.16 ± 0.01 for *K. erythromyxa*, 0.14 ± 0.01 for *M. imperiale* and 0.15 ± 0.05 for *T. saccharophilus* bacterized-plants, while control non-bacterized plants showed a relative rate of 0.20 after photolysis (Fig. 8).

Fig. 9 shows the oxidation velocity of tryptophan in ethanol solution sensitized with irradiated-Rf and with the extracts plants. The extracts of +*T. saccharophilus* decreased 18% the tryptophan oxidation at the end of the assay (0.82 relative oxidation rate considering oxidation of tryptophan as 1; Fig. 10), showing the largest decline in percentage. Extracts of plants bacterized with *M. imperiale* and *K. erythromyxa* diminished the oxidation 15 and 11% respectively (0.85 ± 0.08 and 0.89 ± 0.1 relative oxidation rates), while the oxidation of tryptophan in presence of control leaf extract decreased only 2% (Fig. 10). This result is in concordance with the absorption spectra of tryptophan in the presence or not of leaf extracts (Fig. 11).

3.7. Bacteria reduce the incidence of B. cinerea infection in leaves

After 30 dpb, leaves of bacterized plants infected with the pathogen *B. cinerea* showed a reduction in the lesion diameter (Table 4; Fig. 12). In plants bacterized with *T. saccharophilus* the lesion was reduced 53% compared with the positive control (3.28 mm and 7.03 mm respectively). Although the differences are not statistically significant the lesion was also reduced 14.23% and 11.53% in plants bacterized with *M. imperiale* and *K. erythromyxa*

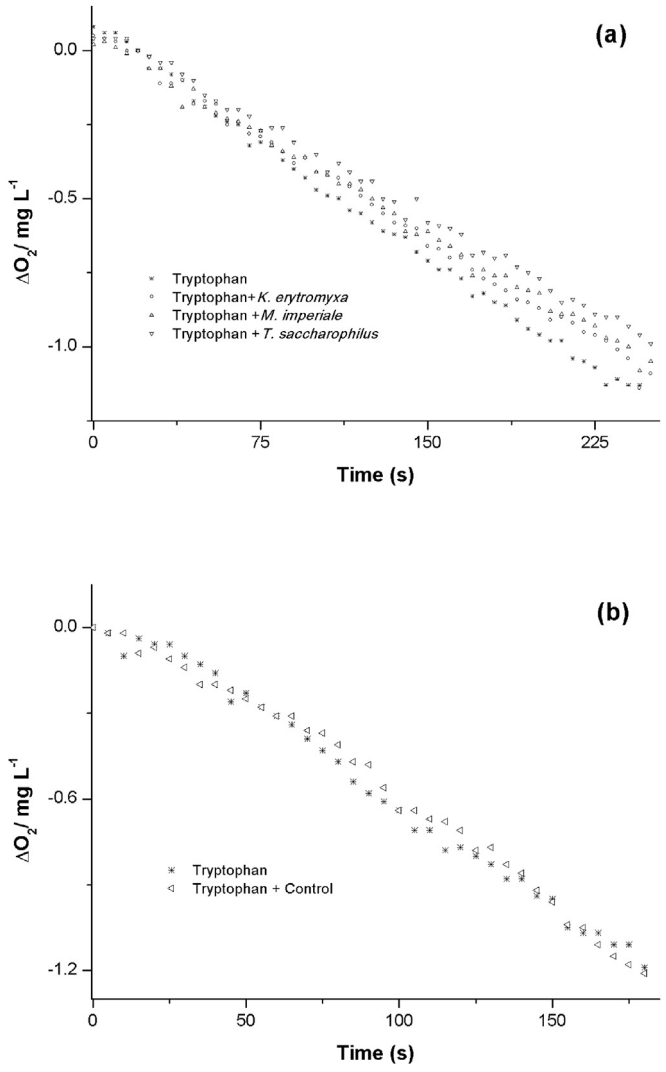

Fig. 9. Oxidation rate of tryptophan in ethanol/distilled water solution (80/20; v/v) with addition of leaf extracts of bacterized plants (a) and leaf extracts of control plants (b), assessed with a specific oxygen electrode. The solutions were sensitized with Rf (0.04 mM concentration) and irradiated with a blue light (445 nm) emitting photolyzer. Values are mean of n = 3 (three leaf extract per treatment) and the experiments were conducted twice.

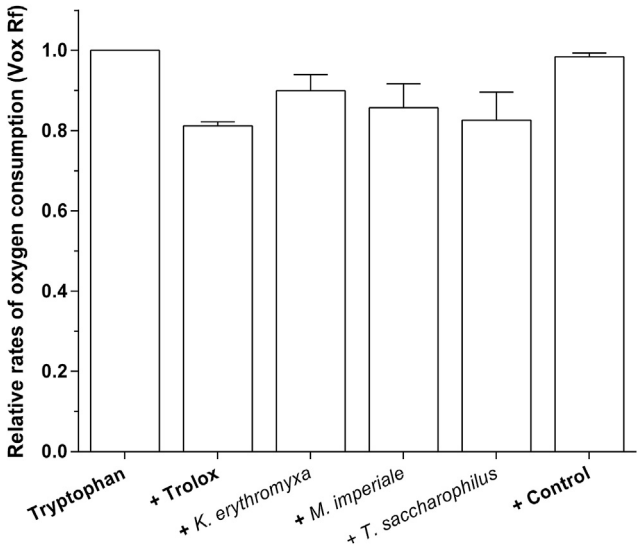

Fig. 10. Relative rates of tryptophan oxidation (V_{ox} Rf) in presence of leaf extracts of bacterized and control plants, and trolox (oxygen consumption rate of tryptophan alone was considered as 1). Measures were assessed by a specific oxygen electrode, in ethanol/water solution (80/20; v/v) sensitized with Rf (0.04 mM concentration) and irradiated with a blue light (445 nm) emitting photolyzer. Values are means of n = 3 ± SE.

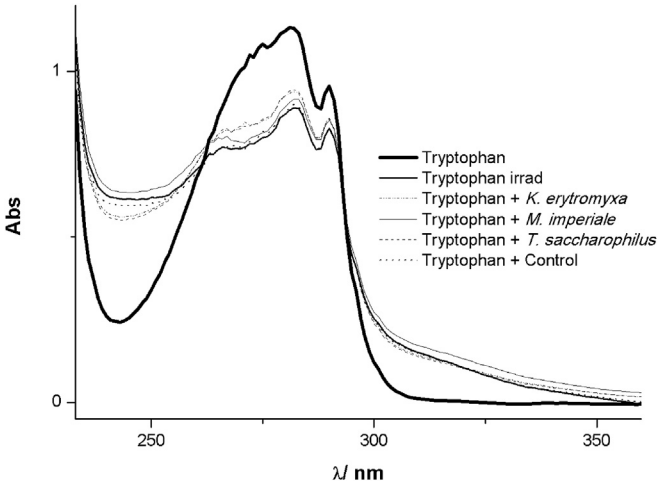

Fig. 11. Changes in absorption spectra of tryptophan in presence or not of leaf extracts of bacterized plants, in ethanol/water (80/20; v/v) solution sensitized with riboflavin and irradiated with a blue light (445 nm) emitting photolyzer. Measures assessed with UV–Vis spectrophotometer were done at the beginning and after 180 s of irradiation. The measurements were conducted with n = 3 (three leaf extract per treatment) and the showed spectrum is representative of them.

(6.03 and 6.22 mm) compared with the positive control (Table 4).

4. Discussion

Previously we isolated *Microbacterium imperial* Rz19M10, *Kocuria erythromyxa* Rt5M10 and *Terribacillus saccharophilus* Rt17M10 from roots and rhizosphere of *Vitis vinifera* L. cv. Malbec (Salomon et al., 2014). These bacteria belong to genera commonly found in soils, two of them were found in association with wild grape plants (Karagöz et al., 2012), but they have been scarcely studied as PGPR. It was shown that *Terribacillus saccharophilus* associated to *Paeonia ostii* is able to tolerate elevated NaCl conditions (An et al., 2007; Han et al., 2011). On the other hand, it was observed that *Microbacterium* increased yield and growth of apple (Karlidag et al., 2007); and may be potentially used in bioremediation (Sathyavathi et al., 2014). Respect to genera *Kocuria*, Godinho et al. (2010) reported that *Kocuria* sp. increased plant nutrient element in eggplants.

Bacteria have been used to elicit induced systemic responses in plants that enhance their defense capacity, helping them to face adverse conditions. In the present work, bacterization of grapevine at root levels with *M. imperiale* Rz19M10, *K. erythromyxa* Rt5M10 and *T. saccharophilus* Rt17M10 induced synthesis or increased the concentration of different terpenes in leaves, suggesting a systemic response since bacteria were barely detectable in leaf tissues (data not shown). Moreover, the results found in bacterized plants suggested de novo synthesis of the monoterpenes α-pinene (1), 4-carene (3) and terpinolene (2), and the sesquiterpene nerolidol (4), since these compounds were not detected in controls. Banchio et al. (2009) reported that *Bacillus subtilis* GB03 releases volatile chemicals that elevate essential oil accumulation and emissions in the terpene-rich herb sweet basil (*Ocimum basilicum*). In a previous

Table 4
Incidence of *B. cinerea* infection on in vitro grapevine leaves collected 30 days pos-bacterization with *Kocuria erythromyxa*, *Microbacterium imperiale* and *Terribacillus saccharophilus* and in controls. The incidence was measured as the average of the maximum diameter of the lesions. Different letters indicate significant differences ($p \leq 0.05$). Values are means ± SE of n = 10 and the experiments were conducted twice.

	Lesion diameter (mm)	Disease reduction (%)
Negative control (−*B. cinerea*)	*Nd*	–
Positive control (+ *B. cinerea*)	7.03 ± 1.27 ***b***	–
+ *K. erythromyxa* (+ *B. cinerea*)	6.22 ± 1.6 ***ab***	11.53
+ *M. imperial* (+ *B. cinerea*)	6.03 ± 1.26 ***ab***	14.23
+ *T. saccharophilus* (+ *B. cinerea*)	3.28 ± 0.88 ***a***	53.35

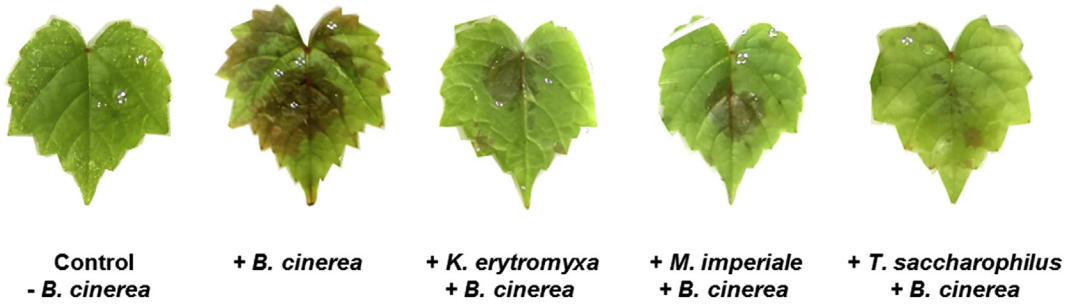

Fig. 12. In vitro grapevine leaves after 5 days' infection with the fungus *B. cinerea*. Control (*-B. cinerea*), positive control (+ *B. cinerea*) and, leaves with each bacteria and *B. cinerea* (+*K. erythromyxa* +*B. cinerea*, +*M. imperiale* +*B. cinerea* and *T. saccharophilus* +*B. cinerea*). The experiments were conducted twice with n = 10 and leaves in the photo are representative.

study we reported that *Pseudomonas fluorescens* and *Bacillus licheniformis* induce de novo synthesis of mono and sesquiterpenes that potentially improved defense in leaves of grapevine (Salomon et al., 2014), being nerolidol (4) the most abundant compound 30 dpb. In the present study *T. saccharophilus* treated-plants showed the same pattern but nerolidol (4) concentration was lower than those of *P. fluorescens* and *B. licheniformis* treated-plants; in contrast plants treated with *K. erythromyxa* and *M. imperiale* showed more accumulation of monoterpenes (78.59% and 85.99%, respect to 53.59% of *T. saccharophilus* treated-plants), being α-pinene (1) and terpinolene (2) the most abundant. Although it can be assume that all bacteria produce an impact in the interaction with the plant, to propose a bacterium as PGPR it is important to evaluate the magnitude of that impact. There are studies in which *Escherichia coli* DH5R was used as negative control because it does not trigger ISR in relation to volatiles and monoterpene production in plants (Banchio et al., 2009; Ryu et al., 2004). Changes in volatile emission patterns under stress conditions provide circumstantial evidence that volatiles are linked with stress responses (Vickers et al., 2009); in this report, it seems that the population of bacteria, dead and alive, is responsible to elicit terpene synthesis as defense compounds that may help plant to battle with pathogens attack or other abiotic stress. In fact, it has been shown increases in their content after pathogen infection (Zhao et al., 2010), and there are evidences that they inhibit pathogen incidence (Himejima et al., 1992) via different mechanisms (Park et al., 2009; Pontin et al., 2015); similar results were found in the biocontrol assay performed in this work, where the lesion diameter (caused by *B. cinerea*) was reduced in plants with higher terpenes content (bacterized plants). Furthermore, other researches provided evidences that volatiles (terpenes) emission play an important role in responses to abiotic stresses (Alonso et al., 2015; Vickers et al., 2009). Loreto et al. (1998) found that monoterpenes reduce the decline of photosynthesis in plants exposed to heat, suggesting that they help plants to cope this stress by enhancing the membrane stability, thus providing protection of the photosynthetic and respiratory processes. Similarly, Peñuelas et al. (2005) reported that emission of monoterpenes along with other antioxidants (e.g. tocopherols, ascorbic acid) is related with dissipation of excess in excitation energy conferring protection to the photosynthetic system against heat damage. It has also been shown that monoterpenes are very efficient ozone scavengers in detoxifying leaves, thus photosynthesis is less affected in monoterpenes-emitting plants when they are expose to ozone (Fares et al., 2008). Other study has shown that high light and temperature conditions also stimulate the emission of volatile sesquiterpenes by plants, and these volatiles can combine rapidly with ROS (Vickers et al., 2009). In field-grown grapevines cv. Malbec of a high altitude vineyard (1450 m a.s.l.), Alonso et al. (2015) found that α-pinene, 3-carene, terpinolene, and nerolidol were augmented by all, UV-B radiation, water deficit or sprayed ABA eliciting mechanisms of acclimation with antioxidant and antifungal properties, therefore enhancing plant defensive mechanisms towards both biotic and abiotic signals. After studying the antioxidant properties of the monoterpene α-pinene (1) and the sesquiterpene nerolidol (4), the most abundant in leaves of bacterized plants, it has been found that both terpenes were able to protect tryptophan from photo-oxidation, notwithstanding they were less oxidized than trolox (less oxygen consumption rate). These results suggest that ROS might be intercepted by the studied terpenes through the mechanisms proposed in this work. These findings also provide evidence that volatiles monoterpenes are involved in protection against abiotic stress. Moreover it is important to note that the leaf extracts of bacterized plants with *T. saccharophilus* and *M. imperiale* with the major levels of α-pinene, and nerolidol, were the most effective in decreasing tryptophan oxidation (18 and 15% respectively vs. 2% of control).

Tocopherols are a group of lipid-soluble compounds (belong to Vitamin E) present in several plant organs well known for their antioxidant activity. They protect membranes against lipid peroxidation caused by ROS, due to their ability to scavenge lipid peroxy radicals and to quench singlet oxygen and others ROS (DellaPenna and Pogson, 2006). In this study, tocopherols content was significantly augmented in leaves of bacterized plants, mainly α-

tocopherol (5) which is the most antioxidant form among tocopherols (Kamal-Eldin and Appelqvist, 1996). Increases of tocopherols content may be elicited by ROS as a plant response to pathogenic infection (Wojtaszekp, 1997), so bacterization in our experiments would promote a similar (although harmless) response. In high light exposure, α-tocopherol (5) should contribute to reduce ROS levels in photosynthetic membranes (and diminished lipid peroxidation), so dissipating excess energy in thylakoids during photosynthesis and thus conferring photoprotection (Munné-Bosch, 2005). Under high ambient UV-B radiation increase of tocopherols concentration was observed in grapevine as a mechanism that protects chloroplastic membranes (Gil et al., 2012). In plants subjected to drought stress, α-tocopherol (5) beyond to collaborate in diminishing oxidative damage, it improves membrane integrity because of the complementary shapes of its prenyl side chain in the asymmetric phospholipids membrane, resulting in more controlled water efflux (Liu et al., 2008). In this work, leaf extracts of bacterized plants with higher levels of tocopherols (compared to control plants) decreased tryptophan oxidation; mainly the leaf extracts that correspond to the treatments +*T. saccharophilus* and +*M. imperiale* (coinciding also with the highest concentration of volatile terpenes).

Other compounds analyzed in response to bacterization were plant membrane sterols. Two of the most common sterols in plants are sitosterol (8) and stigmasterol (10). Sitosterol (8) restricts motion of fatty acyl chains in membranes regulating their fluidity so adapting membranes to temperature and water stresses. Associated with the increment of sitosterol (8) and lupeol (9) in leaves of bacterized plants, also it was observed an increment of squalene (11). This molecule is precursor in the biosynthesis of phytosterols and triterpenes in plants (Lee et al., 2004). In other words, the association of grapevine with these non-deleterious microorganisms may be considered a sort of "priming" signal that prepares tissues to cope with harder situations. *K. erythromyxa* Rt5M10, *M. imperiale* Rz19M10 and *T. saccharophilus* Rt17M10 are able to promote growth of *Vitis vinifera* L. cv. Malbec via two independent mechanisms; directly by producing phytohormones, but also indirectly inducing a systemic response that stimulate synthesis of different terpenes that help the plant to afford oxidative stress. The experimental evidence indicated that α-pinene (1) and nerolidol (4) showed moderate capacity of intercept ROS and are acceptable candidates for effective antioxidant protection, thus together with tocopherols (with well known antioxidant capacity) the antioxidant capacity of leaves of bacterized plants were enhanced. In general, the increases on monoterpenes, sesquiterpenes, tocopherols and membrane sterols may help grapevine to cope with both biotic (pathogen infections) and abiotic (drought, high light, UV-B radiation and high temperatures) stresses.

Contributions

MVS produced bacterial cultures and the in vitro plants, conducted the inoculation experiments, carried out GC-EIMS analysis as well as interpreted all experiments. RP carried out and interpreted the photolysis and antioxidant experiments. RB and PP designed and interpreted all experiments, and wrote the manuscript together with MVS.

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