Planta (2016) 243:1055–1070
DOI 10.1007/s00425-015-2460-8

ORIGINAL ARTICLE

Defense responses in plants of *Eucalyptus* elicited by *Streptomyces* and challenged with *Botrytis cinerea*

Tamiris D. Salla[1] · Leandro V. Astarita[1] · Eliane R. Santarém[1]

Received: 27 September 2015 / Accepted: 21 December 2015 / Published online: 21 January 2016

Abstract

Main conclusion **Elicitation of *E. grandis* plants with *Streptomyces* PM9 reduced the gray-mold disease, through increasing the levels of enzymes directly related to the induction of plant defense responses, and accumulation of specific phenolic compounds.**

Members of *Eucalyptus* are economically important woody species, especially as a raw material in many industrial sectors. Species of this genus are susceptible to pathogens such as *Botrytis cinerea* (gray mold). Biological control of plant diseases using rhizobacteria is one alternative to reduce the use of pesticides and pathogen attack. This study evaluated the metabolic and phenotypic responses of *Eucalyptus grandis* and *E. globulus* plants treated with *Streptomyces* sp. PM9 and challenged with the pathogenic fungus *B. cinerea*. Metabolic responses were evaluated by assessing the activities of the enzymes polyphenol oxidase and peroxidase as well as the levels of phenolic compounds and flavonoids. The incidence and progression of the fungal disease in PM9-treated plants and challenged with *B. cinerea* were evaluated. Treatment with *Streptomyces* sp. PM9 and challenge with *B. cinerea* led to changes in the activities of polyphenol oxidase and peroxidase as well as in the levels of phenolic compounds in the plants at different time points. Alterations in enzymes of PM9-treated plants were related to early defense responses in *E. grandis*. Gallic and chlorogenic acids were on average more abundant, although caffeic acid, benzoic acid and catechin were induced at specific time points during the culture period. Treatment with *Streptomyces* sp. PM9 significantly delayed the establishment of gray mold in *E. grandis* plants. These results demonstrate the action of *Streptomyces* sp. PM9 in inducing plant responses against *B. cinerea*, making this organism a potential candidate for biological control in *Eucalyptus*.

Keywords Biocontrol · Peroxidases · Plant defense · Phenolic compounds · Plant growth promoting rhizobacteria

Abbreviations

AUDPC	Area under the disease progress curve
dpi	Days after inoculation
ISR	Induced systemic resistance
PGPR	Plant growth promoting rhizobacteria
POX	Peroxidases
PPO	Polyphenol oxidases

Introduction

Species of *Eucalyptus* (family Myrtaceae) are the most widely planted hardwood forest trees in the world and consisted of renewable resources for the production of pulp, paper, biomaterials and bioenergy, while mitigating human pressures on native forests (Myburg et al. 2014). *Eucalyptus globulus* Labill. is one of the most widely planted and economically important hardwood species in temperate regions of the world, and *E. grandis* Hill ex Maiden is most often cultivated for industrial purposes in South Africa and Brazil (FAO 2001). *Eucalyptus* species

✉ Eliane R. Santarém
esantarem@pucrs.br

[1] Laboratory of Plant Biotechnology, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul - PUCRS, Avenida Ipiranga, 6681, 12C, sala 213, Porto Alegre, RS 90619-900, Brazil

are targeted by a broad range of pathogens, especially fungi, which infect plants throughout their life cycles. Among the principal pathogens that attack *Eucalyptus* is the gray mold *Botrytis cinerea*, a typical necrotrophic fungus. When the soil is contaminated with the fungus, it can directly infect the aerial parts of susceptible plants in contact with the soil (Leyronas et al. 2015). *B. cinerea* causes programmed cell death in the host during the course of infection, by secreting toxic molecules and lytic enzymes and subsequently consuming the plant tissues to support its own growth (Dean et al. 2012).

One alternative for promoting plant health and development is the use of microorganisms as biological control agents (Postma et al. 2003; Ashraf et al. 2013). Among these microorganisms are bacteria that live attached to plant roots, named plant growth promoting rhizobacteria (PGPRs) (Kloepper et al. 1980). *Streptomyces* (Actinomycetes) are part of the PGPR group, and comprise Gram-positive filamentous bacteria that are known for their ability to control plant diseases by inducing defense responses in colonized plants (Schrey and Tarkka 2008; Gopalakrishnan et al. 2011). These microorganisms show mechanisms of pathogen suppression through production of antibiotics, competition for colonization sites and nutrients, production of siderophores, and production of cell wall-degrading enzymes (Palaniyandi et al. 2013).

In plants, induction of resistance is typically achieved through two physiological pathways, termed systemic acquired resistance (SAR) and induced systemic resistance (ISR), which can be differentiated by the regulatory pathways and the nature of the elicitor. While SAR is triggered by necrotizing pathogens, ISR is activated by nonpathogenic rhizobacteria, such as specific PGPR (Conn et al. 2008). ISR is dependent on jasmonic acid and ethylene signaling in colonized plants, and is generally associated with a physiological state in which plants can react more efficiently to pathogen attack, a defense mechanism referred to as *priming* (Kurth et al. 2014). Priming plants with PGPRs can provide systemic resistance against a broad spectrum of plant pathogens (Palaniyandi et al. 2013). The efficiency of *Streptomyces* rhizobacteria as biocontrol agents has been reported in numerous studies. *Streptomyces hygroscopicus* was efficient against grapevine downy mildew caused by *B. cinerea,* through production of antimicrobial molecules (Nair et al. 1994), and *S. cavourensis* SY224 reduced anthracnose in pepper, a result attributed in part to the production of chitinase and glucanase (Lee et al. 2012). Inoculation of roots of Norway spruce with *Streptomyces* GB 4–2 provided systemic resistance to *B. cinerea* (Lehr et al. 2008). Culture filtrates from *S. bikiniensis* HD-087 were able to induce ISR against *Fusarium* wilt in cucumber, and the treatments increased the activities of peroxidase, phenylalanine ammonia lyase and β-1,3-glucanase (Zhao et al. 2012). Isolates of *Pseudomonas* sp. were efficient in reducing rust in *Eucalyptus* sp. (Teixeira et al. 2005) and in suppressing bacterial wilt in *E. urophylla* (Ran et al. 2005). *Streptomyces* sp. PM9 was proven to modulate secondary metabolism of *E. grandis* and *E. globulus* plants (Salla et al. 2014). To date, these are the only reports demonstrating the use of rhizobacteria on *Eucalyptus*.

The development of inducible resistance in plants is associated with various defense responses, including synthesis of pathogenesis-related proteins, phytoalexins, rapid alterations in cell walls and enhanced activities of several enzymes (Małolepsza 2006). Generally, enzymes of the phenylpropanoid pathway are involved in the production of phytoalexins and phenolic compounds are associated with ISR (Alizadeh et al. 2013). Enzymes that are commonly related to defense responses include phenylalanine ammonia lyase (PAL), chitinase, β-1,3-glucanase, peroxidase (POX), polyphenol oxidase (PPO), superoxide dismutase (SOD), catalase (CAT), lipoxygenase (LOX), ascorbate peroxidase (APX) and proteinase inhibitors (van Loon 1997; Alizadeh et al. 2013).

This study evaluated the ability of the isolate *Streptomyces* sp. PM9 as an elicitor of defense responses against *B. cinerea* in in vitro-grown plants of *E. grandis* and *E. globulus*. Activity of defense-related enzymes and compounds of secondary metabolism were quantified. Progression of gray mold disease in plants pretreated with *Streptomyces* was also evaluated.

Materials and methods

Plant material and microorganisms

Seeds of *Eucalyptus grandis* and *E. globulus* were provided by Suzano Papel e Celulose, Brazil. Seeds were surface-disinfected in 70 % ethanol for 60 s followed by immersion in the fungicide Ridomil Gold® MZ (8 g L^{-1}) for 20 min, and immersion in 1 % sodium hypochlorite solution for 10 min. Seeds were rinsed three times with sterile distilled water and sown on MS culture medium (Murashige and Skoog 1962) with the salt concentration reduced to one quarter (1/4 MS), supplemented with 10 g L^{-1} sucrose and 6 g L^{-1} agar. Seedlings were maintained at 25 ± 2 °C with light intensity of 31 μmol m^{-2} s^{-1} under a 16-h photoperiod. Ninety-day-old plants were used in the experiments.

Streptomyces sp. PM9 (GenBank accession number: PM9-HM460337) was grown from stock cultures initiated from samples collected in an Araucaria Forest at

29°29′18.4″S, 50°12′23.5″W. The isolate was cultivated in ISP_4 liquid medium (Shirling and Gottlieb 1966), shaken at 100 rpm for 7 days (stationary phase), and centrifuged at 2500*g* for 10 min at room temperature. The pellet was resuspended in sterile distilled water with further standardization to 10^6–10^7 CFU mL^{-1} ($OD_{600nm} = 1$) (Salla et al. 2014). The plant pathogenic fungus *Botrytis cinerea* was grown for 15 days on potato agar (PDA), and a suspension of its hyphae was prepared with sterile distilled water and adjusted to $OD_{600nm} = 0.2$ and 0.5, representing 3050 and 9520 hyphae mL^{-1}, respectively.

Disease evaluation

Plants of *E. grandis* or *E. globulus* were cultivated in an in vitro system according to Salla et al. (2014). Briefly, 25 mL of 1/4 MS medium was poured into a Petri dish (9 cm in diameter), and after the agar solidification, a semicircle of medium was discarded and one plant was placed on the remaining medium semicircle. Treatments consisted of (1) plants inoculated with sterile distilled water (control); (2) plants inoculated with *B. cinerea* (treatment F) on the roots ($OD_{600nm} = 0.2$ or 0.5); and (3) plants treated with *Streptomyces* sp. PM9 ($OD_{600nm} = 1$) and challenged with *B. cinerea* after 4 days ($OD_{600nm} = 0.2$ and 0.5; treatments PM9 + F 0.2 and PM9 + F 0.5). Inoculations were performed by placing 200 µL of either the rhizobacterium or the fungus on the root surface. In this condition, the fungus can form a mycelium web, covering the plant and making it collapse.

Disease incidence and development of gray mold symptoms were evaluated in the shoots, which were divided into three equal parts. Disease incidence in a completely infected shoot was considered to be 100 %. Data were collected from observations made every 2 days from the beginning of the experiment. The area under the disease progress curve (AUDPC) was estimated. AUDPC values were normalized and corrected (AUDPC-nc) by dividing the values by the number of days until the final severity reading for each treatment, and multiplying the resulting values by the number of days until the final severity evaluation (Graichen et al. 2010; Zambonato et al. 2012), as shown below:

$$\text{AUDPC-nc} = \{\{\Sigma[(y_i + 1 + y_i \times 0.5)] * [t_i + 1 - t_i]\}/n\} * c,$$

where y_i = percentage of shoot affect by gray mold (severity at the *i*th observation); t_i = time (in days) after inoculation of *B. cinerea* at the *i*th observation; n = number of days between the disease onset and the last disease assessment; c = longest period of epidemic duration among the plants evaluated.

Evaluation of secondary metabolism of *Eucalyptus* spp. treated with *Streptomyces* sp. PM9

Plants of *E. grandis* and *E. globulus* were transferred to the in vitro system described above. The treatments consisted of (1) plants inoculated with sterile distilled water (absolute control); (2) plants inoculated with *Streptomyces* sp. PM9 ($OD_{600} = 1$; treatment PM9) on the roots; (3) plants infected with *B. cinerea* ($OD_{600nm} = 0.5$) on the roots (control for disease; treatment F); and (4) plants treated with *Streptomyces* sp. PM9 ($OD_{600nm} = 1$) and challenged with *B. cinerea* ($OD_{600nm} = 0.5$; treatment PM9 + F) after 4 days. Inoculations were performed by placing 200 µL of either the rhizobacterium or the fungus on the root surface.

Components of plant secondary metabolism, such as defense-related enzymes and phenolic compounds were analyzed as part of defense responses mediated by *Streptomyces* spp. Plants were evaluated for basal secondary metabolism before and 4 days after inoculation with *Streptomyces* PM9, named B and BS, respectively. Plant responses were then evaluated at 1, 3, 9 and 15 days post-inoculation with *B. cinerea*. The induced levels of secondary metabolites (total phenolics and flavonoids) and the activity of polyphenol oxidases (PPO) and peroxidases (POX) were analyzed. Each treatment consisted of 20 plants per time-course point, totaling 380 plants. Shoots and roots of *Eucalyptus* plants were analyzed separately. Shoots or roots from each treatment and each time course were pooled, kept on ice and cut into small pieces. A minimum of three biological repetitions was used; each repetition was analyzed in three replicates for the colorimetric reactions, and in duplicate for the chromatographic analysis.

Determination of defense enzyme activity and secondary compounds

The activities of the enzymes polyphenol oxidase (PPO; EC 1.14.18.1) and peroxidases (POX; EC 1.11.17) were determined according to Salla et al. (2014). Briefly, extracts were prepared from shoots and roots (0.4 g) ground in 2.5 mL of 50 mM sodium phosphate buffer (pH 7) and polyvinylpyrrolidone (PVP; 1:6, w/v). Before grinding, the plant material was thoroughly rinsed in distilled water to remove any excess of medium or microorganisms. PPO activity was determined in a reaction containing chlorogenic acid (1 mM) as substrate at 400 nm. Specific enzyme activity was defined as the change in absorbance min^{-1} mg^{-1} protein. The activity of peroxidases was determined in a reaction mixture containing 50 mM sodium phosphate buffer (pH 6), 1 % (v/v) guaiacol as substrate and 10 mM hydrogen peroxide, using the crude extract described above. Oxidation of guaiacol

was measured by the increase in absorbance at 420 nm for 30 s at an interval of 5 s. Specific enzyme activity was expressed as μkatal mg^{-1} protein. Total protein concentration was determined according to Bradford's method (Bradford 1976), using bovine serum albumin as standard.

For analysis of secondary compounds, samples of shoots and roots of *Eucalyptus* plants (0.1 g of fresh weight; FW) were taken from each treatment, dried by blotting on sterile filter paper, and ground in 10 mL of 80 % (v/v) methanol at room temperature. Extracts were filtered and centrifuged at 1250*g* for 15 min. Total phenolic compounds were analyzed in the supernatant by the colorimetric Folin-Ciocalteu method, as described previously (Sartor et al. 2013). Gallic acid was used as the standard. The contents of total phenolic compounds were expressed as mg g^{-1} FW. The fraction of quercetin-derived flavonoids was determined by the colorimetric method using the reaction with 96 % ethanol, 10 % aluminum nitrate and 1 M potassium acetate, measured at 415 nm. Quercetin was used as standard for the calibration curve. The flavonoid content was expressed as mg quercetin equivalents g^{-1} FW (Salla et al. 2014).

Identification and quantification of the phenolic compounds in *Eucalyptus* sp. plants were determined by High Liquid Performance Chromatography (HPLC). Analyses were carried out in an Agilent Technologies, 1200 Series liquid chromatograph operated at 45 °C, and separations were performed on a MetaSil ODS column (5 μm; 150 × 4.6 mm). Detection was achieved with a UV/V detector set at 280 nm. A gradient was formed between two mobile phases: phase A consisted of 2 % formic acid in water, and phase B of 100 % methanol. The analysis followed a linear gradient programmed as 10–20 % of eluent B from 0 to 15 min, 20 to 40 % from 15 to 20 min, 40 to 60 % from 20 to 25 min and 60 to 100 % from 25 to 25.1 min. The flow rate was kept constant at 1 mL min^{-1} and the injection volume was 20 μL. HPLC analysis was performed using a five-point calibration curve generated with authentic phenolic standards (gallic acid, caffeic acid, chlorogenic acid, 2-hydroxybenzoic acid, benzoic acid, catechin and coumarin). HPLC analysis was carried out with two replicates obtained from the pool of root and shoot samples prepared from the total phenolic compounds analysis. Data were expressed as mean ± standard deviation (SD).

Statistical analysis

Experiments for evaluation of secondary metabolism and disease were performed in a fully randomized design, tested for variance homogeneity by Levene's test and subjected to one-way ANOVA. Means were separated by Tukey Test at a significance level of $\alpha \leq 0.05$. All statistical analyses were performed using the software SPSS v. 17.5. Data from enzymatic activities and secondary metabolites were expressed as mean ± standard error.

Results

Disease evaluation

The area under the disease progress curve (AUDPC) was evaluated in plants of *E. grandis* and *E. globulus* pretreated with *Streptomyces* and challenged with *B. cinerea*. Plants of *E. grandis* infected with *B. cinerea* at $OD_{600nm} = 0.2$ resulted in a larger area (12,032.80) than the plants treated with PM9 and challenged with the pathogen (PM9 + F0.2; 1338.76). A similar variation was recorded for the $OD_{600nm} = 0.5$ (Table 1). In contrast, no effect of *Streptomyces* was observed on *E. globulus*-treated plants (Table 1). The response observed with AUDPC could be confirmed when the disease incidence was recorded as percentages of incidence during the cultivation period. The lowest disease incidence (13.3 %) was recorded in *E. grandis* plants from the PM9 + F0.2 treatment, with the first diseased plant being observed at 12 dpi (Fig. 1a). Increase of disease incidence (33 %) was recorded on treatment PM9 + F0.5, although the timing of disease appearance was the same than PM9 + 0.2 (Fig. 1a, b). The earliest incidence of disease was observed in F-treated (0.2) plants at 7 dpi, reaching 66.7 % at 20 dpi. In *E. globulus,* no differences were observed between the optical densities used for fungus inoculation, either in F or PM9 + F treatments (Fig. 1c, d).

Table 1 Mean AUDPC-nc values of *Eucalyptus* plants elicited with *Streptomyces* sp. PM9 and challenged with *B. cinerea* in different optical densities (0.2 and 0.5), cultivated for 20 days post-inoculation with the pathogen

Treatments[a]	*E. grandis* AUDPC	*E. globulus* AUDPC
Control	0 ± 0.0 (b)*	0 ± 0.0 (b)
F 0.2	12,032.8 ± 3610.4 (a)	13,828.6 ± 2227.2 (a)
PM9 + F 0.2	1338.7 ± 886.8 (b)	4652.6 ± 6922.89 (a)
Control	0 ± 0.0 (b)	0 ± 0.0 (b)
F 0.5	5818.5 ± 1552.2 (a)	18,426.7 ± 9555.1 (a)
PM9 + F 0.5	3755.2 ± 1676.9 (b)	13,845.3 ± 4314.2 (a)

* Different letters indicate significantly differences among treatments within the species (Tukey test, $P \leq 0.05$)

[a] Control (distilled water), F = plants infected with *B. cinerea* ($OD_{600nm} = 0.2$ and 0.5), and PM9 + F = plants elicited with *Streptomyces* sp. PM9 and challenged with *B. cinerea* ($OD_{600nm} = 0.2$ and 0.5)

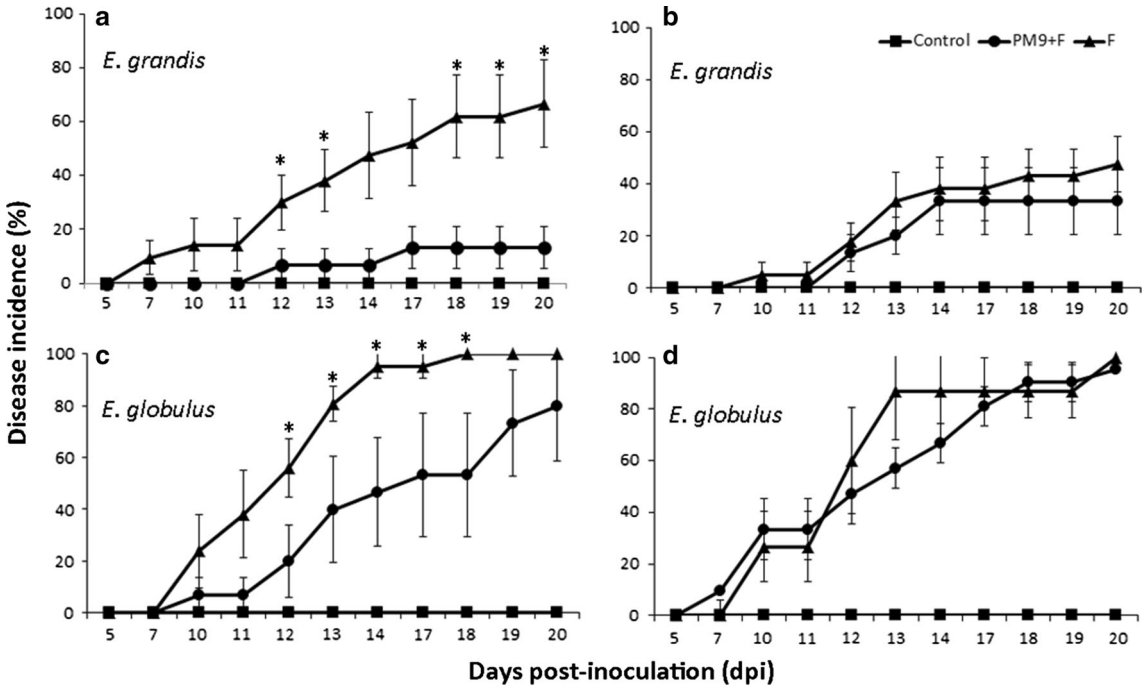

Fig. 1 Percentage of disease incidence over 20 days after inoculation with *B. cinerea.* Treatments consisted of control (C), *Streptomyces* sp. PM9 (PM9), pathogenic fungus *B. cinerea* (F) and plants elicited with *Streptomyces* sp. PM9 challenged with *B. cinerea* (PM9 + F) and plants infected with *B. cinerea* (F) in different optical density (0.2 and 0.5) in plants of **a**, **b** *E. grandis* and **c**, **d** *E. globulus,* respectively. *Asterisk* significant difference between PM9 + F and F treatments within a time point (*t* test, $P \leq 0.05$)

Determination of enzymatic activities

Differences in plant response between roots and shoots were observed when plants established contact with *Streptomyces* PM9 in both species. In general, enzymatic activities were observed to be stronger in roots than in shoots (Figs. 2, 3).

In *E. grandis* roots, the PPO activity did not differ at the first time points of analysis (BS and 1 dpi; Fig. 2a). At 3 dpi, roots inoculated with PM9 (treatment PM9) showed increased PPO activity compared to roots infected with *B. cinerea* (treatment F) and to roots treated with PM9 and challenged with the pathogen (treatment PM9 + F) (Fig. 2a). However, in this treatment, the activity was significantly increased in PM9-treated roots at 9 dpi, showing a similar response to those from the PM9 treatment. In roots, POX activity was triggered earlier (at 1 dpi) in PM9 + F compared to PPO, and thereafter showed significant differences from the F treatment (Fig. 2b). At 9 dpi, POX activity was suppressed in the F plants (roots and shoots).

In *E. globulus*, treating roots with PM9 + F resulted in twofold increase of PPO activity when compared to the control (C) at 1 dpi in (Fig. 3a). Nonetheless, a reduction in this enzyme activity in PM9 + F plants was observed at 3 dpi in the roots and no difference was noted among the treatments at 9 dpi. At 15 dpi, PPO activity increased in the F and PM9 + F treatments (Fig. 3a). On the contrary, at 1 dpi, POX activity in F roots showed a marked increase comparing to the PM9 + F treatment (Fig. 3b). Although the levels of POX for all treatments were reduced when compared to the control plants, differences were evident between PM9/PM9 + F and fungus-infected plants at 3 and 15 dpi. However, a marked decrease of POX activity was detected in PM9 + F treatment at 9 dpi. Roots from the F treatment showed steady levels of POX activity from 3 dpi of *B. cinerea* and thereafter (Fig. 3b).

The shoots of *Eucalyptus* sp. were also affected by the interaction with the microorganisms indicating a systemic response. In *E. grandis* the PPO activity showed a slight increase in the F treatment at 3 dpi, and this response persisted until 9 dpi (Fig. 2a). No significant difference was observed among the treatments at 15 dpi. On the other hand, although levels of POX activity were significantly reduced at 1 dpi for all treatments, an increase was recorded in PM9 + F at 3 and 9 dpi. These plants then showed a marked decrease in POX activity at 15 dpi (Fig. 2b). POX activity in shoots from PM9 + F plants increased immediately after the initial responses had occurred in the roots.

In *E. globulus* PM9 + F-shoots, PPO activity showed a slight increase compared to the control plants at 3 dpi, and this response was enhanced at 9 dpi (Fig. 3a). At 15 dpi, the treatments differed significantly from the control, and the PPO specific activity in F-infected plants reached 8.3 Δ

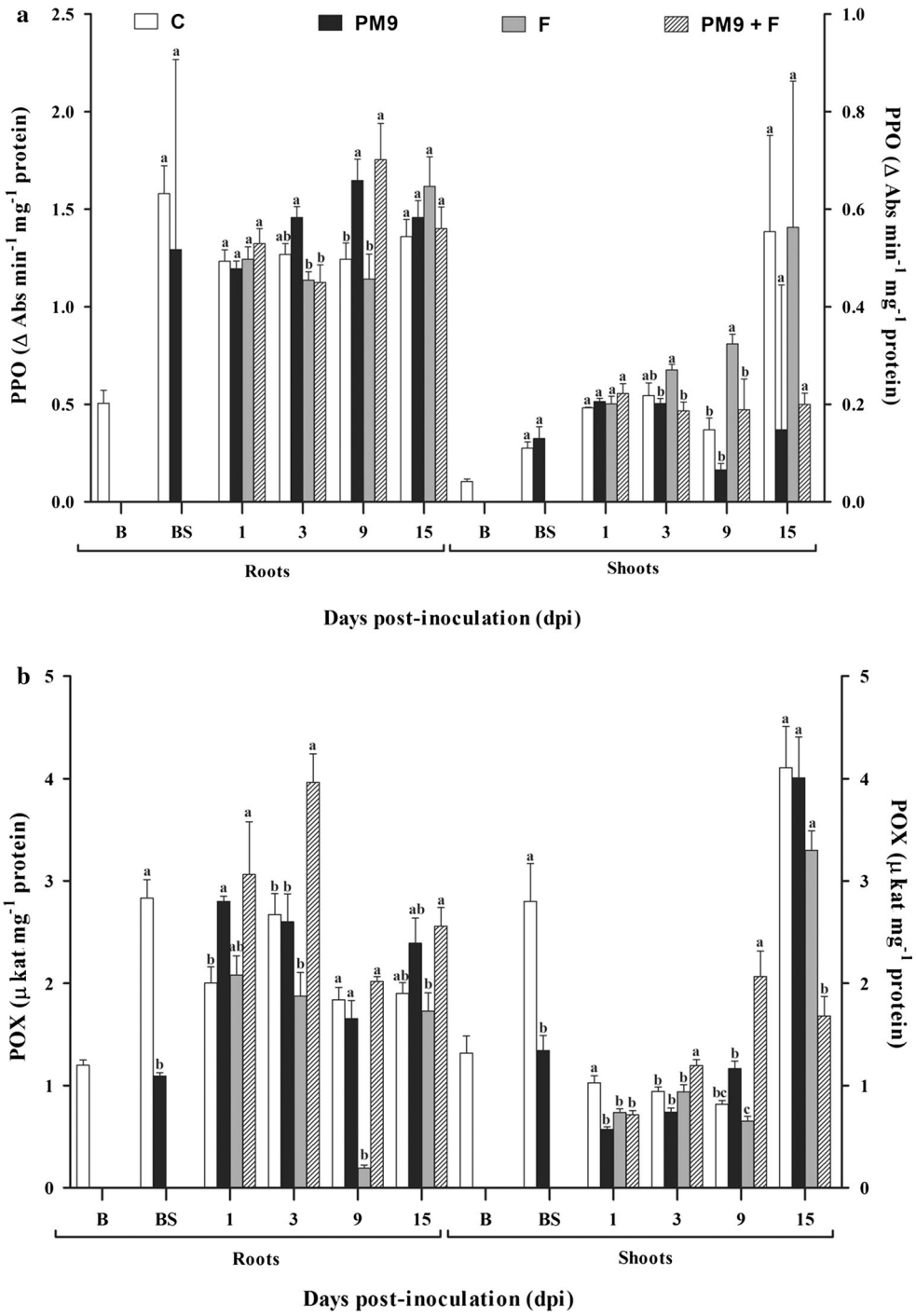

Fig. 2 Activities of the enzymes **a** polyphenol oxidase and **b** peroxidases in the roots and shoots of *E. grandis* plants. Control (C), *Streptomyces* sp. PM9 (PM9), pathogenic fungus *B. cinerea* (F) and plants elicited with *Streptomyces* sp. PM9 and challenged with *B. cinerea* (PM9 + F). Responses were evaluated at 1, 3, 9 and 15 days post-inoculation with *B. cinerea*. *Bars* represent ± SE of the mean ($n = 9$). *Different letters* indicate significantly differences among treatments within a time point (Tukey test, $P \leq 0.05$)

Abs min^{-1} mg^{-1} of protein, whereas in PM9 + F plants activity was 2.8 Δ Abs min^{-1} mg^{-1} of protein (Fig. 3a). By this time point, plants were already showing strong disease incidence (Fig. 1c, d). POX activity was higher in PM9-treated shoots at 3 and 15 dpi, whereas in PM9 + F plants, a marked increase was detected at 9 dpi (Fig. 3b). When compared to *E. grandis*, PPO and POX activities were higher overall in *E. globulus* shoots (Figs. 2, 3).

Quantification of phenolic compounds

Production and accumulation of phenolic compounds were observed in roots of *Eucalyptus* sp. in response to the microorganisms (Fig. 4). A decrease in the levels of phenolics was detected in *E. grandis* roots after 4 dpi with *Streptomyces* (BS) in PM9-plants and at 1 dpi in PM9 + F (Fig. 4a). Levels of phenolics increased in PM9 + F roots

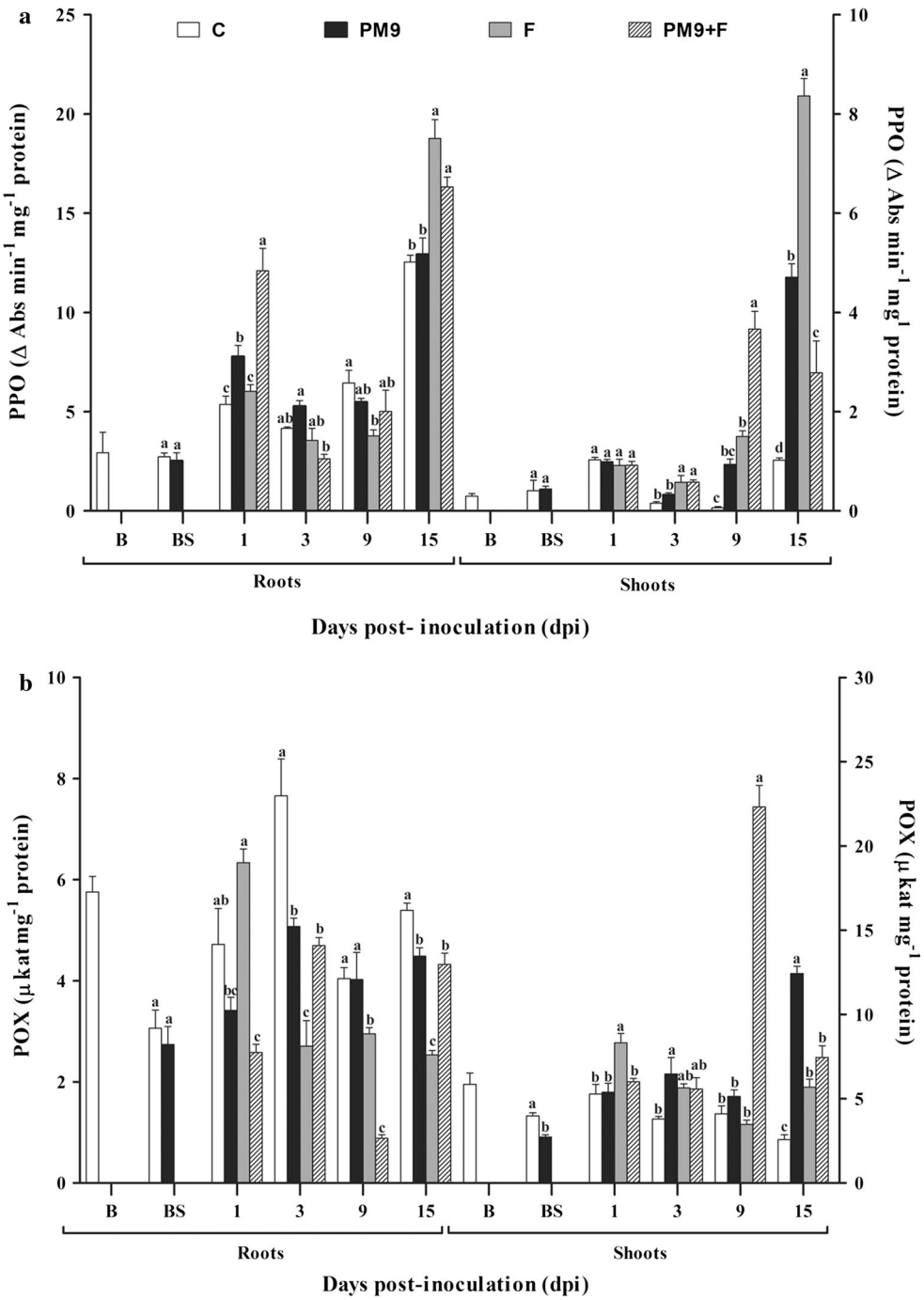

Fig. 3 Activities of the enzymes **a** polyphenol oxidase and **b** peroxidases in the roots and shoots of *E. globulus* plants. Control (C), *Streptomyces* sp. PM9 (PM9), pathogenic fungus *B. cinerea* (F) and plants elicited with *Streptomyces* sp. PM9 and challenged with *B. cinerea* (PM9 + F). Responses were evaluated at 1, 3, 9 and 15 days post-inoculation with *B. cinerea*. *Bars* represent ± SE of the mean ($n = 9$). *Different letters* indicate significantly differences among treatments within a time point (Tukey test, $P \leq 0.05$)

at 3 dpi, which was coincident with the highest POX activity in roots at this time point (Fig. 2b). At 9 dpi, the lowest level was found in PM9 + F roots (Fig. 4a), coincident with the highest PPO activity (Fig. 2a). Levels of quercetin flavonoids were reduced in roots of plants treated with PM9 and PM9 + F at 9 dpi (Fig. 4b). Overall, PM9 + F plants showed increased content of flavonoids in roots when compared to F plants (Fig. 4b).

Levels of phenolic compounds in *E. globulus* roots were affected by *Streptomyces* sp., and a reduction was observed at BS in PM9-plants (Fig. 5a). However, in this species, a marked reduction in phenolic contents occurred in roots of plants infected with *B. cinerea* at 9 and 15 dpi (Fig. 5a). At 15 dpi, the lowest level of phenolics in the F treatment coincided with the highest activity of PPO (Fig. 3a). The PM9-roots evidenced higher levels of phenolics than the control plants at 3 and 15 dpi (Fig. 5a). Flavonoids were increased in response to root inoculation with PM9 comparing to the control treatment (BS and thereafter; Fig. 5b).

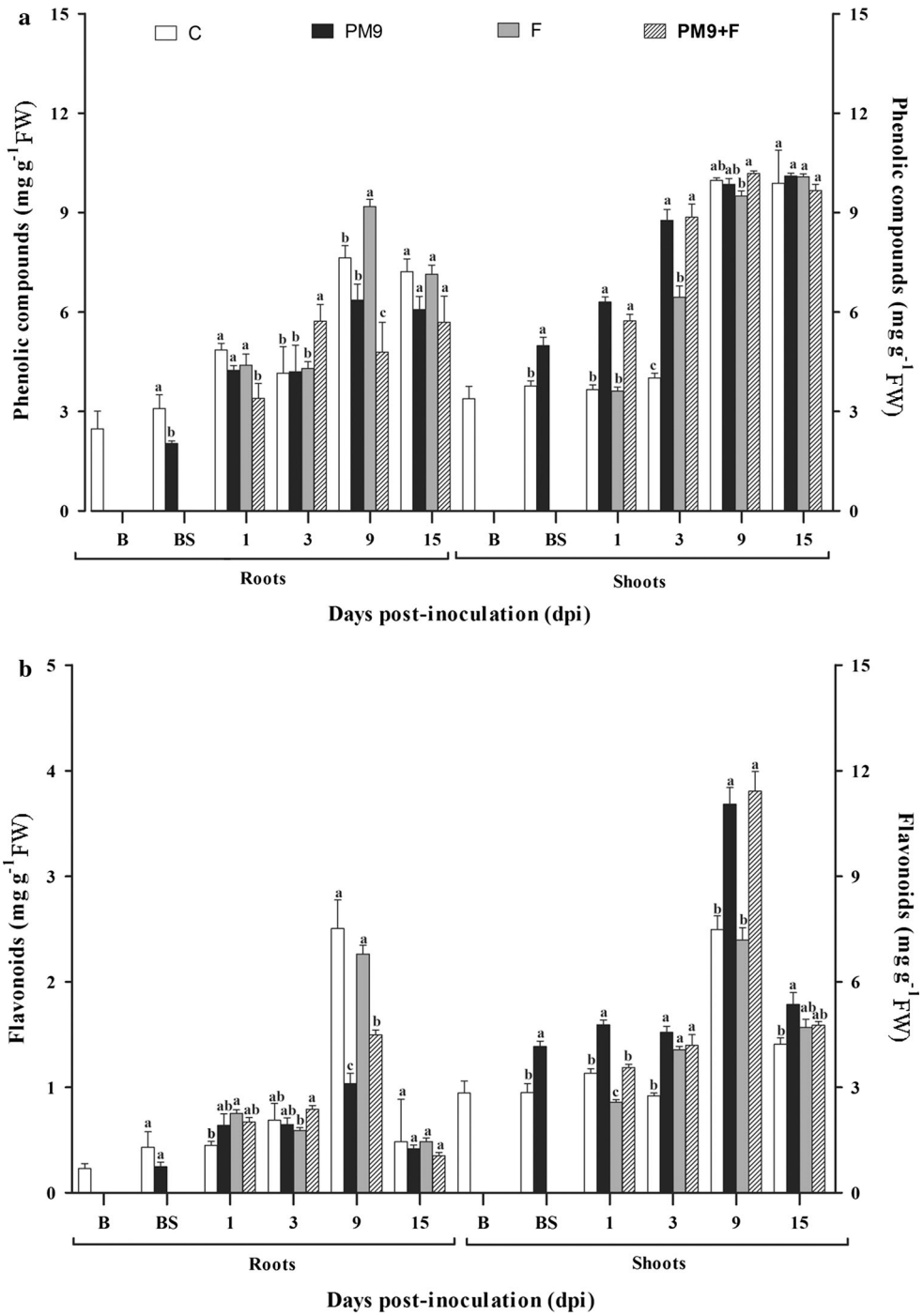

Fig. 4 Levels of total **a** phenolic compounds and **b** flavonoids in the roots and shoots of *E. grandis* plants. Control (C), *Streptomyces* sp. PM9 (PM9), pathogenic fungus *B. cinerea* (F) and plants elicited with *Streptomyces* sp. PM9 and challenged with *B. cinerea* (PM9 + F). Responses were evaluated at 1, 3, 9 and 15 days post-inoculation with *B. cinerea*. *Bars* represent ± SE of the mean ($n = 9$). *Different letters* indicate significantly differences among treatments within a time point (Tukey test, $P \leq 0.05$)

Shoots of both *E. grandis* and *E. globulus* showed variation on phenolic compounds with the treatments and time of culture. Similar results were observed in shoots of both species at 1 and 3 dpi, when the PM9 and PM9 + F plants showed the highest amounts (Figs. 4a, 5a). No significant differences among the treatments were apparent at the last two time points in *E. grandis*, although in *E. globulus,* all treatments differed from the control (Figs. 4a, 5a). Specifically for *E. globulus*, shoots from the PM9 treatment showed a constant high level of phenolics from 1 dpi until the end of cultivation compared to the control plants.

Increased levels of phenolic compounds in *E. grandis* shoots could be related to the reduced levels of POX activity at 1 dpi (Figs. 2b, 4a). Shoots from PM9- and PM9 + F-treated plants showed higher levels of phenolics than plants infected with *B. cinerea* (F) at 1 and 3 dpi, which may have played a role as substrates for POX at 3

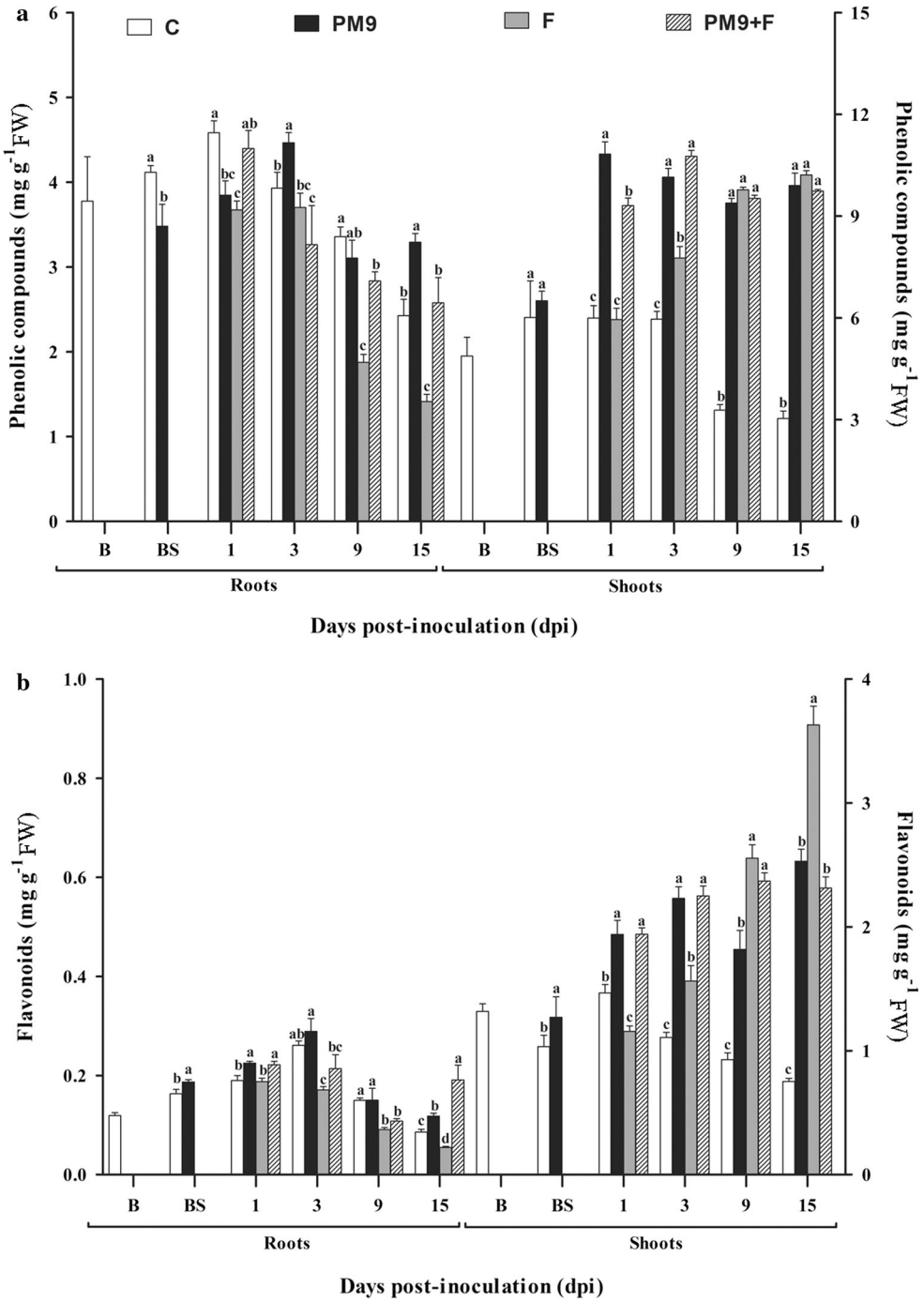

Fig. 5 Levels of total **a** phenolic compounds and **b** flavonoids in the roots and shoots of *E. globulus* plants. Control (C), *Streptomyces* sp. PM9 (PM9), pathogenic fungus *B. cinerea* (F) and plants elicited with *Streptomyces* sp. PM9 and challenged with *B. cinerea* (PM9 + F). Responses were evaluated at 1, 3, 9 and 15 days post-inoculation with *B. cinerea*. *Bars* represent ± SE of the mean ($n = 9$). *Different letters* indicate significantly differences among treatments within a time point (Tukey test, $P \leq 0.05$)

and 9 dpi for both species (Figs. 2b, 3b, 4a, 5a). Levels of quercetin flavonoids in PM9 shoots were significantly higher than control plants during the culture period, in both species (Figs. 4b, 5b). Comparing the concentration of flavonoids in shoots from F treatment, an increase of these compounds was observed in PM9 + F-shoots at 1 and 9 dpi in *E. grandis* and at 1 and 3 dpi in *E. globulus* (Fig. 5b).

Identification and quantification of the phenolic compounds by HPLC

Chromatographic analysis of phenolic compounds showed wide variation for each compound and species tested (Tables 2, 3). Amongst the metabolites assessed, chlorogenic and gallic acids were the phenolics detected in both species at the basal level, before any contact with

Table 2 Contents of phenolic compounds (mg g^{-1} FW) in roots and shoots of *E. grandis*

Phenolic compounds	Treatments	Roots (dpi)					Shoots (dpi)				
		BS	1	3	9	15	BS	1	3	9	15
Chlorogenic acid	C[a]	2.3 ± 0.2	2.1 ± 0.3	4.8 ± 1.1	4.0 ± 0.1	7.4 ± 0.1	1.9 ± 0.2	2.4 ± 0.01	1.5 ± 0.01	13.4 ± 2.2	13.4 ± 3.0
	PM9	2.3 ± 0.01	2.5 ± 0.2	2.4 ± 0.2	nd	nd	5.5 ± 1.2	4.1 ± 0.4	6.5 ± 1.9	1.2 ± 0.1	15.4 ± 0.1
	F		32.8 ± 1.0	3.5 ± 0.6	11.3 ± 1.0	7.8 ± 0.1		3.2 ± 0.4	4.2 ± 0.9	10.2 ± 0.6	9.0 ± 7.0
	PM9 + F		1.9 ± 0.01	2.1 ± 0.01	5.3 ± 0.001	5.2 ± 1.0		3.9 ± 0.9	4.6 ± 1.1	11.6 ± 5.6	11.9 ± 3.9
Gallic acid	C	9.8 ± 2.5	nd	33.2 ± 4.9	18.4 ± 11.7	50.6 ± 5.4	8.6 ± 2.3	12.6 ± 1.9	8.2 ± 0.2	129.3 ± 27.4	116.7 ± 98.0
	PM9	9.5 ± 1.0	3.2 ± 0.9	12 ± 0.9	27.2 ± 2.4	nd	5.7 ± 2.3	27.0 ± 0.4	53.5 ± 10.7	12.3 ± 4.7	171.2 ± 15.5
	F		21.2 ± 1.1	12.2 ± 3.2	79.2 ± 6.1	54.0 ± 2.5		20.7 ± 1.2	27.3 ± 8.9	100.9 ± 5.2	208.9 ± 17.6
	PM9 + F		10.5 ± 0.0	29.5 ± 5.3	21.2 ± 2.2	20.9 ± 5.5		23.5 ± 5.2	25.6 ± 5.9	144 ± 39.2	101.6 ± 56.0
2-Hydroxybenzoic acid	C	4.6 ± 0.1	4.4 ± 2.4	7.4 ± 3.5	nd	15.4 ± 0.5	nd	5.0 ± 1.6	3.3 ± 0.5	34.1 ± 9.6	32.0 ± 11.4
	PM9	3.4 ± 0.3	3.5 ± 1.9	4.1 ± 0.01	7.9 ± 0.4	nd	nd	nd	nd	22.3 ± 0.9	35.0 ± 5.6
	F		6.6 ± 1.4	7.7 ± 3.1	21.8 ± 6.7	13.6 ± 0.4		nd	nd	22.0 ± 1.1	48.6 ± 5.7
	PM9 + F		2.6 ± 0.1	9.2 ± 0.2	7.0 ± 0.4	5.7 ± 0.7		6.9 ± 0.01	5.1 ± 2.8	26.1 ± 6.6	25.4 ± 8.3
Caffeic acid	C	4.3 ± 0.01	nd	5.5 ± 1.6	4.8 ± 0.3	13.9 ± 1.9	10.7 ± 5.1	6.5 ± 2.1	8.2 ± 0.2	39.7 ± 9.9	32.1 ± 10.8
	PM9	nd	nd	nd	5.1 ± 0.8	nd	14.3 ± 4.5	13.8 ± 0.8	nd	26.1 ± 9.9	35.8 ± 8.6
	F		7.7 ± 1.4	nd	18.4 ± 7.5	10.2 ± 0.7		nd	17.6 ± 4.7	29.7 ± 0.01	46.9 ± 4.1
	PM9 + F		nd	10.4 ± 1.4	6.9 ± 1.7	7.2 ± 2.5		16.1 ± 1.9	nd	33.9 ± 4.1	23.6 ± 2.6
Benzoic acid	C	nd	nd	nd	nd	nd	nd	nd	nd	3.5 ± 2.0	3.2 ± 1.4
	PM9	nd	nd	nd	nd	nd	1.0 ± 0.3	1.2 ± 0.1	1.6 ± 0.2	2.3 ± 1.2	2.8 ± 0.6
	F		nd	0.9 ± 0.2	2.9 ± 1.4	nd		nd	1.0 ± 0.3	2.3 ± 0.2	3.7 ± 0.5
	PM9 + F		nd	0.9 ± 0.1	3.8 ± 1.9	nd		1.0 ± 0.2	nd	3.3 ± 2.0	1.7 ± 0.5
Catechin	C	10.2 ± 0.8	nd	nd	nd	24.0 ± 3.3	nd	17.5 ± 3.1	21.3 ± 0.3	79.4 ± 9.8	78.5 ± 17.6
	PM9	nd	nd	nd	10.0 ± 1.6	nd	28.9 ± 4.4	nd	27.7 ± 2.0	41.8 ± 2.3	67.2 ± 13.5
	F		nd	nd	45.4 ± 2.6	20.9 ± 0.2		nd	26.2 ± 5.3	75.9 ± 11.8	90.3 ± 4.2
	PM9 + F		12.3 ± 0.2	16.9 ± 3.0	12.9 ± 1.4	14.0 ± 18		32.0 ± 2.1	17.8 ± 5.7	51.7 ± 16.4	56.1 ± 12.7
Coumarin	C	1.2 ± 0.2	0.9 ± 0.3	2.0 ± 0.5	2.6 ± 0.1	10.8 ± 0.7	0.2 ± 0.1	0.8 ± 0.4	1.3 ± 0.1	13.8 ± 4.3	11.5 ± 5.1
	PM9	0.7 ± 0.0	1.5 ± 0.3	1.6 ± 0.01	1.4 ± 0.6	1.8 ± 2.3	2.9 ± 1.0	0.9 ± 0.2	4.7 ± 0.7	6.3 ± 0.5	23.6 ± 0.5
	F		1.3 ± 0.6	nd	13.5 ± 2.4	nd		1.6 ± 0.4	4.0 ± 2.0	11.8 ± 0.5	26.7 ± 3.9
	PM9 + F		1.3 ± 0.1	7.9 ± 1.4	3.2 ± 1.8	3.2 ± 0.8		2.3 ± 0.9	3.3 ± 1.1	27.2 ± 2.1	15.9 ± 3.2

nd not detected

[a] C, control; PM9, *Streptomyces* sp. PM9; F, pathogenic fungus B. *cinerea*; PM9 + F, elicited plants with PM9 and challenged with *B. cinerea*. Quantifications were performed 4 days after inoculation with *Streptomyces* PM9 (BS) and at 1, 3, 9 and 15 days post-inoculation (dpi) with *B. cinerea*

Table 3 Contents of phenolic compounds (mg g^{-1} FW) in roots and shoots of *E. globulus*

Phenolic compounds	Treatments	Roots (dpi)					Shoots (dpi)				
		BS	1	3	9	15	BS	1	3	9	15
Chlorogenic acid	C[a]	3.7 ± 1.0	4.7 ± 0.01	2.0 ± 4.6	1.2 ± 0.1	1.6 ± 0.2	7.8 ± 0.9	7.0 ± 0.4	30.0 ± 21.3	6.1 ± 3.1	1.7 ± 0.1
	PM9	3.5 ± 1.2	3.6 ± 0.1	2.1 ± 0.6	2.5 ± 1.9	6.2 ± 0.3	4.4 ± 0.3	6.1 ± 1.8	10.1 ± 0.1	14.6 ± 3.1	147.5 ± 8.5
	F		2.7 ± 1.3	2.8 ± 1.1	2.4 ± 1.1	1.8 ± 0.1		5.5 ± 0.6	7.8 ± 0.4	12.5 ± 0.1	17.7 ± 1.3
	PM9 + F		4.6 ± 0.5	2.7 ± 0.5	4.1 ± 0.8	5.7 ± 1.0		7.2 ± 0.4	12.9 ± 1.6	14.5 ± 1.0	15.6 ± 2.2
Gallic acid	C	nd	38.3 ± 0.3	17.8 ± 0.4	2.2 ± 1.6	21.3 ± 5.6	45.6 ± 0.9	16.9 ± 6.3	32.3 ± 6.4	9.8 ± 6.9	27.6 ± 8.1
	PM9	19.2 ± 8.8	27.0 ± 0.9	nd	2.4 ± 0.6	nd	nd	79.5 ± 36.1	67.9 ± 2.0	26.5 ± 5.9	nd
	F		18.6 ± 14.9	11.7 ± 4.0	nd	3.5 ± 0.6		48.6 ± 7.8	59.9 ± 23.9	8.2 ± 0.4	53.4 ± 14.7
	PM9 + F		25.6 ± 3.1	17.3 ± 6.3	nd	nd		65.8 ± 4.6	76.2 ± 22.6	16.5 ± 9.0	nd
2-Hydroxybenzoic acid	C	nd	15.5 ± 0.8	3.1 ± 1.1	nd	7.8 ± 3.1	nd	34.5 ± 6.6	11.4 ± 1.7	nd	12.1 ± 4.9
	PM9	25.3 ± 9.9	22.5 ± 9.4	nd	2.5 ± 0.7	nd	nd	92.5 ± 3.7	19.8 ± 0.8	nd	67.3 ± 11.1
	F		nd	nd	nd	nd		37.9 ± 4.6	24.1 ± 10.9	14.2 ± 0.8	113.7 ± 40.6
	PM9 + F		21.5 ± 2.7	nd	nd	4.8 ± 2.4		45.0 ± 3.0	22.1 ± 6.5	27.9 ± 3.9	43.1 ± 14.6
Caffeic acid	C	nd	11.4 ± 1.6	nd	nd	nd	nd	10.6 ± 0.2	15.2 ± 4.4	12.2 ± 0.1	nd
	PM9	5.3 ± 1.4	6.9 ± 1.4	nd	nd	6.2 ± 0.001	nd	42.9 ± 3.5	23.9 ± 2.5	16.2 ± 4.0	28.4 ± 1.2
	F		nd	nd	nd	nd		10.9 ± 0.6	20.7 ± 4.3	27.8 ± 8.2	59.8 ± 15.1
	PM9 + F		10.6 ± 1.3	nd	nd	nd		14.3 ± 0.7	30.0 ± 4.9	32.1 ± 8.6	nd
Benzoic acid	C	0.6 ± 0.3	1.3 ± 0.0	nd	nd	nd	1.7 ± 0.7	1.5 ± 0.2	0.8 ± 0.1	1.0 ± 0.2	0.6 ± 0.09
	PM9	0.5 ± 0.08	1.2 ± 0.3	nd	nd	nd	0.8 ± 0.2	2.3 ± 1.2	1.3 ± 0.2	1.3 ± 0.3	1.1 ± 0.02
	F		nd	nd	nd	nd		1.9 ± 0.05	1.5 ± 0.5	0.9 ± 0.1	0.9 ± 0.2
	PM9 + F		2.4 ± 0.5	nd	nd	nd		1.9 ± 0.2	1.7 ± 0.2	1.6 ± 0.2	1.1 ± 0.5
Catechin	C	nd	17.7 ± 0.8	nd	nd	11.7 ± 2.9	nd	14.9 ± 1.0	22.3 ± 6.4	nd	nd
	PM9	nd	nd	nd	nd	nd	nd	28.1 ± 10.4	46.0 ± 3.0	21.5 ± 10.7	85.7 ± 3.1
	F		nd	nd	nd	nd		14.0 ± 0.7	41.4 ± 0.1	28.1 ± 0.2	92.5 ± 0.8
	PM9 + F		20.5 ± 0.0	nd	nd	nd		22.6 ± 1.3	44.1 ± 11.1	39.5 ± 8.2	118.2 ± 1.8
Coumarin	C	2.2 ± 0.4	2.5 ± 0.1	4.4 ± 0.4	0.5 ± 0.2	nd	4.7 ± 1.4	0.9 ± 0.02	3.1 ± 0.4	1.2 ± 0.5	3.0 ± 0.9
	PM9	2.7 ± 0.2	2.6 ± 0.4	3.6 ± 0.9	nd	2.7 ± 1.0	4.0 ± 1.1	17.2 ± 1.6	5.8 ± 0.5	1.9 ± 0.3	13.7 ± 0.2
	F		1.0 ± 0.01	2.3 ± 0.6	nd	nd		4.2 ± 0.1	4.8 ± 2.7	1.5 ± 0.3	6.2 ± 1.0
	PM9 + F		3.4 ± 0.7	2.5 ± 0.5	nd	3.4 ± 0.5		4.8 ± 0.7	6.2 ± 0.7	3.0 ± 2.0	4.5 ± 1.3

nd not detected

[a] C, control; PM9, *Streptomyces* sp. PM9; F, pathogenic fungus B. *cinerea*; PM9 + F, elicited plants with PM9 and challenged with *B. cinerea*. Quantifications were performed 4 days after inoculation with *Streptomyces* PM9 (BS) and at 1, 3, 9 and 15 days post-inoculation (dpi) with *B. cinerea*

microorganisms. Chlorogenic acid was found in 1.1 mg g^{-1} FW in roots and shoots of *E. grandis*, while in *E. globulus*, its concentration reached 3 and 3.7 mg g^{-1} -FW, respectively. Levels of gallic acid were 1.1 and 3.1 mg g^{-1} FW in roots and shoots of *E. grandis*, respectively; this compound was only detected in shoots of *E. globulus* (8.3 mg g^{-1} FW).

Four days after inoculation with *Streptomyces* (BS), the phenolic profile changed for both species. In the roots of *E. grandis,* a decrease on 2-hydroxybenzoic acid and coumarin was detected, whereas the presence of benzoic acid and coumarin was first observed on shoots (Table 2). In roots of *E. globulus*, however, gallic acid, 2-hydroxybenzoic acid and caffeic acid were induced when compared to the control treatment, although no differences were observed in shoots among the treatments (Table 3).

Several changes in the phenolic profile were detected when plants were treated with *Streptomyces* and challenged with *B. cinerea*. Roots of *E. grandis* plants (PM9 + F) showed production of catechin at 1 and 3 dpi, and an increase of coumarin at 3 dpi when compared to F and PM9 treatments (Table 2). Overall, roots of *E. grandis* plants from treatment F showed the highest concentrations of chlorogenic acid, gallic acid and caffeic acid at 1 dpi (Table 2). All phenolics, with exception of benzoic acid, were abundant at 9 dpi in plants from the same treatment (Table 2). In roots of *E. globulus,* little variation was observed in phenolic compounds (Table 3). However, benzoic acid, catechin and coumarin had their synthesis induced in PM9 + F plants at 1 dpi. Chlorogenic acid and coumarin were detected in highest concentrations in PM9 and PM9 + F roots at 15 dpi (Table 3). Shoots of both species also showed variation on either the presence or concentration of specific phenolics (Tables 2, 3). In *E. grandis*, catechin and coumarin was significantly accumulated in PM9 + F-shoots at 1 dpi. Moreover, shoots from PM9 and PM9 + F plants showed increase in caffeic acid concentration at 1 dpi. In PM9 + F *E. globulus* shoots, 2-hydroxybenzoic acid was accumulated in higher concentration than the control plants along the period of culture. The highest catechin concentration was detected in PM9-treated plants, followed by F and PM9 plants at 15 dpi (Table 3).

Discussion

The ability and performance of *Streptomyces* species in promoting plant development are unquestionable, either directly by the production of phytohormones, or indirectly by antagonizing plant pathogens. The indirect effect is commonly related to production of siderophores, antibiotics, β-1-3-glucanase, chitinase, fluorescent pigments and cyanide (Pal et al. 2001), as well as to promoting ISR against a number of plant diseases (Jetiyanon and Kloeppe 2002). PGPRs, including *Bacillus* spp. (Lin et al. 2014), *Streptomyces* spp. (Zhao et al. 2012; Kurth et al. 2014) and *Pseudomonas* spp. (Ran et al. 2005) have been previously used to control several antagonistic microorganisms.

Beneficial rhizobacteria, as PGPRs, have been reported as mediators for reducing disease severity and enhancing yields of many crops (Murphy et al. 2000; Kim et al. 2014). Increasing levels of defense-related enzymes during early stages of defense play a crucial role in plant host resistance (Jain and Choudhary 2014; Kurth et al. 2014) and is usually linked to responses including cell-wall reinforcement (Mandal and Mitra 2007) and production of secondary metabolites (Yedidia et al. 2001; Kurth et al. 2014).

The indirect mode of action of *Streptomyces* sp. PM9 on plants of *E. grandis* and *E. globulus* was determined by the analysis of components of secondary metabolism and disease evaluation. Enzymatic alterations (PPO and POX) occurred in both roots and shoots when plants were treated with *Streptomyces* sp. or challenged with *B. cinerea*. Furthermore, plant responses on roots and shoots treated with microorganisms were also different in strength and time. Roots are the organs establishing the first contact with the microorganism, and therefore their biochemical responses are expected to be the strongest at the time of colonization. It is noteworthy that the similarity of response between plants inoculated with *Streptomyces* sp. and not challenged with the pathogen (PM9), and those elicited and challenged (PM9 + F) at several time points indicates that *Streptomyces* sp. PM9 raised the basal activity levels of defense-related enzymes and secondary metabolites both locally and systemically. Although the levels of enzymatic activities had varied between the species studied, and *E. grandis* showed the lowest levels of basal activities when compared to *E. globulus*, the increase in the enzymes activity compared to the basal level has to be taken in consideration. In this regard, *E. grandis*-treated plants showed the earliest response against *B. cinerea*. Moreover, the combination of changes in enzymatic activity and disease delay suggests that defense response in *Eucalyptus* sp. is being mediated by *Streptomyces* sp. PM9. Studies have reported systemic response elicited by streptomycetes against *Colletotrichum gloeosporioides* (anthracnose) in pepper and cherry tomato (Kim et al. 2014), against *C. musae* in banana (Taechowisan et al. 2009), and against oak powdery mildew (Kurth et al. 2014).

Changes in secondary metabolism are often evidenced in plant defense responses. Induction of defense enzymes increases plant resistance against pathogen invasion (van Loon et al. 1998). Peroxidases are key enzymes in plant defense, since they promote oxidation of phenolic compounds using H_2O_2 as an electron donor for the reaction

(Zámocky et al. 2001), and are involved in the biosynthesis of lignin, which plays a direct role in mechanical protection against pathogens by fortification of the cell wall (Mandal and Mitra 2007). PGPRs were shown to induce POX in various species, such as cucumber (Chen et al. 2000), tomato (Ramamoorthy et al. 2002) and soybean (Jain and Choudhary 2014). Although the generation of reactive oxygen species (ROS) has been related to plant defense by causing hypersensitivity response and plant cell death, these molecules facilitate root colonization by necrotrophic fungi, such as *B. cinerea* (van Kan 2006; Asselbergh et al. 2007). The low POX activity in *E. grandis* and *E. globulus* roots from fungus-infected plants (F) along the time of culture is likely due to the susceptibility of eucalyptus to *B. cinerea*. However, when activated early, POX plays an important role in plant resistance against *B. cinerea* (Małolepsza 2006; Senthilraja et al. 2013). This forehand response was observed in roots and shoots of *E. grandis* PM9-treated and challenged plants (PM9 + F) at 1 and 3 dpi of *B. cinerea*, respectively. However, in PM9 + F roots of *E. globulus*, POX activity was reduced when compared to the control and F treatment. Moreover, significant POX activation in the shoots was delayed to 9 dpi, indicating a later systemic response. Oxidation of phenolic compounds by POX may be related to the production of lignin in elicited plants, which is a well-known defense response against fungi (Ramamoorthy et al. 2001).

Polyphenol oxidases oxidize a broad group of phenolic compounds without H_2O_2 and are involved in the oxidation of polyphenols into quinones (antimicrobial compounds) and lignification of plant cells during the microbial invasion (Lattanzio et al. 2006). Due to this property, this enzyme is reported to play a role in disease resistance (Li and Steffens 2002; Mohammadi and Kazemi 2002). Chen et al. (2000) demonstrated that PPO is involved in ISR mediated by PGPR in cucumber. In *E. grandis* shoots, the activation of PPO in plants infected with *B. cinerea* at 3 and 9 dpi was coincident with reduced concentrations of total phenolic compounds. This enzyme might be metabolizing phenolics to produce toxic molecules against fungus infection. Nevertheless, later at 9 dpi, roots from PM9 + F plants showed significantly increased PPO activity from those plants infected with the fungus (F), which, combined with a decrease of phenolic compounds, suggests that *Streptomyces* sp. PM9 plays a role in modulating the metabolism against *B. cinerea*. In *E. globulus*, activation of PPO was observed to occur earlier than in *E. grandis* in PM9 + F plants. Activity of PPO showed a peak at 1 and 9 dpi in roots and shoots, respectively.

Plants of *E. grandis* and *E. globulus* showed differences in the production of phenolic compounds. In non-treated roots of *E. globulus* (B), levels of phenolic compounds were higher than observed in *E. grandis*. In contrast, during contact with microorganisms (either PM9-treatment or challenging), production of phenolics in *E. grandis* reached 9 mg g^{-1} FW in F plants, while in *E. globulus* the phenolic content in roots did not exceed 4.6 mg g^{-1} FW. In shoots of both species, the accumulation of phenolics differed from the control in all treatments until at least 3 dpi. Moreover, PM9- and PM9 + F plants also respond with increased production of phenolics in comparison to F plants up to 3 and 9 dpi in *E. globulus* and *E. grandis*, respectively. This result strengthens the hypothesis of a systemic response in these plants.

Different from phenolics, quercetin flavonoids increased 4 days after inoculation with PM9 (BS) in roots and shoots of *E. globulus* which may suggest a response to the interaction between plant and rhizobacterium. Roots of PM9 + F plants contained more flavonoids than the fungus-infected plants in *E. globulus* and *E. grandis* at 1 and 3 dpi, respectively. Shoots of *E. globulus* also respond to PM9 + F treatment showing increase in flavonoid concentrations at 1 and 3 dpi. Certain flavonoids may influence the association with PGPRs and are also involved in host defense against pathogens, exhibiting antifungal properties and acting as phytoalexins (Jeong et al. 2014). At 9 dpi, levels of flavonoids increased in the PM9 + F-shoots of *E. grandis* plants, which at this time had not showed disease symptoms yet. In *E. globulus*, at the same time point, the flavonoids in the PM + F-shoots decreased from 9 to 15 dpi, and plants showed disease symptoms at 10 dpi of *B. cinerea*. It is likely that flavonoids are playing a role as antagonistic compounds against *B. cinerea* in *E. grandis* and not in *E. globulus* plants.

The individual phenolic compounds showed variation among treatments during the culture period. Treatment with *Streptomyces* induced synthesis at phenolic compounds that were absent in the plants before contact with the rhizobacteria, indicating the modulation of phenylpropanoid metabolism after plant–microbe interaction. Otherwise, some interesting reduction on 2-hydroxybenzoic and coumarin levels was detected on the BS-roots of *E. grandis*, which might be a result of the colonization process, since these phenolics are known as antimicrobial molecules, and their reduced level would allow a more efficient interaction between plant and *Streptomyces*. In PM9 + F plants of both species, most of the phenolics were produced or accumulated at 1 and 3 dpi. Catechin was present in roots of PM9 + F-*E. grandis* plants during all time points assayed, while in fungus-treated plants this compound appeared from 9 dpi onwards. In *E. globulus* roots, catechin was scarcely detected. In *E. grandis* shoots, the early accumulation of catechin was also observed in BS and in PM9 + F at 1 dpi, although in *E. globulus* the differential production of this phenolic to occur later in the culture (9 and 15 dpi). The early induction of this

compound in *E. grandis* may be related to the best efficiency on decreasing disease in these plants, since catechin is known to present antibacterial and antifungal properties and its biosynthesis and accumulation was related to defense responses in leaves of wheat upon attack by *Puccinia triticina* (Ghassempour et al. 2010). Benzoic acid was produced in shoots of *E. grandis* in response to PM9 or PM9 + F at BS and 1 dpi. Otherwise, 2-hydroxybenzoic acid was detected in shoots under PM9 + F treatment at 1 and 3 dpi. In *E. globulus*, 2-hydroxybenzoic acid was only increased at 1 and 15 dpi, in PM9 and F-shoots, respectively. Low-molecular-weight phenols such as benzoic acids and other phenylpropanoids are also formed in the initial response to infection (Niemann et al. 1991), and *p*-hydroxybenzoic acid was involved in the initial defense reactions of *Phoenix dactylifera* to brittle leaf disease (Latreche and Rahmania 2010). Indeed, *p*-hydroxybenzoic acid, the salicylic acid analog, is known to function as a phytoalexin. Its accumulation in plants is associated with antimicrobial activity and fungitoxicity (Chong et al. 2009). Its role in defense may be related to the increase of 2-hydroxybenzoic acid in PM9 + F *E. grandis* plants. In *E. grandis*, caffeic acid was evident in roots of fungus-infected plants at 1 dpi and in PM9 + F plants at 3 dpi, and might be prone to oxidation into *o*-quinones, which are toxic to microorganisms (Lattanzio et al. 2006). In *E. globulus,* this compound was not detected in roots of F plants. Although some specific phenolic compounds such as 2-hydroxybenzoic acid, caffeic acid and gallic acid had been induced in roots of *E. globulus*, they are not necessarily related to defense, and might have been produced in response to plant-microorganism interaction.

Different responses against *B. cinerea* were seen in the *Eucalyptus* plants, and disease symptoms were significantly delayed in *E. grandis*. This response coincided with the biochemical variations recorded for both enzymatic activity and phenolic compounds. Basal levels of secondary metabolism in *E. globulus* were overall higher than in *E. grandis*. On the other hand, when *E. grandis* roots were inoculated with *Streptomyces* and challenged with the pathogen, POX was activated before disease was established and the enzymatic levels were maintained up to 15 dpi. Differently, in roots of *E. globulus* an increase of POX activity was only detected at 1 dpi when compared to control plants. Likewise, POX activity in shoots of *E. grandis* increased at 3 and 9 dpi, indicating the systemic action of *Streptomyces* in *Eucalyptus* plants. PPO activity was less significant as part of plant response to *Streptomyces,* since few time points showed some alteration in enzyme activity, either in roots or shoots. These results indicate that efficient modulation of secondary metabolism in *E. grandis* took place, reducing and delaying the development of gray mold. Although some alteration was observed in the secondary metabolism of *E. globulus*, the influence of *Streptomyces* was less effective.

The reduction in the severity of gray mold disease in *E. grandis* might be the consequence of activation of systemic defense machinery induced by *Streptomyces* sp. PM9. In conclusion, *Streptomyces* sp. PM9 was able to elicit plants of *E. grandis*, increasing the basal levels of two enzymes (PPO and POX) that are directly related to disease response, as well as promoting the synthesis of specific phenolic compounds as a part of defense strategies. Our results showed that *Streptomyces* sp. PM9 is a candidate for using as a biological control agent against *B. cinerea* in the early cultivation of *E. grandis*. Further studies will be carried out to determine the period of effectiveness of *Streptomyces* sp. PM9 in vivo, and to test other pathosystems, since *B. cinerea* is such an aggressive necrotrophic fungus.

Author contribution statement TDS and ERS conceived and designed the research. TDS performed the experiments. LVA contributed to data analyses and discussion of the results. The manuscript was written by TDS and ERS and approved by LVA.

Acknowledgments Authors thank Dr Hans-Peter Fiedler and Dr Rüdiger Hampp from Tübingen University, Germany, for providing the rhizobacteria isolates, to Suzano Papel e Celulose for providing the Eucalyptus seeds and to Janaina Belquis da S. P. Langois for technical assistance. This work was supported by PROBOLSAS-PUCRS (fellowship of first author) and by the National Council for Scientific and Technological Development (CNPq)/Brazil through the Program for Undergraduate Students.

References

Alizadeh O, Azarpanah A, Ariana L (2013) Induction and modulation of resistance in crop (arbuscular mycorrhiza) and hormonal elicitors and plants against disease by bioagent fungi plant growth promoting bacteria. Int J Farming Allied Sci 2:982–998

Ashraf MA, Asif M, Zaheer A, Malik A, Ali Q, Rasool M (2013) Plant growth promoting rhizobacteria and sustainable agriculture: a review. Afr J Microbiol Res 7:704–709

Asselbergh B, Curvers K, França SC, Audenaert K, Vuylsteke M, Breusegem FV, Höfte M (2007) Resistance to *Botrytis cinerea* in *sitiens*, an abscisic acid-deficient tomato mutant, involves timely production of hydrogen peroxide and cell wall modifications in the epidermis. Plant Physiol 144:1863–1877

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Chen C, Belanger RR, Benhamou N, Paulitz TC (2000) Defense enzymes induced in cucumber roots by treatment with plant growth promoting rhizobacteria (PGPR) and *Pythium aphanidermatum*. Physiol Mol Plant Pathol 56:13–23

Chong KP, Rossall S, Atong M (2009) In vitro fungitoxicity and antimicrobial activity of syringic acid, caffeic acid and 4-hydroxybenzoic acid against *Ganoderma boninense*. J Agric Sci 1:15–20

Conn VM, Walker AR, Franco CMM (2008) Endophytic actinobacteria induce defense pathways in *Arabidopsis thaliana*. Mol Plant Microbe Interact 21:208–218

Dean R, Di Pietro A, Kahmann R, van Kan JAL, Spanu PD, Ellis J, Foster GD, Pretorius ZA (2012) The top 10 fungal pathogens in molecular plant pathology. Mol Plant Pathol 13:414–430

FAO (2001) Mean annual volume increment of selected industrial forest plantation species. In: Ugalde L, Pérez O (eds) Forest plantation thematic papers, Working paper 1. Forest Resources Development Service, Forest Resources Division. FAO, Rome

Ghassempour A, Mollayi S, Farzaneh M, Sharifi-Tehrani A (2010) Variation of catechin, epicatechin and their enantiomers concentrations before and after wheat cultivar-*Puccinia triticina* infection. Food Chem 125:1287–1290

Gopalakrishnan S, Pande S, Sharma M, Humayun P, Kiran BK, Sandeep D, Vidya MS, Deepthi K, Rupela O (2011) Evaluation of actinomycete isolates obtained from herbal vermicompost for biological control of *Fusarium* wilt of chickpea. Crop Prot 30:1070–1078

Graichen FAS, Martinelli JA, Federizzi LC, Pacheco MT, Chaves MS, Wesp CL (2010) Inheritance of resistance to oat crown rust in recombinant inbred lines. Sci Agric 67:435–440

Jain S, Choudhary DK (2014) Induced defense-related proteins in soybean (*Glycine max* L. Merrill) plants by Carnobacterium sp. SJ-5 upon challenge inoculation of *Fusarium oxysporum*. Planta 239:1027–1040. doi:10.1007/s00425-014-2032-3

Jeong SW et al (2014) Variation in flavonoid levels in *Citrus benikoji* Hort. ex. Tan. infected by *Colletotrichum gloeosporioides*. Food Chem 148:284–288

Jetiyanon K, Kloeppe JW (2002) Mixtures of plant growth-promoting rhizobacteria for induction of systemic resistance against multiple plant diseases. Biol Control 24:285–291

Kim HJ, Lee EJ, Park SH, Lee H, Chung N (2014) Biological control of anthracnose (*Colletotrichum gloeosporioides*) in pepper and cherry tomato by *Streptomyces* sp. A1022. J Agric Sci 6:54–62

Kloepper JW, Schroth MM, Miller TD (1980) Effects of rhizosphere colonization by plant growth-promoting rhizobacteria on potato plant development and yield. Phytopatology 70:1078–1082

Kurth F, Mailänder S, Bönn M, Feldhahn L, Herrmann S, Große I, Buscot F, Schrey SD, Tarkka MT (2014) *Streptomyces*-induced resistance against oak powdery mildew involves host plant responses in defense, photosynthesis, and secondary metabolism pathways. Mol Plant Microbe Interact 27:891–900

Latreche K, Rahmania F (2010) GC/MS screening of volatile compounds profiles from healthy and brittle leaf disease (BLD) affected date palm leaves (*Phoenix dactylifera* L.): a comparative study. Acta Hortic 882:903–908. doi:10.17660/ActaHortic.882.105

Lattanzio V, Lattanzio VMT, Cardinali A (2006) Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. In: Imperato F (ed) Phytochemistry: advances in research. Research Signpost, Kerala, pp 26–67

Lee SY, Tindwa H, Lee YS, Naing KW, Hong SH, Nam Y, Kim KY (2012) Biocontrol of anthracnose in pepper using chitinase, β-1, 3 glucanase, and 2-furancarboxaldehyde produced by Streptomyces cavourensis SY224. J Microbiol Biotechnol 22:1359–1366

Lehr NA, Schrey SD, Hampp R, Tarkka MT (2008) Root inoculation with a forest soil streptomycete leads to locally and systemically increased resistance against phytopathogens in Norway spruce. New Phytol 177:965–976

Leyronas C, Duffaud M, Pàres L, Jeannequin B, Nicot PC (2015) Flow of *Botrytis cinerea* inoculum between lettuce crop and soil. Plant Pathol 64:701–708

Li L, Steffens JC (2002) Overexpression of polyphenol oxidase in transgenic tomato plants results in enhanced bacterial disease resistance. Planta 215:239–247

Lin Y, Du D, Si C, Zhao Q, Li Z, Li P (2014) Potential biocontrol *Bacillus* sp. strains isolated by an improved method from vinegar waste compost exhibit antibiosis against fungal pathogens and promote growth of cucumbers. Biocontrol 71:7–15

Małolepsza U (2006) Induction of disease resistance by acibenzolar-S-methyl and *o*-hydroxyethylorutin against *Botrytis cinerea* in tomato plants. Crop Prot 25:956–962

Mandal S, Mitra A (2007) Reinforcement of cell wall in roots of *Lycopersicon esculentum* through induction of phenolic compounds and lignin by elicitors. Physiol Mol Plant Pathol 71:201–209

Mohammadi M, Kazemi M (2002) Changes in peroxidase and polyphenol oxidase activities in susceptible and resistant wheat heads inoculated with *Fusarium graminearum* and induced resistance. Plant Sci 162:491–498

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

Murphy JF, Zehnder GW, Schuster DJ, Sikora EJ, Polstan JE, Kloepper JW (2000) Plant growth-promoting rhizobacteria mediated protection in tomato against tomato mottle virus. Plant Dis 84:779–784

Myburg AA, Grattapaglia D, Tuskan GA, Hellsten U, Hayes RD et al (2014) The genome of *Eucalyptus grandis*. Nature 510:356–362

Nair MG, Chandra A, Thorogood DL (1994) Gopalamicin, an antifungal macrolide produced by soil actinomycete. J Agric Food Chem 42:2308–2310

Niemann GH, van der Kerk A, Niessen MA, Versluis K (1991) Free and cell wall-bound phenolics and other constituents from healthy and fungus infected carnation (*Dianthus caryophilus* L.) stems. Physiol Mol Plant Pathol 38:417–432

Pal KK, Tilak KVB, Saxena AK, Dey R, Singh S (2001) Suppression of maize root disease caused by *Macrophomina phaseolina*, *Fusarium moniliforme* and *Fusarium graminearum* by plant growth-promoting rhizobacteria. Microbiol Res 156:209–223

Palaniyandi SA, Yang SH, Zhang L, Suh J-W (2013) Effects of actinobacteria on plant disease suppression and growth promotion. Appl Microbiol Biotechnol 97:9621–9636

Postma J, Montanari M, van den Boogert PHJF (2003) Microbial enrichment to enhance the disease suppressive activity of compost. Eur J Soil Biol 39:157–163

Ramamoorthy V, Viswanathan R, Raguchander T, Prakasam V, Samiyappan R (2001) Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and diseases. Crop Prot 20:1–11

Ramamoorthy V, Raguchander T, Samiyappan R (2002) Induction of defense-related proteins in tomato roots treated with *Pseudomonas fluorescens* Pf1 and *Fusarium oxysporum* f. sp. *lycopersici*. Plant Soil 239:55–68

Ran LX, Li NZ, Wu GJ, van Loon LC, Bakker AHM (2005) Induction of systemic resistance against bacterial wilt in *Eucalyptus urophylla* by fluorescent *Pseudomonas* spp. Eur J Plant Pathol 113:59–70

Salla TD, Silva TR, Astarita LV, Santarém ER (2014) *Streptomyces* rhizobacteria modulate the secondary metabolism of *Eucalyptus* plants. Plant Physiol Biochem 85:14–20

Sartor T, Xavier VB, Falcão MA, Mondin CA, Santos MA, Cassel E, Astarita LV, Santarém ER (2013) Seasonal changes in phenolic compounds and in the biological activities of *Baccharis dentata* (Vell.) G.M. Barroso. Ind Crop Prod 51:355–359

Schrey SD, Tarkka MT (2008) Friends and foes: streptomycetes as modulators of plant disease and symbiosis. Antonie Van Leeuwenhoek 94:11–19

Senthilraja G, Anand T, Kennedy JS, Raguchander T, Samiyappan R (2013) Plant growth promoting rhizobacteria (PGPR) and entomopathogenic fungus bioformulation enhance the expression of defense enzymes and pathogenesis-related proteins in groundnut plants against leafminer insect and collar rot pathogen. Physiol Mol Plant Pathol 82:10–19

Shirling EB, Gottlieb D (1966) Methods for characterization of *Streptomyces* species. Int J Syst Bacteriol 16:313–340

Taechowisan T, Chuaychot N, Chanaphat S, Wanbanjob A, Tantiwachwutikul P (2009) Antagonistic effects of *Streptomyces* sp. SRM1 on *Colletotrichum musae*. Biotechnology 8:86–92

Teixeira DA, Alfenas AC, Mafia RG, Maffia LA, Ferreira EM (2005) Evidências de indução de resistência sistêmica à ferrugem do eucalipto mediada por rizobactérias promotoras do crescimento. Fitopatol Bras 30:350–356

van Kan JAL (2006) Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends Plant Sci 11:247–253

van Loon LC (1997) Induced resistance in plants and the role of pathogenesis-related proteins. Eur J Plant Pathol 103:753–765

van Loon LC, Bakker PAHM, Pieterse CMJ (1998) Systemic resistance induced by rhizosphere bacteria. Annu Rev Phytopathol 36:453–483

Yedidia I, Srivastva AK, Kapulnik Y, Chet I (2001) Effect of *Trichoderma harzianum* on microelement concentrations and increased growth of cucumber plants. Plant Soil 235:235–242

Zambonato F, Federizzi LC, Pacheco MT, Arruda MP, Martinelli JA (2012) Phenotypic and genetic characterization of partial resistance to crown rust in *Avena sativa* L. Crop Breed Appl Biotechnol 12:261–268

Zámocky M, Regelsberger G, Jakopitsch C, Obinger C (2001) The molecular peculiarities of catalase-peroxidases. FEBS Lett 492:177–182

Zhao S, Du CM, Tian CY (2012) Suppression of *Fusarium oxysporum* and induced resistance of plants involved in the biocontrol of cucumber *Fusarium* wilt by *Streptomyces bikiniensis* HD-087. World J Microbiol Biotechnol 28:2919–2927