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PRODUÇÃO DE ÁCIDO 3-INDOLACÉTICO, POTENCIAL RIZOGÊNICO E INDUÇÃODE RESPOSTAS DE DEFESA POR *STREPTOMYCES* SP. EM PLANTAS DE EUCALIPTO PARA O CONTROLE DE *BOTRYTIS CINEREA*

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RESUMO

O eucalipto é uma espécie lenhosa economicamente importante, destacando-se como matéria-prima em diversos setores industriais. O Brasil ocupa a segunda posição mundial em área plantada, totalizando aproximadamente três milhões de hectares. As espécies de eucalipto são muito suscetíveis a patógenos como *Botrytis cinerea* (mofo-cinzento), o qual leva à mortalidade de estacas em fase de enraizamento. O controle biológico de doenças em plantas utilizando microrganismos do solo tem sido considerado uma alternativa para reduzir o uso de agroquímicos e o ataque de patógenos. Rizobactérias promotoras de crescimento vegetal podem agir diretamente no desenvolvimento das plantaspela produção de fitormôniosou indiretamente, como antagonistas a fungos patogênicos, além de causar alterações no metabolismo secundário, com consequente indução de resistência sistêmica. Neste trabalho, avaliou-se a ação direta no desenvolvimento vegetal de isolados rizobacterianos do gênero *Streptomyces* através da produção de auxinas e potencial rizogênico de *Eucalyptus grandis* e *E. globulus*, bem como oefeito modulador no metabolismo secundárioe a indução de resistência sistêmica em plantas eliciadas com *Streptomyces* sp. e desafiadas com o fungo patogênico *B. cinerea*. As respostas metabólicas foram avaliadas através das atividades de enzimas realacionadas à defesa vegetal (PPO e POX) e dos compostos secundários induzidos (compostos fenólicos totais e fração flavonóides quercetínicos). A incidência e progressão da doença mofo cinzento em plantas eliciadas com *Streptomyces* sp. PM9, e cocultivo destes microrganismos (*Streptomyces* e *B.cinerea*) também foram avaliados. Os isolados de *Streptomyces*sp.PM5 e PM9 apresentaram maior produção de auxina que os demais isolados testados. *Streptomyces* sp.PM9 apresentou o maior potencial rizogênico em plantas de *Eucalyptus* sp. emodulou o metabolismo secundário destas plantas. Oantagonismo deste isolado sobre *B. cinerea* foi evidenciado.As plantas eliciadas com *Streptomyces* sp. PM9 e desafiadas com *B. cinerea*apresentaram alterações nas

enzimas PPO e POXe nos níveis de compostos fenólicos totais em diferentes tempos de análise, as quais foram relacionadas à resposta inicial de defesa.Os compostos fenólicos ácido gálico e clorogênico foram, em média, os mais abundantes, embora os ácidos cafeico e benzoico e a catequina tenham sido induzidos em momentos específicos. O retardo no estabelecimento da doença foi significativo em plantas de *E. grandis* eliciadas com *Streptomyces*. Os resultados de indução de resistência, retardo da doença eantagonismocontra *B.cinerea*, demonstram a capacidade de ação de *Streptomyces*sp. PM9 como indutor de resistência sistêmica vegetal, colocando este microrganismo como potencial candidato aos programas de controle biológico em viveiros de mudas de *Eucalyptus*. A interação da planta de eucalipto com a rizobactéria, bem como a modulação dos mecanismos de defesa podem contribuir para o estabelecimento de novas estratégiasde biocontrole aplicado à silvicultura.

Palavras chaves: Biocontrole, Metabolismo secundário, Peroxidases, Resistência sistêmica induzida.

ABSTRACT

Eucalyptus is an economically important woody species, especially as a raw material in many industrial sectors. Brazil ranks the second worldwide position in acreage, totalizing approximately three million hectares. Eucalyptus species are very susceptible to pathogens such as *Botrytis cinerea* (gray mold), which leads to mortality of cuttings in rooting phase. Biological control of plant diseases using soil microorganisms has been considered an alternative to reduce the use of pesticides and pathogen attack. Plant growth promoting rhizobacteria can act directly on plant development for production of phytohormones or indirectly as antagonists to pathogens, as well as promote changes in secondary metabolism, and hence inducing of systemic resistance. In this study, the direct roleof *Streptomyces* isolates in plant development was evaluated through the production of auxin and rhizogenic potential in *Eucalyptus grandis* and *E. globulus* plants, as well as indirectly, by modulation of the secondary metabolism, and induction of sistemic resistence in plantselicited with *Streptomyces* sp. and challenged with the pathogenic fungus *B. cinerea*. Metabolic responses were evaluated throughactivity of plant defense enzymes (PPO and POX) and induced secondary compounds (total phenolics and quercetinic-flavonoids fraction). The incidence and progression of gray mold disease on plants elicited *Streptomyces* sp. PM9, and coculture of these microorganisms (*Streptomyces* and *B. cinerea*) were also evaluated. *Streptomyces* sp. PM5 and PM9 isolates produced more auxin than the other isolates tested. *Streptomyces* sp. PM9 showed the highest rhizogenic potential on *Eucalyptus* sp. and modulated secondary metabolism of these plants. Antagonism of this isolated over *B. cinerea* was evidenced. Plants elicited with *Streptomyces* sp. PM9 and challenged with *B. cinerea* showed changes in PPO and POX enzymes and levels of phenolic compounds at different time points of analysis, which may be related to initial defense response. Phenolic compounds chlorogenic acid and gallic acid were, on average, the most abundant.

while caffeic acid, benzoic acidand catechin were induced at specific time points. A delay in the onset of disease was significant in plants of *E. grandis* elicited with *Streptomyces*. The induction of resistance, disease delay and antagonism against *B. cinerea*indicate the capacity of *Streptomyces* sp. PM9 as an inducer of plant systemic resistance, and poses this microorganism as a potential candidate for biological control programs in nurseries of *Eucalyptus*. Interaction of rhizobacteria with eucalyptus plant, as well as the modulation of defense mechanisms may contribute to the establishment of new biocontrol strategies applied to forestry.

Keywords: Biocontrol, Induced systemic resistance, Peroxidases, Secondary metabolism.

LISTA DE ABREVIATURAS

AIA -Ácido 3-indolacético

EROs - Espécies reativas de oxigênio;

ETI - Imunidade desencadeada por efetores;

ISR - Resistência sistêmica induzida;

MAMPs - Padrões moleculares associados a microrganismos;

PAMPs - Padrões moleculares associados a patógenos;

PAL - Fenilalanina amônia liase;

PGPRs - Rizobactérias promotoras de crescimento vegetal;

POX – Peroxidase;

PPO - Polifenol oxidase;

PRRs - Receptores de reconhecimento de padrões;

PRs - Proteínas relacionadas à patogenicidade;

PTI - Imunidade desencadeada por PAMPs;

SAR - Resistência sistêmica adquirida;

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Capítulo I

INTRODUÇÃO E OBJETIVOS

1. INTRODUÇÃO

1.1. Importância econômica de *Eucalyptus spp.*

O gênero *Eucalyptus*, pertencente à família *Myrtaceae*, é conhecido por sua ampla variedade genética, englobando mais de 900 espécies. É originário da Austrália, de clima temperado e subtropical, mas de fácil adaptação a outras condições climáticas (1). Considerado uma espécie lenhosa economicamente importante, destaca-se como matéria-prima em diversos setores industriais. O Brasil ocupa a segunda posição mundial em área plantada, totalizando aproximadamente três milhões de hectares. A madeira oriunda das plantações de eucalipto é utilizada para produção de chapas, lâminas, compensados, aglomerados, carvão vegetal, madeira serrada, celulose, móveis, além de óleos essenciais e mel (2, 3). *Eucalyptus globulus* e *E. grandis* predominam na região sul do Brasil e estas espécies e seus híbridos *E. urophylla*, *E. viminalis e E. dunnii* são cultivados em diferentes regiões do mundo, principalmente para obtenção de madeira e celulose (4).

Mudas até árvores adultas de várias espécies de *Eucalyptus* são alvo constante de uma ampla gama de patógenos, principalmente fungos. A intensidade da doença depende da espécie atacada e da época do ano, e resulta em impactos econômicos significativos.

1.2. Fungo patogênico *Botrytis cinerea*

Os fitopatógenos são classificados de acordo com o modo de infecção, podendo ser biotróficos, necrotróficos ou hemibiotróficos. Os biotróficos têm como característica viver dentro de tecidos do hospedeiro, sem causar a morte, ao contrário dos necrotróficos, que causam

morte celular (5). Entre os fitopatógenos que atacam as espécies de *Eucalyptus* destaca-se *Botrytis cinerea* (mofo cinzento), fungo necrotrófico típico(6). *B. cinerea* ocupa o segundo lugar entre os fungos fitopatogênicos, característica atribuída devido à ampla gama de hospedeiros, podendo infectar, além de *Eucalyptus* spp. (Figura 1A), mais de 200 espécies de plantas. Por esse motivo, causa severos danos pré e pós-colheita e apresenta grande impacto em muitos agrossistemas (7).

O mofo-cinzento está entre as principais doenças em viveiros florestais de *Eucalyptus* spp.na região sul do Brasil, sendo encontrado facilmente em canteiros com alta densidade de mudas (700 mudas/m^2), sob condições de alta umidade (acima de 70%) e temperaturas amenas (outono e inverno). A infecção se dá pelo desenvolvimento dos conídios, que podem invadir a célula vegetal por pressão ou através do poro estomático. Durante o curso de infecção, o fungo promove a morte celular programada no hospedeiro através da secreção de moléculas tóxicas e enzimas líticas, que, posteriormente, consomem tecidos de plantas para o seu crescimento (7). Inicialmente, as folhas apresentam-se enroladas, e em seguida secam e caem. As partes afetadas apresentam características acinzentadas, coloração atribuída às estruturas assexuadas deste patógeno (conídios e conidiósporos), utilizadas para dispersão na natureza (Figura 1B). A doença se caracteriza por afetar mudas e tecidos jovens da parte aérea, causando morte da parte apical ou da planta como um todo. Este patógeno causa grandes infestações e leva à mortalidade de estacas e microestacas de plantas em fase de enraizamento (8,9).

A infestação por *B. cinerea* pode ocorrer em qualquer fase de desenvolvimento da planta. Por isso, os custos dos danos causados por *Botrytis* são difíceis de estimar. Apesar do uso cada vez mais eficaz do controle biológico em algumas culturas (10), a aplicação de fungicidas continua a ser o método mais empregado para controlar a infecção por este fitopatógeno.

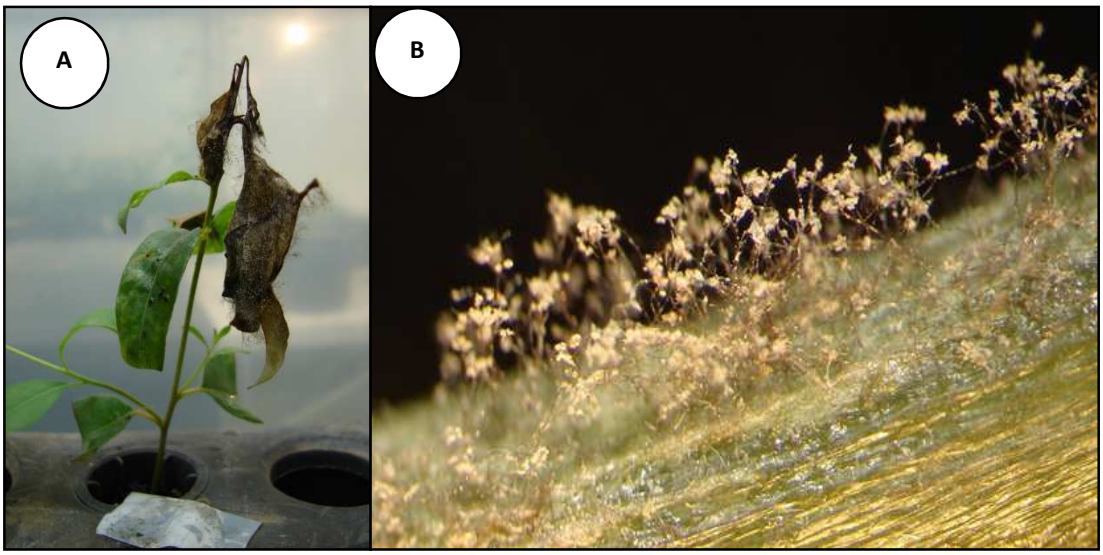

Figura 1: (A) Planta de *Eucalyptus* sp. infectada com o fungo patogênico *Botrytis cinerea*. (B) Partes afetadas da planta apresentando características acinzentadas (conídios e conidióforos). (A) Barra: 1 cm; (B) 40×. Fonte: Banco de imagens do Departamento de Fitosanidade da Faculdade de Agronomia - UFRGS <http://www.ufrgs.br/agrofitossan/galeria/index.asp.> (74).

1.3.Defesa vegetal

As plantas, ao contrário dos mamíferos, não apresentam células móveis de defesa e um sistema imune adaptativo, mas contam com a imunidade inata de cada célula e sinais que emanam de sítios de infecção, podendo desencadear uma resposta sistêmica (11). Em geral, a complexidade e as estratégias encontradas nos patossistemas são decorrentes da coevolução entre planta e patógeno. As plantas respondem aos patógenos através de diferentes mecanismos de resistência, que inclui o reconhecimento, o qual é traduzido em uma resposta apropriada de defesa. São capazes de se defender dos ataques de patógenos de forma efetiva, devido à multiplicidade e eficiência desses mecanismos de defesa, de maneira que, na natureza, a resistência é uma regra e a suscetibilidade, uma exceção (12). Sendo assim, as plantas não permitem de forma passiva a entrada de patógenos. A patogenicidade se dá, principalmente, pela entrada do patógeno, que desarma e suspende as respostas de defesa da planta através da

secreção de moléculas efetoras (virulentas), que permitem o crescimento, reprodução e, por fim, a propagação do patógeno (13).

A imunidade inata é a primeira linha de defesa contra microrganismos invasores em plantas. Padrões moleculares associados a patógenos (PAMPs – do inglês *Pathogen-Associated Molecular Patterns*) são os ativadores clássicos de respostas imunes. Em microrganismos não patogênicos, estes padrões são chamados de MAMPs (MAMPs – do inglês, *Microorganisms-Associated Molecular Patterns*), que são estruturas como flagelina bacteriana, peptideoglicanos e lipopolissacarídeos. Essas estruturas diferenciam-se entre os microrganismos, e estão relacionadas com o modo de infecção e o estilo de vida de cada um (14).

A primeira barreira encontrada pelos patógenos é a parede celular vegetal, onde se encontram os receptores denominados PRR (do inglês, *Pattern Recognition Receptors*). Estes receptores atuam no reconhecimento de MAMPs/PAMPs. A primeira interação entre a planta e o patógeno envolve o reconhecimento das MAMPs através de PRRs, gerando uma resposta que inclui o aumento da síntese de hormônios do estresse, a síntese de compostos antimicrobianos, a produção de espécies reativas de oxigênio (EROs), a deposição de calose na parede celular, a ativação de cascatas de transdução de sinais, a alteração nos níveis hormonais e a indução da expressão de genes de defesa. Esta primeira resposta de defesa formada é denominada de imunidade desencadeada por PAMPs (PTI – do inglês, *PAMP-Triggered Immunity*) ou resistência basal (13).

Mesmo a PTI sendo considerada uma resposta de defesa eficiente, patógenos bem sucedidos (causadores de doença), desenvolveram estratégias de infecção através de novas moléculas efetoras citoplasmáticas (13). As plantas, ao detectarem esses efetores citoplasmáticos, disparam uma resposta denominada de imunidade desencadeada por efetores

(ETI – do inglês, *Effector-Triggered Immunity*), sendo uma versão amplificada da PTI, relacionada a proteínas de resistência (PRs) (15).

As plantas são capazes de integrar sinais de diferentes vias relacionadas à defesa. Os principais sinalizadores da defesa vegetal são o ácido salicílico, ácido jasmônico e etileno. As rotas de sinalização se relacionam, fornecendo às plantas um grande potencial de regulação para uma defesa refinada (16). Um composto, para ser considerado um sinalizador, deve possuir características específicas, como ser sintetizado pela própria planta, aumentar os seus níveis após o ataque de patógenos ou após um tratamento com um indutor, ser móvel pelo floema, induzir a síntese de substâncias de defesa, como PRs, peroxidases, fitoalexinas e aumentar a resistência a patógenos (17,18).

1.3.1.Resistência sistêmica adquirida (SAR) e resistência sistêmica induzida (ISR)

A indução de resistência em plantas envolve a ativação de mecanismos inativos ou latentes na planta. Pode ser induzida pela utilização de indutores bióticos ou abióticos, ocorre de forma inespecífica, por meio da ativação de genes que codificam para diversas respostas de defesa (19). A indução de resistência nas plantas pode ocorrer de forma sistêmica e seu efeito pode ser observado em locais distantes do local de infecção. Esse fenômeno é denominado Resistência Sistêmica Adquirida (SAR – do inglês, *Systemic Acquired Resistance*) (20), e pode resultar na imunização temporária contra futuras infecções e também contra diferentes patógenos. Considera-se que a SAR envolve o acúmulo de PRs e é salicilato-dependente, podendo resultar em alterações visuaisna planta que sofreu a indução. Por outro lado, a resistência sistêmica induzida (ISR – do inglês, *Induced Systemic Resistance*) é definida como o aumento da capacidade de defesa da planta contra amplo espectro de patógenos.

Édesencadeada após um estímulo apropriado (21-23) e baseia-se no reconhecimento de um invasor e em eventos subsequentes de transdução de sinal, que levam à ativação das defesas (24). Diferentemente da SAR, a ISR não envolve o acúmulo de PRs, não promove alterações na planta que sofreu a indução, envolve sinalização porjasmonato e etileno (25) e a participação de microrganismos não patogênicos (26,27).

1.4. Rizobactérias promotoras de crescimento vegetal (PGPRs)

As PGPRs (PGPR - do inglês, *Plant Growth Promoting Rhizobacteria*) são bactérias encontradas na rizosfera, as quais podem agir diretamente no desenvolvimento vegetal através da produção de reguladores de crescimento, do aumento da fixação do nitrogênio e disponibilidade de nitrato, da solubilização do fósforo, da oxidação do enxofre, pelo aumento da permeabilidade das raízes ou pela competição por substrato (28,29).Muitas PGPRs são capazes de produzir ácido indol-3-acético (AIA),hormônio quecontrola a divisão e expansão celular e desempenha um importante papel no desenvolvimento das raízes(30).Desta forma, a produção de AIA é uma importante e eficiente maneira pela qualas rizobactérias podem modular o crescimento de raízes das plantas hospedeiras(31,32). Além disso, as PGPRs atuamna supressão de doenças, produzindo antibióticos, bacteriocinas, enzimas líticas, ácido cianídrico, sideróforos e induzindo resistência sistêmica(33).

O gênero *Streptomyces* (actinobactérias) é considerado por muitos como PGPR, apresentando características típicas desta classe de microrganismos, como não patogenicidade, efeito benéfico no crescimento das plantase supressão de doença (34,35). Este gênero, que compreende 10% do total de microrganismos do solo, apresenta um papel ecológico adaptativo

significativo na rizosfera. Estes microrganismos são capazes de quebrar polímeros biológicos complexos, como quitina e lignina, e apesar de serem bactérias de solo, vários relatos indicam que apresentam associações com plantas e hifas de fungos (36). Existe também a possibilidade destas associações induzirem grande variedade de metabólitos secundários nos vegetais (37). Efeitos como a solubilização de fósforo, a produção de auxina, o aumento da biomassa, a antibiose e a indução de respostas de defesa nas plantasforam relatados após a colonização radicular por *Streptomyces* spp. (29, 34,38,39,40).

O potencial de rizobactérias do gênero *Streptomyces* na promoção de crescimento de plantas foi relatado em eucalipto (41). Estirpes deste gênero aumentaram significativamente o desenvolvimento radicular em plantas de arroz (*Oryza sativa*), milho (*Zea mays*) e*Bruguiera parviflora*, através da ação de auxinas (42,43).

1.4.1.Respostas metabólicas relacionadas à interação com PGPRs

Os mecanismos de defesa vegetal decorrentes da ISR mediada por PGPRs podem ser estruturais ou bioquímicos (44), eresultam de alterações metabólicas relacionadas com mudanças na atividade de enzimas chaves do metabolismo, como as peroxidases (POXs), polifenoloxidases (PPOs) e fenilalanina amônia-liase (PAL).

As peroxidases (POX) pertencem a uma família de enzimas com diversas funções nos vegetais. Participam da oxidação de compostos fenólicos, utilizando o peróxido de hidrogênio (H_2O_2) como doador de elétrons para a reação (45). Esta família de enzimas está envolvida em diversos processos fisiológicos, como na formação de lignina, no metabolismo oxidativo da auxina (oxidação doAIA), na biossíntese de etileno, na cicatrização de ferimentos e regulação do alongamento das células (crescimento e senescência). A lignina parece estar envolvida em

respostas de defesa contra organismos patogênicos (46), atuando como barreira mecânica e interferindo no crescimento de patógenos (12). Embora a lignificação ainda seja pouco estudada, sabe-se que esta síntese é uma resposta de resistência, potencialmente induzida por agentes bióticos e abióticos, e que pode estar intrinsecamente associada à resistência sistêmica induzida (47,48). A comparação entre *Eucalyptus calophylla* (resistente) e *E. marginata* (suscetível) mostrou que a resposta de defesa ao fungo *Phytophthora cinnamomi* envolve alterações na atividade da PAL, de lignina e de compostos fenólicos nas raízes das plantas infectadas (49).

As polifenol oxidases(PPO) oxidam um amplo grupo de compostos fenólicos sem a necessidade de H_2O_2. Por meio desta oxidação, produzem quinonas, compostos mais tóxicos aos patógenos do que os compostos fenólicos originais (50,51). Em geral, a atividade da PPO é elevada em tecidos infectados por patógenos e apresenta grande importância para as plantas, pois está envolvida nos mecanismos de defesa e na senescência (12).

A PALcatalisa a desaminação da L-fenilalanina a ácido *trans*-cinâmico e amônia, reação da primeira etapa na via dos fenilpropanóides. A PAL também é considerada como o ponto de ramificação entre o metabolismo primário, via metabólica do chiquimato (19), e o metabolismo secundário, via dos fenilpropanóides (52-54). O aumento na atividade das enzimas PAL e POX foi observado em resposta ao ataque de *Rhizoctonia solani* em plantas de feijão-caupi tratadas com ácido salicílico, um eliciador de resposta de defesa (55). Resposta semelhante foi observada em tomateiro desafiado com o fungo *Fusarium oxysporum* (56).

1.4.2.PGPRscomoagentes de biocontrole

As doenças de plantas são responsáveis por grandes perdas na agricultura. Os métodos convencionais de controle são baseados na aplicação de agentes químicos e melhoramento genético para obtenção de resistência. O uso de agentes químicos e sua presença no solo podem ser nocivos ao meio ambiente, especialmente quando esses químicos são aplicados repetitivamente de modo exagerado para o controle de patógenos. Os métodos clássicos de melhoramento dependem da disponibilidade de genes de resistência, os quais podem ser perdidos ao longo das gerações. Além disso, o melhoramento e a utilização de agentes químicos no controle de doenças estão frequentemente direcionados a um patógeno ou a um grupo pequeno de microrganismos.Uma das grandes preocupações atuais na agricultura é buscar alternativas para aumentar a produção e diminuir a utilização de defensivos agrícolas, sendo o controle biológico de doenças em plantas considerado uma das estratégias para reduzir o uso de agroquímicos (57).

A resistência induzida através de agentes de biocontrole é baseada na ativação de mecanismos para o desenvolvimento vegetal e de resistência existentes na planta e no efeito contra um amplo espectro de patógenos vegetais (22). Dentre os microrganismos que atuam como agentes de controle biológico e que estão envolvidos na resistência induzida, estão algumas PGPRs (58), as quais têm mostrado resultados promissores no controle de doenças contra fungos, bactérias e vírus em várias culturas (59-62). Os mecanismos mais estudados de controle biológico mediado por PGPRs incluem a competição por um substrato, a produção de aleloquímicos inibidores e a resistência sistêmica induzida em plantas. ISR mediada por PGPR pode potencializar várias respostas de defesa celular, que são posteriormente induzidas pelo patógeno (63) em um mecanismo conhecido como *priming* (64). As respostas potencializadas

incluem reforço da parede celular (65), o acúmulo das enzimas PPO e POX relacionadas à defesa (66) e a produção de metabólitos secundários (67).

As actinobactérias têm sido utilizadas no controle contra *Rhizoctonia solani* e *Pseudomonas solanacearum* em tomate (68), no controle de *Fusarium oxysporum* em trigo, de *Colletotrichum musae* em banana (69), e *Fusarium* e *Armillaria* em *Pinus taeda* (70). A aplicação de *Streptomyces plicatus* em raízes de tomate antes do plantio reduziu significativamente a incidência de doenças nessas plantas (71). Da mesma maneira, *S. hygroscopicus* demonstrou atividade antagônica contra *B. cinerea* causador da doença mofo cinzento em videiras (73). No híbrido *Eucalyptus grandis* x *E. urophylla* e em *E. urophylla*, ISR mediada por *Pseudomonas* sp. foi comprovada contra a ferrugem causada por *Puccinia psidii* e murcha causada por *Ralstonia solanacearum*(25), bem como a supressão da murcha bacteriana em *E. urophylla* por *Pseudomonas* spp. (72).

2. HIPÓTESE

I) Oisolado *Streptomyces* sp.PM9 modula o metabolismo de plantas de *Eucalyptus grandis* e *E. globulus.*

II) Plantas de *Eucalyptus* spp. eliciadas com *Streptomyces* sp.PM9 retardam os sintomas da doença mofo cinzento, quando infectadas com o fungo *Botrytis cinerea*.

3. OBJETIVOS

3.1. Objetivo geral

Avaliar a produção de AIA, o potencial rizogênico e a modulação do metabolismo secundário induzido por rizobactérias do gênero *Streptomyces* em plantas de *Eucalyptus grandis* e *E. globulus* cultivadas *in vitro*. Avaliar o efeito direto do isolado*Streptomyces* sp.PM9 sobre o fungo *Botrytis cinerea* e indireto nas respostas de defesa nas plantas de *E. grandis* e *E. globulus* eliciadas com S*treptomyces*e desafiadas com *B.cinerea*.

3.2. Objetivos específicos

- Determinar aprodução de AIA dos isolados de rizobactérias *Streptomyces*sp. PM1, PM3, PM4, PM5, PM6 e PM9;
- Determinar o potencial rizogênico dos isolados de *Streptomyces*sp.nas plantas de *E. grandis* e *E. globulus*;
- Determinar a concentração dos compostos fenólicos totais e da fração flavonóides quercetínicos nas plantas de *E. grandis* e *E. globulus* inoculadas com *Streptomyces* sp.;
- Quantificar as atividades enzimáticas da polifenoloxidase e peroxidase nas plantas inoculadas com *Streptomyces* sp. e relacionar com a concentração de metabólitos secundários*;*
- Avaliar a interação entre os microrganismos*Streptomyces* sp. e fungo patogênico *B. cinerea*, através da técnica de co-cultivo;
- Avaliar o efeito modulador de*Streptomyces* sp. no metabolismo secundário de defesa (compostos secundários e atividades enzimáticas) de plantas de *E. grandis* e *E. globulus*eliciadas com *Streptomyces* sp. e desafiadas com *B. cinerea*;
- Avaliar o potencial deeliciação de defesa de*Streptomyces* sp. em plantas desafiadas com *B. cinerea*, quanto ao estabelecimento da doença.

CAPÍTULO II

MANUSCRITO SUBMETIDO:

Streptomyces rhizobacteria modulate the secondary metabolism of *Eucalyptus* plants

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Streptomyces rhizobacteria modulate the secondary metabolism of *Eucalyptus* plants

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Abstract

The genus *Eucalyptus* comprises economically important species, such as *E. grandis* and *E. globulus*, used especially as a raw material in many industrial sectors. Species of *Eucalyptus* are very susceptible to pathogens, mainly fungi, which leads to mortality of plant cuttings in rooting phase. One alternative to promote plant health and development is the potential use of microorganisms that act as agents for biological control, such as plant growth-promoting rhizobacteria (PGPR). Rhizobacteria *Streptomyces*spp have been considered as PGPR. This study aimed at selecting strains of *Streptomyces* with ability to promote plant growth and modulate secondary metabolism of *E. grandis*and *E. globulus in vitro* plants. The experiments assessed the development of plants (root number and length), changes in key enzymes in plant defense (polyphenol oxidase and peroxidase) and induction of secondary compounds (total phenolic and quercetinic flavonoid fraction). The isolate *Streptomyces* PM9 showed highest production of indol-3-acetic acid and the best potential for root induction. Treatment of

Eucalyptus roots with *Streptomyces* PM9 caused alterations in enzymes activities during the period of co-cultivation (1 to 15 days), as well as in the levels of phenolic compounds and flavonoids. Shoots also showed alteration in the secondary metabolism, suggesting induced systemicresponse. The ability of *Streptomyces* sp. PM9 on promoting root growth, through production of IAA, and possible role on modulation of secondary metabolism of *Eucalyptus* plants characterizes this isolate as PGPR and indicates its potential use as a biological control in forestry.

Keywords: Actinomycetes, PGPRs, peroxidases, polyphenol oxidases, phenolic compounds.

Introduction

Eucalyptus, a genus native toAustralia, belongs to the family Myrtaceae and comprises about 900 species and subspecies (Brooker and Kleinig 2004). Wood from several economically important members of the genus is used as a raw material in many industrial sectors. Easily adapting to different climatic conditions, some species, such as *E. globulus* and *E. grandis*, are grown in different regions of the world for timber and pulp production (Eldridge et al. 1994).

In Brazil, cloning of *Eucalyptus* spp. is done mainly by rooting mini-cuttings under high humidity and temperature. These conditions favor the attack of a wide variety of pathogens, mainly fungi, which cause extensive losses, especially of young plants thatare more susceptible to pathogen attack (Ribeiro and Cardoso 2012). In forestry, promotion of plant development and reduction of infectious diseases have been achieved by the use of microorganisms that play a role in biological control (Ashraf et al. 2013).

Rhizobacteria are rhizosphere-competent bacteria that aggressively colonize plant roots (Antoun and Prévost 2005). Among this group of microorganisms are some root-associated bacteria, termed plant growth-promoting rhizobacteria (PGPR), which is a class of non-pathogenic soil microorganisms that have a beneficial effect on plant growth (Kloepper et al. 1980). PGPR bacteria may directly influence plant growth by either synthesizing plant hormones such as indol-3-acetic acid (IAA) (Ashraf et al. 2013), or facilitating uptake of nutrients from the soil through different mechanisms, such as solubilization of phosphorus and potassium, as reported for *Fraxinusamericana* (Liu et al. 2013) and synthesis of siderophores for iron sequestration (Adesemoye et al. 2009).PGPR can also indirectly affect plants through antagonism between bacteria and soil-borne pathogens (Pal et al. 2001), as well as by inducing systemic resistance (ISR) in plants against both root and foliar pathogens. Induced resistance constitutes an increase in the level of basal resistance, whereby the plant's innate defenses are potentiated against several pathogens and parasites (Adesemoye et al. 2009).

Members of *Streptomyces* (order Actinomycetales) are a group of Gram-positive bacteria that are commonly found in soil. Comprising ~10% of total soil microbiota, these organisms play important roles in plant-microbial community associations (Schrey and Tarkka 2008). A great deal of interest in *Streptomyces* has centered on their potential for producing a wide variety of secondary metabolites, including antibiotics and extracellular enzymes (Inbar et al. 2005). Many *Streptomyces* are considered PGPR due to their capacity for increasing plant growth, antibiosis and inducing defense responses in *Streptomyces*-colonized plants (Lehr et al. 2007; 2008). *Streptomyces*species have also been widely used for biological control of soil-borne plant pathogens (Inbar et al. 2005; Gopalakrishnan et al. 2014).

Mechanisms of plant response to PGPR may be physical or biochemical. These mechanisms can include reinforcement of the plant cell wall, production of antimicrobial phytoalexins, and pathogenesis-related proteins (PRs), as well as an enhanced capacity to express defense

responses upon challenge with a pathogen, a mechanism referred to as ‘priming’ (Conrath et al. 2006). Plant defense responses result, in part, from metabolic alterations, including changes in the activity of central enzymes of the secondary metabolism, such as peroxidases (POXs) and polyphenol oxidases (PPOs), and in the synthesis of secondary metabolites (Dalmas et al. 2011).

The aim of this study was to select rhizobacteria strains of the genus *Streptomyces* with the capacity to promote plant growth and modulate the secondary metabolism of *E. grandis*and *E. globulus*plants *in vitro*.

Materials and methods

Plant material

Seeds of *E. grandis* and *E. globulus* were surface-disinfected in 70% ethanol for 60 s, followed by immersion in solution ofthe fungicide Ridomil Gold® MZ (8 g L^{-1}) for 20 min, and immersion in sodium hypochlorite (1%) 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 32 µmol m^{-2} s^{-1} under a 16 h photoperiod. Ninety-day-old plants were used in all experiments.

Culture of *Streptomyces* isolates

For inoculum preparation, six isolates of rhizobacteria *Streptomyces* sp. (PM1, PM3, PM4, PM5, PM6 and PM9) were grown from stock cultures initiated from samples collected in an Araucaria Forest at the Pró-Mata Center for Research and Conservation of Nature

(29°29'18.4"S, 50°12'23.5" W), São Francisco de Paula, Rio Grande do Sul, Brazil.DNA sequences are deposited in GenBankunder the following access numbers: Streptomyces spp. PM1-HM460335, PM3 – KM196121, PM4-HM460336, PM5 – KM196122, and PM9-HM460337.Suspensions of isolates were prepared in Erlenmeyer flasks containing 10 mL of ISP_4 liquid medium (Shirling and Gottlieb 1966), at 100 rpm for 7 days (stationary phase). Suspensions were centrifuged at 2,500 x*g* for 10 min at room temperature, the pellet was resuspended in sterile distilled water, and the suspension was adjusted to 10^6–10^7 CFU mL^{-1} (OD_{600nm} = 1), used for the experiments.

Determination of IAA levels produced by *Streptomyces* sp. isolates

The production of IAA by the six isolates of *Streptomyces* sp. was determined according to the Salkowski method (Salkowski 1885). Isolates PM1, PM3, PM4, PM5, and PM9 were cultured on semi-solid ISP_4medium, and samples of rhizobacteria (50 mg) were extracted with 0.5 mL of 96% ethanol and centrifuged at 2,500 x*g* for 6 min at room temperature. The supernatant (0.3 mL) was analyzed with 1 mL of Salkowski's reagent (180 mL concentrated H_2SO_4 dissolved in 150 mL H_2O, with addition of 9 mL of 1.5 M $FeCl_3.6H_2O$), after incubation for 60 min at room temperature in the dark. Optical densities were read at 530 nm. The level of IAA produced was estimated against the IAA standard (Dalmas et al. 2011). Production of IAA was determined from three replicates for each isolate of *Streptomyces* sp.

Effect of *Streptomyces* sp. on rhizogenesis of *Eucalyptus* plants

The isolates that produced the highest levels of IAA were used to evaluate the induction of secondary roots and the growth of the tap root. *Eucalyptus* plants were cultivated in an *in vitro* system according to Lehr et al. (2008), with modifications. Briefly, 25 mL of ¼ MS medium was poured in a Petri dish (9 cm in diameter), and after the agar solidified, a semicircle of

121 medium was discarded and one plant was placed on the remaining semicircle (Figure 1).The
122 root surface was covered with 200 µL of *Streptomyces* suspension. Sterile distilled water was
123 used on the control plants. Wetted filter paper was placed over the roots and medium in order
124 to maintain humidity. Plates were maintained at 25 ± 2 °C and 16 h photoperiod. The length of
125 the tap root and the number of secondary roots were evaluated at the onset of the experiment
126 and 30 days after the beginning of cultivation. Ten plants were used for each treatment.
127
128 Evaluation of secondary metabolism of *E. grandis* and *E. globulus* in the presence of
129 *Streptomyces* sp. (PM9)
130 The isolate that produced the highest level of IAA and induced rhizogenesis in the*Eucalyptus*
131 plants was used to evaluate the capacity of *Streptomyces*to modulatethe secondary metabolism
132 as a result of plant-microbe interaction. Plants of *E. grandis* and *E. globulus* were transferred
133 to the culture system described above. A suspension of rhizobacteria (200 µL) was inoculated
134 on the roots;sterile distilled water was applied to the roots of the control plants. The modulation
135 effect of the rhizobacteria on secondary plant metabolism was evaluated at 0, 1, 3, 9 and 15
136 days post-inoculation (dpi), as changes in the enzymatic activity of polyphenol oxidases and
137 peroxidases as well as in the levels of total phenolic compounds and quercetinic flavonoids.
138 Each treatment contained 20 plants per time-course point, totaling 200 plants. Shoots and roots
139 of *Eucalyptus* plants were analyzed separately. Shoots or roots at each time-course point and
140 each treatment were pooled and cut into small pieces on ice. A minimum of three repetitions
141 were used, and each repetition was analyzed in three replicates for the colorimetric reaction.
142 The activities of the enzymes polyphenol oxidase (PPO; EC 1.14.18.1) and peroxidases
143 (POX; EC 1.11.17) were determined according to Sávio et al. (2012), with modifications.
144 Briefly, extracts were prepared from shoots and roots (0.4 g) ground in 2.5 mL of 50 mM
145 sodium phosphate buffer (pH 7.0) and polyvinylpyrrolidone (PVP; 1:6 w/v). The extracts were

146 filtered and centrifuged at 2,500 x*g* for 15 min at 5ºC, and the supernatant was collected for
147 determination of the protein content and for the enzyme assays. The polyphenol oxidase activity
148 was determined spectrophotometrically at 400 nm, using chlorogenic acid as substrate. Specific
149 enzyme activity was defined as the change in absorbance min^{-1} mg^{-1} protein. The activity of
150 peroxidases was determined in a reaction mixture containing 50 mM sodium phosphate buffer
151 (pH 6.0), 1% (v/v) guaiacol as substrate, and 10 mM hydrogen peroxide, using the crude extract
152 described above. Enzyme activity was determined in a spectrophotometer (420 nm) by
153 oxidation of guaiacolfor 30 s at intervals of 5 s. Specific enzyme activity was expressed as
154 µkatal mg^{-1} protein.Total protein concentration was determined according to Bradford's
155 method (Bradford 1976), using bovine serum albumin as the standard.
156 For quantification of the total phenolic compounds, samples of shoots and roots (0.1 g of
157 fresh mass)of *Eucalyptus* plants were taken from each treatment, blotted on sterile filter paper,
158 and ground in 10 mL of 80% (v/v) methanol at room temperature. Extracts were filtered and
159 centrifuged at 1,250 x*g* for 15 min.Total phenolic compounds were analyzed in the supernatant
160 by the colorimetric Folin-Ciocaulteau method as described previously (Sartor et al. 2013).
161 Gallic acid was used as the standard. The contents of total phenolic compounds were expressed
162 as mg g^{-1} of fresh mass (FM). The fraction of quercetinic-derived flavonoids was determined
163 by the colorimetric method using the reaction with 96% ethanol, 10% aluminum nitrate and 1
164 M potassium acetate, measured at 415 nm. Quercetin was used as the standard for the
165 calibration curve. The flavonoid content was expressed as mg quercetin equivalents g^{-1}
166 FM(Poiatti et al. 2009).

167 Statistical analysis

168 Experiments were independently repeated twice under the same conditions. Results of IAA
169 production, length and number of roots were analyzed by one-way ANOVA, and the means
170 were separated by Tukey Test at a significance level of $\alpha \leq 0.05$. When homogeneity of the
171 variances was not achieved, the data were transformed by log x+1. The experiments on the
172 modulation of secondary metabolism useda fully randomized design, and the data were
173 analyzed by Student's t test ($\alpha \leq 0.05$). All statistical analyses were performed using the
174 software SPSS v. 17.5.

175

176 **Results**

177 Determination of IAA levels produced by *Streptomyces*sp. isolates

178 The isolate *Streptomyces* sp. PM9 showed the highest production of IAA (0.991 mg g^{-1} FM),
179 followed by isolate PM5 (0.724 mg g^{-1} FM). Between isolates PM3 and PM4, no significant
180 difference was observed in IAA production; isolate PM1 showed the lowest level of IAA
181 compared to the other isolates (Table 1).

182

183 Effect of *Streptomyces* sp. on rhizogenesis

184 The rhizogenic potential of plants of *E. grandis* and *E. globulus* was significantly affected by
185 the presence of rhizobacteria *Streptomyces* sp. In *E. grandis*, proliferation of roots inoculated
186 with *Streptomyces* sp. PM9 and PM5 wasapproximately twice as high compared to the control
187 treatment. Similarly, plants of *E. globulus* showed no difference in their responses to isolates
188 PM9 and PM5 regarding proliferation of secondary roots, and the presence of rhizobacteria
189 increased the number of lateral roots by approximately 5 times compared to the control

treatment. Tap-root growth was not influenced by the presence of rhizobacteria in either*Eucalyptus* species tested (Table 2).

Modulation of secondary metabolism of *Eucalyptus* plants by *Streptomyces* sp.

Since *Streptomyces* PM9 showed the highest production of IAA and promoted rhizogenesisin*E. grandis* and *E. globulus*, this isolate was used for testing the modulation of the secondary metabolism resulting from the plant-rhizobacteria interaction.Both the roots and shoots of eucalyptus were affected by the treatment with rhizobacteria, showing alterations in PPO and POX activity as well as in the levels of total phenolic compounds and quercetinic flavonoids, although the timing and intensity of the responses differed between the species (Figs. 2-5).

In general, plants of *E. grandis*and *E. globulus*in culture at day 0 (before the treatments) showed a lower level of enzymatic activity than plants treated with *Streptomyces* or water (control) at 1 dpi (Figs. 2 and 4). However, the levels of total phenolic compounds and quercetinic-flavonoids did not differ (Figs. 3 and 5).

Roots of *E. grandis*showed differences in enzymatic activities of PPO and POX along the time points evaluated. In plants treated with *Streptomyces* sp. PM9, the PPO activity showed no difference from the control at 1 dpi. However, the PPO activity showed aslight increase at 3 dpi, followed by a marked decrease at 9 dpi(Fig. 2a). At 15 dpi, the PPO activity in PM9-treated roots was significantly higher than in the control roots. The POX activity in the roots increased at 1 dpi, and similarly to PPO, decreased at 9 dpi (Fig. 2b).The enzymatic activity indicated that the accumulation of phenolic compounds also responded to the inoculation of roots with *Streptomyces* sp. (Fig. 3a). At 1 and 3 dpi, lower levels of total phenolic compounds were observed, followed by an increase at 9 dpi, simultaneously with the PPO and POX activities at the same time points (Figs. 2a, b; 3a).No alterationsin the levels of quercetinic flavonoids were detected in the roots during the culture period(Fig. 3b).

Shoots of *E. grandis*also responded to the inoculation with *Streptomyces*. Differences in enzymatic activities and phenolic compounds were observed during the time points. Compared with control plants, shoots from PM9-treated plants showed significantly higher PPO activity at 1, 3 and 15 dpi (Fig. 2a), whereas the POX activity increased from 9 and 15 dpi compared to the control (Fig. 2b). Phenolic compounds also accumulated in the shoots from plants treated with PM9 at 1, 3 and 9 dpi. Differently from what was observed in the roots, levels of flavonoids were significantly higher in shoots from the PM9-treated plants than in shoots from the control plants at 9 and 15 dpi (Fig. 3b).

Similarly to the responses of *E. grandis* to *Streptomyces* sp., variations in enzymatic activities and phenolic compounds were observed in *E. globulus*. In *E. globulus* roots,PPO activity increased at 3 dpi followed by a marked decrease at 9 dpi (Fig. 4a), a similar response to that of*E. grandis* (Fig. 3a). At 15 dpi, the PPO activity in PM9-treated roots was significantly lower than in control roots (Fig. 4a). The POX activity was significantly higher in PM9-treated roots at 1 dpi compared with control plants (Fig. 4b). In the roots, little variation was detected in phenolic compounds and flavonoids, although a decrease was observed at 1 dpi (Fig. 5a,b),which might be related to the increase in POX activity at the same time point (Fig. 4b).

Shoots of *E. globulus* showed less variation in secondary metabolism than the variation detected in the roots. A change in the PPO activity was detected only at 15 dpi (Fig. 5a), whereas the POX activity was reduced at 3 dpi, and increased at 9 dpi (Fig. 4b). Phenolics and flavonoids decreased at 1 dpi and increased at 15 dpi compared with control plants (Fig. 5b).

Discussion

The ability to produce the plant hormone IAA is widespread among microorganisms that are commonly associated with plant surfaces, and almost 80% of rhizosphere bacteria can secrete this common natural auxin (Manulis et al. 1994;Ashraf et al. 2013). Production of plant-growth

239 regulators is one of the modes of action by which PGPR stimulates plant growth. The
240 *Streptomyces* sp. isolates tested differedin the production of IAA, ranging from 0.102 to 0.991
241 mg g^{-1} FM. *Streptomyces* sp. isolates PM9 and PM5 showed the highest production of this
242 plant-growth regulator. Production of IAA by *Streptomyces* spp. has been reported in isolates
243 recovered from soil associated with medicinal plants,with concentrations ranging from 11 to
244 144 µg ml^{-1} (Khamna et al. 2010). Mansour et al. (1994) evaluated 24 strains
245 of*Streptomyces*spp. for their ability to produce plant hormones, and all the strains synthesized
246 auxin, gibberellins and cytokinins in liquid medium. A previous test of *Streptomyces* sp. PM1
247 showed low production of IAA (Dalmas et al. 2011).
248 *Streptomyces* sp. PM5 and PM9were efficient in inducing roots in both*Eucalyptus* species
249 compared to the control plants. The enhancement of root growth observed in the*Streptomyces*-
250 treated plants is likely related to the ability of isolates PM5 and PM9 to produce IAA.
251 Rhizobacteria can release phytohormones that can be absorbed by plant roots and thus promote
252 plant growth (Hussain and Hasnain 2011). Since rhizogenesisin *Eucalyptus* was increased by
253 the rhizobacteria, the *Eucalyptus–Streptomyces* sp. association might result in more vigorous
254 plants, affecting productivity, as previously reported for PGPRs (Van Loon 2007). Plant growth
255 promotion by*Streptomyces* was reported for tomato (El-Tarabily 2008), wheat (Sadeghi et al.
256 2012), apple (Aslantaş et al. 2007), and previously on eucalyptus (Mafia et al. 2009).
257 In addition to the beneficial effect on root growth in *Eucalyptus*, *Streptomyces* sp. PM9 was
258 able to modulate the secondary metabolism of *E. grandis*and *E. globulus* during 15 days of
259 culture, suggesting that this PGPR has an indirect effect on the plants. More significant
260 alterations were observed in the PPO and POX activity than in the phenolic compounds and
261 flavonoids levels. PPO is a copper-containing enzyme that catalyzes the oxidation of phenols
262 to highly toxic quinones (Kim et al. 2001). PPO is involved in several other important
263 physiological processes in plant cells, such as pigment formation, oxygen scavenging and

pseudocyclic phosphorylation in the chloroplast, and defense mechanisms against insects and plant pathogens (Yoruk and Marshall 2003). POX is a key enzymeknown to be involved with defense responses(Lehr et al. 2007; Appu and Muthukrishnan 2014), which participates in the biosynthesis of lignin by the oxidation of phenolic compounds, thus strengthening the cell wall (Mandal and Mitra 2007). The activity of these enzymes was modified following inoculation by *Streptomyces* PM9 on the roots of*Eucalyptus* plants. Roots of *E. grandis*and *E. globulus*responded to the presence of *Streptomyces* PM9, initially activating POX (1 dpi) and later PPO (3 dpi). However, at 9 dpi the activity of both enzymes decreased significantly compared to non-treated plants. Increase of POX and PPO in roots as an early response to the presence of *Streptomyces* on the roots, and the subsequent temporal variation might result from the attempt to establish an interaction between the two organisms. Similar results were found in *Araucaria angustifolia* treated with *Streptomyces* sp. (Dalmas et al. 2011). Several mechanisms are involved in the plant–rhizobacteria interaction, and its success involves colonization of the roots, including recognition, adhesion, and in some cases, cell invasion (Berg 2009). Non-pathogenic rhizobacteria such as PGPR interact with roots without activating the defense responses in the host plants, and only cause the accumulation of transcription factors related to defense genes that reduce the response time to pathogen attack (Van der Ent et al. 2009). The decrease in enzyme activity at 9 dpi in the PM9-treated roots coincided with the accumulation of phenolics at the same time point. As these compounds are substrates for PPO and POX, the results suggest that the activity of these enzymes was suppressed, and this metabolic response may favor the rhizobacterium during the process of root colonization.

Similarly to the root responses, alterations in the basal levels of the enzymes PPO and POX were observed in the shoots, although the changes in *E. grandis* were more significant than in *E. globulus*. Because the inoculation was carried out on the roots, this result indicates the

288 presence of an induced systemic response in *Eucalyptus* sp. plants inoculated with
289 *Streptomyces* sp. PM9. The activities of PPO and POX differed between the*Streptomyces*-
290 treated shoots ofthe two species tested. In *E. grandis*, *Streptomyces* inoculation led to increased
291 PPO activity at 1 and 3 dpi, whereas in *E. globulus* no alteration was observed until 15 dpi. On
292 the other hand, POX activity was similar in the two species, decreasing at 3 dpi andincreasing
293 at 9 dpi. Changes in peroxidases elicited by PGPR have been reported in cucumber (Chen and
294 Kirkbride 2000) and tomato (Ramamoorthy et al. 2002). Peroxidase activity in diseased plants
295 and its effects on resistance or susceptibility in many host-pathogen interactions have also been
296 studied. The infection of Norway spruce with *Heterobasidionannosum* was associated with a
297 plant defense-related increase in peroxidase activity and the*PaSpi2* peroxidase and
298 *PaChi4*chitinase gene expression levels (Fossdal et al. 2001;Hietala et al. 2004; Lehr et al.
299 2008). Otherwise, the increase in POX activity detected at 9 and 15 dpi, combined with the
300 high levels of secondary metabolites induced in the shoots of both species, might be related to
301 plant development, since POX is involved with lignin formation during plant growth, in
302 addition to defense responses (Datta and Muthukrishnan 1999).

303 In conclusion, the isolates tested in this study exhibited some features of PGPR.
304 *Streptomyces* sp. PM9 directly promoted root proliferation in *E. grandis*and*E. globulus*through
305 IAA production, and modulated the secondary metabolism, increasing the basal levels of
306 defense-related enzymes, which suggeststhe induction of a systemic response. Although several
307 studies have examined the efficiency of microorganisms as biocontrol agents, few studies have
308 assessed rhizobacteria from the Mixed Ombrophilous Forest as plant-growth and ISR-
309 promoters (Vasconcellos and Cardoso 2009; Dalmas et al. 2011). Further studies on
310 thepotential of this isolate as an antagonist against pathogenic fungi would strengthen the
311 possibility of using *Streptomyces* sp. PM9 as an alternative forbiocontrol, with a concomitant
312 reduction in pesticide use.

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Table 1 Concentration of the indol-3-acetic acid (IAA) in different isolates of *Streptomyces* sp. cultivated in semisolid ISP_4 medium.

***Streptomyces* sp.** **Isolates**	**IAA (mg g^{-1} FM)**[*]
PM1	0.102 (0.06) d[**]
PM3	0.539 (0.04) c
PM4	0.410 (0.01) c
PM5	0.724 (0.02) b
PM6	0.415 (0.11) c
PM9	0.991 (0.05) a

[*]Values are means of three replicates.Numbers in parentheses represent standard error of the mean.

[**]Different letters indicate significant difference among isolates at $\alpha \leq 0.05$ by Tukey Test.

Table 2 Number and length of roots of *E. grandis* and *E. globulus* plants inoculated with suspensions of *Streptomyces* (isolates PM5 and PM9) after 30 days of co-culture.

Species	Treatments	Root length (cm)*	Number of roots[a]
E. grandis	Control	1.93 (0.69) a**	8.5 (1.5) b
	Streptomyces sp. PM5	1.77 (0.34) a	18.9 (2.8) a
	Streptomyces sp. PM9	0.72 (1.55) a	17.8 (3.5) a
E. globulus	Control	0.61 (0.67) a	7.0 (7.03) b
	Streptomyces sp. PM5	0.37 (0.47) a	32.6 (14.8) a
	Streptomyces sp. PM9	0.55 (0.68) a	36.7 (16.6) a

*Values are means of 10 replicates.Numbers in parentheses represent standard error of the mean.

**Different letters indicate significant difference within the species at $\alpha \leq 0.05$ by Tukey Test. Data were transformed by log x+1.

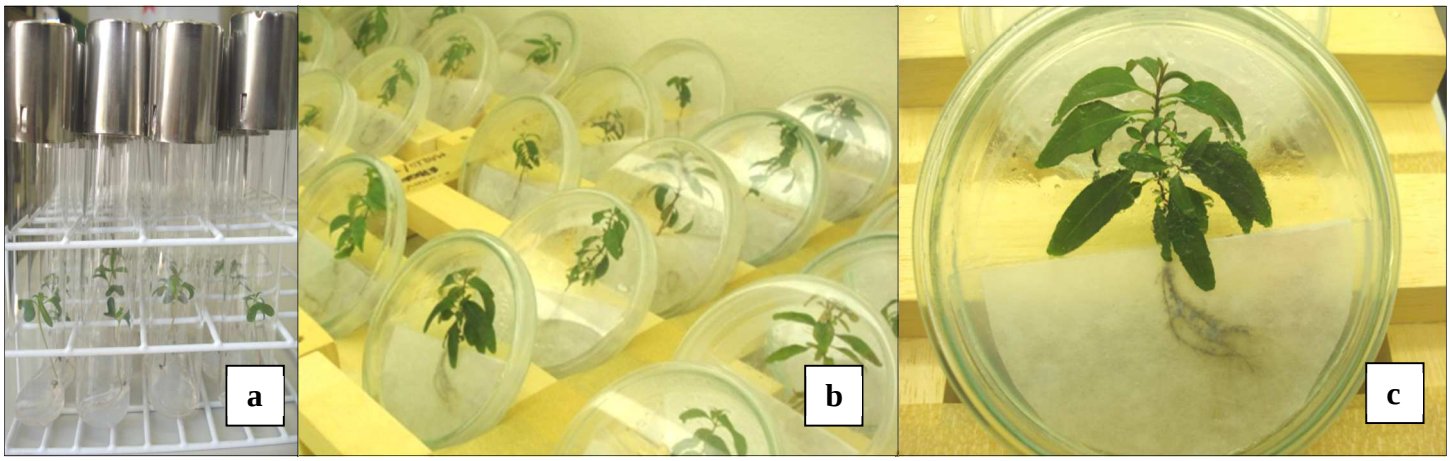

Fig. 1 Cultivation of plant material. (a) Germinated seeds of *E. grandis*. (b) Plants arranged in plates containing a semicircle of $^{1}/_{4}$ MS semisolid culture medium as support. (C) Plants under cultivation after treatments inoculation.

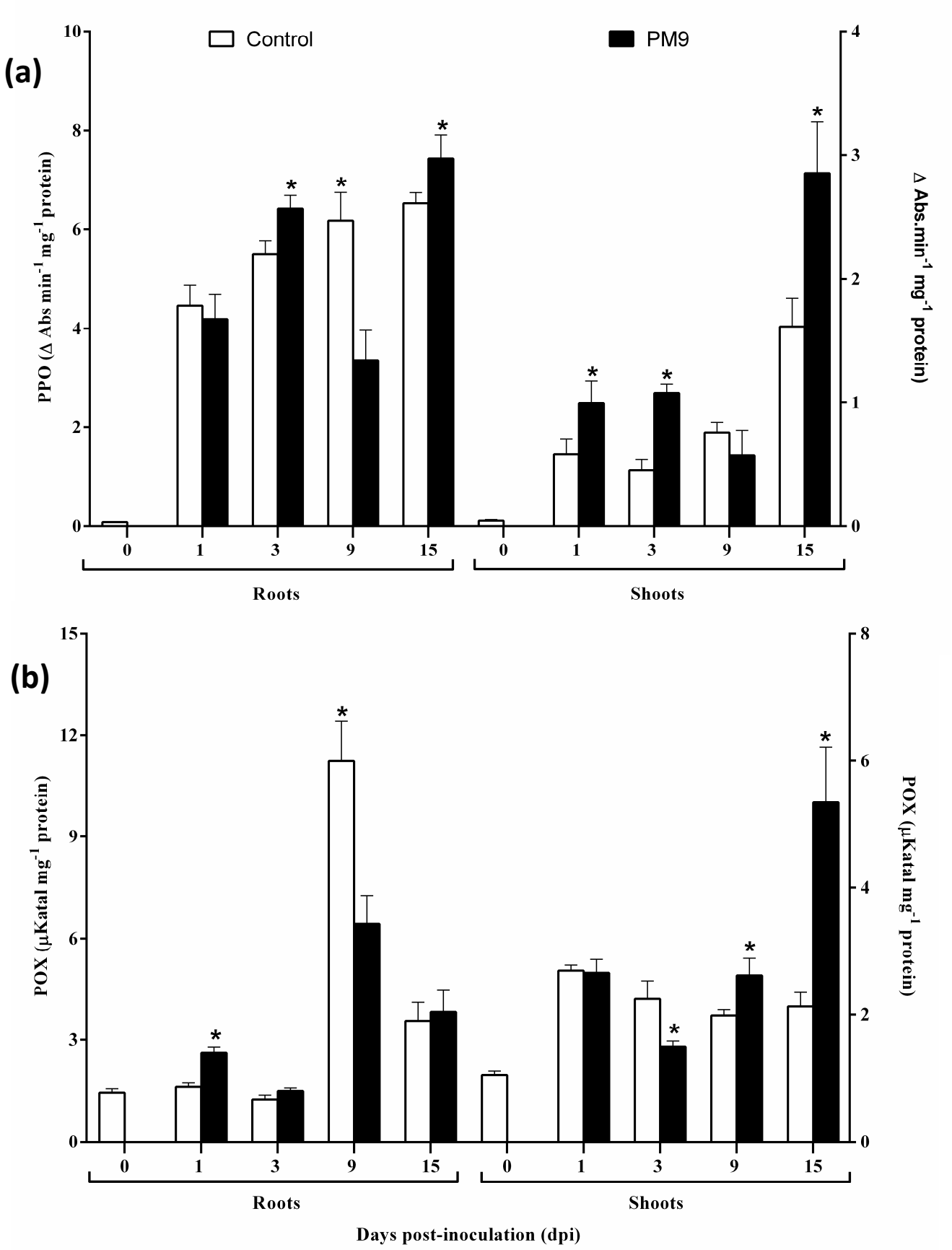

Fig. 2 Activities of the enzymes (a) polyphenol oxidase, and (b) peroxidases in shoots and roots of *E. grandis* plants inoculated with *Streptomyces* sp. PM9. Distilled water was used as control. Analyses were carried out at 1, 3, 9 and 15 dpi. Bars represent standard error of the mean. The asterisks indicate difference between treatments (Student T-test $P \leq 0.05$).

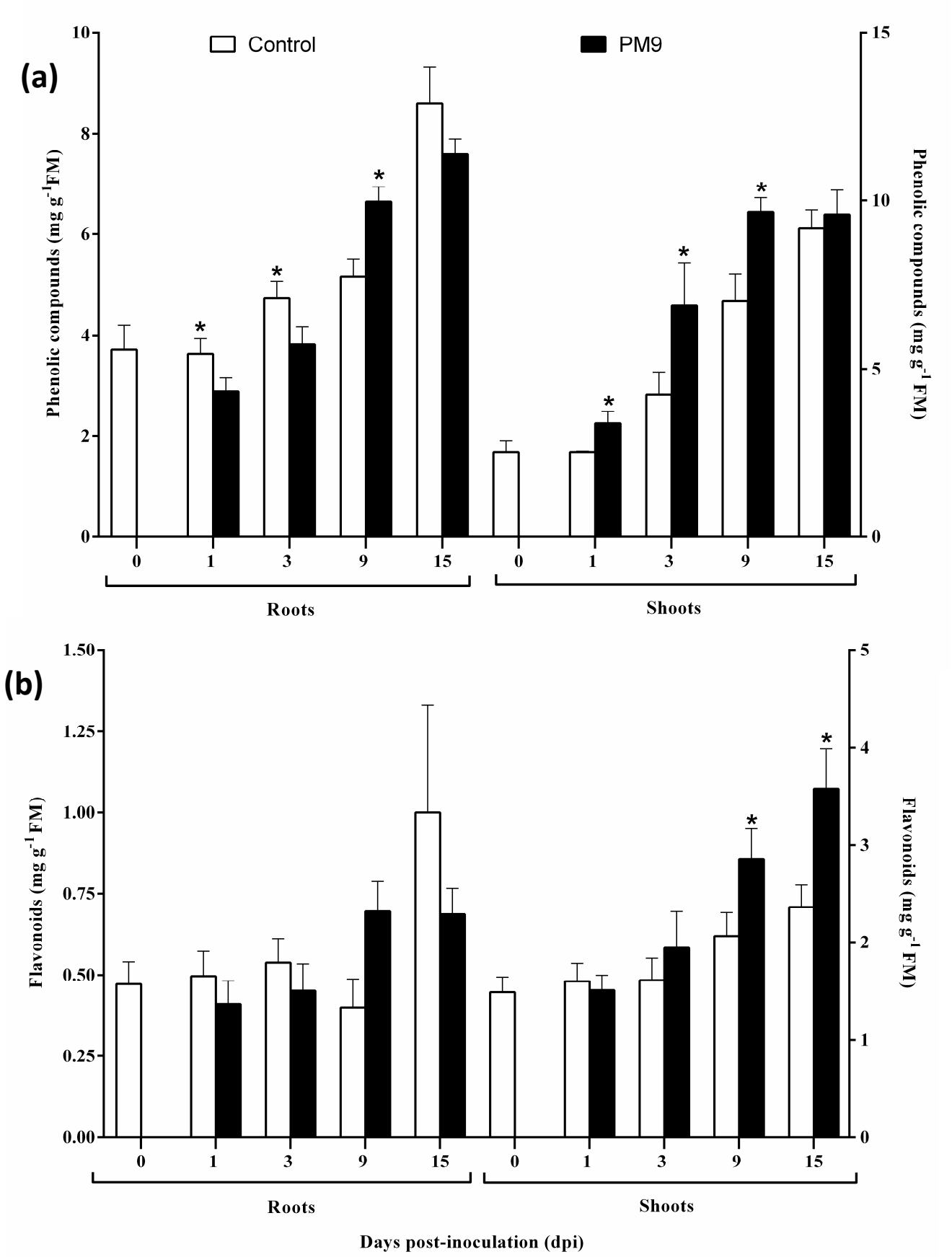

Fig. 3 (a) Phenolic compounds, and (b) flavonoids in shoots and roots of *E. grandis* plants inoculated with *Streptomyces* sp. PM9. Distilled water was used as control. Analyses were carried out at 1, 3, 9 and 15 dpi. Bars represent standard error of the mean. Asterisks indicate difference between treatments at $P \leq 0.05$ by Student T-test.

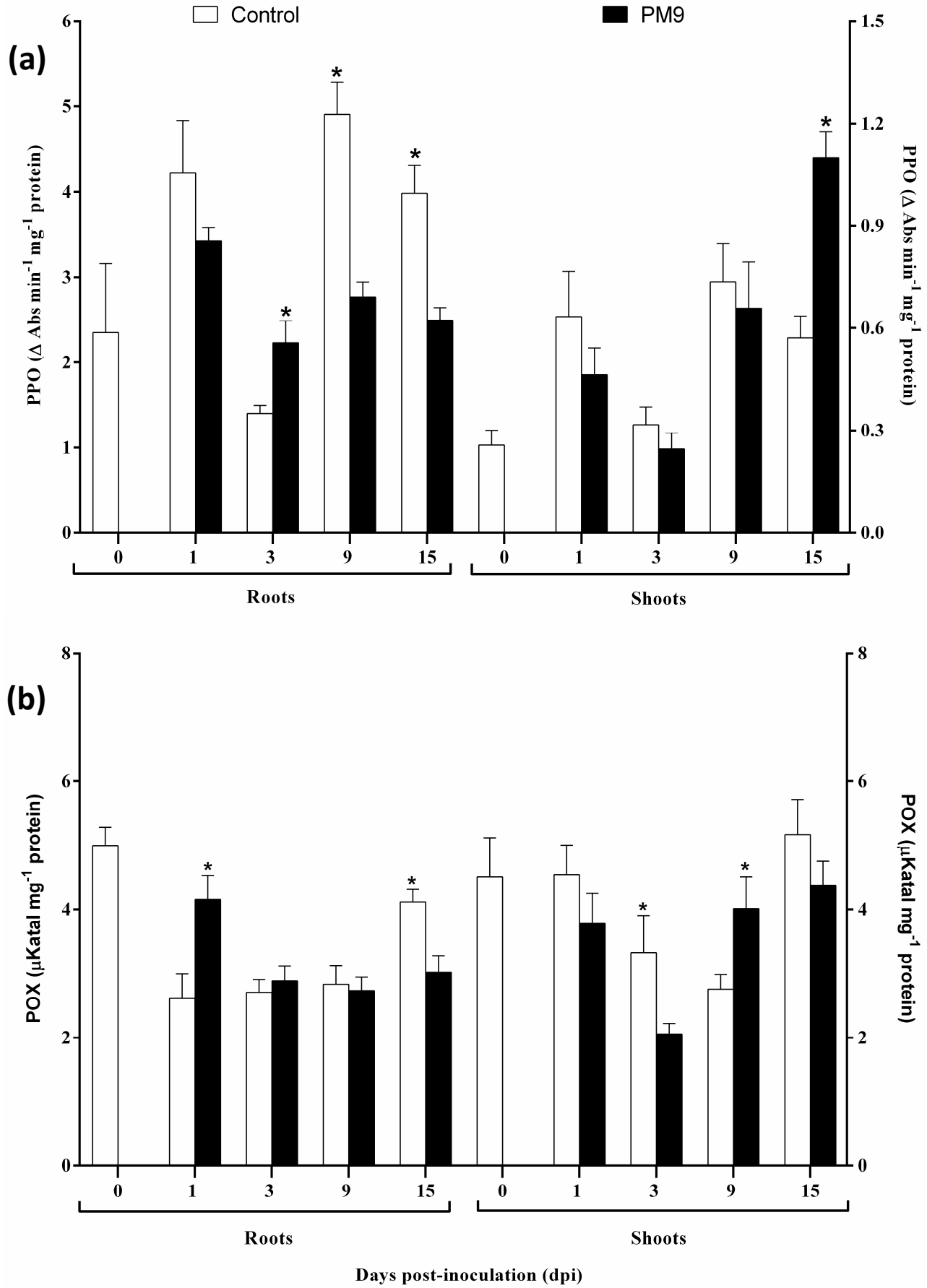

Fig. 4 Activities of the enzymes (a) polyphenol oxidase, and (b) peroxidases in shoots and roots of *E. globulus* plants inoculated with *Streptomyces* sp. PM9. Distilled water was used as control. Analyses were carried out at 1, 3, 9 and 15 dpi. Bars represent standard error of the mean. Asterisks indicate difference between treatments at $P \leq 0.05$ by Student T-test.

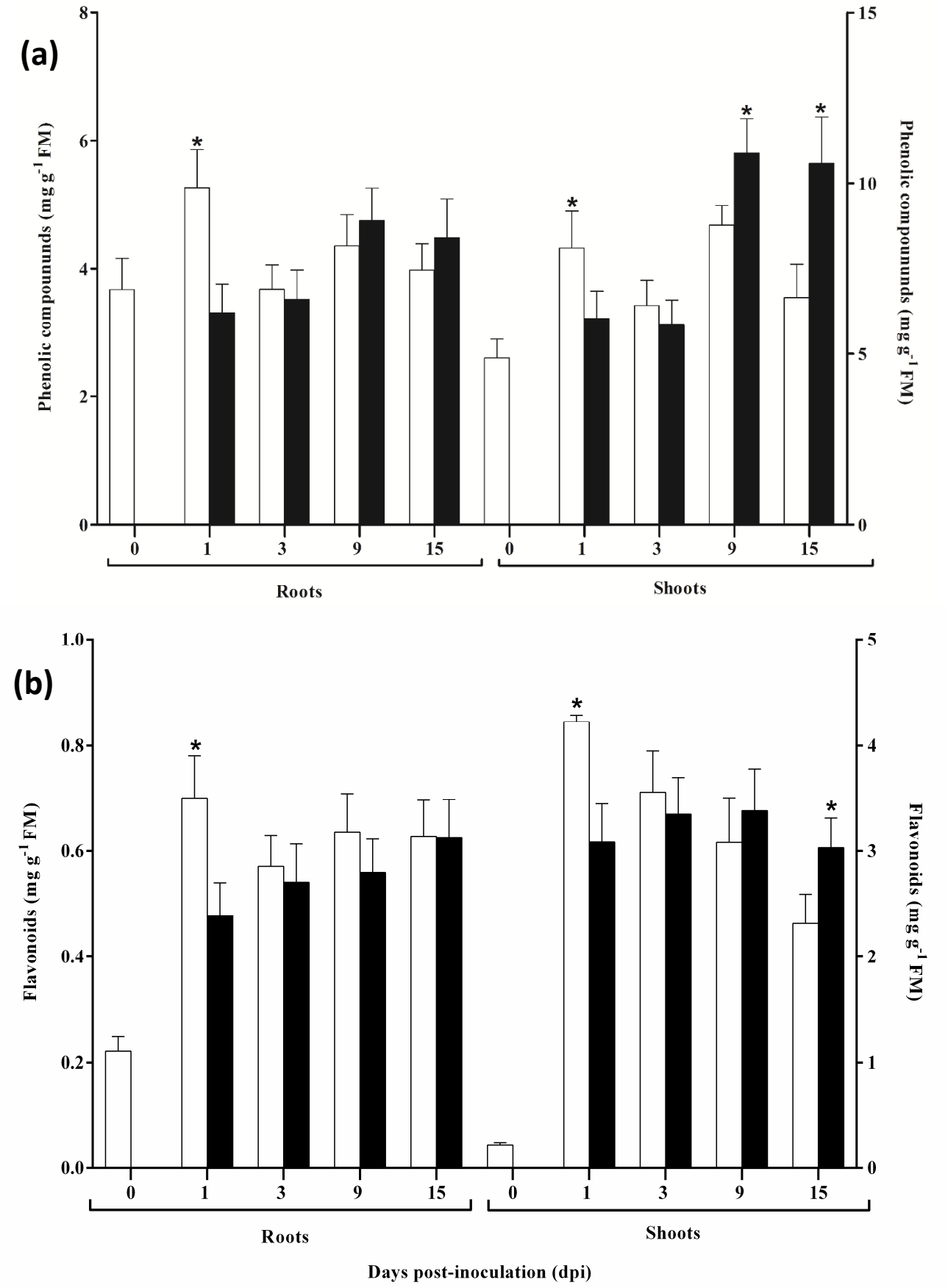

Fig. 5 (a) Phenolic compounds, and (b) flavonoids in shoots and roots of *E. globulus* plants inoculated with *Streptomyces* sp. PM9. Distilled water was used as control. Analyses were carried out at 1, 3, 9 and 15 dpi. Bars represent standard error of the mean. Asterisks indicate difference between treatments at $P \leq 0.05$ by Student T-test.

CAPÍTULO III

MANUSCRITO A SER SUBMETIDO:

Defense responses in plants of *Eucalyptus grandis* Hill ex Maiden and *E. globulus* Labill elicited by *Streptomyces* and challenged with *Botrytis cinerea*

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Defense responses in plants of *Eucalyptus grandis* Hill ex Maiden and *E. globulus* Labill elicited by *Streptomyces* and challenged with *Botrytis cinerea*

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Abstract

Eucalyptus is an economically important woody species, especially as a raw material in many industrial sectors. The species of this genus are very susceptible to pathogens such as *Botrytis cinerea* (gray mold) which lead to mortality of *Eucalyptus*cuttings in rooting phase. Biological control of plant diseases using soil microorganisms has been considered an alternative to reduce the use of pesticides and pathogen attack. Rhizobacteria can cause changes in secondary metabolism, inducing systemic resistance in plants, and therefore leading to enhanced plant defense. This study evaluated the modulating effect of *Streptomyces* sp. PM9 in the secondary metabolism of plants of *Eucalyptus grandis* and *E. globulus*, determining the metabolic and phenotypic responses of plants elicited with *Streptomyces* sp. PM9 and challenged with the pathogenic fungus *B. cinerea*. Metabolic responses were evaluated assessing the enzymatic activities of polyphenol oxidase and peroxidase involved as well as the levels of induced secondary matabolites, phenolic compounds and flavonoids. Disease incidence and progression in elicited plants, as well as co-culture between *Streptomyces*sp. PM9 and *B. cinerea* were evaluated. Antagonistic potential of this isolate against *B. cinerea* was found. Elicitation with *Streptomyces* sp. PM9 and challenging with *B. cinerea* led to changes in polyphenol oxidase and peroxidase activities as well as in the levels of phenolic

compounds in plants at different times of analysis. Alterations in enzymes of elicited plants were related to early defense responses. Phenolic compounds such as gallic and chlorogenic acids were, on average, more abundant, although caffeic acid, benzoic acid and catechin were induced at specific time points. A delay on the establishment of gray mold was significant in *E. grandis* plants elicited with *Streptomyces*sp. PM9. These results combined with the antagonistic effect against *B. cinerea*, demonstrate the action of *Streptomyces* sp. PM9 on inducing plant systemic resistance, making this organism a potential candidate for biological control in *Eucalyptus*.

Keywords: Biocontrol, Induced systemic resistance, Peroxidases, Plant growth-promoting rhizobacteria.

Introduction

Eucalyptus is a perennial tree native to Australia, successfully introduced worldwide and widely cultivated in many countries, including Brazil (Bruneton 1995). *Eucalyptus* is a large genus of the Myrtaceae family, comprising about 900 species and subspecies (Brooker and Kleinig 2004) with high economic value, such as *E. globulus* and *E. grandis*, whichare grown in different regions of the world for timber and pulp production (Cotterill and Brolin 1997; Eldridge et al. 1994). *Eucalyptus* species are constant targets for a broad range of pathogens, especially fungi, which infect plants throughout the life cycle. Among the pathogens that attack *Eucalyptu*s stands out *Botrytis cinerea* (gray mold), considered a typical necrotrophic fungus, which promotes programmed cell death in the host during the course of infection, by secreting toxic molecules and lytic enzymes, subsequently consuming plant tissues for its own growth (Dean et al. 2012).

One alternative for promoting plant health and development is the use of microorganisms as biological control agents (Ashraf et al. 2013; Postma et al. 2003). Amongst these microorganisms are some bacteria that live attached to plant roots, named Plant Growth Promoting Rhizobacteria (PGPRs) (Kloepper et al. 1980). *Streptomyces* (Actinomycetes) are considered to be part of the group PGPR and comprises Gram positive filamentous bacteria that are well known for their ability to control plant diseasesby inducing defense responses in colonized plants (Gopalakrishnan et al. 2011; Schrey and Tarkka 2008; van der Ent et al. 2009). 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 (Berg 2009).

In plants, induction of resistance is typically achieved through two physiological pathways namely systemic acquired resistance (SAR) and induced systemic resistance (ISR), mechanisms that can be differentiated by the

regulatory pathways and the nature of the elicitor. Whilst SAR is triggered by necrotizing pathogens (Conrath et al. 2002), ISR is activated by non pathogenic rhizobacteria, such as specific PGPR (van der Ent et al. 2009). Furthermore, ISR is dependent on jasmonic acid and ethylene signaling in colonized plants (van Loon et al. 1998), 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* (Conrath et al. 2006). Priming plants with PGPRs can provide systemic resistance against a broad spectrum of plant pathogens (Compant et al. 2005). The efficiency of rhizobacteria *Streptomyces* as biocontrol agents has been reported in numerous studies. *S. hygroscopicus* was efficient against downy mildew grape caused by *B. cinerea* through production of antimicrobial molecules (Nair et al. 1994), and *S. cavourensis* SY224 reduced anthracnose in pepper, result attributed in part by the production of chitinase and glucanase (Lee et al. 2012). Roots of Norway spruce inoculated with *Streptomyces* GB 4–2 provided systemic resistance to *B. cinerea* (Lehr et al. 2008). Furthermore, culture filtrates from *S. bikiniensis* HD-087 were able to induce ISR in cucumber against *Fusarium* wilt, and treatments increased the activities of peroxidase, phenylalanine ammonia lyase, and β-1, 3-glucanase (Zhao et al. 2012). Moreover, 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. unpublish data).Up to date, those are the only two 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 from the phenylpropanoid pathway and hence, the production of phytoalexins and phenolic compounds, are associated to 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 (Alizadeh et al. 2013; van Loon 1997).

The aim of this work was to evaluate the ability of the isolate *Streptomyces* sp. PM9 as elicitor of defense responses against *B. cinerea* in plants of *E. grandis* and *E. globulus in vitro*. Secondary metabolism and disease progression were analyzed. The potential of antibiosis was also evaluated as a possible component of disease control.

Materials and methods

Plant material and microorganisms

Seeds of*E. grandis*and *E.globulus*were surface disinfectedin70% ethanolfor 60sfollowed by treatment with fungicideRidomilGold®MZ(8 g L^{-1})for 20 min, and immersion in sodium hypochlorite (1%) solution for 10min. Seeds were rinsed three times with sterile distilled water and sown on MS culture medium (Murashige and Skoog 1962) with salt concentration reduced to one quarter (1/4 MS), supplemented with 10 g L^{-1} sucrose and 6 g L^{-1} of 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 was grown from stock cultures initiated from samples collected in the Araucaria Forest at Pró-Mata Centre for Research and Conservation of Nature (29°29'18.4"S, 50°12'23,5" W), São Francisco de Paula, Rio Grande do Sul, Brazil. Isolate was cultivated in ISP_4 liquid medium (Shirling and Gottlieb 1966), shaken at 100 rpm for seven days (stationary phase), centrifuged at 2,500 *xg* 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) (Dalmas et al. 2011). The plant pathogenic fungus *Botrytis cinerea* was grown for 15 days on potato agar (PDA), and a suspension consisting of hyphae was prepared with sterile distilled water and adjusted to OD_{600nm}= 0.2 and 0.5, representing 3,050 and 9,520 hyphae mL^{-1}.

Co-cultivation of *Streptomyces* sp. PM9 and *B.cinerea*

The antagonism between *Streptomyces* sp. PM9 and *B. cinerea* was verified by co-cultivation. In Petri dishes containing semi-solid medium ISP_4, 200 µL of *Streptomyces* sp. PM9were inoculated, establishing a line 1 cm away from the edge of the plate. In the opposite position, 1 cm away from the edge, a disk (1 cm diameter) containing *B. cinerea* was positioned. In a control plate, rhizobacteria was replaced by 200 µL of sterile distilled water. Co-cultivation was maintained for 30 days and size of the inhibition zone (mm) was recorded.

Disease evaluation

Plants of *E. grandis* of *E. globulus* were cultivated in an *in vitro* system according to Lehr et al. (2008), with modifications. Briefly, 25 mL of ¼ MS medium were poured in a Petri dish (9 cm in diameter), and following agar

115 solidification, a semicircle of medium was discarded and one plant was placed onto the remained medium
116 semicircle. Treatments consisted of (i) plants inoculated with sterile distilled water (control); (ii) plants inoculated
117 with *B. cinerea* (treatment F) on the roots (OD_{600nm}=0.2 or 0.5) and (iii) plants elicited with *Streptomyces* sp. PM9
118 (OD_{600nm}=1) and challenged with *B. cinerea* four days after elicitation (OD_{600nm}=0.2 and 0.5; treatments PM9+F
119 0.2 and PM9+F 0.5). Inoculations were performed disposing 200 μLof either rhizobacteria or fungus on root
120 surface.

121 Disease incidence and development ofgray mold symptoms were evaluated in shoots, which were divided in
122 three parts, being each part accounted for 33.33%. Disease incidence in a shoot completely infected was considered
123 100%. Data were collected from observations at each two days from the experiment onset. The area under the
124 disease progress curve (AUDPC) was estimated. AUDPC values were normalized and corrected (AUDPC-nc) by
125 dividing the values by the number of days until the final severity reading for each treatment and multiplying the
126 resulting values by the number of days until the final severity evaluation (Graichen et al. 2010; Zambonato et al.
127 2012), as shown below:

128

129 AUDPC-nc = {{Σ[(yi+1 + yi) x 0,5]*[ti+1 - ti]}/n}*c,

130

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

134

135 Evaluation ofsecondarymetabolism of *Eucalyptus* spp. elicited with *Streptomyces* sp. PM9

136 Plants of *E. grandis* and *E. globulus* were transferred to the *in vitro* system described above. Treatments consisted
137 of (i) plants inoculated with sterile distilled water (absolute control); (ii) plants inoculated with *Streptomyces* sp.
138 PM9 (OD_{600}=1; treatment PM9) on the roots; (iii) plants infected with *B. cinerea* (OD_{600nm}=0.5) on the roots
139 (control for disease; treatment F) and (iv) plants elicited with *Streptomyces* sp. PM9 (OD_{600nm}=1) and challenged
140 with *B. cinerea* (OD_{600nm}=0.5; treatment PM9+F) four days after elicitation. Inoculations were performed
141 disposing 200 μL of either rhizobacteria or fungus on root surface.

142 Plants were evaluated for basal secondary metabolism before and after four days of inoculation with
143 *Streptomyces* PM9, named B and BS, respectively. Defense responses were evaluated at 1, 3, 9 and 15 days post-
144 inoculation with *B. cinerea*. The induced levels of secondary compounds (total phenolics and flavonoids) and the
145 activity of polyphenol oxidases (PPO) and peroxidases (POX) were the parameters analyzed. Each treatment
146 consisted of 20 plants per time course point, totalizing 380 plants. Shoots and roots of *Eucalyptus* plants were
147 analyzed separately. Shoots or roots from each treatment and each time course were pooled, kept on ice and cut in
148 small pieces. A minimal of three biological repetitions was used and each repetition was analyzed in three
149 replicates for the colorimetric reactions and in duplicate for chromatographic analysis.
150
151 Defense enzymes activity determination
152 Activities of the enzymes polyphenol oxidase (PPO; EC 1.14.18.1) and peroxidases (POX; EC 1.11.17) were
153 determined according Savio et al. (2012), with little modification. Briefly, extracts were prepared from shoots and
154 roots (0.4 g) ground in 2.5 mL of 50 mM sodium phosphate buffer (pH 7) and polyvinylpyrrolidone (PVP; 1:6
155 w/v). Before grinding, plant material was extensively rinsed in distilled water to remove any excess of medium or
156 microorganisms. Extracts were filtered and centrifuged at 2,500 *xg* for 15 min at 5 ºC, and the supernatant was
157 collected for determination of protein content and enzyme assays. PPO activity was determined in a reaction
158 containing chlorogenic acid (1 mM) as substrate at 400 nm. Specific enzyme activity was defined as the change in
159 absorbance min^{-1} mg^{-1} protein. The activity of peroxidases was determined in a reaction mixture containing 50 mM
160 sodium phosphate buffer (pH 6), 1% (v/v) guaiacol as substrate and 10 mM hydrogen peroxide, using the crude
161 extract described above. Oxidation of guaicol was measured by the increase in absorbance at 420 nm for 30 s at
162 an interval of 5 s. Specific enzyme activity was expressed as µkatal mg^{-1} protein. Total protein concentration was
163 determined according to Bradford's method (Bradford 1976), using bovine serum albumin as standard.
164

165 Analysis of secondary compounds

166 Shoots and roots samples of *Eucalyptus* plants (0.1 g of fresh mass; FM) were taken from each treatment, blot
167 dried on sterile filter paper and ground in 10 mL of 80% (v/v) methanol at room temperature. Extracts were filtered
168 and centrifuged at 1,250 *xg* for 15 min. Total phenolic compounds were analyzed in the supernatant by the
169 colorimetric Folin-Ciocaulteau method as described previously (Sartor et al. 2013). Gallic acid was used as the
170 standard. The contents of total phenolic compounds were expressed as mg g^{-1} FM. The fraction of quercetinic-
171 derived flavonoids was determined by the colorimetric method using the reaction with 96% ethanol, 10%
172 aluminum nitrate and 1 M potassium acetate, measured at 415 nm. Quercetin was used as standard for the
173 calibration curve. Flavonoid content was expressed as mg quercetin equivalents g^{-1} FM (Poiatti et al. 2009).

174 Identification and quantification of the main phenolic compounds in *Eucalyptus* sp. plants were determined
175 by High Liquid Performance Chromatography (HPLC). Analyses were carried out an Agilent Technologies, 1200
176 Series operated at 45 ºC, and separations were performed on a MetaSil ODS column (5 µm; 150 x 4.6 mm).
177 Detection was achieved with a UV/V detector set at 280 nm. Gradient was formed between two mobile phases:
178 phase A consisted of 2% of formic acid in water and phase B in methanol (100%). The analysis followed a linear
179 gradient programmed as 10% to 20% of eluent B from 0 to15 min, 20 to 40%, from 15 to 20 min, 40 to 60% from
180 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 injection
181 volume was 20 µL. HPLC analysis was performed by using a five point calibration curve generated with authentic
182 phenolic standards (gallic acid, caffeic acid, chlorogenic acid, 2-hydroxybenzoic acid, benzoic acid, catechin, and
183 coumarin).
184
185 Statistical analysis
186 Experiments for evaluation ofsecondarymetabolism were performed in completely randomized design, tested for
187 variance homogeneity by Levene's test and subjected to one-way ANOVA. Means were separated by Tukey Test
188 at significance level of $\alpha \leq 0.05$. All statistical analyzes were performed using the software SPSS v. 17.5. Data
189 from enzymatic activities and secondary metabolites were expressed as mean ± standard error. HPLC analysis was
190 carried out with two replicates obtained from the pool of root and shoot samples prepared from total phenolic
191 compounds analysis. Data was expressed as mean ± standard deviation.
192

Results

Co-cultivation of *Streptomyces* sp. PM9 and *Botrytiscinerea*

In the co-cultivation of *Streptomyces* sp. PM9 x *B. cinerea* an inhibition zone (20 mm) was observed between the two microorganisms. In the control, fungus grew uniformly on the plate (Fig. 1).

Disease evaluation

Evaluation of the area under disease progress curve (AUDPC) in plants of *E. grandis* and *E. globulus* showed difference among the treatments. Plants of *E. grandis* infected with *B. cinerea* at OD_{600nm}= 0.2 resulted in greater area (12,032.80 ± 3,610.40) than the plants elicited and challenged with pathogen (PM9+F0.2; 1,338.76 ± 886.81). Similar result was recorded for the OD_{600nm}=0.5 (Table 1). Contrary, *E. globulus* elicited-plants were similar in response to both densities of pathogen, although differences from control plants were recorded (Table 1). The response observed with AUDPC could be confirmed when disease incidence was recorded as percentages of incidence during cultivation period. The lowest disease incidence (13.3%) was recorded in *E. grandis* plants from PM9+F0.2 treatment, with the first diseased plant observed at 12 dpi (Fig. 2a). Highest and earliest incidence (66.7%; 7 dpi) was observed in F-treated plants. Highest density of fungus in elicited plants (PM9+F0.5) resulted in higher disease incidence (33%), when compared with the OD_{600nm}=0.2, although the timing of disease appearance was the same (12 dpi) (Fig. 2a and b). In *E. globulus* no differences were observed between optical densities used for fungus inoculation, either in F or PM9+F treatments. However, disease was seen in elicited-plants at 10 dpi (Fig. 2c e d). In *E. globulus* plants, percentage of mortality was higher than in *E. grandis*, reaching 100% within 18 dpi.

Enzymatic activity and secondary compounds

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

In *E. globulus* roots, the highest PPO activity was detected at 1 dpi in PM9+F, followed by PM9. Nonetheless, a reduction on the enzyme activity on PM9+F plants was observed at 3 dpi. At 15 dpi, an increase on PPO activity was observed in both F and PM9+F treatments (Fig. 5a). On the other hand, different response was observed for POX (Fig. 5b). At 1 dpi, roots infected with the fungus (F) showed a markedly increase on POX activity, whereas a reduced level of enzymatic activity was observed in PM9+F. Although levels of POX for the treatments were lower than the control plants, differences were evident between PM9/PM9+F and fungus-infected plants at 3 and 15 dpi. Roots on F treatment showed steady levels of POX activity from 3 dpi of *B. cinerea* and thereafter (Fig. 5b).

Production and accumulation of phenolic compounds were observed in roots of *Eucalyptus* sp. in response to the microorganisms. A decrease on the phenolics levels was detected in *E. grandis* roots four days after inoculation with *Streptomyces* (BS) in PM9-plants and at 1 dpi in PM9+F (Fig. 4a). Increased levels of phenolics on PM9+F were observed at 3 dpi, which was coincident with the highest POX activity at this time point. At 9 dpi, the lowest level was found on PM9+F plants, coincident to the highest PPO activity (Fig. 3a and 4a). Similarly, the lowest level was coincident to the highest POX activity on elicited plants (Fig. 3b and 4a). Levels of quercetinc flavonoids were reduced in plants treated with PM9 and PM9+F at 9 dpi (Fig. 4b). Infection with *Botrytis* resulted in increased levels of flavonoids at 1dpi (Fig. 4b).

Phenolic compounds in *E. globulus* roots were affected by *Streptomyces* sp. and reduction was observed at BS on PM9-plants (Fig. 6a). However, on this species, the lowest levels resulted from plants infected with *B. cinerea*, along the time of the experiment (Fig. 6a). At 15 dpi, the lowest level of phenolics on F treatment coincided with highest activity of PPO (Fig. 5a and 6a). PM9-roots evidenced higher levels of phenolics than control plants at 3 and 15 dpi (Fig. 6a). Flavonoids were increased in response to inoculation with PM9 (BS and thereafter; Fig. 6b). At 15 dpi PM9+F plants showed the highest concentration of these metabolites.

Similar to the roots, shoots of *Eucalyptus* sp. were affected by treatments. In *E. grandis* PPO activity showed slightly increment in F treatment at 3 dpi and this response persisted until 9 dpi (Fig. 3a). No significant difference was observed among 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 on PM9+F at 3 and 9 dpi (Fig. 3b). However, these plants showed a markedly decrease on POX activity at 15 dpi (Fig. 3b). POX activity in shoots from plants PM9+F raised immediately after the initial responses had taken place on the roots.

In *E. globulus*PM9+F-shoots, PPO activity showed slightly increase when compared to control plants at 3 dpi, and such response was highlighted at 9 dpi (Fig. 5a). At 15 dpi, treatments differed significantly from the control and the highest activity was recorded in F infected-plants, followed by PM9 treatment (Fig. 5a). POX activity was higher in PM9-treated shoots at 3 and 15 dpi, whereas in PM9+F plants, a markedly increase was detected at 9 dpi (Fig. 5b). When compared to *E. grandis*, PPO and POX activities were overall higher in *E. globulus* shoots (Fig. 3 and 5).

Phenolic compounds varied with the treatments and time of culture in shoots of both *E. grandis* and *E. globulus*. Similar results were observed in shoots of both species at 3 dpi, when PM9 and PM9+F plants showed the highest amounts (Fig. 4a and 6a). No significant differences among the treatments were evidenced at the last two time points in *E. grandis*, although in *E. globulus* all treatments differed from the control (Fig. 4a and 6a). Specifically for *E. globulus*, shoots from PM9 treatment showed a constant high level of phenolics from 1 dpi until the end of cultivation. Increased levels of these compounds in *E. grandis* shoots could be related to the reduced levels of POX activity at 1 dpi (Fig. 4a and 3b). Overall, shoots from elicited plants showed higher levels of phenolics than plants infected with *B. cinerea*(F) at 1 and 3 dpi, which may have play a role as substrates for POX at 3 and 9 dpi for both species (Fig. 3b, 4a, 5b, 6a). Quercetinic flavonoids were maintained elevated in PM9 plants along the culture in both species assayed (Fig.4b, 6b). At 3 dpi, however, shoots from PM9 and PM9+F showed similar levels of these compounds (Fig.4b, 6b). Differences between species were recorded at 9 and 15 dpi, although all treatments showed higher levels than the control shoots.

Chromatographic analysis of phenolic compounds showed wide variation for each compound and species tested (Tables 2 and 3). At basal level (B), before any contact with microorganisms, only chlorogenic acid was detected in shoot and roots of both species, reaching the highest levels in *E. globulus*. Gallic acid was detected in shoot and roots of *E. grandis* and shoot of *E. globulus*, and in all it was present in higher levels than chlorogenic acid.Coumarin was present in *E. globulus*, whereas caffeic acid, 2-hydroxybenzoic acid, benzoic acid, catechin were absent in plants at this time point (Table 2 and 3). Upon contact with microorganisms (*Streptomyces*, *B. cinerea* or both), concentrations of phenolic showed variation, which, at some extension, could reflect part of the response to plant-microorganism interaction.

Overall, in roots of *E. grandis* plants from F treatment show higher concentrations of chlorogenic acid, caffeic acid and gallic acid at 1 dpi (Table 2). Chlorogenic acid, caffeic acid, catechin, 2-hydroxybenzoic acid and coumarin were abundant at 9 dpi. Roots of elicited plants showed the highest concentration catechin at 1 and 3

dpi, in addition of coumarin at 3 dpi. In shoots, gallic acid showed higher concentrations when compared to control plants at 1 dpi and 3 dpi. Caffeic acid was not detected at 3 dpi in PM9 and PM9+F shoots. In plants infected with *Botrytis* (F or PM9+F), coumarin was observed in higher concentrations than in PM9 and control plants at 1 dpi (Table 2).

In roots of *E. globulus* little variation was observed on phenolic compounds (Table 3). However, benzoic acid, catechin and coumarin were the compounds found in highest concentrations in PM9+F plants at 1 dpi. Chlorogenic acid and coumarin were detected in PM9 and PM9+F at 15 dpi. A significant decrease in caffeic acid at 1 dpi was observed. In shoots from PM9 and PM9+F plants caffeic acid, coumarin and 2-hidroxibenzoic acid were increased in PM9-treated plants at 1 dpi. In PM9+F plants, differences were observed in gallic acid and caffeic acid at 3 dpi and in chlorogenic acid, 2-hidroxibenzoic acid, caffeic acid, benzoi acid at 9 dpi. However, gallic acid was not detected in PM9 and elicited plants at 15 dpi. Catechin was elevated in elicited plants, followed by F and PM9 plants at 15 dpi (Table 3).

Discussion

The ability and performance of *Streptomyces* species on 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 promote ISR against a number of plant diseases (Jetiyanon and Kloepper 2002). PGPRs, including *Bacillus* spp. (Lin et al. 2014), *Streptomyces* spp. (Zhao et al. 2012), *Pseudomonas* spp. (Ran et al. 2005), and *Trichoderma* spp. (John et al. 2010) have been already used to control several antagonistic microorganisms.

Co-cultivation between *Streptomyces* and *B. cinerea* showed antibiosis activity of the isolate PM9 against the pathogenic fungus, since inhibition of mycelial growth was observed. Similarly, *S. padanus* TH-04 showed antifungal activity against *Monilinia fructicola* (Lim et al. 2007), whereas antifungal activity of purified compounds produced by *Streptomyces anulatus* S37 against *B. cinerea* was reported forboth *in vitro* and *in vivo* seedlings of vines (Couillerot et al. 2014).

Beneficial rhizobacteria, as PGPR, trigger ISR by priming the plant for potentiated activation of various cellular defense responses, which are further induced by the pathogen (Conrath et al. 2006; Ramamoorthy et al. 2002). They reduce disease severity and enhance yield of many crops (Kim et al. 2014; Murphy et al. 2000). Increased

levels of defense-related enzymes during ISR are known to play a crucial role in plant host resistance (Chen et al. 2000) and are usually linked to responses including cell-wall reinforcement (Mandal and Mitra 2007) and production of secondary metabolites (Yedidia et al. 2001).

Indirect mode of action of *Streptomyces* sp. PM9 on plants of *E. grandis* and *E. globulus* was determined by the analysis of secondary metabolism and disease evaluation. Intense enzymatic alterations (PPO and POX) occurred in both roots and shoots when plants were elicited with *Streptomyces* sp. and challenged with *B. cinerea*. The similarity of response between plants inoculated with *Streptomyces* sp. and not challenged with the pathogen and those elicited and challenged indicates that *Streptomyces* sp. PM9 raised the basal activity levels of defense-related enzymes and secondary metabolites both locally and systemically. Combination of changes in enzymatic activity and disease delay suggests that ISR in *Eucalyptus* sp. is being mediated by *Streptomyces* sp. PM9. Studies have reported ISR elicited by streptomycetes against *Colletotrichumgloeosporioides* (anthracnose) in pepper and cherry tomato (Kim et al. 2014), and against *C. musae* in banana (Taechowisan et al. 2009).

Changes in secondary metabolism are often evidenced in plant defense responses. Induction of defense enzyme increases plant resistance against pathogen invasion (van Loon et al. 1998). Peroxidases are key enzymes in the plant defense since promote oxidation of phenolic compounds using hydrogen peroxide (H_2O_2) as an electron donor for 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 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 ragi (Radjacommare et al. 2004).

Notwithstanding the generation of reactive oxygen species (ROS) had been related to plant defense responses by causing hypersensitivity response and plant cell death, these molecules facilitate root colonization by necrotrophic fungi, such as *B. cinerea* (Asselbergh 2002; van Kan 2006).The significant decreased POX activity in *E. grandis* and*E. globulus* roots at 9 dpi in fungus-infected plants might be consequence of a plant effort of blocking the fungus infection by suppressing the production of ROS, likely diminishing the H_2O_2 availability for POX. However, in *E. grandis* later on the process hyphae are already established in the plant tissue, setting the disease, and thus POX activity was resumed. On the other hand, when early activated, POX plays an important role in plant resistance against *B. cinerea* (Małolepsza 2006; Senthilraja et al. 2013). This response was observed in roots and shoots of *E. grandis* elicited plants at 3 dpi of *B. cinerea*. However, in PM9+F plants of *E. globulus*, significant activation of POX occurs in the shoots at 9 dpi, demonstrating a later systemic response. At 15 dpi this

enzyme is still activated in elicited plants of both species compared to the control plants, indicating that some level of response triggered by *Streptomyces* sp. PM9 is taking place. 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). Involvement of PPO in ISR mediated by PGPR in cucumber has been demonstrated (Chen et al., 2000). In *E. grandis* shoots, activity of PPO was higher in plants infected with *B. cinerea*at 3 and 9 dpi, coincident with lowest concentrationsof total phenolic compounds. This enzyme might be metabolizing phenolics to produce toxic molecules against fungus infection. Nevertheless, later at 9 dpi roots from elicited plants showed PPO activity significantly different from those plants infected with the fungus, which combined with a decrease of phenolic compounds, suggest the effect of *Streptomyces* sp. PM9 on modulating the metabolism against *B. cinerea*. In *E. globulus*, highest activity of PPO was observed at 1 and 3 dpi, in roots and shoots, respectively. In contrast to *E. grandis*, activity of this enzyme was earlier triggered.

In cases where activation of defenses enzymes is coincident with highest levels of phenolic compounds, such as responses of POX and PPO in elicited plants of *Eucalyptus* sp. at 3 or 9 dpi, other enzymes of the phenylpropanoid pathway might be activated. Synthesis of phenolic compounds and flavonoids is catalyzed by phenylalanine ammonia lyase (PAL), known to be involved on ISR mediated by PGPRs. Chithrashree et al. (2011) showed increased synthesis of PAL, POX and PPO enzymes in two PGPR (*Bacillus* sp.) treated plants and challenged with pathogen *Xanthomonas oryzae* pv. *oryzae*. Similarly, *Carnobacterium* sp. SJ-5 was found to be significantly eliciting ISR in soybean plant leading to accumulation of defense-related proteins PAL, PPO and POX in different parts of plants (Jain and Choudhary 2014).

Plants of *E. grandis* and *E. globulus* showed differences on production ofphenolic compounds. In *E. globulus* non-treated roots, levels of phenolic compounds were shown to be higher than observed in *E. grandis*. On the contrary, during after contact with microorganisms (either elicitation or challenging) variation of phenolic in *E. grandis* was more significant than in *E. globulus*. In shoots, accumulation of phenolics was different from the control in all treatment tested at least until 3 dpi in both species, strengthening the hypothesis of induced systemic response in these plants. Differently from phenolics, quercetinic flavonoids were increased in 4 days after

366 inoculation with PM9 (BS) in *E. globulus*, which may suggest a response of interaction between plant and
367 *Streptomyces* sp. At 1 and 3 dpi, PM+F plants showed more flavonoids than fungus infected plants in *E. globulus*
368 and *E. grandis*, respectively. Certain flavonoids may influence the association with PGPRs and are involved in
369 host defense against pathogens, exhibiting antifungal properties and acting as phytoalexins (Jeong et al. 2014). At
370 9 dpi, levels of flavonoids were increased in shoots ofelicited plants, which at this time course, still did not show
371 disease symptoms. Likelihood, flavonoids are playing a role as antagonistic compound against to *B.*
372 *cinerea.*Although significant difference had been observed in the levels of flavoinoids in *E. globulus* at the same
373 time point, this metabolic alteration was not efficient to delay the gray mold in this species.
374 Individually phenolic compounds showed variation among treatments along the time of culture. Gallic acid
375 was the most abundant phenolic detected in both species in non-treated plants. When elicited plants were taken in
376 consideration, most of the phenolics were produced or accumulated at 1 and 3 dpi for both species. Roots of *E.*
377 *grandis* showed presence of chlorogenic acid, gallic acid, catechin, caffeic acid and coumarin, whereas in *E.*
378 *globulus* catechin, benzoic acid and coumarin were the most abundant phenolics. Catechin was present in roots of
379 elicited *E. grandis* plants during all time points assayed while in fungus-treated plants this compound appeared at
380 9 dpi. On the other hand, in shoots of *E. globulus* catechin was markedly increased at 15 dpi. This compound is
381 known to present antibacterial and antifungal activities and its biosynthesis and accumulation was related to
382 defense responses in leaves of wheat upon *Puccinia triticina* attack (Ghassempour et al. 2010). Likewise, gallic
383 acid was also reported as antifungal compound against *Fusarium solani* (Nguyen et al. 2013). Benzoic acid seemed
384 to be produced in roots of both species in response to either fungus infection or elicitation with *Streptomyces* sp.
385 PM9. Similarly, 2-hydroxybenzoic acid was detected in shoots at same treatments in different time points.
386 Accumulation of low molecular weight phenols, such as benzoic acids and other phenylpropanoids, are also formed
387 in the initial response to infection (Niemann et al. 1991), and*p*-hydroxybenzoic acid was involved in the initial
388 defense reactions of *Phoenix dactylifera* to the brittle leaf disease (Latreche and Rahmania 2010). Indeed, *p*-
389 hydroxybenzoic, the salicylic acid analog, is known to function as a phytoalexin. Its accumulation in plant is
390 associated with antimicrobial activity and fungitoxicity (Chong et al. 2009). In *E. grandis*, caffeic acid was evident
391 in roots of fungus infected-plants at 1 dpi and in elicited plants at 3 dpi and might be prone to oxidation into *o*-
392 quinones, which are toxic to microorganisms (Lattanzio et al. 2006). In *E. globulus*, this compound was not
393 detected in roots of F-plants.

Different responses against *B. cinerea* were seen in plants of *Eucalyptus* and disease symptoms were significantly delayed in *E. grandis*. This response might be related to 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*. However, changes on defense-related enzymes were more expressive in elicited *E. grandis* plants. Results indicate that efficient modulation of secondary metabolism in *E. grandis* took place, reducing and delaying gray mold development. Although some alteration had been observed on secondary metabolism of *E. globulus*, it seems that the influence of *Streptomyces* was less effective.

Notwithstanding, reduction on severity of gray mold disease in *E. grandis* is not only related to the induction of ISR by *Streptomyces* sp. PM9, but could also be consequence of the antagonism against *B. cinerea*. In conclusion, *Streptomyces* sp. PM9 was able to elicit plants *E. grandis*, increasing the basal levels of two enzymes (PPO and POX) directly related to induction of systemic resistance, as well as promoting synthesis of phenolic compounds. Our results showed that *Streptomyces* sp. PM9 poses as a candidate for biological control agent against *B. cinerea* in the 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 considered an aggressive necrotrophic fungus.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TDS designed and performed the experiments, analyzed the data, and wrote the manuscript. TRS assisted with the experiments and analyses. LVA gave technical advice and contributed to the study design. ERS conceived the idea, designed and coordinated the study, and edited the manuscript. All authors read and approved the final manuscript.

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Table 1AUDPC-nc values of *Eucalyptus* plants elicited with *Streptomyces* sp. PM9 and challenged with *B. cinerea*, 20 days post-inoculation with the pathogen.

Treatments*	***E. grandis***	***E. globulus***
	AUDPC	**AUDPC**
Control	0±0.0 (b)**	0±0.0 (b)
F 0.2	12032.80±3610.40 (a)	13828.61±2227.26 (a)
PM9+F 0.2	1338.76±886.81 (b)	4652.56±6922.89 (a)
Control	0±0.0 (b)	0±0.0 (b)
F 0.5	5818.46±1552.19 (a)	18426.72± 9555.13(a)
PM9+F 0.5	3755.18±1676.89 (b)	13845.28± 4314.18(a)

*C= control (distilled water), F= plants infected with *B. cinerea* ($OD_{600nm=}$ 0.2 and 0.5), and PM9+F= elicited plants with *Streptomyces* sp. PM9 and challenged with *B. cinerea* ($OD_{600nm=}$ 0.2 and 0.5). Data expressed as mean±standard error.

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

Table 2. Quantification of phenolic compounds (mg g^{-1} FM) in plants by HPLC in *E. grandis* rootsand shoots.

Phenolic Compounds	Treatments*	Roots						Shoots					
		B	BS	1 dpi	3 dpi	9 dpi	15 dpi	B	BS	1 dpi	3 dpi	9 dpi	15 dpi
Chlorogenic acid	**C**	1.1±0,01	2.3±0.2	2.1±0.3	4.8±1.1	4.0±0.1	7.4±0.1	1.2±0.2	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**	1.1±0.01	9.8±2.5	ND	33.2±4.9	18.4±11.7	50.6±5.4	3.1±1.2	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**	ND	4.6±0.1	4.4±2.4	7.4±3.5	ND	15.4±0.5	ND	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**	ND	4.3±0.01	ND	5.5±1.6	4.8±0.3	13.9±1.9	ND	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	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±4.9	ND	-	-	1.0±0.2	ND	3.3±2.0	1.7±0.5
Catechin	**C**	ND	10.2±0.8	ND	ND	ND	24.0±3.3	ND	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**	ND	1.2±0.2	0.9±0.3	2.0±0.5	2.6±0.1	10.8±0.7	ND	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

*C= control, PM9= *Streptomyces* sp. PM9, F= pathogenic fungus B. *cinerea* and PM9+F= elicited plants with PM9 and challenged with *B. cinerea*. Quantifications were performed at 1, 3, 9 and 15 days post-inoculation with *B.cinerea*. ND: not detected.Responses were evaluated at 1, 3, 9 and 15 days post-inoculation with *B. cinerea*. B and BS: basal secondary metabolism before and after four days of inoculation with *Streptomyces* PM9, respectively.

Table 3. Quantification of phenolic compounds (mg g^{-1} FM) in plants by HPLC in *E. globuluss* rootsand shoots.

Phenolic Compounds	Treatments*	Roots						Shoots					
		B	BS	1 dpi	3 dpi	9 dpi	15 dpi	B	BS	1 dpi	3 dpi	9 dpi	15 dpi
Chlorogenic acid	**C**	3.0±1.1	3.7±1.0	4.7±0.001	2.0±4.6	1.2±0.1	1.6±0.2	3.7±1.0	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	ND	38.3±0.3	17.8±0.4	2.2±1.6	21.3±5.6	8.3±0.1	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	ND	15.5±0.8	3.1±1.1	ND	7.8±3.1	ND	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	ND	11.4±1.6	ND	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**	ND	0.6±0.3	1.3±0.0	ND	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	ND	17.7±0.8	ND	ND	11.7±2.9	ND	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**	ND	2.2±0.4	2.5±0.1	4.4±0.4	0.5±0.2	ND	1.1±0.006	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

*C= control, PM9= *Streptomyces* sp. PM9, F= pathogenic fungus B. *cinerea* and PM9+F= elicited plants with PM9 and challenged with *B. cinerea*. Quantifications were performed at 1, 3, 9 and 15 days post-inoculation with *B.cinerea*. ND: not detected.Responses were evaluated at 1, 3, 9 and 15 days post-inoculation with *B. cinerea*. B and BS: basal secondary metabolism before and after four days of inoculation with *Streptomyces* PM9, respectively.

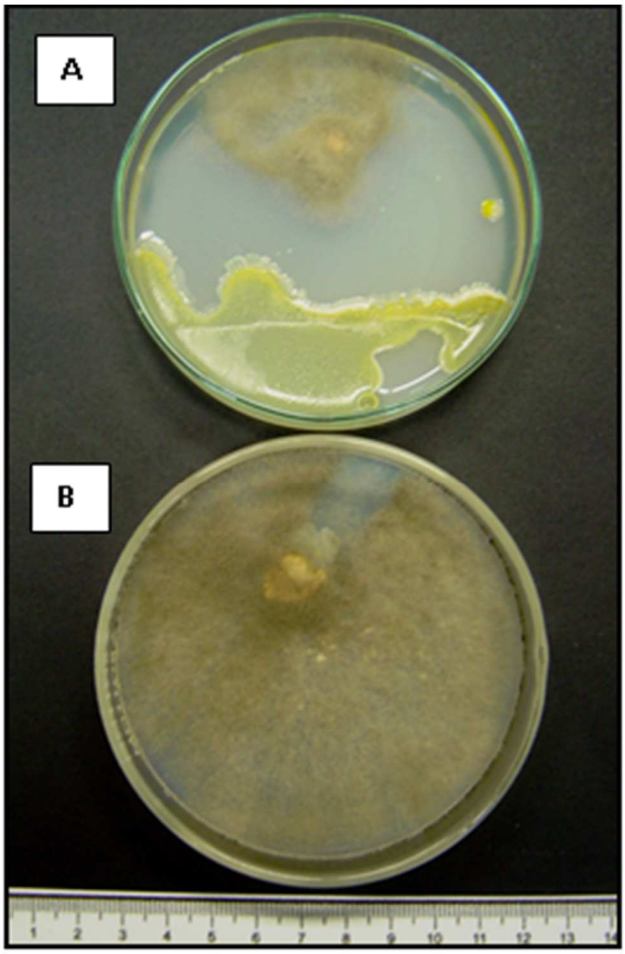

Fig. 1 Experiment of co-cultivation of (a) *Streptomyces* sp. PM9 x *B. cinerea* and (b) *B. cinerea* (control plate).

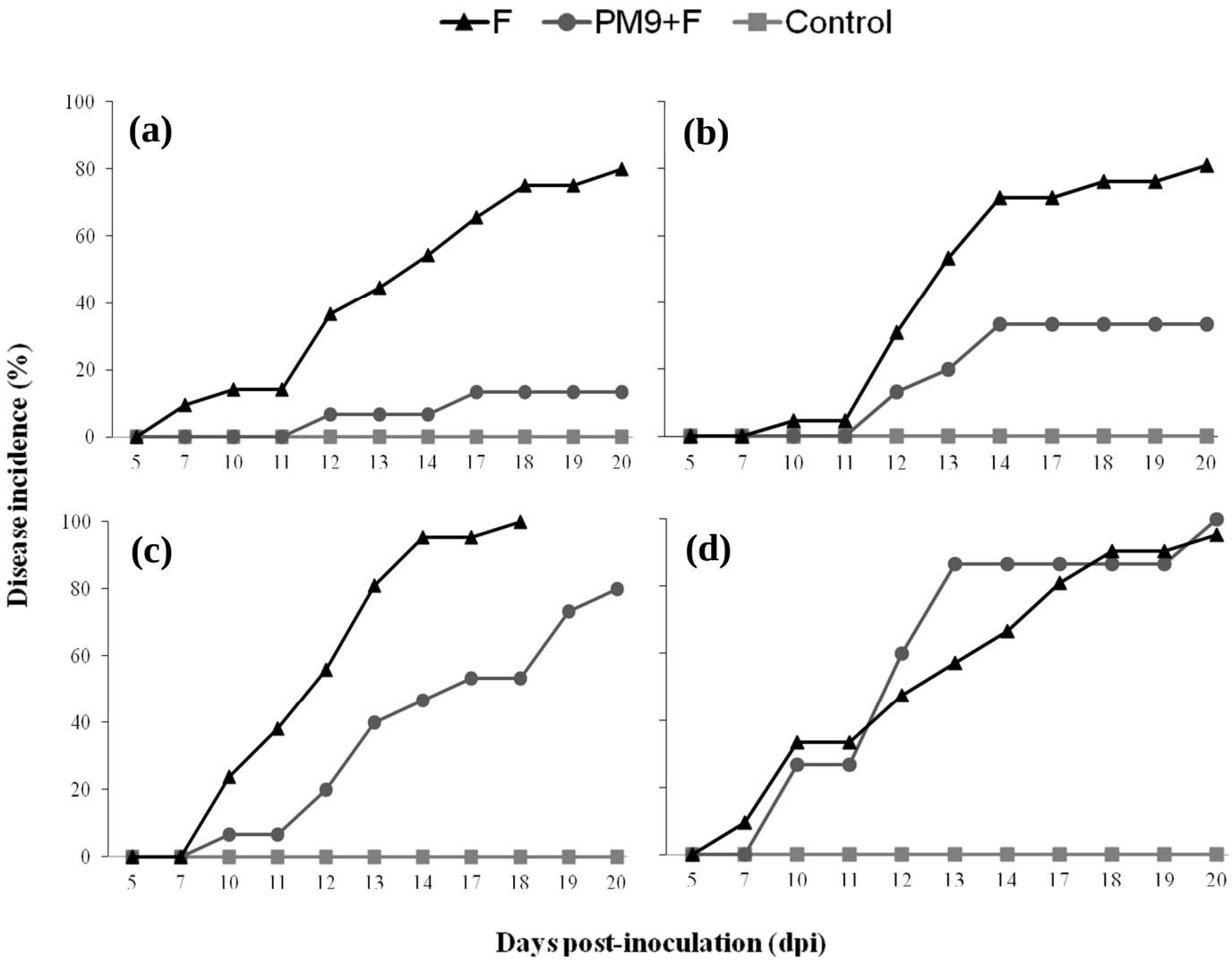

Fig. 2 Percentage of disease incidence during 20 days after inoculation with *B. cinerea*. (a, b) *E. grandis*; (c, d) *E. globulus*. Treatments consisted of Control, F= plants infected with *B. cinerea* andPM9+F= elicited plants with *Streptomyces* sp. PM9 and challenged with *B. cinerea*. Different optical densitieswere tested: (a, c) OD_{600nm}= 0.2; (b, d). OD_{600nm}= 0.5.

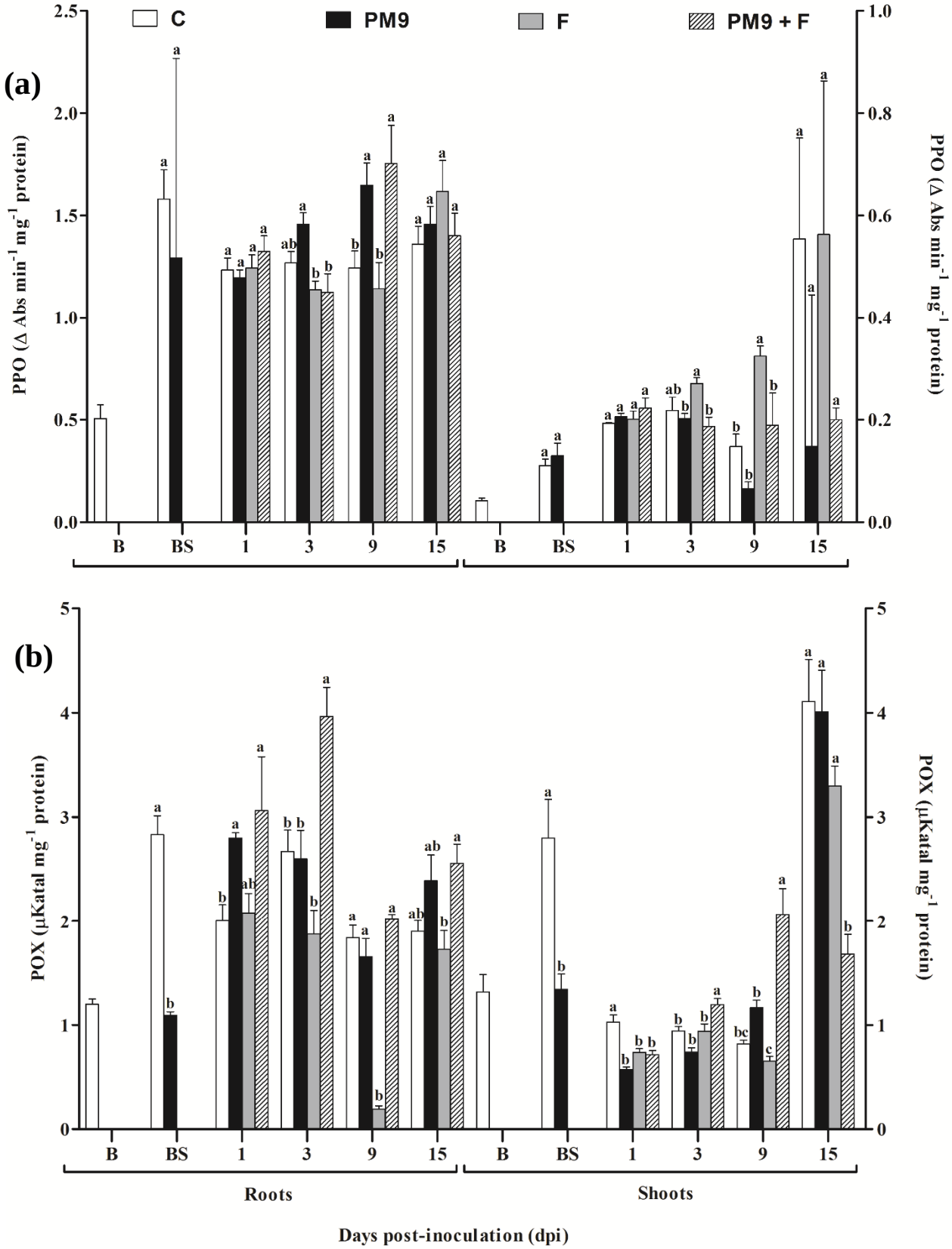

Fig 3 Activities of the enzymes (a) polyphenol oxidase and (b) peroxidases in roots and shoots of *E. grandis*. C= control, PM9= *Streptomyces* sp. PM9, F= pathogenic fungus *B. cinerea* and PM9+F= elicited plants with *Streptomyces* sp. PM9 and challenged with *B. cinerea*. Responses were evaluated at 1, 3, 9 and 15 days post-inoculation with *B. cinerea*. B and BS: basal secondary metabolism before and after four days of inoculation with *Streptomyces* PM9, respectively.Bars represent standard error of the mean. Different letters indicate significantly differences among treatments within a time point (Tukey test, $\alpha \leq 0.05$).

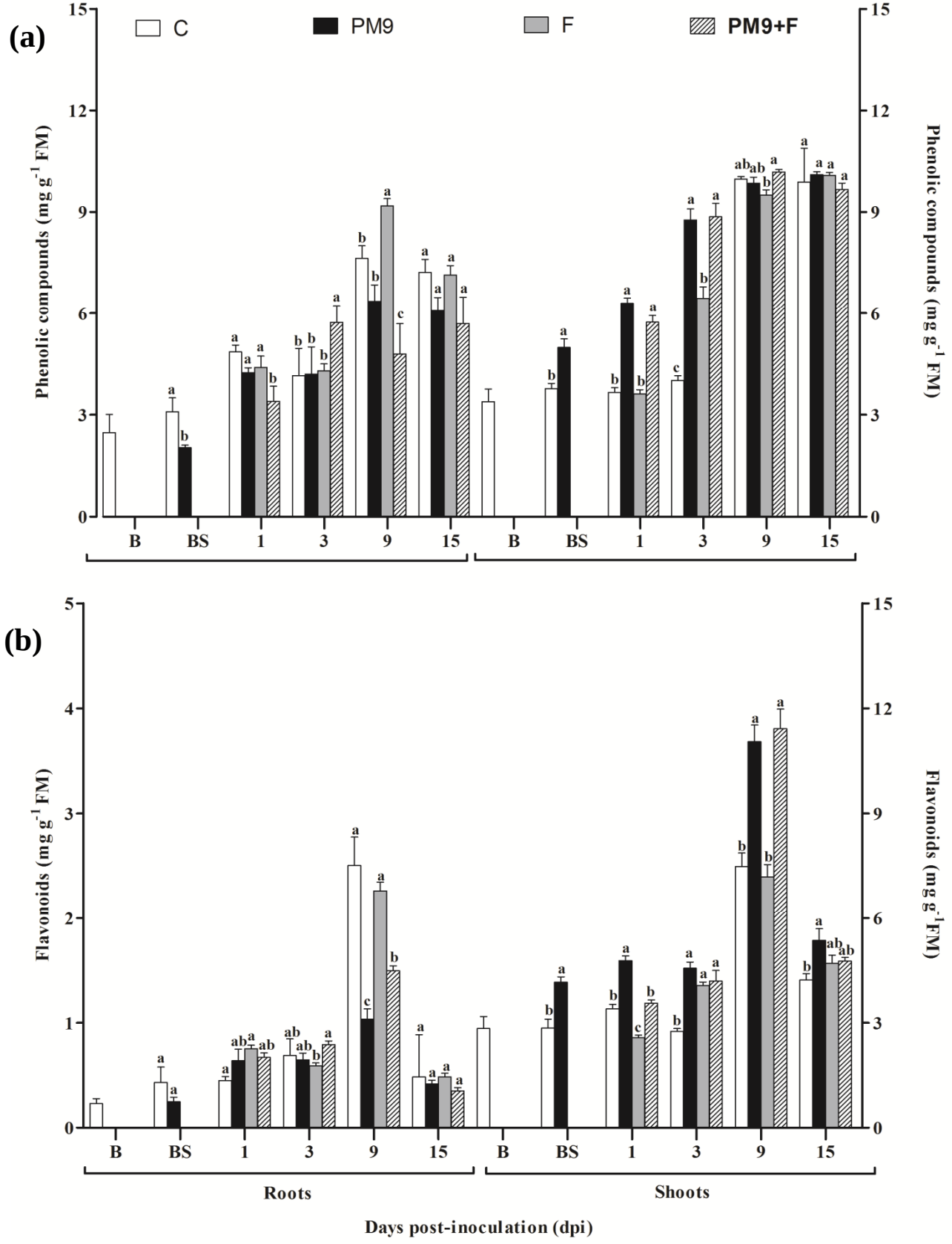

Fig. 4 Levels of total (a) phenolic compounds and (b) flavonoids in roots and shoots of *E. grandis*. C= control, PM9= *Streptomyces* sp. PM9, F= pathogenic fungus *B. cinerea* and PM9+F= elicited plants with *Streptomyces* sp. PM9 and challenged with *B. cinerea*. Responses were evaluated at 1, 3, 9 and 15 days post-inoculation with *B. cinerea*. B and BS: basal secondary metabolism before and after four days of inoculation with *Streptomyces* PM9, respectively.Bars represent standard error of the mean. Different letters indicate significantly differences among treatments within a time point (Tukey test, $\alpha \leq 0.05$).

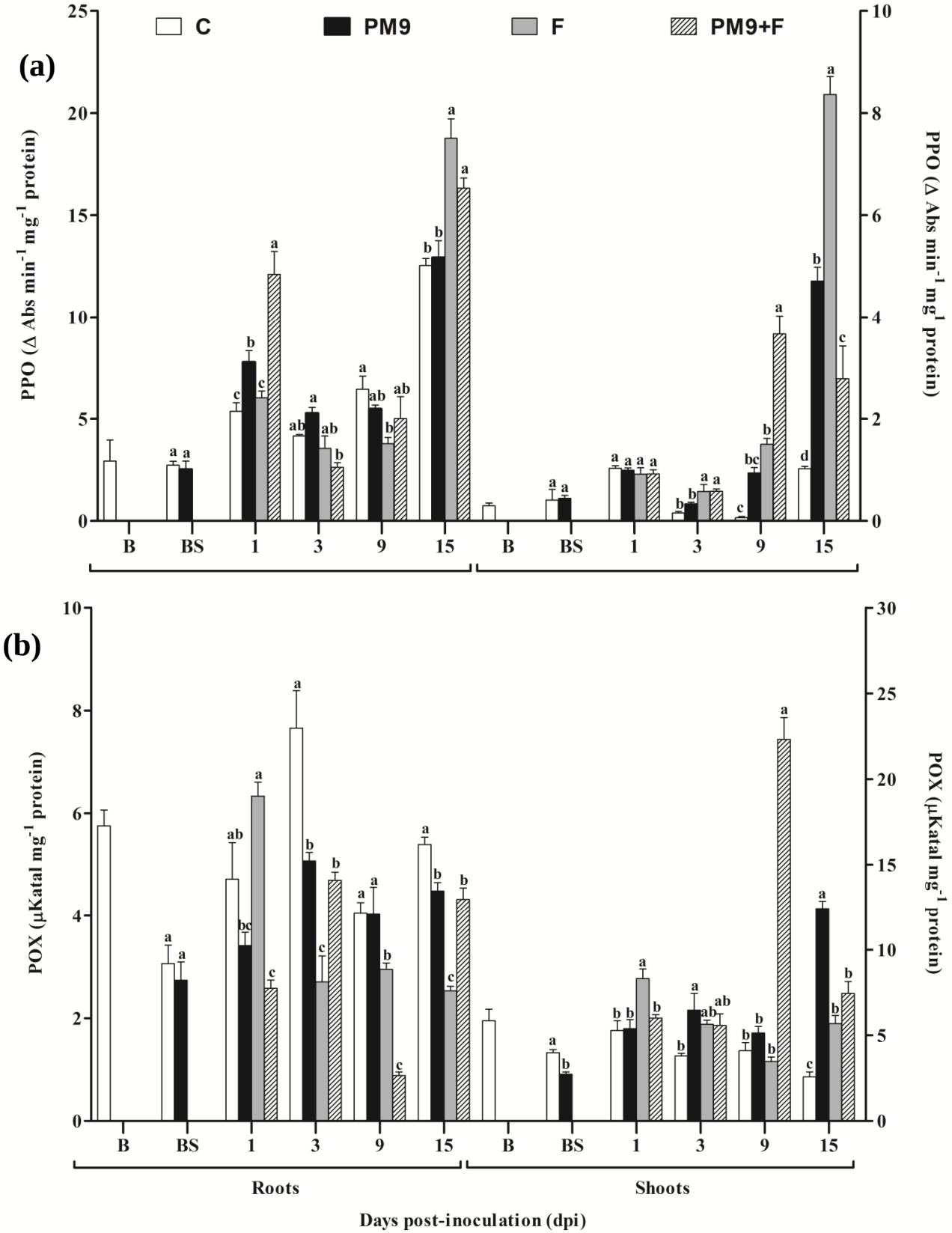

Fig 5 Activities of the enzymes (a) polyphenol oxidase and (b) peroxidases in roots and shoots of *E. globulus*. C= control, PM9= *Streptomyces* sp. PM9, F= pathogenic fungus *B. cinerea* and PM9+F= elicited plants with *Streptomyces* sp. PM9 and challenged with *B. cinerea*. Responses were evaluated at 1, 3, 9 and 15 days post-inoculation with *B. cinerea*. B and BS: basal secondary metabolism before and after four days of inoculation with *Streptomyces* PM9, respectively.Bars represent standard error of the mean. Different letters indicate significantly differences among treatments within a time point (Tukey test, $\alpha \leq 0.05$).

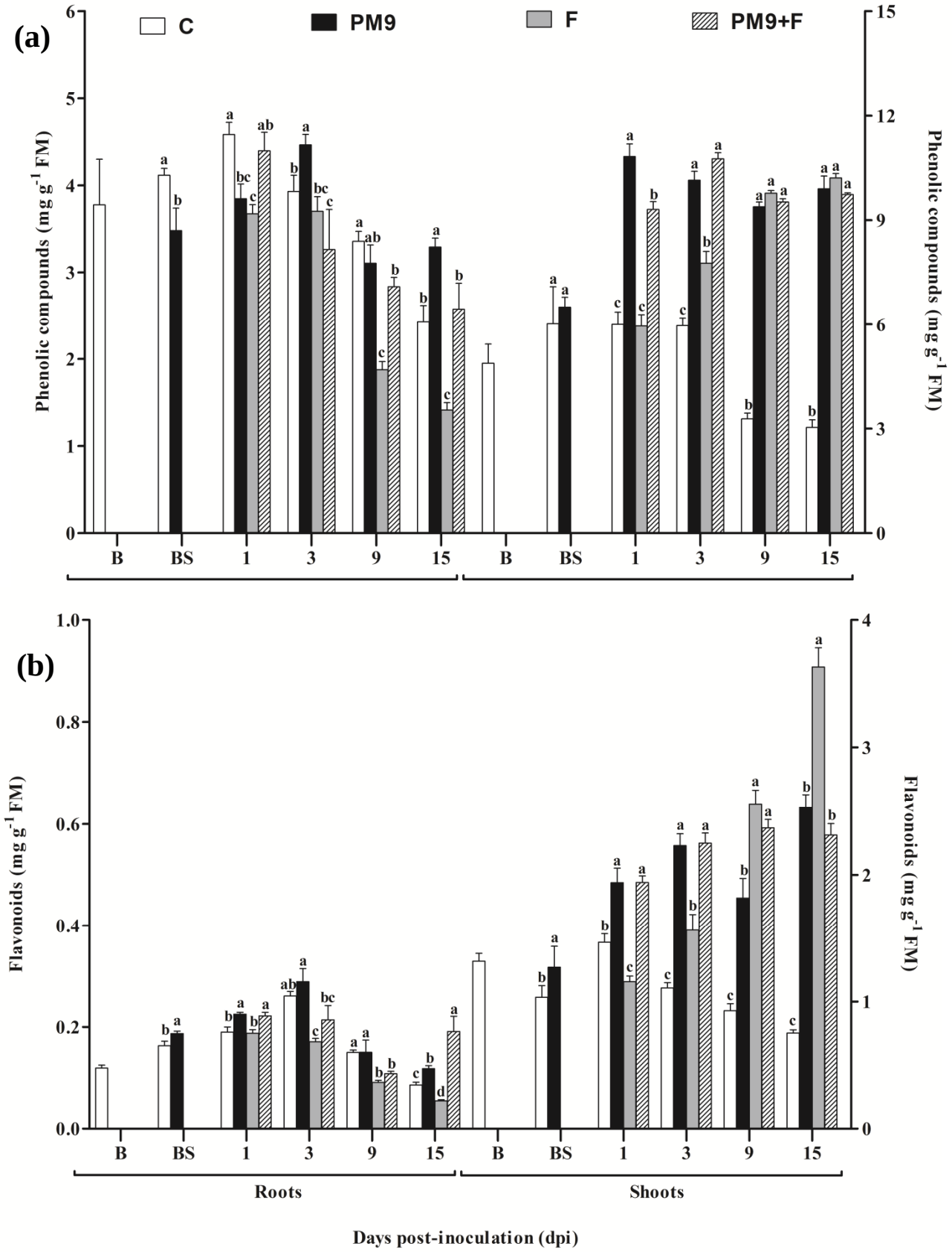

Fig. 6 Levels of total (a) phenolic compounds and (b) flavonoids in roots and shoots of *E. globulus*. C= control, PM9= *Streptomyces* sp. PM9, F= pathogenic fungus *B. cinerea* and PM9+F= elicited plants with *Streptomyces* sp. PM9 and challenged with *B. cinerea*. Responses were evaluated at 1, 3, 9 and 15 days post-inoculation with *B. cinerea*. B and BS: basal secondary metabolism before and after four days of inoculation with *Streptomyces* PM9, respectively.Bars represent standard error of the mean. Different letters indicate significantly differences among treatments within a time point (Tukey test, $\alpha \leq 0.05$).

CAPÍTULO IV

CONSIDERAÇÕES FINAIS

CONSIDERAÇÕES FINAIS

Este estudo demonstrou a capacidade de atuação dos isolados de rizobactérias do gênero *Streptomyces* como PGPRs. *Streptomyces* sp. PM9 induziu a proliferação de raízes adventícias através da produção de auxina (AIA), bem como modulouo metabolismo secundário de plantas de *E. grandis* e *E. globulusin vitro*. Esta modulação foi evidenciadaatravés de alterações nas atividades das enzimas PPO e POX, além de mudanças nos compostos secundários induzidos ao longo do tempo de cultivo das plantas eliciadas. Estas respostasforam observadas tanto nas raízes (local de inoculação) quanto nas partes aéreas das plantas de *Eucalyptus,*indicando uma resposta sistêmica decorrente da interação planta-microrganismo.

Streptomyces sp. PM9 também demonstrou efeito antagônico contra *B. cinerea*. Além disso, plantas de *Eucalyptus*sp. eliciadas com este isolado e desafiadas com *B. cinerea*apresentaram alterações metabólicas logo após a infecção com o fungo, tanto na atividade enzimática quanto na concentração e tipo de compostos fenólicos induzidos, alguns destes com reconhecida ação fungicida. Foi observado o atraso no estabelecimento da doença mofo cinzento em *E. grandis*, sugerindo que as plantas eliciadas com *Streptomyces* sp. foram sensibilizadas ao possível ataque por fitopatógenos.

O efeito sinérgico da associação de*Strepyomyces* sp. PM9 com plantas de *Eucalyptus* sp.e seu antagonismo contra *B. cinerea*, colocam este isolado como potencial agente de biocontrole, podendo reduzir as quantidades de pesticidas utilizadas na silvicultura.

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CC: tamiris_salla@acad.pucrs.br, thanise.silva@acad.pucrs.br, astarita@pucrs.br

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Dear Co-Author(s),

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