ANGELICA NUNES TIEPO

BACTÉRIAS PROMOTORAS DO CRESCIMENTO VEGETAL INFLUENCIAM ROTAS METABÓLICAS E AUMENTAM A TOLERÂNCIA AO DÉFICIT HÍDRICO EM ESPÉCIES ARBÓREAS NEOTROPICAIS: POTENCIAL PARA RECUPERAÇÃO DE ÁREAS DEGRADADAS

Londrina
2020

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Estadual de Londrina, como requisito parcial para a obtenção do título de Doutora em Ciências Biológicas.

Orientador: Profa. Dra. Renata Stolf Moreira.
Coorientador: Prof. Dr. Halley Caixeta de Oliveira.

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Londrina, 26 de Novembro de 2020.

“*Não é apenas sobre compreender a fisiologia das plantas,*
é sobre saber como usá-la em prol de um mundo melhor.”

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RESUMO

O desmatamento tem sido crescente nas últimas décadas, o que ocasiona eventos de seca prolongados. A reversão dessa situação pode acontecer por meio do desenvolvimento de programas de restauração florestal. No entanto, os eventos de seca podem afetar negativamente o estabelecimento das mudas de espécies arbóreas utilizadas na restauração desses ambientes. Uma das estratégias biotecnológicas para a produção de mudas mais tolerantes a estresses abióticos é a associação com Bactérias Promotoras do Crescimento em Plantas (BPCP). O objetivo deste estudo foi avaliar se a associação de mudas de *Cecropia pachystachya* Trécul e *Cariniana estrellensis* (Raddi) Kuntze com *Azospirillum brasilense* (Ab-V5) e *Bacillus velezensis* (ZK) gera mudanças fisiológicas, bioquímicas e biométricas que aumentem a tolerância dessas espécies arbóreas ao déficit hídrico. Para isso, mudas de *C. pachystachya* e *C. estrellensis* foram associadas com Ab-V5 e com ZK e submetidas ao déficit hídrico moderado por 30 dias. No primeiro capítulo, foram analisados parâmetros biométricos e a resposta antioxidante enzimática e não enzimática nas folhas de ambas as espécies vegetais. Foi observado que a associação com BPCP levou ao aumento na atividade de enzimas antioxidantes (ascorbato peroxidase, superóxido dismutase e peroxidases) em mudas de *C. pachystachya* e de *C. estrellensis*. As BPCP também influenciaram positivamente o metabolismo antioxidante não enzimático, levando ao aumento de compostos fenólicos, como o ácido clorogênico, ácido gálico, rutina, ácido sinápico e catequina, e do alcalóide trigonelina em ambas as espécies vegetais. A associação com as BPCP também influenciou parâmetros biométricos, em *C. pachystachya* houve aumento da massa seca de raiz, da massa seca de parte aérea, e da razão raiz:parte aérea. Em mudas de *C. estrellensis* tratadas com BPCP foi verificado a redução da massa seca de raiz e massa seca de parte aérea. Foi observada também, em ambas as espécies vegetais, a redução da área foliar específica e da razão de área foliar induzida pela associação com as BPCP. No segundo capítulo, trocas gasosas e parâmetros relacionados com o metabolismo do carbono e do nitrogênio foram avaliados nas folhas de ambas as espécies vegetais. Em *C. pachystachya*, as BPCP induziram aumento na taxa fotossintética líquida (*A*), na condutância estomática (g_s), na eficiência de carboxilação (*k*), na atividade da enzima glutamina sintetase, na quantidade de aminoácidos e na quantidade de lactato. Também houve uma influência negativa nas concentrações de malato, succinato, citrato, glicose e sacarose. O déficit hídrico influenciou positivamente as concentrações de proteína, hidroxibutirato, malato e glicose. Em mudas de *C. estrellensis*, a associação com as BPCP ocasionou aumento na *k*, e *Bacillus velezensis* induziu positivamente as concentrações de hidroxibutirato, malato, succinato e ácido quínico; enquanto que o déficit hídrico induziu acúmulo de glicose e proteínas. No terceiro capítulo, uma análise fitoquímica do extrato hidroalcoólico das folhas de *C. estrellensis* foi realizada. As análises por Ressonância Magnética Nuclear (RMN) e Cromatografia líquida indicaram que os principais compostos secundários encontrados foram: ácido quínico e ácidos hidroxicinâmicos, como por exemplo ácido *p*-cumárico e ácido ferúlico. Também foram identificados flavonoides como o kaempferol e a quercetina. Esses resultados são a primeira descrição fitoquímica para a espécie e serão importantes para fundamentar estudos futuros em ecologia e metabolômica de *C. estrellensis*. Os resultados obtidos no primeiro e segundo capítulos evidenciam o potencial da associação com BPCP em gerar

respostas fisiológicas, bioquímicas e biométricas que aumentem a tolerância das espécies arbóreas ao déficit hídrico. Essa ferramenta biotecnológica pode ser utilizada para a produção de mudas nativas mais tolerantes e com maiores taxas de sobrevivência, aumentando o sucesso dos programas de restauração florestal.

Palavras-chave: espécie arbórea; estresse abiótico; mata atlântica; microrganismos; reflorestamento; restauração florestal; tolerância à seca.

Tiepo, Angelica Nunes. **Plant growth-promoting bacteria influence on metabolic pathways and improve the drought tolerance in neotropical trees: potential for the recovery of degraded areas** 2020. 107 f. Thesis (Doctorate in Biological Sciences) – Universidade Estadual de Londrina, Londrina, 2020.

ABSTRACT

Deforestation has been increasing in recent decades, which has led to prolonged drought events. This situation can be reversed through the development of forest restoration programs. However, drought events can negatively affect the establishment of tree species seedlings used to restore these environments. One of the biotechnological tools for development of seedlings more tolerant to abiotic stresses is the association with plant growth-promoting bacteria (PGPB). The aim of this study was to evaluate whether the association of *Cecropia pachystachya* Trécul and *Cariniana estrellensis* (Raddi) Kuntze seedlings with *Azospirillum brasilense* (Ab-V5) and *Bacillus velezensis* (ZK) generates physiological, biochemical and biometric changes that increase the tolerance of these tree species to moderate drought. For this, seedlings of *C. pachystachya* and *C. estrellensis* were associated with Ab-V5 and ZK and submitted to moderate drought for 30 days. In the first chapter, biometric parameters and the enzymatic and non-enzymatic antioxidant response in the levaes of both plant species were analyzed. It was observed that the association with PGPB led to an increase in the antioxidant enzymes activity (ascorbate peroxidase, superoxide dismutase and peroxidases) in *C. pachystachya* and *C. estrellensis* seedlings. PGPB also influenced positively the non-enzymatic antioxidant metabolism, leading to an increase in the level of phenolic compounds, such as chlorogenic acid, gallic acid, rutin, synapic acid and catechin, and on the alkaloid trigonelline in both plant species. The association with PGPB also influenced biometric parameters, in *C. pachystachya* there was an increase in root dry weight, shoot dry weight and root:shoot ratio. In seedlings of *C. estrellensis* inoculated with PGPB it was observed the reduction of root dry weight and shoot dry weight. It was also observed, in both plant species, the reduction of the specific leaf area and the leaf area ratio induced by PGPB. In the second chapter, gas exchange and parameters related to carbon and nitrogen pathways were evaluated in the leaves of both plant species. In *C. pachystachy*, PGPB induced an increase in net photosynthesis (*A*), stomatal conductance (g_s), carboxylation efficiency (*k*), glutamine synthetase activity, amino acids amount and lactate amount. There was also a negative influence on the amounts of malate, succinate, citrate and sucrose. The moderate drought positively influenced the amounts of protein, hydroxybutyrate, malate and glucose. In *C. estrellensis*, the associtation with PGPB led to an increase in *k*, and *Bacillus velezensis* indued positively the amounts of hydroxybutirate, malate, succinate and quinic acid; while the moderate drought induced accumulation of glucose and proteins. In the third chapter, a phytochemical analysis of the hydroalcoholic extract from leaves of *C. estrellensis* was carried out. Analyzes through Nuclear Magnetic Resonance (NMR) and high performance liquid chromatography indicated that the main secondary compounds found were: quinic acid and hydroxycinamic acids, such as *p*-coumaric acid and ferulic acid. Flavonoids such as kaempferol and quercetin have also been identified. These results are the first phytochemical description for the species and will be important to support future studies in ecology and metabolomics for *C. estrellensis*. The results from the first and second chapters showed the potential of the association with PGPB in generating physiological, biochemical and biometric responses that increase the tolerance of tree species to moderate drought. This biotechnological tool can be used to produce native seedlings that are more tolerant and have higher survival rates, increasing the succes of restoration and reforestation

programs.

Key words: abiotic stress; atlantic forest; drought tolerance; microorganisms; reforestation; forest restoration; tree species.

LISTA DE FIGURAS

INTRODUÇÃO GERAL

CAPÍTULO 1

CAPÍTULO 2

LISTA DE TABELAS

CAPÍTULO 1

CAPÍTULO 2

CAPÍTULO 3

LISTA DE ABREVIATURAS E SIGLAS

A	Assimilação líquida de CO_2, net photosynthesis
AAS	Aminoácidos, amino acids
Ab-V5	*Azospirillum brasilense*, estirpe Ab-V5
ALA	Alanina, alanine
APX	Ascorbato peroxidase, ascorbate peroxidase
ASC	Ácido ascórbico, ascorbic acid
BPCP	Bactéria promotora do crescimento em plantas
CAT	Catequina, catechin
CHLO	Ácido clorogênico, chlorogenic acid
C_i	Concentração intercelular de CO_2, intercelular CO_2 concentration
CIT	Citrato, citrate
EDTA	Ácido etilenodiamina tetra-acético, Ethylenediamine tetraacetic acid
EPICAT	Epicatequina, epicatechin
ERO	Espécie reativa de oxigênio
FA	Área foliar, leaf area
FC	Capacidade de campo, field capacity
FLA	Flavonoides, flavonoids
GALL	Ácido gálico, gallic acid
GLU	Glicose, glucose
g_s	Condutância estomática, stomatal conductance
GS	Glutamina sintetase, glutamine synthetase
HYD	Hidroxibutirato, hydroxybutyrate
k	Eficiência instantânea de carboxilação, instantaneous carboxylation efficiency
LAC	Lactato, lactate
LAR	Razão de área foliar, leaf area ratio
MAL	Malato, malate
MD	Seca moderada, moderate drought
NBT	Azul de nitrotetrazólio, nitro blue tetrazolium
NI	Não inoculado, not inoculated
PGPB	Plant growth-promoting bacteria
PHE	Fenólicos totais, total phenolics

POD	Peroxidase
PROT	Proteínas, protein
PVPP	Polivinilpolipirrolidona, polyvinylpolypyrrolidone
QUIN	Ácido quínico, quinic acid
RDW	Massa seca de raiz, root dry weight
ROS	Reactive oxygen species
Rubisco	Ribulose-1,5-bifosfato carboxilase / oxigenase, Ribulose-1,5-bisphosphate carboxylase/oxygenase
RUT	Rutina, rutin
SDW	Massa seca de parte aérea, shoot dry weight
SLA	Área foliar específica, specific leaf area
SOD	Superóxido dismutase, superoxide dismutase
SUCC	Succinato, succinate
SUCR	Sucrose, sacarose
SYN	Ácido sinápico, synapic acid;
TDW	Massa seca total, total dry weight
TRIGO	Trigonelina, trigonelline
UEL	Universidade Estadual de Londrina
ZK	*Bacillus velezensis*, estirpe ZK

SUMÁRIO

INTRODUÇÃO GERAL

Eventos de seca prolongada têm sido recorrentes ao redor do mundo nas últimas décadas, os quais são consequência de mudanças climáticas decorrentes do desmatamento e da emissão de gases causadores do efeito estufa (Hossain et al. 2016; IPCC, 2019). A retirada das florestas causa modificações nos padrões de trocas gasosas da vegetação, o que afeta a quantidade de vapor de água em escala global, levando a um aumento nos episódios de seca no mundo (Lawrence and Vandecar 2015; Khanna et al. 2017).

Além disso, o desmatamento tem sido um dos causadores do aumento da emissão de gases do efeito estufa, e a reversão desse desmatamento, por meio do reflorestamento, tem um potencial reconhecido na recuperação dos estoques de carbono armazenados em biomassa vegetal e no solo (Nave et al. 2018; Locatelli et al. 2015). A recuperação do estoque de carbono em florestas surge como uma ferramenta com potencial para mitigar mudanças climáticas; e ainda contribuir para a manutenção dos ciclos hidrológicos, garantindo o suprimento e a qualidade da água (Locatelli et al. 2015).

As características climáticas e os eventos de seca podem ocasionar o estresse fisiológico nas plantas, o qual afeta negativamente o estabelecimento e a sobrevivência da mudas utilizadas no reflorestamento (Allen et al. 2010; Young et al. 2017). Os regimes alterados de precipitação e temperatura desencadeiam efeitos mais severos nas plantas em estágios inicias de desenvolvimento e estabelecimento (Wright et al. 2018). Em vista disso, tornam-se necessárias ferramentas biotecnológicas que proporcionem um aumento da tolerância dessas mudas a estresses abióticos como a seca. Um exemplo é a associação com Bactérias Promotoras do Crescimento em Plantas (BPCP), que está entre as ferramentas biotecnológicas disponíveis para o aumento da tolerância a estresses abióticos em plantas (Vurukonda et al. 2016).

Estudos com plantas cultivadas mostram que a interação com BPCP leva ao aumento de metabólitos primários em *Zea mays* (L.) (Bano et al. 2013) e em *Solanum lycopersicum* Mill. (Shintu and Jayaram 2015); e do crescimento em *Oriza sativa* L. (Cassán et al. 2009). As BPCP influenciam também rotas metabólicas enzimáticas e não enzimáticas envolvidas com a tolerância ao déficit hídrico, como o metabolismo antioxidante de plantas. Em *Zea mays*, foi observado um aumento na atividade de enzimas antioxidantes como Ascorbato Peroxidase (APX), Superóxido Dismutase (SOD) e Catalase (CAT) (Fukami et al. 2018c). Também em *Zea mays* e em *Vitis vinifera* L., observou-se um aumento na quantidade de polifenóis em resposta à inoculação das plantas com BPCP (Cho et al. 2003; Barka et al. 2006).

Visando ao uso de BPCP como ferramenta biotecnológica para o aumento do sucesso de programas de restauração florestal, objetivou-se neste estudo verificar as respostas fisiológicas, bioquímicas e biométricas de espécies arbóreas nativas do Bioma Mata Atlântica, submetidas ao déficit hídrico e associadas com BPCP.

1.1 O bioma Mata Atlântica e as espécies vegetais utilizadas no estudo

O bioma Mata Atlântica já foi uma das maiores florestas tropicais do mundo, cobrindo aproximadamente 150 milhões de hectares. Essa área estava distribuída em uma ampla faixa longitudinal, o que gera diferenças na composição da floresta devido à diminuição da precipitação longe das áreas costeiras (Ribeiro et al. 2009). Essa característica da distribuição geográfica da Mata Atlântica favoreceu uma alta diversidade e endemismo de espécies, de modo que a fauna e flora deste Bioma podem incluir de 1 a 8% da biodiversidade mundial (Ribeiro et al. 2009).

Contudo, eventos de desmatamento ao longo dos anos reduziram drasticamente a área da Mata Atlântica, e os remanescentes caracterizam-se por pequenos fragmentos de floresta secundária (< 50 ha) em estágios iniciais ou médios de sucessão, que estão isolados e perto da borda das florestas (Ribeiro et al. 2009). Essa fragmentação fez com que a vasta biodiversidade esteja ameaçada de extinção, restando uma área de aproximadamente 11% da vegetação original (SOS Mata Atlântica, 2008; Ribeiro et al. 2009).

As condições microclimáticas de uma floresta madura são amplamente diferentes de uma área desmatada, de modo que o desmatamento leva a um aumento da incidência luminosa, aumento da temperatura do solo, aumento no déficit de pressão de vapor e ainda a flutuações no potencial de água do solo (Craven et al. 2011). Mudanças como as citadas foram observadas na Mata Atlântica; assim, a ampla taxa de desmatamento modificou o balanço de água entre a superfície terrestre e a atmosfera, levando a um aumento nas taxas de evapotranspiração da vegetação remanescente, a um aumento na temperatura, e a uma redução nos regimes de precipitação na Mata Atlântica em 30 anos (1981 – 2010) (Salazar et al. 2016).

Esses fatores influenciam negativamente a sobrevivência da fauna e da flora, o que torna crescente a necessidade de implementação de ações de conservação e restauração para mitigar esta situação (Ribeiro et al. 2009). Uma das alternativas é a produção e utilização de mudas nativas para restauração das áreas degradadas.

As mudas de espécies arbóreas nativas podem ser vulneráveis aos estresses ambientais nessas áreas desmatadas; portanto, torna-se necessária a produção de mudas que

sejam aclimatadas e mais tolerantes a estresses abióticos (Craven et al. 2011). Uma das maneiras de aclimatação de mudas ocorre em viveiros, nos quais as mudas são transferidas para locais com maior incidência luminosa, o que faz com que as mudas desenvolvam traços anatômicos e biométricos que aumentam a sua tolerância a estresses (como a alta irradiância, a seca e a alta temperatura); proporcionando uma maior taxa de sobrevivência quando transplantadas para o campo (Calzavara et al. 2015).

Além disso, é essencial que as espécies vegetais sejam nativas do Bioma a ser restaurado, o que possibilita a recuperação de características naturais dos ecossistemas, a preservação da biodiversidade, a manutenção dos serviços ecossistêmicos e, ainda, o reestabelecimento da homeostase do Bioma (Hall et al. 2011). Dentre as espécies arbóreas nativas da Mata Atlântica que são empregadas em programas de reflorestamento estão as espécies utilizadas neste estudo: *Cecropia pahystachya* Trécul e *Cariniana estrellensis* (Raddi) Kuntze (Fig. 1, Fig. 2 e Fig. 3).

Cecropia pachystachya, conhecida popularmente como Embaúba-do-brejo (Fig. 1 A), é caracterizada por ser uma árvore de médio porte (4 a 8 metros), é pioneira, possui crescimento rápido, e está presente nas fases iniciais da sucessão ecológica (Lorenzi 1998). Tem ampla distribuição geográfica e está presente na maioria dos estados do Brasil (Gaglioti e Aguiar, 2020). Ocorre em solos úmidos nas bordas das matas e em clareiras; é ideal para o início de reflorestamento, uma vez que possibilita sombreamento para o estabelecimento de outras espécies vegetais, e ainda é atrativa para fauna como pássaros e formigas (Lorenzi 1998).

Cariniana estrellensis, conhecida popularmente como Jequitibá-branco (Fig. 1 B), é caracterizada como árvore de grande porte (35 a 45 metros), é não-pioneira, possui crescimento lento e está presente nas fases mais avançadas da sucessão (Lorenzi 1998). Apresenta distribuição disjunta entre a floresta Amazônica e a Mata Atlântica, estando presente no Acre, na Bahia, em Goiás, no Espírito Santo, em Minas Gerais, no Rio de Janeiro, em São Paulo, no Paraná e em Santa Catarina (Ribeiro et al. 2020). Ocorre em solos úmidos e profundos, está presente nas florestas clímax, suas sementes são consumidas por macacos e possuem importante função na sobrevivência de epífitas, sendo essenciais para a manutenção da homeostase no ambiente em que se encontram (Lorenzi 1998; Reis e Fontoura, 2009).

1.2 Efeitos do déficit hídrico na fisiologia e no crescimento das plantas

O desmatamento leva à redução da disponibilidade hídrica no solo, o que ocasiona mudanças em traços fisiológicos, morfológicos, ecológicos, bioquímicos e moleculares das

plantas, e afeta negativamente o crescimento e desenvolvimento das mesmas (Hossain et al. 2016). Esta redução da disponibilidade hídrica e, consequentemente, do potencial de água da planta causa mudanças no balanço hormonal, levando a um aumento na síntese de ácido abscísico (ABA) (Hossain et al. 2016). A sinalização metabólica pelo ABA começa nas raízes e é transmitida ao longo da planta para as folhas, onde este hormônio se liga aos receptores RCAR/PYR/PYL presentes nas membranas plasmáticas das células-guarda. Essa ligação induz a formação de um complexo com proteínas fosfatases do tipo 2C (PP2Cs), inibindo-as. A inibição da atividade das PP2C ativa quinases que fosforilam e ativam NADPH-oxidases, as quais catalisam a formação de espécies reativas de oxigênio (EROs) apoplásticas, desencadeando a abertura de canais de influxo de Ca_2^+ e a liberação de Ca^{2+} dos vacúolos para o citoplasma (Taiz et al. 2017).

A ativação das NADPH-oxidases juntamente com o aumento do Ca^{2+} citoplasmático induzem a síntese de peróxido de hidrogênio (H_2O_2), o que ativa uma via de transdução de sinais que leva à síntese de óxido nítrico (NO). O NO induz uma cascata de sinalização pela ativação do mensageiro cGMP e por meio da S-nitrosação de grupos tióis em resíduos de cisteína, que modula a atividade de proteínas celulares em situação de estresse hídrico (Desikan et al. 2004; Neill et al. 2008; Nabi et al. 2019).

A síntese de NO e o aumento de Ca^{2+} no citoplasma ativam proteínas quinases que ativam canais de efluxo de ânions (Cl^- e malato), o que leva à despolarização da membrana plasmática das células-guarda e à ativação de canais de efluxo de potássio (K^+). Essa resposta também inibe a atividade da H^+-ATPase responsável pela hiperpolarização da membrana plasmática da célula-guarda. Além disso, enzimas fosfatases são estimuladas e inibem canais de influxo de K^+ nas células-guarda, simultaneamente há inibição do simporte Cl^-H^+. Essas mudanças nos canais iônicos da membrana plasmática ocasionam um desbalanço eletroquímico nas células-guarda, o que leva ao aumento no potencial osmótico e consequentemente no potencial de água das células-guarda, fazendo com que haja movimento líquido de água para fora dessas células e consequentemente o fechamento estomático hidroativo (Taiz et al. 2017; Lawson and Vialet-Chabrand 2019).

O fechamento estomático causa a redução no influxo de CO_2 para o mesofilo, o que reduz a quantidade de carbono disponível para a síntese de gliceraldeído-3-fosfato, e afeta negativamente a atividade carboxilase da enzima Ribulose 1-5-bisfosfato carboxilase/oxigenase (Rubisco) (Taiz et al. 2017). A manutenção da incidência luminosa associada à menor razão CO_2:O_2 induz um aumento da atividade oxigenase da Rubisco (fotorrespiração), o que ocasiona perda parcial do carbono fixado pelo ciclo de Calvin-Benson,

e leva à menor síntese de carboidratos, afetando negativamente o crescimento das plantas (Nogués e Baker 2000; Lawson and Vialet-Chabrand 2019).

A fotorrespiração irá atuar como um dreno para o excesso de equivalentes redutores (NADPH) provenientes da cadeia de transporte de elétrons da fotossíntese em plantas em déficit hídrico (Das and Roychoudhury 2014). No entanto, a fotorrespiração não é totalmente eficiente para equilibrar a razão NADPH:$NADP^+$ (Taiz et al. 2017). A limitação da entrada de CO_2 associada ao acúmulo de NADPH em plantas submetidas ao déficit hídrico afeta negativamente o transporte de elétrons na cadeia de transporte de elétrons da fotossíntese e expõe os cloroplastos ao excesso de energia de excitação luminosa (Bettaieb et al. 2011).

O excesso de NADPH, ocasionado pelo déficit hídrico, leva à síntese e acúmulo de EROs por meio da atividade de enzimas NADPH-oxidases, que reduzem o oxigênio molecular (O_2) (Taiz et al. 2017), ou pela transferência de energia de excitação eletrônica, como a reação da clorofila tripleto ($^3Chl^*$) com o O_2 levando à síntese de oxigênio singleto ($^1O_2$) (Triantaphylidès e Havaux 2009).

A menor disponibilidade hídrica pode gerar uma condição de fotoinibição, e induzir o FSI a doar elétrons para uma molécula de O_2 gerando o ânion superóxido ($O_2^{\bullet-}$) (Jaleel et al. 2009; Bettaieb et al. 2011; Das e Roychoudhury 2014). Na presença do $O_2^{\bullet-}$ pode haver a síntese do radical hidroxila ($OH^\bullet$), que é a ERO mais tóxica conhecida; a sua formação ocorre por meio da reação entre H_2O_2 e $O_2^{\bullet-}$ e é catalisada por metais de transição como o Ferro (Fe^{2+}, Fe^{3+}) (Jaleel et al. 2009).

As EROs desempenham um importante papel de sinalização no metabolismo vegetal, porém em situação de estresse abiótico há uma superprodução, tornando-as prejudiciais à manutenção da homeostase celular (Hossain et al. 2016). Quando em excesso, as EROs induzem dano oxidativo e consequente degradação de componentes celulares como: pigmentos, proteínas, lipídeos, carboidratos, membrana e DNA, o que pode leva à morte celular (Gómez-Caravaca et al. 2014).

As plantas possuem mecanimos de manutenção do balanço entre produção e eliminação de EROs, o que auxilia na manutenção das vias bioquímicas celulares. Esses mecanimos consistem em duas rotas metabólicas antioxidantes: enzimática e não-enzimática; e ambas são responsáveis pela eliminação do excesso de EROs.

A rota metabólica enzimática é composta pela Superóxido Dismutase (SOD), a qual atua na eliminação do $O_2^{\bullet-}$, Ascorbato peroxidase (APX), Catalase (CAT) e Guaiacol peroxidase (GPX), que são responsáveis pela eliminação do H_2O_2. Há ainda a monodehidroascorbato redutase (MDHAR) e a dihidroascorbato redutase (DHAR) e ambas

atuam na regeneração do ácido ascórbico utilizado pela APX (Das and Roychoudhury 2014).

A via bioquímica não-enzimática compreende compostos como ácido ascórbico, α-tocoferol, carotenoides (Das and Roychoudhury 2014), polifenóis, alcaloides e alguns aminoácidos como a prolina (Gómez-Caravaca et al. 2014). O ácido ascórbico atua doando elétrons para uma ampla gama de reações enzimáticas e não enzimáticas, e sua localização no apoplasto o torna a primeira linha de defesa contra o dano oxidativo por EROs; atua, ainda, na eliminação do $^1O_2$ uma vez que é doador de elétrons para o ciclo das xantofilas (Triantaphylidès and Havaux 2009).

O α-tocoferol pertence à classe de antioxidantes lipofílicos, sendo eficiente na eliminação de EROs e radicais lipídicos; protege lipídeos e outros constituintes de membrana dos cloroplastos, reduz o excesso de energia de excitação, protegendo o FSII funcional e estruturalmente, e também participa de reações que eliminam o $^1O_2$ (Das and Roychoudhury 2014; Gómez-Caravaca et al. 2014).

Dentro dos polifenóis, encontram-se ácidos fenólicos e flavonoides, os quais já estão presentes nas plantas e podem ser acumulados durante situações de estresse (Das and Roychoudhury 2014; Gómez-Caravaca et al. 2014). Os ácidos fenólicos são primeiramente acumulados no apoplasto ou nos vacúolos, e desempenham um papel de sinalização ou de defesa direta das células. Ao serem oxidados, esses compostos atuam na eliminação de EROs por meio da doação de elétrons para enzimas antioxidantes, ou de um átomo de hidrogênio para as EROs, o que estabiliza as taxas de oxidação lipídica das membranas (Sgherri et al. 2004; Bettaieb et al. 2011; Kumar and Goel 2019).

Os flavonoides atuam como sequestradores de radicais livres por meio da doação rápida de um átomo de hidrogênio do grupo hidroxil (Amic et al. 2003; Mierziak et al. 2014). Aqueles presentes nos cloroplastos eliminam $^1O_2$, o que reduz os danos causados à membrana externa dos cloroplastos durante a desidratação. Os presentes em outros compartimentos celulares inibem enzimas que geram EROs; quelam íons de metais de transição, os quais poderiam catalizar a síntese de EROs; e ainda extinguem cascatas de reações livres na peroxidação lipídica. Além do seu papel como antioxidantes e eliminadores de EROs, os flavonoides podem atuar como fotoprotetores, por meio da limitação da entrada de luz nas células do mesofilo, o que reduz a excitação da clorofila. Todas essas atividades dos polifenóis são essenciais para a manutenção do estado redox das células e, assim, da homeostase das plantas mesmo em situações de estresse (Nogués e Baker 2000; Bettaieb et al. 2011)

A tolerância ao déficit hídrico é um fenômeno que exige um alto custo energético para a sobrevivência das plantas. Portanto, condições de estresse, como a falta de água,

induzem mudanças no metabolismo do Carbono; o que afeta o ciclo dos ácidos tricarboxílicos (TCA) e a biossíntese de ATP (Hossain et al. 2016).

Já foi relatado que o déficit hídrico está diretamente relacionado a diversas mudanças em vias metabólicas celulares do metabolismo primário, como a síntese de carboidratos, aminoácidos e proteínas (Das et al. 2017). Além da redução do influxo e fixação de CO_2, há mudanças na destinação do carbono fixado, o qual tende a ser destinado para o incremento de compostos não estruturais relacionados ao ajuste osmótico e à defesa antioxidante, afetando negativamente o crescimento das plantas (Hasibeder et al. 2015)

A condição de seca muda a quantidade e o tipo de proteínas e aminoácidos sintetizados; isso ocorre devido a mudanças na expressão gênica e à quebra de proteínas, que levam à maior síntese de enzimas, aminoácidos e proteínas relacionadas com proteção osmótica e com a detoxificação das EROs (Hildebrandt et al. 2015). O acúmulo de solutos compatíveis aumenta o movimento líquido de água de solos menos hidratados para a planta, além de proteger contra a perda excessiva de água e contra a desestabilização de macromoléculas essenciais à manutenção da vida (Das et al. 2017)

Os solutos compatíveis são divididos entre quatro grupos: açúcares (glicose, frutose, sacarose, trealose); aminoácidos (prolina); compostos iônicos, incluindo amônio terciário e quaternário (glicina-betaína); e polióis e álcoois de açúcar (manitol, pinitol, glicerol) (Hossain et al. 2016). O acúmulo de glicose, frutose e sacarose já foi relatado em *Glycine max* L. sob seca, indicando o aumento da síntese de carboidratos com função osmótica em detrimento da destinação dos fotossintatos para o crescimento (Das et al. 2017).

O déficit hídrico é um dos fatores que afeta negativamente a nutrição mineral das plantas, uma vez que a seca pode influenciar diretamente os fatores fisicoquímicos do solo, reduzindo a mobilidade e o aporte de nutrientes para a planta (Ahmad et al. 2016). As respostas fisiológicas à seca, como a síntese de compostos osmoticamente ativos e as mudanças no aporte e transporte de nutrientes, estão relacionadas ao metabolismo do nitrogênio (N). (Wang et al. 2017). Sob déficit hídrico, ocorre a limitação do aporte e do transporte de N das raízes para as folhas pois os compostos nitrogenados (NO_3^- e NH_4^+) são usualmente solubilizados em água e transportados pela solução do solo até a superfície das raízes, onde ocorre a absorção por meio de transportadores específicos (Wang et al. 2017).

A disponibilidade de N é um dos fatores limitantes para o crescimento e desenvolvimeno das plantas, uma vez que ele é um dos mais importantes componentes de aminoácidos, proteínas, coenzimas, clorofila e ácidos nucleicos (Meng et al. 2016a). As raízes das plantas absorvem nitrato (NO_3^-) e amônio (NH_4^+). O nitrato absorvido é reduzido à nitrito

(NO_2^-) por uma reação catalisada pela enzima nitrato redutase (NR), o qual é reduzido a NH_4^+ pela nitrito redutase (NiR). Este NH_4^+ é incorporado a moléculas orgânicas, como aminoácidos e proteínas, em reações catalisadas pela glutamina sintetase (GS) e pela glutamato sintase (GOGAT) (Wang et al. 2017). Esses processos são regulados por sinais internos e externos, levando a diferenças no metabolismo e respostas em plantas com requisitos ecológicos distintos (Huang et al. 2018).

Além da absorção de íons, o déficit hídrico também afeta o metabolismo vegetal, de modo que influencia negativamente a atividade de enzimas como a NR, a GS e a GOGAT. Assim como observado em *Zea mays*, em *Populus simonii* Carrièri e em *Malus prunifolia* (Willd.) Borkh, isso ocorre pois a expressão dos genes que codificam a síntese dessas enzimas, assim como a sua atividade, são dependentes dos substratos e do fluxo de compostos orgânicos (Meng et al. 2016a; Hung et al. 2018).

Ao afetar o metabolismo do nitrogênio, o déficit hídrico influencia diretamente na síntese de aminoácidos, os quais podem ser intermediários de rotas metabólicas de tolerância à seca nos vegetais e também importantes no ajuste osmótico. A alteração nas quantidades de aminoácidos, como a prolina, alanina, glicina e tirosina, já foi relatada em raízes de mudas de *Zea mays*, e em folhas de *Trema micrantha* (L.) Blume e de *Cariniana estrellensis* submetidas ao déficit hídrico (Wang et al. 2017; Tiepo et al. 2018).

O fato de o déficit hídrico afetar os metabolismos do carbono e do nitrogênio reflete em respostas biométricas em plantas nativas e cultivadas; como já foi observado em *Zea mays*, em *Populus simonii*, em *Malus prunifolia*, em *Trema micrantha* e em *Carinianna estrellensis* a redução de massa seca e do comprimento de raiz e parte aérea (Meng et al. 2016a, Tiepo et al. 2018, Hung et al. 2018)

Desse modo, a baixa disponibilidade hídrica, ao afetar negativamente a atividade de enzimas de assimilação do carbono e do nitrogênio, leva à redução na síntese de fotossintatos. Há ainda um desvio dos fotossintatos disponíveis para o metabolismo antioxidante, no qual uma eritrose-4-fosfato se une ao fosfoenolpiruvato para sintetizar ácido chiquímico; sendo este precursor na síntese de polifenóis e alcaloides, por exemplo (Dias et al. 2016). Esses fatores induzidos pela seca diminuem a disponibilidade de compostos orgânicos destinados para a produção de biomassa, evidenciando que o déficit hídrico induz *trade-off* entre crescimento e sobrevivência nas espécies vegetais (Fernandez et al. 2016).

1.3 Mecanismos de tolerância ao déficit hídrico mediados por Bactérias Promotoras do Crescimento em Plantas

Processos de seca e desertificação têm sido mais comuns nas últimas décadas (IPCC 2019), e o déficit hídrico é considerado um dos fatores limitantes para o estabelecimento e sobrevivência de espécies vegetais (Marulanda et al. 2006). Além da aclimatação das mudas em viveiros, a associação com Bactérias Promotoras do Crescimento em Plantas (BPCP) tem sido uma importante ferramente biotecnológica capaz de induzir ou melhorar vias bioquímicas de tolerância à seca em plantas. A escolha de inóculos adequados para cada espécie vegetal auxilia no estabelecimento e sobrevivência de mudas em áreas sujeitas a estresses abióticos (Armada et al. 2014).

As BPCP compreendem um grupo de microrganismos que são benéficos para as plantas e possuem a capacidade de colonizar a rizosfera, a superfície das raízes e tecidos internos das plantas (Hungria et al. 2010). A ação das BPCP na promoção da tolerância a estresses, do crescimento e da sobrevivência em plantas tem sido largamente estudada, principalmente em espécies cultivadas (Fukami et al. 2018a). Dentre as BPCP, podemos citar os gêneros *Azospirillum*, *Pseudomonas*, *Bacillus, Agrobacter* e *Herbaspirillum*, os quais possuem meios de ação que promovem o crescimento e a sobrevivência das plantas. Essas ações podem ocorrer por mecanismos indiretos, que são relacionados com a proteção vegetal contra fatores que influenciam negativamente o crescimento, como a incidência de doenças e estresses ambientais; ou por mecanismos diretos de promoção do crescimento, como a maior disponibilização e assimilação de nutrientes e melhoria de absorção de água do solo (Khan et al. 2018).

Esses mecanismos de ação ocorrem por intermédio da influência das BPCP na modulação de diversas vias bioquímicas do metabolismo vegetal, como a síntese dos fitormônios auxinas, citocininas e giberelinas, os quais regulam processos fisiológicos e estão associados ao estímulo do crescimento e proteção contra estresses bióticos e abióticos (Bashan e de-Bashan 2010). Enquanto o déficit hídrico pode levar à redução do crescimento de raiz e parte aérea, como observado em *Lactuca sativa* L. (Kohler et al. 2008), as vias de síntese de fitormônios induzidas pelas BPCP estimulam o crescimento de diferentes espécies vegetais, como relatado em mudas de *Lactuca sativa* e de *Solanum lycopersicum* L. associadas com *Pseudomonas putida* e *Pseudomonas fluorescens*, e em mudas de *Brassica oleracea* L. associadas com *Bacillus megaterium* (Khan et al. 2018). Essas respostas levam a um aumento na área de superfície para absorção de água na raiz, o que é um mecanismo de tolerância ao déficit hídrico (Tiepo et al. 2018).

Uma outra característca que leva ao aumento da tolerância à seca é a promoção da

síntese de moléculas nitrogenadas, a qual acontece por meio da fixação biológica do nitrogênio por BPCP diazotróficas, e pelo aumento da atividade de enzimas vegetais da rota de assimilação desse nutriente (Ahemad e Kibret 2014; Fukami et al. 2018b). Por exemplo, mudanças induzidas pela associação com BPCP na atividade das enzimas nitrato redutase (NR), glutamina sintetase (GS) e glutamato sintase (GOGAT) já foram relatadas em *Vigna unguiculata* (L.) Walp e em *Zea mays* sob condições de estresse abiótico (Calzavara et al. 2018; Santos et al. 2018). Além disso, a via bioquímica de assimilação do nitrogênio é afetada pela associação com as BPCP por meio de alterações na síntese e degradação de aminoácidos e proteínas, como relatado em *Capsicum annuum* L. associadas com *Microbacterium* sp. (Vílchez et al. 2018), em mudas de *Trema micrantha* associadas com *Azospirillum brasilense* e em mudas de *Cariniana estrellensis* associadas com *Azomonas* sp. (Tiepo et al. 2018).

Outro atributo que promove o crescimento de plantas é a capacidade que as BPCP possuem de solubilizar fosfatos, como já relatado em mudas de *Triticum aestivum* L. associadas com *Bacillus cereus, Planomicrobium chinense* e *Pseudomonas fluorescens* (Khan et al. 2016), e, também, em estirpes de *Azospirillum brasilense* (Bashan e de-Bashan 2010). Essa característica aumenta a disponibilidade de fosfato nos solos, o que leva à manutenção de nutrição adequada para o estabelecimento, sobrevivência e crescimento das plantas (Khan et al. 2016; Khan et al. 2018).

A ação das BPCP ainda pode estar relacionada com as vias bioquímicas do metabolismo fotossintético. Como observado em *Trema micrantha* e em *Cariniana estrellensis*, a associação com *A. brasilense* levou à manutenção da eficiência de carboxilação em mudas submetidas ao déficit hídrico (Tiepo et al. 2018). Ainda, em mudas de *Sorghum bicolor* L. Moench submetidas ao déficit hídrico, a associação com *Bacillus* sp. preveniu a redução da taxa fotossintética líquida (Santana et al. 2020). Essas respostas podem ter ocorrido devido à influência positiva das BPCP na atividade da Rubisco (Tiepo et al. 2018). Em mudas de *Avena sativa* L. sob déficit hídrico, a associação com *Azotobacter vinelandii*, *Pantoea agglomerans* e *Pantoea putida* influenciou positivamente a síntese de clorofila *a*, clorofila *b* e carotenoides (Delshadi et al. 2017).

Outro fator importante é que a associação com BPCP induz vias bioquímicas de partição e síntese de carboidratos, as quais podem estar relacionados à degradação de amido ou ao aumento da taxa fotossintética (Tiepo et al. 2018). Uma maior quantidade de açúcares solúveis foi relatada em mudas de *Vigna unguiculata* inoculadas com *Bradyrhizobium* sp., *Paenibacillus graminis* e *Bacillus* sp. sob condições de estresse abiótico (Santos et al. 2018). Essa resposta pode aumentar a destinação de fotossintatos para a produção de energia

metabólica ou pode estar relacionada ao ajuste osmótico, uma vez que solutos osmoticamente ativos protegem as células contra a desidratação e contra danos causados pelo déficit hídrico (Tiepo et al. 2018).

Quando esses mecanismos de tolerância nas plantas são relacionados com o ajuste osmótico, mudanças na fisiologia dos estômatos podem ser induzidas, o que ocasiona uma melhor eficiência do uso da água. Assim, a utilização de microorganismos simbióticos têm sido uma importante estratégia para auxiliar na conservação de água em plantas submetidas ao déficit hídrico (Khan et al. 2018).

Além disso, o déficit hídrico leva ao aumento na síntese de EROs, e a associação com BPCP aprimora mecanismos de eliminação dessas EROs e proteção contra o dano oxidativo em tecidos vegetais (Khan et al. 2018). A associação com *A. brasilense* induziu um aumento na atividade das enzimas Catalase, Superóxido Dismutase e Ascorbato Peroxidase em mudas de *Zea mays* submetidas a estresse (Fukami et al. 2018b); e a associação com *Pseudomonas mendocina* e fungos micorrízicos arbusculares levaram à um aumento na atividade da Catalase em *Lactuca sativa* sob déficit hídrico (Kohler et al. 2008). Além disso, a associação com BPCP pode induzir mecanismos antioxidantes não enzimáticos, como o aumento de polifenóis observado em mudas de *Zea mays* e de *Vitis vinifera* submetidas a estresses abióticos e associadas com *Azobacter* sp. e com *Burkholderia phytofirmans*, respectivamente (Barka et al. 2006; Rojas-Tapias et al. 2012).

Fica evidente que a associação com diferentes espécies de BPCP induz mecanismos de respostas distintos nas espécies vegetais submetidas a estresses. No presente estudo duas espécies de BPCP foram utilizadas em associação com mudas de *C. pachystachya* e *C. estrellensis* submetidas ao déficit hídrico (Fig. 3 e Fig. 4): *Azospirillum brasilense* (estirpe Ab-V5) e *Bacillus velezensis* (estirpe ZK).

1.4 Azospirillum brasilense

Azospirillum é um importante gênero de BPCPs, cujo processo de colonização das raízes requer vários passos, como a síntese de biofilme, síntese de exopolissacarídeos e motilidade celular (Fukami et al. 2017b). No Brasil, *A. brasilense* (incluindo a estirpe Ab-V5) tem sido usada em inoculantes comerciais para plantas cultivadas, como milho, trigo e soja (Fukami et al. 2017b).

A promoção do crescimento vegetal ocorre pois essa BPCP possui rotas bioquímicas de fixação biológica de nitrogênio, de síntese de compostos como fitormônios e

sideróforos, e ainda estimula mecanismos de tolerância a estresses bióticos e abióticos no metabolismo vegetal (Ardakani et al. 2011). A fixação biológica do nitrogênio ocorre por meio do complexo de enzimas nitrogenase presentes nas BPCP, o qual converte nitrogênio atmosférico em NH_4^+, uma forma assimilável pelas plantas (Ahemad e Kibret 2014); sendo então uma alternativa ao uso de fertilizantes químicos como já observado em *Saccharum* spp., *Zea mays*, *Triticum aestivum* e *Oryza sativa* L. (Fukami et al. 2016; Fukami et al. 2018b).

Outro importante mecanismo de promoção do crescimento vegetal realizado por *A. brasilense* inclui o controle biológico de fitopatógenos, que ocorre por meio da síntese de sideróforos, limitação da disponibilidade de ferro para os patógenos e pela síntese de ácido salicílico. Ainda, pode ocorrer mediante a indução da resistência sistêmica, na qual há estímulo da síntese de metabólitos secundários pelas plantas, os quais aumentam a resistência vegetal à infecção e a estresses abióticos (Fukami et al. 2018b). Esses mecanismos induzidos por *A. brasilense* foram observados em *Z. mays*, *Arabdopsis* sp. e *Fragaria* sp. (Fukami et al. 2017a, Fukami et al. 2018a)

Os fitormônios que são sintetizados por *Azospirillum*, como auxinas, citocininas e giberelinas, podem agir como sinalizadores no metabolismo vegetal ou induzir respostas de crescimento em parte aérea e raízes (Fukami et al. 2017a). Além disso, estudos relatam uma importante ação de *A. brasilense* na promoção do crescimento vegetal por meio da influência em vias bioquímicas das plantas (Bashan e de-Bashan 2010). Dentre os mecanimos induzidos por essa BPCP, está o aumento da absorção de nutrientes como Nitrogênio, Fósforo e Potássio em *Triticum aestivum* (Ardakani et al. 2011).

A bactéria *A. brasilense* também já foi relacionada com o aumento da tolerância ao estresse salino por meio de mudanças no acúmulo de solutos em *Z. mays* (Fukami et al. 2017a), ou ainda mediante a restauração do crescimento e redução do acúmulo de solutos em *Triticum durum* L. (Fukami et al. 2018a). Também induziu aumento na quantidade de açúcares solúveis e de aminoácidos em mudas de *Zea mays* cultivadas sob diferentes disponibilidades de Nitrogênio (Calzavara et al. 2018).

Os efeitos positivos de *A. brasilense* na tolerância à seca podem ser mediados pela síntese de ABA, que induz fechamento estomático e maior eficência do uso da água. Ainda, podem estar ligados ao aumento da taxa fotossintética e da quantidade de pigmentos fotoprotetores, como observado em *Arabidopsis* sp. (Cohen et al. 2015), e tambérm à indução do acúmulo de açúcares solúveis e aminoácidos, que auxiliam a reduzir a desidratação (Bashan e de Bashan 2010; Cohen et al. 2015).

As vias metabólicas de eliminação das EROs e proteção contra o dano oxidativo

também são estimuladas pela associação com *A. brasilense* em espécies vegetais. Essa BPCP pode induzir a síntese e a atividade de enzimas antioxidantes, como a SOD, a APX e a CAT, reduzindo os efeitos deletérios das EROs (Fukami et al. 2018b).

Outra característica de tolerância induzido por esta BPCP é o aumento do comprimento e biomassa de raízes e parte aérea em *Arabidopsis* sp., *Glycine max* e *Triticum aestivum* (Cohen et al. 2015; Hungria et al. 2015; Fukami et al. 2016). Essa alteração de traços biométricos relaciona-se à síntese de auxinas e auxilia na absorção de água e consequentemente na tolerância ao déficit hídrico. Diante disso, torna-se evidente que o uso de *A. brasilense* como inoculante tem sido amplamente difundido e utilizado em espécies cultivadas a fim de aumentar a tolerância a estresses e também o rendimento das colheitas. Todavia, seu uso em mudas de espécies arbóreas nativas da Mata Atlântica é escasso e os mecanismos de ação nessas espécies são pouco conhecidos.

1.5 Bacillus velezensis

Algumas estirpes de *Bacillus velezensis* vem sendo utilizadas como biopesticidas, promotores do crescimento vegetal e probióticos veterinários (Reva et al. 2019). *Bacillus velezensis* já foi classificado como *Bacillus subtilis* e *Bacillus amyloliquefaciens*, isso ocorria devido à alta similaridade genômica do DNA, porém, avaliações recentes de determinação pangenômica proporcionaram uma melhor compreensão das diferenças filogenéticas em representantes do gênero *Bacillus* (Adeniji et al. 2019).

Análises genômicas revelaram que essa bactéria possui genes relacionados com a biossíntese de metabólitos secundários, os quais atuam na supressão de patógenos e também na promoção do crescimento de plantas (Rabbee et al. 2019). Estudos com essa BPCP confirmaram a capacidade de síntese de diversos compostos, como antibióticos, antifúngicos, enzimas, fitormônios e antioxidantes que promovem o crescimento vegetal (Adeniji et al. 2019).

Os metabólitos secundários produzidos por *B. velezensis* podem provocar uma resistência sistêmica induzida nas plantas, fazendo com que mecanismos próprios de defesa contra microrganismos virulentos sejam ativados (Rabbee et al. 2019). Além disso, efeitos antagonistas a fitopatógenos foram relatados para essa BPCP, como a inibição da infecção por *Streptomyces scabies* em *Raphanus sativus* L. por meio da síntese de antibióticos, a inibição do crescimento de *Fusarium graminearum* em *Triticum aestivum* pela síntese de antifúngicos, e também a redução do número de ovos de nematódeos nas raízes de *Solanum lycopersicum* e

de nematódeos juvenis no solo devido à síntese de um nematicida pela bactéria (Meng et al. 2016b; Chen et al. 2018; Rabbee et al. 2019).

Algumas estirpes de *B. velezensis* sintetizam auxinas, citocininas, giberelinas e ácido jasmônico, os quais estão relacionados com a promoção do crescimento vegetal e tolerância contra estresses. Além disso, a síntese de ACC desaminase também já foi encontrada em *B. velezensis*, o que reduz a quantidade de etileno produzido pelas plantas (Meng et al. 2016b). A produção desses compostos está relacionada a efeitos positivos no crescimento vegetal. Já foi observado que a associação com *B. velezenzsis* induziu aumento no comprimento de parte aérea, na massa fresca e massa seca de folhas e ainda na massa seca de raiz de beterraba, cenoura, pepino, pimenta, batata, rabanete, tomate e nabo (Meng et al. 2016b).

Genes relacionados com a interação planta-bactéria foram encontrados na estirpe LM2303 de *B. velezensis*, dentre os quais pode-se citar genes que codificam a formação de biofilme, a assimilação de nitrogênio, ferro e potássio, e a síntese de promotores do crescimento vegetal (Chen et al. 2018). Além disso, efeitos positivos da associação com essa estirpe foram observados no aumento da taxa de germinação, no comprimento da parte aérea e também na quantidade de clorofilas em mudas de *Triticum aestivum* (Chen et al. 2018). Os resultados apresentados para espécies cultivadas tornam evidente a capacidade do uso de *B. velezensis* em substituição a biofertilizantes tradicionais e a pesticidas químicos, o que pode gerar menos danos ao meio ambiente (Adeniji et al. 2019).

A estirpe ZK de *Bacillus velezensis* foi caracterizada como BPCP por Goes et al. (2012), possuindo a capacidade de sintetizar auxinas e sideróforos, que são compostos importantes para a promoção do crescimento vegetal; porém, essa estirpe não é diazotrófica. Em mudas de *Zea mays* submetidas à diferentes disponibilidades de nitrogênio, a associação com *Bacillus* sp. (posteriormente caracterizado como *B. velezensis* – estirpe ZK) influenciou positivamente a taxa fotossintética líquida, a eficiência quântica máxima do fotossistema II, o comprimento e o número de vasos do metaxilema da raiz (Calzavara et al. 2018).

A associação com *Bacillus* sp. (posteriormente caracterizado como *B. velezensis* - estirpe ZK) também induziu resultados positivos em espécies arbóreas neotropicais, como o aumento da porcentagem de germinação, redução do tempo médio de germinação e aumento do índice de vigor em *Cecropia pachystachya* (Souza et al. 2019). Além disso, efeitos positivos na atividade da enzima fenilalanina amonia liase (PAL) foram observados em *Heliocarpus popayanensis* Kunth, e na atividade da enzima polifenol oxidase (PPO) em *Trema micrantha* e em *Cariniana estrellensis* (Souza et al. 2019). Esses resultados indicam um aumento na tolerância a estresses induzido por *B. velezensis* (estirpe ZK), uma vez que a atividade da PAL

e da PPO estão relacionadas à eliminação de EROs (Souza et al. 2019).

O aumento da tolerância ao déficit hídrico também já foi induzido em espécies arbóreas neotropicais por meio da associação com *Bacillus* sp. (posteriormente caracterizado como *B. velezensis* – estirpe ZK), com a redução dos níveis de H_2O_2 e da peroxidação lipídica, e pela manutenção da massa seca de raiz e aumento da razão raiz:parte aérea em *T. micrantha* (Tiepo et al. 2018). Os mecanismos de tolerância mediados por *B. velezensis* em espécies arbóreas neotropicais ainda não estão completamente elucidados, sendo necessário o desenvolvimento de mais estudos para que as vias metabólicas envolvidas sejam conhecidas. E esta estirpe ainda não é registrada para uso em inoculantes comercias; contudo, os estudos apresentados indicam que a ação como promotora do crescimento em plantas a torna uma importante opção como inoculante.

Diante do exposto, observa-se que a restauração da Mata Atlântica é uma necessidade crescente, e ainda que as áreas degradadas muitas vezes estão sujeitas a períodos prolongados de seca. Assim, a inoculação com BPCP apresenta-se como uma importante ferramenta biotecnológica para aumentar a tolerância de mudas de espécies nativas Neotropicais a este estresse abiótico. Torna-se então essencial que mais estudos sejam desenvolvidos a fim de avaliar os efeitos da associação dessas bactérias com plantas nativas utilizadas em programas de reflorestamento.

Portanto, o presente estudo testou a seguinte hipótese geral: “A associação entre Bactérias Promotoras do Crescimento em Plantas e espécies arbóreas nativas neotropicais induz mudanças em parâmetros fisiológicos, bioquímicos e biométricos, tornando as espécies vegetais mais tolerantes ao déficit hídrico.”

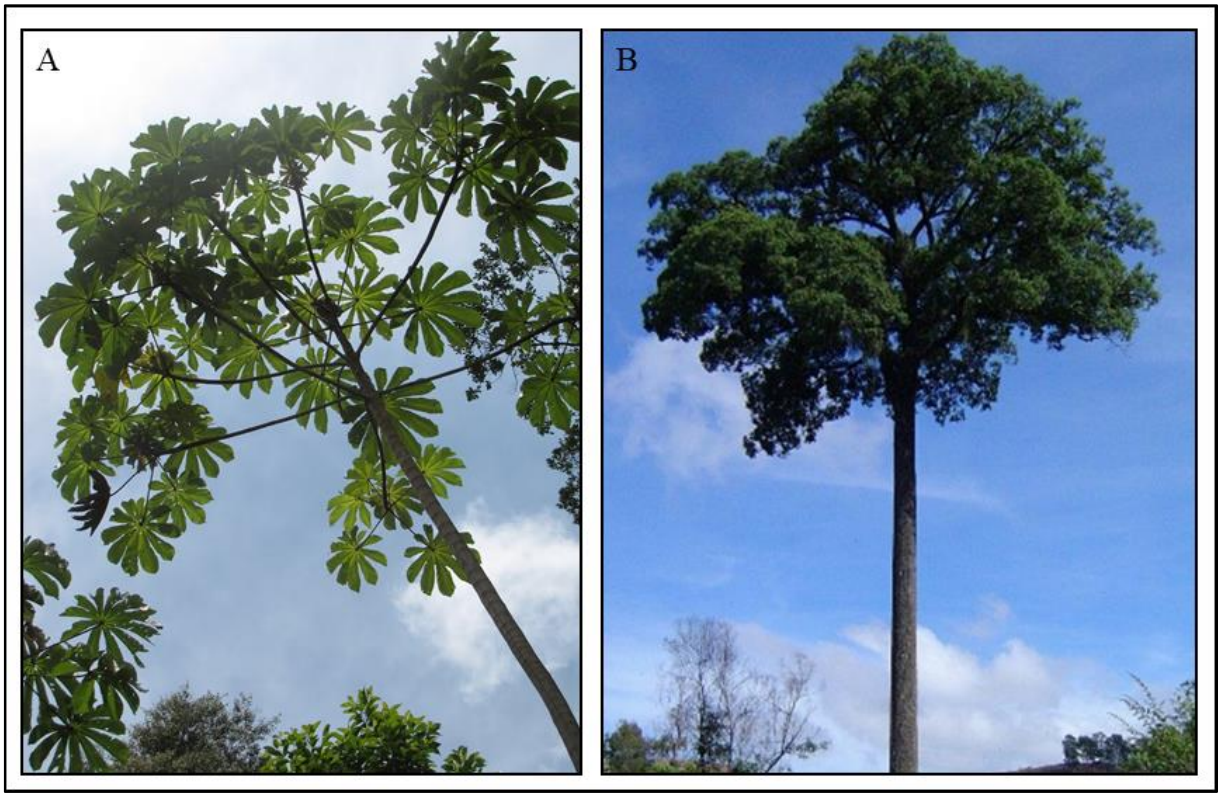

Fig. 1: Espécies arbóreas utilizadas no presente estudo. A: *Cecropia pachystachya*. B: *Cariniana estrellensis*. Imagens disponíveis respectivamente em: tudosobreplantas.wordpress.com/2014/01/22/sistsp-embaba-cecropia-pachystachya/; soflor.com.br/produto/jequitiba-branco-cariniana-estrellensis-5-sementes/

Fig. 2: Experimento com mudas de *Cecropia pachystachya.* A: Mudas mantidas em capacidade de campo. B: Mudas submetidas ao déficit hídrico. C: Mudas inoculadas com *Azospirillum brasilense* e submetidas ao déficit hídrico. D: Mudas inoculadas com *Bacillus velezensis* e submetidas ao déficit hídrico. Escala: 10 cm. Fotos: a autora.

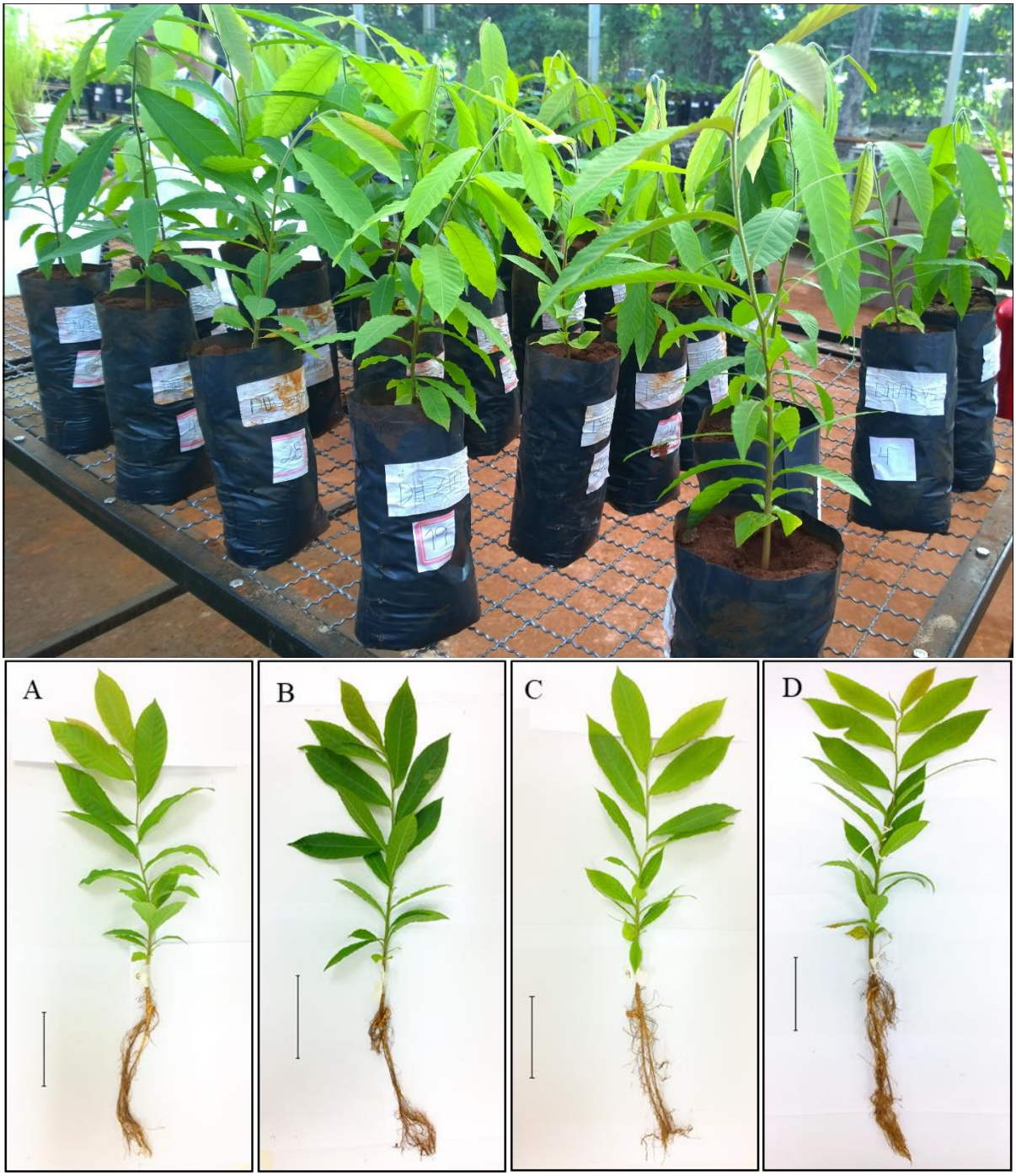

Fig. 3: Experimento com mudas de *Cariniana estrellensis.* A: Mudas mantidas em capacidade de campo. B: Mudas submetidas ao déficit hídrico. C: Mudas inoculadas com *Azospirillum brasilense* e submetidas ao déficit hídrico. D: Mudas inoculadas com *Bacillus velezensis* e submetidas ao déficit hídrico. Escala: 10 cm. Fotos: a autora.

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CAPÍTULO 1

Plant growth-promoting bacteria improve leaf antioxidant metabolism of drought-stressed Neotropical trees

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ORIGINAL ARTICLE

Plant growth-promoting bacteria improve leaf antioxidant metabolism of drought-stressed Neotropical trees

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Abstract

Main Conclusion **Plant growth-promoting bacteria association improved the enzymatic and non-enzymatic antioxidant pathways in Neotropical trees under drought, which led to lower oxidative damage and enhanced drought tolerance in these trees.**

Abstract Water deficit is associated with oxidative stress in plant cells and may, thus, negatively affect the establishment of tree seedlings in reforestation areas. The association with plant growth-promoting bacteria (PGPB) is known to enhance the antioxidant response of crops, but this strategy has not been tested in seedlings of Neotropical trees. We evaluated the effects of inoculation with two PGPB (*Azospirillum brasilense* and *Bacillus* sp.) on the antioxidant metabolism of *Cecropia pachystachya* and *Cariniana estrellensis* seedlings submitted to drought. We measured the activity of antioxidant enzymes and the content of non-enzymatic antioxidants in leaves, and biometrical parameters of the seedlings. In both tree species, drought decreased the activity of antioxidant enzymes and the content of non-enzymatic antioxidant compounds. For *C. pachystachya,* the enzymatic and non-enzymatic pathways were mostly influenced by *A. brasilense* inoculation, which enhanced ascorbate peroxidase (APX) and superoxide dismutase activities and positively affected the level of non-enzymatic antioxidant compounds. In *C. estrellensis*, *A. brasilense* inoculation enhanced APX activity. However, *A. brasilense* and *Bacillus* sp. inoculation had more influence on the non-enzymatic pathway, as both bacteria induced a greater accumulation of secondary compounds (such as chlorogenic acid, gallic acid, rutin and synapic acid) compared to that in non-inoculated plants under drought. For both species, PGPB improved biometrical parameters related to drought tolerance, as specific leaf area and leaf-area ratio. Our results demonstrate that PGPB induced antioxidant mechanisms in drought-stressed Neotropical trees, increasing drought tolerance. Thus, PGPB inoculation provides a biotechnological alternative to improve the success of reforestation programmes.

Keywords Associative bacteria · Drought · Microorganisms · Oxidative stress · Reforestation

Abbreviations

Ab-V5	*Azospirillum brasilense* Ab-V5 strain
APX	Ascorbate peroxidase
PGPB	Plant growth-promoting bacteria
ROS	Reactive oxygen species
SOD	Superoxide dismutase
ZK	*Bacillus* sp. ZK strain

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Introduction

Large-scale deforestation events have become more com- mon in recent decades, leading to a warmer and drier climate over deforested areas, which increases the large-scale vapour pressure deficit and causes impacts around the world (Stokstad 2004; Lawrence and Vandecar 2015). One way to mitigate the negative consequences of deforestation is to improve reforestation at certain sites (Chen et al. 2016). The increased frequency and severity of drought events resulting from climatic changes negatively affect plant growth, physiology and metabolism (Ortiz et al. 2015; Tiepo et al. 2018). Thus, water deficit is one of the major abiotic stress factors that limit the establishment of tree seedlings in the field, leading to high mortality rates and consequently high costs in reforestation programmes (Carvalho-Filho et al. 2003; Nolan et al. 2018).

Water deficit affects plant metabolism through the accumulation of reactive oxygen species (ROS), which can cause damage to membranes, DNA and proteins (Tri- antaphylidès and Havaux 2009; Ortiz et al. 2015; Tiepo et al. 2018). However, plants have mechanisms to maintain ROS homeostasis and to protect cells against oxidative damage. Both enzymatic (such as superoxide dismutase (SOD), catalase and peroxidases) and non-enzymatic (such as ascorbic acid, phenolic compounds and alkaloids) anti- oxidant defence systems act in coordination to eliminate ROS from plant cells (Hasanuzzaman et al. 2018). Thus, the development of management techniques that improve plant antioxidant pathways appears to be a strategy to increase plant tolerance to water stress.

The association with microorganisms has been used as a biotechnological tool to improve plant growth, nutri- ent uptake, resistance against pathogens and tolerance to drought (Saharan et al. 2011; Vejan et al. 2016; Tiepo et al. 2018). In particular, the inoculation of crops with plant growth-promoting bacteria (PGPB) has been shown to increase the antioxidant response, as reported for salt-stressed maize plants, (*Zea mays* L.) associated with *Bacillus aquimaris* (Li and Jiang 2017), cowpea plants (*Vigna unguiculata* (L.) Walp.) associated with *Paeni- bacillus graminis* and *P. durus* (Rodrigues et al. 2013), and drought-stressed wheat plants, (*Triticum aestivum* L.) associated with *Bacillus amyloliquefaciens* and *Azospiril- lum brasilense* (Kasim et al. 2013). These studies have reported the improvement of plant antioxidant defence upon PGPB inoculation, which may enhance the tolerance to abiotic stresses (Saravanakumar et al. 2010; Ishizawa et al. 2017).

The identification of a PGPB strain compatible with a given plant species is a valuable strategy that can be used to develop biological inputs, such as microbial inoculants, that can be applied to produce seedlings with higher adap- tive and developmental potential (Finkel et al. 2017; Tiepo et al. 2018). Despite the potential of PGPB association for increasing drought tolerance, studies on the seedlings of Neotropical trees are scarce. In a recent study, Tiepo et al. (2018) reported that PGPB inoculation led to a reduction in leaf oxidative stress in seedlings of native trees from the Brazilian Atlantic Forest, but the involved mechanisms remain unclear.

Here, we aimed to evaluate the effects of inoculation with two PGPB species [*A. brasilense* (Ab-V5) and *Bacillus* sp. (ZK)] on enzymatic and non-enzymatic antioxidant mechanisms in the leaves of seedlings of *Cecropia pachystachya* and *Cariniana estrellensis*. We hypothesized that PGPB inoculation would improve the antioxidant response of Neotropical tree seedlings under drought stress.

Material and methods

Biological material and experimental design

Two tree species native to seasonal semi-deciduous forest (a phytophysiognomy of the Brazilian Atlantic Forest) were chosen for this study: *C. pachystachya* Trécul (Urticaceae), a shade-intolerant species, and *C. estrellensis* (Raddi) Kuntze (Lecythidaceae), a shade-tolerant species. Both species are commonly used in Atlantic Forest reforestation programmes. The seeds were collected in forest fragments in northern Paraná (southern Brazil) and were kindly provided by the Laboratory of Biodiversity and Ecosystem Restoration at the State University of Londrina (UEL).

The bacterial species used are part of the Plant Growth-Promoting Bacteria Collection of UEL. *Azospirillum brasi- lense* (diazotrophic; Ab-V5 strain) is registered at the Brazil- ian Ministry of Agriculture for use in commercial inoculants (Hungria et al. 2010). *Bacillus* sp. (potential diazotrophic; ZK strain) was characterized as a PGPB species by Goes et al. (2012).

The inoculants were prepared according to Oliveira et al. (2017). PGPB were initially cultured in 5 mL of liq- uid DYGS medium (glucose 2 g L^{-1}; peptone 1.5 g L^{-1}; 2 g L^{-1} yeast extract; K_2HPO_4 0.5 g L^{-1}; $MgSO_4 \times 7H_2O$ 0.5 g L^{-1}) in test tubes kept under orbital shaking (180 rpm) at 28 ± 2 °C for 24 h for the preparation of the preinoculum. The inoculants were prepared by the addition of the pre- inoculum (1 mL) to Erlenmeyer flasks containing 50 mL of FORM15 culture medium (glycerol 100 g L^{-1}; sucrose 50 g L^{-1}; yeast extract 50 g L^{-1}; xanthan gum 1 g L^{-1}; PVP 1 g L^{-1}; FeEDTA 50 mM 2 mL L^{-1}; $MgSO_4 \times 7H_2O$ 1 g L^{-1}; NaCl 0.1 g L^{-1}; KH_2PO_4 4 g L^{-1}; K_2HPO_4 6 g L^{-1}; NH_4NO_3 1.5 g L^{-1}) supplemented with 5 mL L^{-1} of micronutri- ent solution (H_3BO_3 1.4 g L^{-1}; $ZnSO_4 \times 7H_2O$ 1.2 g L^{-1};

$MnSO_4 \times H_2O$ 1.18 g L^{-1}; $Na_2MoO_4 \times 2H_2O$ 1.0 g L^{-1}; $CuSO_4 \times 5H_2O$ 0.04 g L^{-1}), adjusted to pH 6.5 and followed by orbital shaking (180 rpm) at 28 ± 2 °C for 48 h. After the growth period, the cultures were normalized by dilution with deionized water to a final density of 1×10^6 cells mL^{-1}, constituting the inoculants used in the assays.

The seeds were sowed in plastic trays (1 L) containing an inert substrate (sieved sand previously heated at 100 °C for 8 h) and then treated with 800 mL of the inoculants. After germination, seedlings with a completely expanded pair of leaves were transferred to plastic bags (2 L, 15 cm high and 13 cm diameter) containing a mixture (1:1) of the inert substrate and fertile soil (pH 5.8; cation exchange capacity 4.4 $cmol_c$ dm^{-3}) characterized as clayey oxisol. At the time of transplanting and after 30 days, each plastic bag received an additional application of 50 mL of PGPB inoculant. The plants were kept under field capacity for 4 months until beginning the drought treatments. The experi- ments were carried out from July to November 2017 for *C. pachystachya* (winter–spring) and from May to September 2017 for *C. estrellensis* (autumn–spring). For both species, the seedlings were cultivated in a greenhouse. The average daily values and standard deviations of temperature, rela- tive humidity, and accumulated global solar radiation of the outdoor environment were 20.7 ± 3.8 °C, 74.2 ± 16.9%, and 18.2 ± 6.8 MJ m^{-2}, respectively (these data were kindly pro- vided by the Laboratory of Agrometeorology, Embrapa Soja, Londrina).

The experimental design was completely randomized, with four treatments as follows: (1) non-inoculated plants maintained in field capacity (30% gravimetric humidity), (2) non-inoculated (NI) plants submitted to moderate drought, (3) plants inoculated with *A. brasilense* and submitted to moderate drought, (4) plants inoculated with *Bacillus* sp. and submitted to moderate drought. In the moderate drought treatments, the soil was kept at 14% gravimetric humidity for 30 days.

Biometrical measurements

At the end of the experiment, the seedlings were carefully removed from the plastic bags, and the roots were washed in water. The total leaf area was determined using an LI-3000C leaf area meter (LICOR Biosciences, Lincoln, NE, USA). The root, stem, and leaf fresh weights were determined and, after keeping these organs for 3 days at 60 °C, the respective dry weights were measured. Shoot dry weight was calculated as the sum of stem and leaf dry weights. The specific leaf area was calculated as the ratio between leaf area and leaf dry weight, and the leaf-area ratio was calculated as the ratio between leaf area and whole-plant dry weight. Root:shoot ratio was obtained by dividing root dry weight by shoot dry weight.

Biochemical assays

Before proceeding with the biometrical measurements, 400 mg of the third and fourth fully expanded leaves was collected, frozen immediately in liquid nitrogen, and maintained at − 80 °C for posterior biochemical assays. To obtain the respective leaf dry weights sampled in each treatment, the following equation was used: Sampled leaf dry weight = (total leaf dry weight/total leaf fresh weight) × 200 mg.

Antioxidant enzyme activity

For the determination of the activity of antioxidant enzymes, 200 mg of frozen leaves were ground to a powder in liquid N_2 and homogenized with 1.8 mL of phosphate buffer (100 mM, pH 7.5) containing 1 mM EDTA and 2% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 15,000*g* for 15 min. All steps in the prepa- ration of the enzyme extract were carried out at 4 °C. The aliquots of this extract were maintained at − 80 °C until the enzymatic assays. As the treatments differently influenced the protein amount in leaf extracts, the enzymatic activities were normalized by dry weight. All spectrophotometric analyses were conducted on a GENESYS 10S UV–Vis spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

The superoxide dismutase (SOD) activity assay was based on the method described by Giannopolitis and Ries (1977), with modifications. The reaction medium was prepared with phosphate buffer (50 mM, pH 7.8), methionine (13 mM), EDTA (0.1 mM), nitro blue tetrazolium (NBT, 75 μM), and riboflavin (2 μM), and 40 μL of enzymatic extract was added to obtain 2 mL of final volume. The reaction was performed under the light from two fluorescent bulbs (25 W each) for 10 min. An identical solution for each sample that was not illuminated served as blank. As a control, 2 mL of the reac- tion buffer without the enzyme extract was illuminated. One unit was defined as the amount of enzyme required to result in a 50% inhibition of the rate of NBT photoreduction meas- ured at 560 nm.

The ascorbate peroxidase (APX) activity was determined according to Nakano and Asada (1981), with modifications. The reaction mixture (total volume of 3 mL) contained phos- phate buffer (50 mM, pH 7.0), EDTA (0.1 mM), ascorbate (0.5 mM), H_2O_2 (30 mM) and 50 μL of enzyme extract. The H_2O_2-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm in kinetics mode (ε = 2.8 mM^{-1} cm^{-1}).

The peroxidase (POD) activity assay was based on the method described by Peixoto et al. (1999), with modifications. The reaction phosphate buffer (25 mM, pH 6.8) was prepared with pyrogallol (20 mM) and H_2O_2 (20 mM). The

enzyme extract (100 µL) was added to 1.9 mL of the reaction buffer. After 1 min, 200 µL of H_2SO_4 was added to stop the reaction. The enzyme activity was measured at 420 nm to quantify the purpurogallin formation ($\varepsilon = 2.47\ mM^{-1}\ cm^{-1}$).

Non-enzymatic antioxidants

For the determination of non-enzymatic antioxidants, leaves (200 mg) were ground to a powder with liquid N_2 and homogenized with 5 mL of 80% methanol (v/v), followed by stirring (180 rpm) for 1 h at room temperature. The extracts were centrifuged at 1730*g* for 15 min and stored at − 20 °C for the assays. The spectrophotometric analyses were conducted using a Thermo Fisher Scientific (Bio-Mate3) spectrophotometer.

The determination of the total phenolic content was based on Swain and Hills (1959), with modifications. The reaction medium contained Folin–Ciocalteu reagent (10%, v/v), Na_2CO_3 (7.5%, w/v) and 250 µL of the extract. The absorbance was measured at 765 nm, and gallic acid was used as a standard ($r^2 = 0.9978$).

The total flavonoid content was measured based on the procedure described by Lee et al. (1995), with modifications. The reaction medium contained $NaNO_2$ (5%, w/v), $AlCl_3$ (10%, w/v), NaOH (4%, w/v) and 250 µL of the extract. The absorbance was measured at 425 nm, and quercetin was used as a standard ($r^2 = 0.9854$).

The same extract was used for the separation of the compounds by ultrahigh-performance liquid chromatography (UHPLC) using Acquity UPLC I Class Waters© Alliance e2695 equipment (Milford, MA, USA) and an HSS C181.8 µm 2.1 × 100 mm column (Waters©). The injection volume was µL with a flow of 0.4 mL min^{-1} using two solvents as the mobile phase: ultrapure water (A) and meth- anol (B) (JT Baker HPLC grade, Philipsburg, NJ, USA), acidified with 0.05% and 0.1% formic acid (JT Baker HPLC grade), respectively. The mobile phase gradient conditions were as follows: 0–10 min at 95% phase A and 5% phase B; 10–10.10 min at 5% phase A and 95% phase B; and 10.10–13 min at 95% phase A and 5% phase B. The total sample run time was 13 min. A photodiode array detector (UHPLCDAD) was used to read the wavelengths at 270 and 320 nm. The compound quantification was carried out by an external standardization method using the following standards: ascorbic acid, catechin, chlorogenic acid, epicatechin, gallic acid, rutin, synapic acid and trigonelline. All the standards used showed a degree of purity > 99% (Sigma- Aldrich, St. Louis, MO, USA).

Statistical analysis

Seven biological replicates were used for the biometrical determinations and four for the biochemical determinations. After checking the homogeneity of variances and normality, the data were analyzed using ANOVA, and when necessary, the means were compared using Fisher's LSD post hoc test ($P < 0.05$). In addition, the biochemical parameters of the moderate drought plants were assessed with principal component analysis (PCA) and a clustering heat map analysis to detect grouping patterns. The statistical analyses were performed using SAS software version 9.3 (SAS Institute, 2001) and the *Rcmdr* and *pheatmap* packages in R software.

Results

To evaluate how moderate drought and PGPB association influenced plant growth, we measured the biometrical parameters of *C. pachystachya* and *C. estrellensis* seedlings (Table 1). Moderate drought did not affect the root and shoot

Table 1 Biometrical measurements in *Cecropia pachystachya* and *Cariniana estrellensis* seedlings

Biometrical measurements	Tree species	NI FC	NI MD	Ab-V5 MD	ZK MD
Root dry weight (g)	*C. pachystachya*	1.32 ± 0.06 b	1.41 ± 0.15 b	1.88 ± 0.08 a	1.77 ± 0.04 a
	C. estrellensis	1.19 ± 0.06 a	1.08 ± 0.05 ab	0.90 ± 0.06 c	0.94 ± 0.06 bc
Shoot dry weight (g)	*C. pachystachya*	1.54 ± 0.04 a	1.41 ± 0.07 a	1.18 ± 0.06 b	1.45 ± 0.05 a
	C. estrellensis	2.63 ± 0.14 a	1.88 ± 0.12 b	1.51 ± 0.09 c	1.95 ± 0.13 b
Root:shoot ratio ($g\ g^{-1}$)	*C. pachystachya*	0.83 ± 0.04 c	1.01 ± 0.07 b	1.21 ± 0.07 a	1.18 ± 0.03 a
	C. estrellensis	0.55 ± 0.03 a	0.63 ± 0.01 a	0.65 ± 0.02 a	0.58 ± 0.04 a
Specific leaf area ($cm^2\ g^{-1}$)	*C. pachystachya*	158.26 ± 9.15 a	135.63 ± 4.00 ab	116.18 ± 9.15 b	155.29 ± 13.82 a
	C. estrellensis	159.14 ± 9.30 a	164.37 ± 5.21 a	125.91 ± 7.00 b	174.91 ± 7.93 a
Leaf-area ratio ($cm^2\ g^{-1}$)	*C. pachystachya*	45.22 ± 5.90 a	44.81 ± 3.69 a	31.00 ± 3.45 b	55.45 ± 3.12 a
	C. estrellensis	65.73 ± 4.00 a	67.33 ± 1.48 a	40.06 ± 3.10 c	53.82 ± 3.88 b

The values are means ± SE ($n = 7$). The same letters indicate no difference according to Fisher's LSD test ($P < 0.05$)

NI FC non-inoculated in field capacity, *NI MD* non-inoculated under moderate drought, *Ab-V5 MD* inoculated with *Azospirillum brasilense* under moderate drought, *ZK MD* inoculated with *Bacillus* sp. under moderate drought

dry weight of *C. pachystachya* plants. However, this treatment led to an increase of root:shoot ratio in comparison to non-inoculated plants in field capacity. Both bacterial strains increased the root dry weight and the root:shoot ratio of drought-stressed *C. pachystachya* plants. Moderate drought did not affect specific leaf area and leaf-area ratio. *Azospirillum brasilense* association reduced the specific leaf area and the leaf-area ratio of *C. pachystachya* in relation to non-inoculated plants in field capacity or in moderate drought.

In the case of *C. estrellensis*, moderate drought decreased the shoot dry weight in comparison with seedlings in field capacity. The association with *A. brasilense* reduced all biometrical parameters (with the exception of root:shoot ratio) compared to non-inoculated *C. estrellensis* seedlings in field capacity or in moderate drought. *Bacillus* sp. association reduced only the leaf-area ratio in relation to non-inoculated drought-stressed plants.

To evaluate the influence of moderate drought and PGPB in enzymatic antioxidant response, the leaf activity of ascorbate peroxidase (APX), superoxide dismutase (SOD), and peroxidases (POD) was measured. In *C. pachystachya* leaves, moderate drought induced a reduction in SOD and POD activities compared with those in non-inoculated plants in field capacity (Fig. 1b, c). The *A. brasilense* association induced an increase in APX activity in relation to that in non-inoculated plants in moderate drought (Fig. 1a), as well as it prevented the drought-mediated reduction in SOD activity (Fig. 1b). Both bacterial strains were able to prevent the drought-induced decrease in POD activity (Fig. 1c).

The enzymatic analyses in *C. estrellensis* leaves showed that the moderate drought induced a reduction in APX activity in relation to that in the non-inoculated plants in field capacity (Fig. 2a). *Azospirillum brasilense* inoculation prevented this drought-mediated reduction in APX activity (Fig. 2a), and both PGPB induced a reduction in SOD activity in relation to that in the non-inoculated plants under moderate drought (Fig. 2b). The treatments did not affect POD activity (Fig. 2c).

The levels of non-enzymatic antioxidant compounds (ascorbic acid, catechin, chlorogenic acid, epicatechin, gallic acid, rutin, synapic acid and trigonelline) were also determined in leaves of both species to verify whether this pathway was affected by moderate drought and PGPB inoculation.

In both tree species, *A. brasilense* and *Bacillus* sp. inoculation induced an increase in trigonelline content compared with non-inoculated plants in field capacity and in moderate drought (Fig. 3a, b). For all the other non-enzymatic antioxidants, no significant difference was detected among the treatments in *C. pachystachya.*

In *C. estrellensis*, both PGPB prevented the drought-mediated reduction in chlorogenic acid content, and induced an increase in gallic acid and rutin levels in relation to those

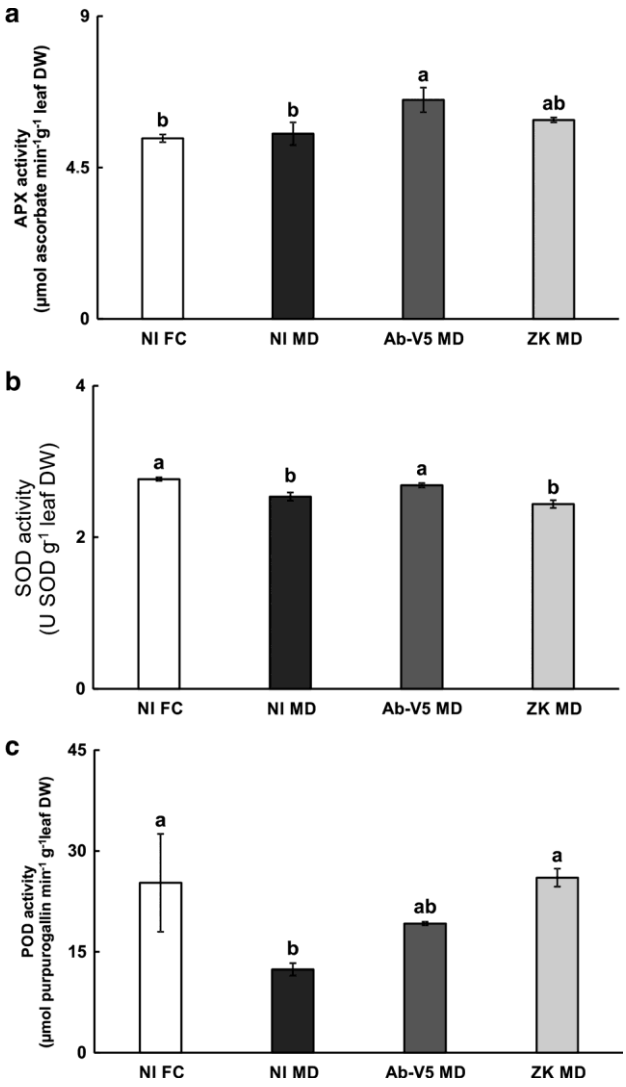

Fig. 1 **a** Ascorbate peroxidase (APX), **b** superoxide dismutase (SOD), and **c** peroxidase (POD) activities in the leaves of *C. pachystachya* seedlings submitted to moderate drought (MD, grey columns) or maintained under field capacity (FC, white columns). The seed- lings were either inoculated with *Azospirillum brasilense* (Ab-V5) or *Bacillus* sp. (ZK) or were not inoculated (non-inoculated, NI). The bars on top of the columns correspond to the standard error ($n = 4$). The columns with the same letters do not differ according to Fisher's LSD test ($P < 0.05$)

in the non-inoculated plants in field capacity and in moder- ate drought. In addition, *Bacillus* sp. association increased the content of synapic acid in relation to that in the non-inoculated plants in field capacity and in moderate drought (Table 2).

When evaluating the biochemical parameters by PCA and cluster heat map analysis, we considered only the plants under moderate drought. For *C. pachystachya*, the PCA explained 60.42 and 37.47% of the variation on axes 1 and 2, respectively (Fig. 4). The PCA and heat map showed that

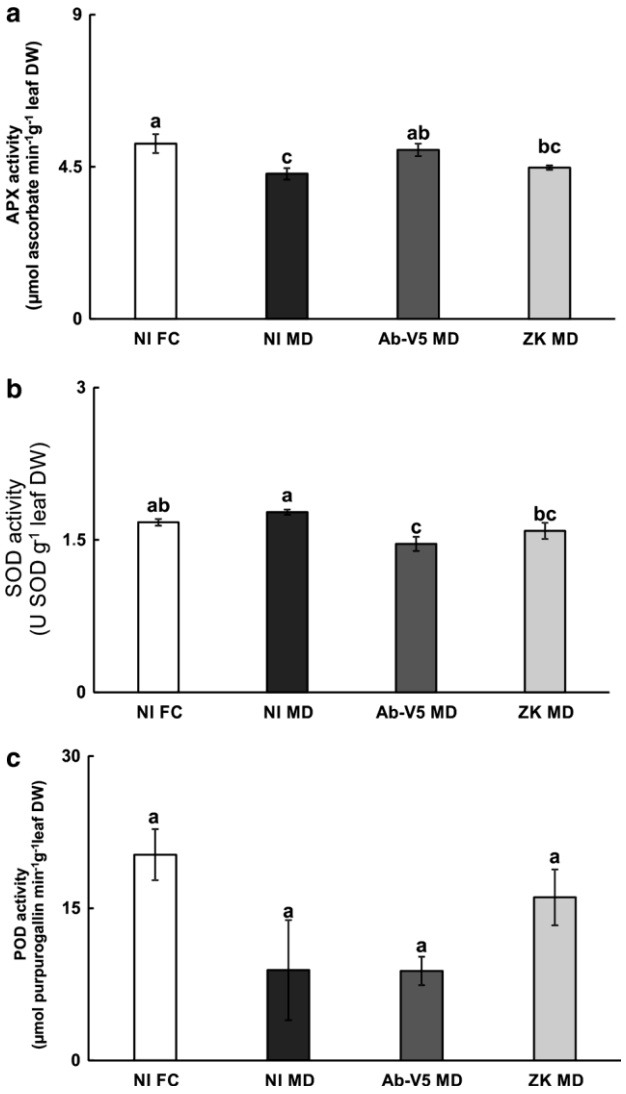

Fig. 2 a Ascorbate peroxidase (APX), **b** superoxide dismutase (SOD), and **c** peroxidase (POD) activities in the leaves of *Cariniana estrellensis* seedlings submitted to moderate drought (MD, grey col- umns) or maintained under field capacity (FC, white columns). The seedlings were either inoculated with *Azospirillum brasilense* (Ab- V5) or *Bacillus* sp. (ZK) or were not inoculated (non-inoculated, NI). The bars on top of the columns correspond to the standard error (n = 4). The columns with the same letters do not differ according to Fisher's LSD test ($P < 0.05$)

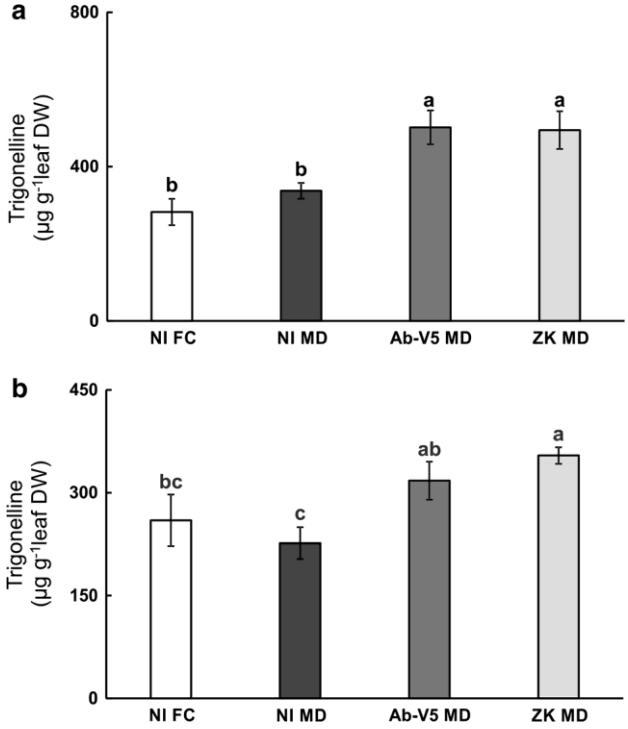

Fig. 3 Trigonelline content in the leaves of **a** *Cecropia pachystachya* and **b** *Cariniana estrellensis* seedlings submitted to moderate drought (MD, grey columns) or maintained under field capacity (FC, white columns). The seedlings were either inoculated with *Azospirillum brasilense* (Ab-V5) or *Bacillus* sp. (ZK) or were not inoculated (non- inoculated, NI). The bars on top of the columns correspond to the standard error (n = 4). The columns with the same letters do not differ according to Fisher's LSD test ($P < 0.05$)

the *A. brasilense* treatment was positively related to chlorogenic acid, synapic acid, catechin and rutin levels and APX and SOD activities, and it was more isolated in relation to the other treatments, which indicates a better discrimination from non-inoculated plants than that of *Bacillus* sp. The *Bacillus sp.* treatment was positively associated with ascorbic acid content and POD activity.

In *C. estrellensis*, the PCA explained 81.72 and 18.28% of the variation in axes 1 and 2, respectively (Fig. 5). The cluster heat map analysis and PCA showed that the non-inoculated treatment was more isolated in relation to the others, which was mainly explained by SOD activity. The *A. brasilense* inoculation was positively associated with APX activity, and *Bacillus* sp. inoculation was positively associated with total phenolic, flavonoids, gallic acid, synapic acid, epicatechin, trigonelline, ascorbic acid and POD activity. However, both inoculations influenced similarly the catechin, chlorogenic acid, and rutin levels in relation to the non-inoculated treatment.

Discussion

The association with PGPB has been reported as an alternative to reduce drought-induced oxidative stress in Neotropical trees (Tiepo et al. 2018). In this sense, the present study aimed to clarify how PGPB influence the enzymatic and non-enzymatic antioxidant pathways in native trees in the Atlantic Forest, which would lead to protection against oxidative stress and improvement of biometrical parameters.

Under environmental stress, SOD forms the first enzymatic line of defence against ROS by catalysing the

Table 2 Non-enzymatic antioxidant compounds in *Cariniana estrellensis*

Non-enzymatic compounds (μg g^{-1} leaf dry weight)	Treatments			
	NI FC	NI MD	Ab-V5 MD	ZK MD
Chlorogenic acid	16.42 ± 2.72 a	9.83 ± 1.08 b	16.55 ± 1.48 a	17.45 ± 0.95 a
Gallic acid	29.79 ± 3.38 c	22.38 ± 5.21 c	58.29 ± 3.45 b	72.79 ± 5.73 a
Rutin	81.66 ± 33.07 b	62.14 ± 9.25 b	202.87 ± 42.11 a	198.61 ± 9.77 a
Synapic acid	5.98 ± 1.25 b	5.53 ± 0.82 b	7.64 ± 0.50 ab	9.73 ± 0.52 a

The values are means ± SE (n = 4). The same letters indicate no difference according to Fisher's LSD test ($P < 0.05$)

NI FC non-inoculated in field capacity, *NI MD* non-inoculated under moderate drought, *Ab-V5 MD* inoculated with *Azospirillum brasilense* under moderate drought, *ZK MD* inoculated with *Bacillus* sp. under moderate drought

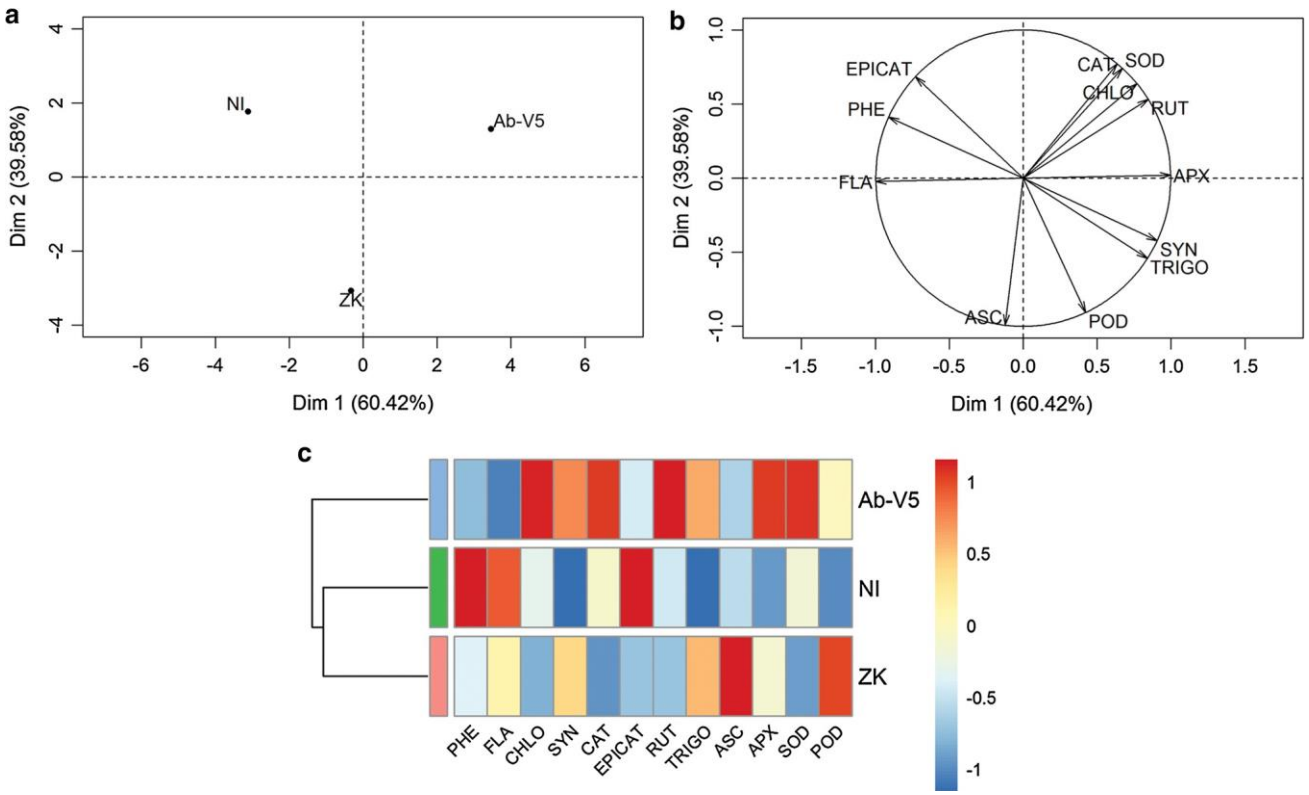

Fig. 4 Principal components analysis (PCA) and heat map of the biochemical parameters of the leaves of *Cecropia pachystachya* seedlings either inoculated with a species of bacteria [either *Azospirillum brasilense* (Ab-V5) or *Bacillus* sp. (ZK)] or not inoculated (non-inoculated, NI). The seedlings were maintained under moderate drought (MD) conditions for 30 days. **a** Treatment diagram. **b** Correlation between the biochemical parameters. **c** Heat map analysis. Biochemical parameters: *POD* peroxidase activity, *SOD* superoxide dismutase activity, *APX* ascorbate peroxidase activity, *ASC* ascorbic acid, *TRIGO* trigonelline, *RUT* rutin, *EPICAT* epicatechin, *CAT* cat- echin, *SYN* synapic acid, *CHLO* chlorogenic acid, *FLA* flavonoids, *PHE* total phenolics

dismutation of superoxide radicals ($O_2^{\bullet-}$) to O_2 and hydrogen peroxide (H_2O_2) (Sarma and Saikia 2014). *Azospirillum brasilense* inoculation improved this mechanism in *C. pachystachya* leaves, but not in *C. estrellensis* leaves. In addition to SOD, H_2O_2 production can occur by other pathways that are favoured under drought conditions, such as photorespiration (Das and Roychoudhury 2014). High intracellular H_2O_2 concentrations have been associated with the oxidation of cysteine and methionine residues, hydroxyl radical ($OH^{\bullet}$) generation and inactivation of Calvin cycle enzymes (Das and Roychoudhury 2014).

APX is closely related to the elimination of H_2O_2, since this enzyme is widely distributed within cells and has a higher affinity for H_2O_2 than catalase does, being a more

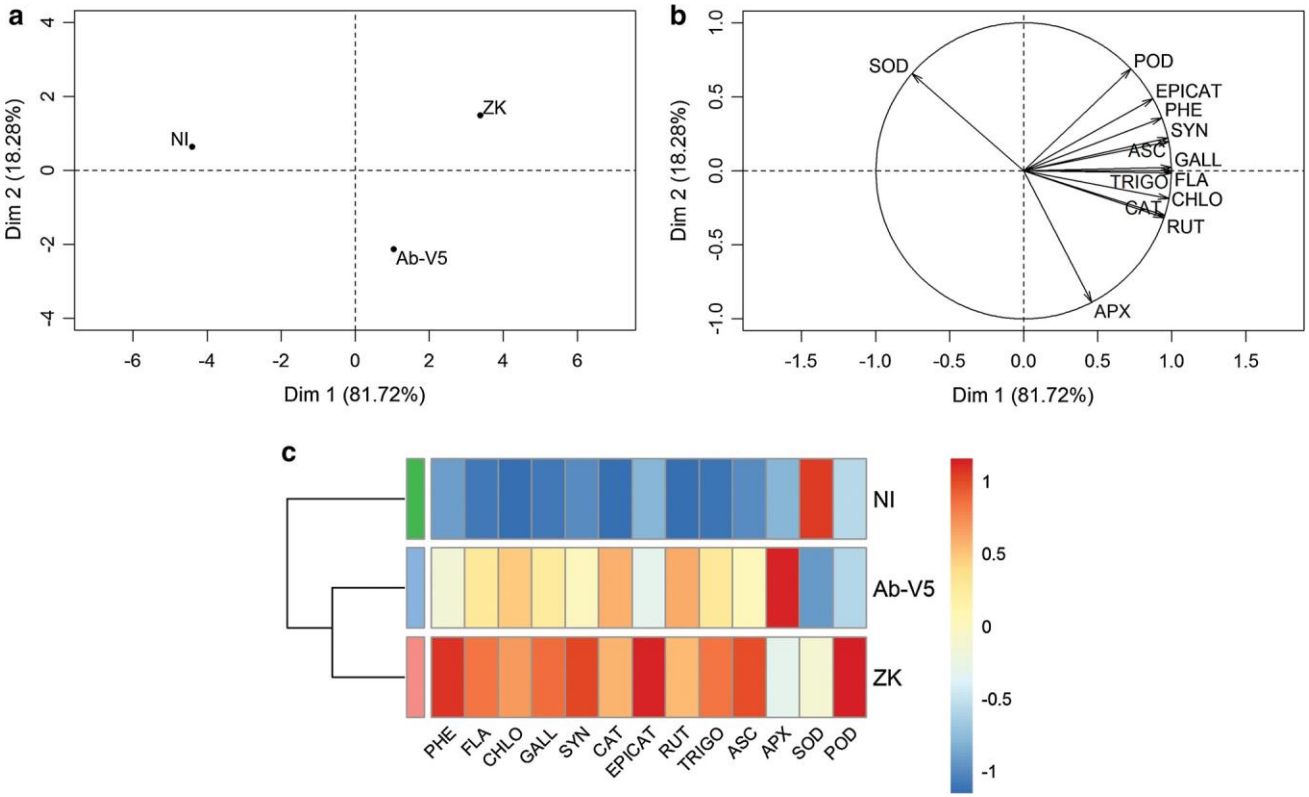

Fig. 5 Principal component analysis (PCA) and heat map of bio- chemical parameters of the leaves of *Cariniana estrellensis* seedlings either inoculated with a species of bacteria [*Azospirillum brasilense* (Ab-V5) or *Bacillus* sp. (ZK)] or not inoculated (non-inoculated, NI). The seedlings were maintained under moderate drought (MD) condi- tions for 30 days. **a** Treatment diagram. **b** Correlation between the biochemical parameters. **c** Heat map analysis. Biochemical param- eters: *POD* peroxidase activity, *SOD* superoxide dismutase activity, *APX* ascorbate peroxidase activity, *ASC* ascorbic acid, *TRIGO* trigo- nelline, *RUT* rutin, *EPICAT* epicatechin, *CAT* catechin, *SYN* synapic acid, *GALL* gallic acid, *CHLO* chlorogenic acid, *FLA* flavonoids, *PHE* total phenolics

efficient H_2O_2 scavenger (Das and Roychoudhury 2014). Significantly, the enhanced APX activity in *C. pachystachya* and in *C. estrellensis* inoculated with *A. brasilense* under moderate drought (Figs. 1, 2) indicates the influence of this bacterium on the enzymatic removal of H_2O_2. Importantly, for both tree species under moderate drought, the *A. brasilense* treatment showed a tendency to negatively influence the ascorbic acid content (Figs. 4, 5). Since two molecules of ascorbic acid are used by APX to reduce H_2O_2 in the ascorbate–glutathione cycle (Smirnoff 2000; Jaleel et al. 2009), the tendency for less ascorbic acid may be a result of the influence of the *A. brasilense* in APX activity. In contrast to *A. brasilense*, *Bacillus* sp. association had a positive influence on POD, another enzyme related to the scavenging of H_2O_2 (Das and Roychoudhury 2014) (Figs. 1c, 4, 5).

Fukami et al. (2018) have demonstrated that the association of *A. brasilense* with maize (*Zea mays* L.) led to an increase in APX, catalase and SOD activities in leaves and roots. Furthermore, improved enzymatic activity has already been related to an improvement of drought tolerance (Timmusk et al. 2014). These authors observed the induction of catalase, SOD and glutathione reductase activities and the enhancement of drought tolerance of wheat (*Triticum aestivum* L. cv. Stava) plants upon association with *Bacillus thuringiensis* AZP2. Heidari and Golpayegani (2012) reported that basil (*Ocimum basilicum* L.) associated with *Pseudomonades* sp., *Bacillus lentus* and *A. brasilense* had increased catalase and APX activities, which were related to improved growth and antioxidant status under drought stress. Moreover, the maintenance of the cellular redox status and leaf growth that was related to the increase in POD activity was observed in a drought-tolerant genotype of *Zea mays*. This was associated with the conservation of growth through the maintenance of meristem size and the number of dividing cells, even under drought (Avramova et al. 2017).

In addition to antioxidant enzymes, in the present study, there was a positive influence of PGPB inoculation on the levels of non-enzymatic antioxidant compounds in leaves of both plant species (Figs. 3, 4, 5 and Table 2). In *C. estrellensis* the PGPB association induced an increase in the content of total phenolics, flavonoids, gallic acid, rutin, chlorogenic acid, synapic acid, and ascorbic acid. For *C. pachystachya*,

we still observed a tendency for an increase in the levels of secondary metabolites in plants associated with *A. brasilense* and of ascorbic acid in plants associated with *Bacillus* sp., which was an important factor for separating treatment groups by PCA (Figs. 4b, c).

The accumulation of phenolic compounds might have a positive influence on protection against ROS damage, by donating electrons to guaiacol peroxidase for the elimination of large amounts of H_2O_2 produced under water deficit (Gómez-Caravaca et al. 2014; Nouman et al. 2018). Flavonoids in the chloroplast eliminate singlet oxygen ($^1O_2$) and alleviate the damage caused to the outer envelope of the chloroplast membrane during cellular dehydration (Fini et al. 2011; Agati et al. 2012; Das and Roychoudhury 2014; Gómez-Caravaca et al. 2014). Additionally, POD acts as an efficient H_2O_2 scavenger in plant vacuoles in the presence of phenolics and reduced ascorbate in a phenolic/ascorbic acid/POD system (Zacani and Nagy 2000; Jaleel et al. 2009; Gómez-Caravaca et al. 2014). Ascorbic acid is the first line of non-enzymatic defence against ROS and reacts with H_2O_2, $OH^{\cdot}$, and $O_2^{\cdot-}$; maintains the dissipation of the excess excitation energy and regenerates tocopherol from tocopheroxyl radicals, protecting the membranes from oxidative damage (Smirnoff 2000; Jaleel et al. 2009; Das and Roychoudhury 2014).

Similar responses have already been found in crops, such as that in *Zea mays* under saline stress, in which an increase in polyphenol was observed in plants associated with *Azobacter* sp. (Rojas-Tapias et al. 2012). In addition, in *Vitis vinifera* L. associated with *Burkholderia phytofirmans* under cold stress (Barka et al. 2006), an increase in total phenolics in leaves was observed. Higher rutin content has already been found in *Fagopyrum esculentum* Moench plants under drought conditions, indicating its protective role against desiccation (Suzuki et al. 2015).

Additionally, both PGPB inoculation induced an accumulation in trigonelline in the leaves of both tree species under moderate drought (Fig. 3). This alkaloid is related to drought tolerance, acting both in the response to oxidative stress and in osmoregulation to prevent water loss within plant cells (Willmon et al. 2017). Similar results have already been reported in the leaves of *Glycine max* in response to drought (Cho et al. 2003), and it can, therefore, be an additional mechanism by which PGPB inoculation influences antioxidant activity and improves drought tolerance in the present study.

For *C. pachystachya*, the changes induced by PGPB in enzymatic and non-enzymatic antioxidants positively influenced the root dry weight and the root:shoot ratio (Table 1). Biometrical responses associated with better nutrient and water uptake have already been reported to be induced by PGPB association, leading to improved drought tolerance (Bashan et al. 2004; Tiepo et al. 2018). Moreover, for both drought-stressed trees species, *A. brasilense* and *Bacillus* sp. inoculation lowered the specific leaf area and leaf-area ratio (Table 1), which may be a phenotypic adjustment related to the enhancement of water use efficiency and competitive ability under environmental stress (Wellstein et al. 2017).

However, the biosynthesis of antioxidant compounds demands energy and carbon skeletons, which are diverted from growth (Dias et al. 2016; Locosselli and Buckeridge 2017). *Cecropia pachystachya*, as other shade-intolerant plants, has high photosynthetic rates (Calzavara et al. 2019). Thus, PGPB could induce the destination of the fixed carbon to the improvement of antioxidant response in this species and still improve root growth and root:shoot ratio. In contrast, the shade-tolerant species *C. estrellensis* has low photosynthetic rates (Calzavara et al. 2019), and the carbon investment in antioxidant mechanisms induced by PGPB association might have hindered the improvement of root growth. Even so, considering the different physiological characteristics of the tree species used in the present study, the effects of PGPB on *C. estrellensis* seedlings can still be considered a strategy to improve drought tolerance, by inducing water-saving biometrical parameters and protecting against oxidative stress as a trade-off trait (Fernandez et al. 2016).

Our results clarify the systemic response induced by PGPB association, following the application of the treatment to the roots and the observation of the responses in leaves. In this sense, the present study demonstrated that the induced responses varied according to the tree and bacterial species. *Azospirillum brasilense* inoculation was more effective than *Bacillus* sp. in inducing protective responses in *C. pachystachya* leaves, whereas the effects of the tested PGPB were more similar in *C. estrellensis* leaves.

We conclude that the association with PGPB improved the enzymatic and non-enzymatic antioxidant pathways, which led to the maintenance of the cellular redox status, improvement of ROS scavenging capacity and biometrical traits, and, consequently, enhancement of drought tolerance. Thus, the present study indicates that the low oxidative damage that has been observed previously for Atlantic Forest native trees under drought conditions (Tiepo et al. 2018) is related to the positive influence of bacteria on the enzymatic and non-enzymatic antioxidant pathways.

Author contribution statement All authors conceived and designed the experiments. ANT, LVC and TBM performed the experiments. All authors analysed the data. ANT drafted the manuscript. All authors revised and approved the manuscript.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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CAPÍTULO 2

Influence of plant growth-promoting bacteria on leaf carbon and nitrogen metabolism of two drought-stressed Neotropical tree species: a metabolomic approach

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Influence of plant growth-promoting bacteria on leaf carbon and nitrogen metabolism of two drought-stressed Neotropical tree species: a metabolomic approach

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Abbreviations

A: Net photosynthesis
Ab-V5: *Azospirillum brasilense* Ab-V5 strain
C_i: intercellular CO_2 concentration
GS: glutamine synthetase
g_s: stomatal conductance
k: instantaneous carboxylation efficiency
MD: moderate drought
NI: not inoculated
PGPB: plant growth-promoting bacteria
Rubisco: Ribulose-1,5-bisphosphate carboxylase/oxygenase
UEL: State University of Londrina
ZK: *Bacillus velezensis* ZK strain

Summary

- Deforestation of Atlantic Forest has caused prolonged drought events in the last decades. The need for reforestation is growing, and the development of native seedlings that are more tolerant is necessary. A biotechnological tool that improves plant tolerance is the use of plant growth-promoting bacteria (PGPB) as inoculant.
- We inoculated two species of PGPB with drought-stressed seedlings of two neotropical tree species that have been used in environmental restoration programs: *Cecropia pachystachya* and *Cariniana estrellensis*. Biometrical, physiological and metabolomic parameters from carbon and nitrogen pathways were evaluated.
- We found that the PGPB influenced positively on photosynthesis and growth parameters in both trees under drought. The enzymes activities, the tricarboxylic acid cycle intermediates, the amino acids and protein contents were also influenced by the PGPB treatments. Furthermore, the results allowed us to find the specific composition of secondary metabolites of each plant species.
- This study provides evidences that there is not a single mechanism involved in drought tolerance and that the association with PGPB promotes a broad-spectrum tolerance response in neotropical trees. The inoculation with PGPB appears as an important strategy to improve drought tolerance in Atlantic Forest native trees and enhances environmental restoration programs success.

Key-words: Associative bacteria**,** Atlantic Forest, Drought, Metabolomic, Plant-microorganism, Reforestation.

1. Introduction

Plants are constantly under environmental stress, which may negatively influence the metabolic patterns, morphophysiological traits, and the establishment of seedlings (Chaves *et al.,* 2009; Pandey *et al.,* 2015). Drought is amongst the critical abiotic stresses that affect plant metabolic pathways, through influence on photosynthesis, nutrient uptake, ion translocation, growth rate, and enzyme activity (Chaves *et al.,* 2009; Shen *et al.,* 2015).

Water deficit can act on photosynthesis and plant carbon (C) balance directly, when CO_2 diffusion is hampered by stomatal limitation, or indirectly, when proteins are inhibited by drought-induced oxidative stress. The low intercellular CO_2 concentration (C_i) compromises the carboxylase activity of Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) which affects the rates of the C fixation, leading to lower concentrations of structural C and reduced growth (Chaves *et al.,* 2009; Hasibeder *et al.,* 2015; Das *et al.,* 2017; Marček *et al.,* 2019). Drought is also associated with alterations in nitrogen (N) metabolism, which may include the inhibition of the activity of N assimilation enzymes, such as nitrate reductase (NR) and glutamine synthetase (GS), alterations of amino acid metabolism, and decreases in leaf N and protein content (Xu and Zhou 2006; Chaves *et al.,* 2009), which were already observed in *Trema micrantha* (L.) Blume, in *Zea mays* (L.) and also in *Glycine max* L. (Das *et al.,* 2017; Wang *et al.,* 2017; Tiepo *et al.,* 2018).

The negative effects of drought on Neotropical trees can be mitigated by the association with plant growth-promoting bacteria (PGPB) (Tiepo *et al.,* 2018; Tiepo *et al.,* 2020). This process, called induced systemic tolerance (IST), involves the induction of antioxidant defense mechanisms and the accumulation of many compounds that act as protectors against the damage caused by drought, such as sugars, amino acids, phenolics, and polyamines (Vílchez *et al.,* 2018; Tiepo *et al.,* 2018, Tiepo *et al.,* 2020).

Drought-stressed plants usually accumulate soluble sugars, such as sucrose, glucose and fructose, to adjust osmotic balance and maintain turgor pressure (Vílchez *et al.,* 2018). In this sense, it has been already reported by Gagné-Bourque *et al.* (2016), Vílchez *et al.* (2018) and Tiepo *et al.* (2018) the increases in the content of several sugars in *Phleum pratense* L. associated with *Bacillus subtilis*, in *Capsicum annuum* L. associated with *Microbacterium* sp. and in *Cariniana estrellensis* (Raddi) Kuntze associated with *Bacillus* sp. (after identified as *B. velezensis*), which has been related to improved plant drought tolerance. The association with PGPB can also positively affect N metabolism of drought-stressed plants, leading to the

accumulation of free amino acids (like glutamine, valine, histidine, leucine, alanine, and proline), which also have an active role in osmotic adjustment and drought tolerance (Gagné-Bourque *et al.,* 2016; Tiepo *et al.,* 2018; Vílchez *et al.,* 2018).

Given that current climate changes models predict drought to increase in frequency and severity in several regions around the world (IPCC 2019), there is an urgent need in know the effect of drought on the metabolic profile of trees and if inoculation with PGPB may mitigate the effects. This information may be used for the development of biotechnological tools that make the trees more tolerant to drought. Still, most analyzes related to C and N metabolism of drought-stressed Neotropical trees lack a metabolomic approach (Tiepo *et al.,* 2018, Tiepo *et al.,* 2020). Thus, in the present study we used NMR analysis, which is a relevant tool to determine the metabolic profile of biological samples, and allows simultaneous measurements of many chemically different metabolites, providing a useful and rapid method for assessing the changes in metabolome as response to the plant interaction to the environment and submitted to different conditions (Charlton *et al.,* 2008). In addition, metabolomic analyzes have made possible to investigate more thoroughly the regulation of metabolic pathways and their influence in plants traits (Khan *et al.,* 2018).

Here, we aimed at evaluating leaf C and N metabolism of drought-stressed seedlings of *Cecropia pachystachya* Trécul and *Cariniana estrellensis* (Raddi) Kuntze inoculated with *Azospirillum brasilense* or *Bacillus velezensis*. We hypothesized that the association with PGPB induces responses in carbon and nitrogen metabolism that improve drought tolerance on neotropical trees.

2. Material and Methods

2.1 Biological material and experimental design

Two native tree species from seasonal semi-deciduous forest (a phytophysiognomy of Brazilian Atlantic Forest) were chosen for this study: *Cecropia pachystachya* Trécul (Urticaceae), a shade-intolerant species, and *Cariniana estrellensis* (Raddi) Kuntze (Lecythidaceae), a shade-tolerant species. Both species are commonly used in Atlantic Forest reforestation programmes. The seeds were collected in forest fragments in northern Paraná (southern Brazil) and were kindly provided by the Laboratory of Biodiversity and Ecosystem Restoration at the State University of Londrina (UEL).

The bacterial species were selected from the Plant Growth-Promoting Bacteria Collection of UEL. *Azospirillum brasilense* (diazotrophic; Ab-V5 strain) is registered at the Brazilian Ministry of Agriculture for use in commercial inoculants (Hungria *et al.*, 2010). *Bacillus velezensis* (no diazotrophic; ZK strain) was characterized as a PGPB species by Goes *et al*. (2012).

The inoculants were prepared according to Oliveira *et al*. (2017). PGPB were initially cultured in 5 mL of liquid DYGS medium in test tubes kept under orbital shaking (180 rpm) at 28 ± 2 °C for 24 h for the preparation of the preinoculum. The inoculants were prepared by the addition of the preinoculum (1 mL) to Erlenmeyer flasks containing 50 mL of FORM15 culture medium, supplemented with 5 mL L^{-1} of micronutrient solution, pH 6.5. Followed by orbital shaking (180 rpm) at 28 ± 2 °C for 48 h. After the growth period, the cultures were normalized by dilution with deionized water to a final density of 1×10^6 cells mL^{-1}, and inoculated in the assays (Tiepo *et al.*, 2020).

The seeds were sowed in plastic trays (1 L) containing an inert substrate (sieved sand previously heated at 100 °C for 8 h), and then treated with 800 mL of the inoculants. After germination, seedlings with a completely expanded pair of leaves were transferred to plastic bags (2 L, 15 cm high, and 13 cm diameter) containing a mixture (1:1) of the inert substrate and fertile soil (pH 5.8; cation exchange capacity 4.4 $cmol_c$ dm^{-3}) characterized as clayey Oxisol. At the time of transplanting and after 30 days, each plastic bag received an additional application of 50 mL of PGPB inoculant. The plants were kept under field capacity for four months until beginning the drought treatments. The experiments were carried out from July to November 2017 for *C. pachystachya* (winter-spring) and from May to September 2017 for *C. estrellensis* (autumn- spring). The seedlings of both species were cultivated in a greenhouse of the State University of Londrina. The average daily values and standard deviations of temperature, relative humidity, and accumulated global solar radiation of the outdoor environment were 20.7 ± 3.8 °C, 74.2 ± 16.9%, and 18.2 ± 6.8 MJ m^{-2}, respectively (these data were kindly provided by the Laboratory of Agrometeorology, Embrapa Soja, Londrina).

The experimental design was completely randomized, with four treatments as follows: (1) non-inoculated plants maintained in field capacity (30% gravimetric humidity), (2) non-inoculated plants submitted to moderate drought, (3) plants inoculated with *A. brasilense* and submitted to moderate drought, (4) plants inoculated with *Bacillus velezensis* and submitted

to moderate drought. In the moderate drought treatments, the soil was kept at 14% gravimetric humidity for 30 days. After that period, the analysis below were performed.

2.2 Physiological and biometrical measurements

Net photosynthesis (A), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and instantaneous carboxylation efficiency ($k = A/C_i$) were measured in the early morning (8-10 a.m.) using an LI-6400XT Portable Photosynthesis System (LI-COR Biosciences, Lincoln, NE, USA). The leaves were placed in a 6 cm^2 6400-02B measuring chamber, where the leaves were exposed to saturating PAR (1,900 μmol m^{-2} s^{-1}) and a flow rate of 400 mL min^{-1}.

At the end of the experiment, the seedlings were carefully removed from the plastic bags, and the roots were washed in water. The total leaf area was determined using a LI-3000C leaf area meter (LICOR Biosciences, Lincoln, NE, USA). The root, stem, and leaf fresh weights were determined and, after keeping these organs for three days at 60ºC, the respective dry weights were measured. The total dry weight was calculated as the sum of root, stem and leaf dry weights.

2.3 Sample preparation and instrumentation for metabolomic analysis

The extracts were obtained from 30 mg of the third and fourth fully expanded lyophilized leaves added with 1 mL of CD_3OD:D_2O (8:2 v/v) containing the TSP-d_4-sodium salt of trimethylsilylpropionic acid (0.264 mmol). The tube contents were mixed using a vortex for 30 s, and then heated at 50 °C in water bath for 10 min. The samples were then centrifuged for 5 min at 4 ºC. The supernatant was cooled to 4 °C for 30 min, and 20 μL of 2.40 mM phosphate buffer was added to each extract (Coutinho *et al.,* 2018). The samples were stored at 4 °C overnight, then 600 μL of each extract were transferred to a tube for the analysis of ^{1}H 1D Nuclear Magnetic Resonance (NMR).

There are two kinds of analyzes that have been performed: target analyzes, in which the concentrations of a predefined set of metabolites are determined; and untargeted analyzes, in which the metabolite fingerprinting is obtained from the sample extracts to reveal specie-specific patterns and allow for comparative studies (Coutinho *et al.,* 2018). Thus, Nuclear Magnetic Ressonance (NMR), and high-performance liquid chromatography coupled to mass

spectrometry (HPLC – MS) were used as important analytical techniques for the metabolomics screening of plants (Gödecke *et al.,* 2013; Coutinho *et al.,* 2018).

The NMR spectra were acquired at 298 K on an Avance III HD spectrometer, operating at 600 MHz and equipped with a 5-mm TCI Prodigy probe. Proton spectra were acquired using NOESY 1D with a 1.50-s presaturation delay and an acquisition time of 2.69 s (298 k points), an accumulation of 256 transients, and a spectral width of 20 ppm. All FIDs were automatically Fourier transformed after the application of an exponential window function with a line broadening of 0.3 Hz. Phase and baseline corrections were carried out within the instrument software. ^{1}H NMR chemical shifts were referenced to TSP-d4 at δ 0.00. The ^{1}D spectra of trees leaves were assigned using identification by public database such as Human Metabolome Database (HMDB) and the established Chenomx NMR Suite (professional version 8.1). Secondary metabolites were identified through 1D and 2D NMR spectra (correlation spectroscopy- COSY), Heteronuclear Single Quantum Correlation (HSQC); besides that, LC-DAD-MS/MS was employed to support the NMR spectrum and metabolite identification.

2.4 Biochemical measurements

To obtain the leaf dry weights sampled in each treatment, the following equation was used: Sampled leaf dry weight = (total leaf dry weight/total leaf fresh weight) * mg of fresh leaves used in respective analyze.

For the determination of glutamine synthetase (GS) activity, the first fully expanded leaves (100 mg) were ground to a powder in liquid N_2 and were homogenized in 0.5 mL of 50 mM HEPES buffer (pH 7.5), 10 mM 2-mercaptoethanol, 2% polyvinylpolypyrrolidone, 1 mM EDTA, 5 mM $MgCl_2$, 114 mM NaCl 1.22 mM $CaCl_2$; 2.35 mM K_2HPO_4. The extract was centrifuged at 18,000 x g for 20 min at 4 °C and 24 μL of the enzyme extract was used to determine GS activity by the hydroximate biosynthetic method according to Ratajczak *et al.,* (1981). Each 1 mL of the reaction medium contained 100 mM Tris-HCl buffer (pH 7.5), 10 mM 2-mercaptoethanol, 40 mM $MgSO_4$, 50 mM glutamate, 10 mM hydroxylamine, and 10 mM ATP. The mixture was incubated at 30 °C for 20 min, and the reaction was stopped by addition of 37 mM $FeCl_3$. The produced amount of Fe-γ-glutamil hydroxymate was assessed at 540 nm.

The second fully expanded leaves (200 mg) were collected for the extraction of amino acids with MCW (methanol:chlorophorm:water solution, 12:5:3, v:v:v), following the procedure described by Oliveira and Sodek (2013). Proteins were extracted from the precipitate resultant from the MCW extraction using NaOH (0.1 M). The total free amino acids were quantified using the ninhydrin assay (Yemm and Cocking, 1955). A mixture of 125 μL of the diluted sample, 62.5 μL of 0.2 M citrate buffer (pH 5.0), 25 μL of 5% ninhydrin (in methyl cellosolve) and 125 μL of 10 mM KCN (in methyl cellosolve) was kept at 100 ^{o}C for 20 min. After dilution with ethanol 60%, the absorbance at 570 nm was measured and compared with a leucine standard curve. Protein content was determined using the Comassie Blue reagent at 595 nm (Bradford 1976).

2.4 Data and statistical analysis

Four biological replicates were used for the biochemical and metabolomic determinations, nine biological replicates were used for physiological measurements and seven biological replicates were used for biometrical determinations. After checking the homogeneity of variances and normality, the data were analyzed using ANOVA, and when necessary, the means were compared using Fisher's LSD post hoc test ($p < 0.05$). In addition, the data were assessed with principal component analysis (PCA) and a clustering heatmap analysis to detect grouping patterns. The statistical analyzes were performed using STATISTICA software version 11.0 (Statsoft Inc., Tulsa, USA) and the *Rcmdr* and *pheatmap* packages in R software.

The ^{1}H NMR data ranging from 0.00 to 10.00 ppm were converted to ASCII files using Bruker TopSpin 3.5. Each dataset was arranged in an X_{IXJ} matrix, where *I* corresponded to the samples, 90, and *J* corresponded to the columns of 32 K variables. The NMR data were aligned using the *icoshift* algorithm (Savorani *et al.*, 2010). Then, the region corresponding to residual signal of water, methanol and TSP was excluded. Chenomx software was used to calculate the levels of the metabolites as measured by ^{1}H NMR

3. Results

3.1 Physiological and biometrical analysis

The association with *B. velezensis* prevented the drought-induced decrease of *A* and g_s in *C. pachystachya* seedlings, as well as it led to an increased *k* compared to the other groups

(Table 1). C_i was not influenced by any treatment. In the case of *C. estrellensis*, moderate drought reduced *A*, g_s, and C_i compared to plants in field capacity (Table 1). The only effect induced by PGPB was the increase of *k* in plants inoculated with *A. brasilense*.

Moderate drought reduced the leaf area and the total dry weight in seedlings of *C. pachystachya*, and the association with *B. velezensis* prevented the drought-induced reduction of the leaf area, while both PGPB association prevented the reduction of the total dry weight. For *C. estrellensis* the association with *A. brasilense* led to a lower leaf area and total dry weight (Table 2). The complete growth profile of these plants was evaluated by Tiepo et al. (2020).

Table 1. Physiological parameters of *Cecropia pachystachya* and *Cariniana estrellensis*. The values are means ± SE (n = 9). The same letters indicate no difference according to Fisher's LSD test ($p < 0.05$). NI FC: non-inoculated in field capacity. NI MD: non-inoculated under moderate drought. Ab-V5 MD: inoculated with *Azospirillum brasilense* under moderate drought. ZK MD: inoculated with *Bacillus velezensis* under moderate drought.

Physiological parameters	Tree species	NI FC	NI MD	Ab-V5 MD	ZK MD
A (μmol m^{-2} s^{-1})	*C. pachystachya*	6.05 ± 0.20 a	4.80 ± 0.50 b	4.25 ± 0.54 b	6.30 ± 0.38 a
	C. estrellensis	3.62 ± 0.12 a	2.21 ± 0.16 b	2.49 ± 0.13 b	2.30 ± 0.25 b
g_s (mol m^{-2} s^{-1})	*C. pachystachya*	0.06 ± 0.004 a	0.04 ± 0.005 b	0.05 ± 0.007 b	0.07 ± 0.004 a
	C. estrellensis	0.05 ± 0.003 a	0.02 ± 0.001 b	0.02 ± 0.001 b	0.02 ± 0.001 b
C_i (μmol mol^{-1})	*C. pachystachya*	226.34 ± 7.61 a	212.27 ± 5.58 a	219.50 ± 2.55 a	216.43 ± 4.63 a
	C. estrellensis	246.29 ± 5.42 a	179.65 ± 4.92 b	183.97 ± 6.16 b	181.97 ± 5.49 b
k (μmol m^{-2} s^{-1} Pa^{-1})	*C. pachystachya*	0.26 ± 0.01 b	0.23 ± 0.02 bc	0.20 ± 0.03 c	0.33 ± 0.02 a
	C. estrellensis	0.14 ± 0.01 ab	0.13 ± 0.01 b	0.17 ± 0.01 a	0.13 ± 0.02 b

Table 2. Biometrical measurements in *Cecropia pachystachya* and in *Cariniana estrellensis*. The values are means ± SE (n = 7). The same letters indicate no difference according to Fisher's LSD test ($p < 0.05$). NI FC: non-inoculated in field capacity. NI MD: non-inoculated under moderate drought. Ab-V5 MD: inoculated with *Azospirillum brasilense* under moderate drought. ZK MD: inoculated with *Bacillus velezensis* under moderate drought.

Biometrical measurements	Tree species	NI FC	NI MD	Ab-V5 MD	ZK MD
Leaf area (cm^2)	*C. pachystachya*	181.44 ± 16.62 a	109.54 ± 8.44 b	69.90 ± 10.89 b	195.89 ± 17.78 a
	C. estrellensis	272.28 ± 22.11 a	211.25 ± 12.91 b	74.87 ± 10.23 c	181.71 ± 19.53 b
Total dry weight (g)	*C. pachystachya*	2.99 ± 0.18 a	2.54 ± 0.19 b	3.12 ± 0.12 a	3.24 ± 0.08 a
	C. estrellensis	3.75 ± 0.27 a	3.19 ± 0.17 ab	2.28 ± 0.15 c	3.04 ± 0.15 b

3.2 Metabolic profile

The NMR ^{1}H was used to screen the leaf metabolic profile of *C. pachystachya* and *C. estrellensis* seedlings. For *C. pachystachya* (Fig. S1) the metabolic groups identified were sugars, organic acids, phenylpropanoids, and flavonoids. The main primary metabolites identified were malic acid (δ 2.66 dd 3.1 and 15.1 Hz), citric acid (δ 2.54 d 16.0 HZ), succinic acid (δ 2.40 s), glucose (δ 5.23 d, 3.70 HZ; δ 4.46 d, 7.92 Hz), fructose (δ 4.10 d), sucrose (δ 5.42 d, 3.80 Hz), and alanine (δ 1.46 d, 7.4 Hz), whilst the main secondary metabolites were chlorogenic acid (δ 7.18 d 1.76 Hz; δ 7.64 d 15.95 Hz; δ 5.30 m; δ 2.19 m), catechin (δ 2.76 dd 2.18 e 17.11 Hz; δ 2.91 dd 4.1 and 16.9 Hz; δ 7.07 d 0.9 Hz), luteolin 8-C-glycoside δ 6.58 s (br), and luteolin 6-C-glycoside δ 6.54 s (br). LC–DAD-UV analysis (Fig. S2) confirmed the secondary metabolites found by NMR ^{1}H. Still, it enabled the identification of luteolin and apigenin derivatives, which were not detected in the NMR ^{1}H spectrum due to their low concentration.

Similarly, in *C. estrellensis* the complete metabolite profile was described in Tiepo *et al.*, 2020b, ahead of print. And the identified metabolic groups were sugars, organic acids, phenolic acids, and flavonoids. The main primary metabolic were malic acid (δ 2,66 dd 3,1 and 15,1 Hz), glucose (δ 5.27 d, 3.70 HZ; δ 4.63 d, 7.92 Hz), sucrose (δ 5.41 d, 3.80 Hz), lactate (δ 1.30 d), and hydroxybutyrate (δ 1.35 s), while the main secondary metabolites were quercetin (δ 7.20), kaempferol (δ 6.99), p-coumaric acid (δ 7.72 d) quinic acid (δ 1.90 m). LC–DAD-UV confirmed the secondary metabolites found by NMR ^{1}H.

3.3 Biochemical and metabolomic analysis

The evaluation of the metabolic parameters by PCA and cluster heat map analysis showed the influence of the treatments in the metabolism of both species of plants (Fig. 1 and Fig. 2). For *C. pachystachya* seedlings, PC1 and PC2 explained 57.32 and 25.25 % of the data variance, respectively. Non-inoculated plants in field capacity were more separated in relation to the other treatments in the positive side of the PC1 axis and negative side of the PC2 axis, as well as it was ungrouped in the heat map analysis. Non-inoculated plants in field capacity was positively related to GS activity, and to alanine, sucrose, and citrate amounts. Non-inoculated plants under moderate drought were in the negative side of the PC1 axis and in the positive side of the PC2 axis and were positively related to protein, hydroxybutyrate, glucose, and malate amounts. Both inoculation treatments formed the closest groups in the PCA and heat map

analysis, being positively related to the amino acids, lactate, and chlorogenic acid amounts. PGPB treatment was also negatively related to malate levels. (Fig. 1)

In *C. pachystachya* (Table 3), moderate drought reduced the leaf GS activity compared to well-hydrated plants. *B. velezensis* prevented the drought-induced reduction of the GS activity, although it reduced the amount of protein of *C. pachystachya* leaves in relation to non-inoculated plants under moderate drought. Moreover, both PGPB enhanced by almost 40% the total amino acids content in the leaf. Metabolomic analyzes showed that both PGPB increased by almost 30% leaf lactate amount of drought-stressed *C. pachystachya* seedlings in relation to those non-inoculated in field capacity. Also, *A. brasilense* induced an about 50% reduction of leaf alanine amount in relation to non-inoculated in field capacity. Significant differences were not observed in the malate, succinate, citrate, glucose, sucrose, hydroxybutyrate and chlorogenic acid amounts among treatments according to ANOVA (Table Supplementary 1).

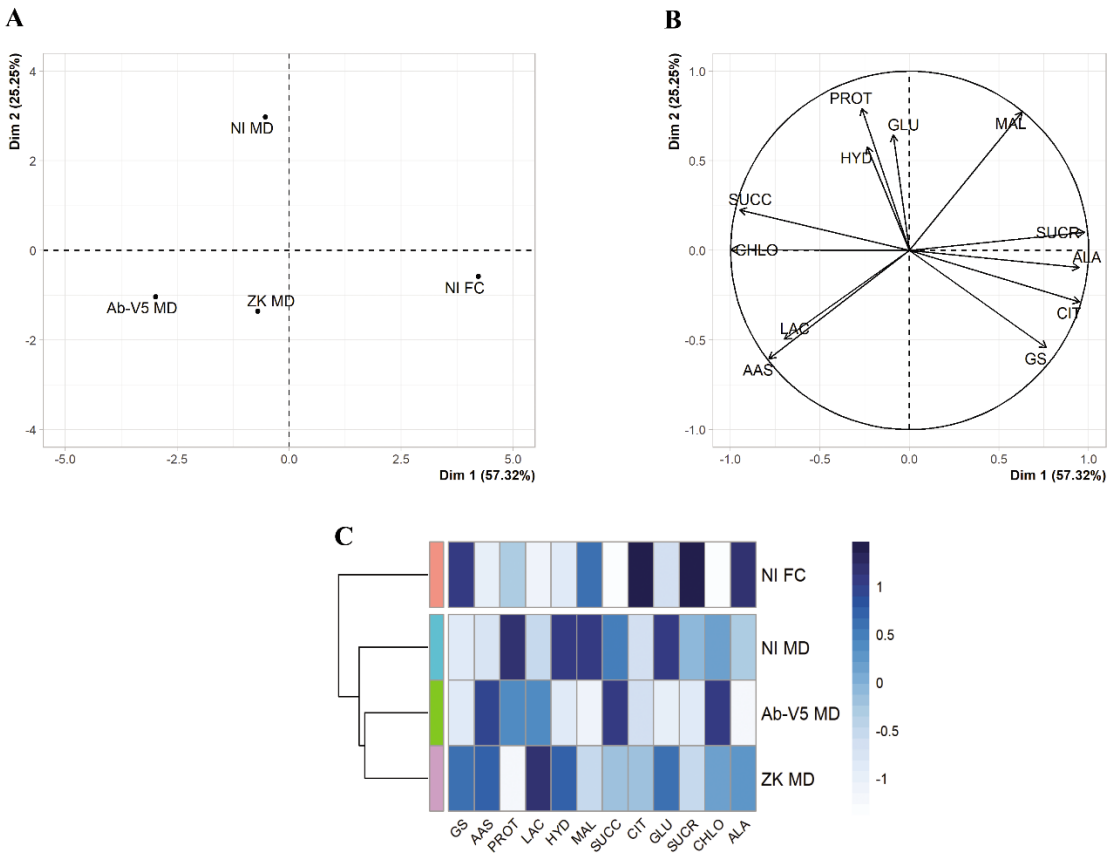

Fig. 1 Principal component analysis (PCA) and heat map of biochemical parameters of the leaves of *Cecropia pachystachya* seedlings. Treatments: NI FC - non-inoculated in field capacity. NI MD - non-inoculated under moderate drought. Ab-V5 MD - inoculated with *Azospirillum brasilense* under moderate drought. ZK MD - inoculated with *Bacillus velezensis* under moderate drought. A: Treatment diagram. B: Correlation between the biochemical parameters. C: Heat map analysis. Biochemical parameters: *GS* glutamine synthetase activity, *AAS* amino acids, *PROT* protein, *LAC* lactate, *HYD* hydroxybutyrate, *MAL* malate, *SUCC* succinate, *CIT* citrate, *GLU* glucose, *SUCR* sucrose, *CHLO* chlorogenic acid, *ALA* alanine.

Table 3. Biochemical parameters of *Cecropia pachystachya* leaves. The values are means ± SE (n = 4). The same letters indicate no difference according to Fisher's LSD test ($p < 0.05$). NI FC: non-inoculated in field capacity. NI MD: non-inoculated under moderate drought. Ab-V5 MD: inoculated with *Azospirillum brasilense* under moderate drought. ZK MD: inoculated with *Bacillus velezensis* under moderate drought. DW: leaf dry weight. GS: glutamine synthetase.

Biochemical parameters	NI FC	NI MD	Ab-V5 MD	ZK MD
GS activity (μmol $g^{-1}min^{-1}$ DW)	7.69 ± 0.59 a	4.23 ± 0.30 b	4.33 ± 0.30 b	6.95 ± 0.57 a
Amino acids (μmol g^{-1} DW)	6.64 ± 0.43 b	7.19 ± 0.89 b	11.01 ± 0.21 a	10.57 ± 0.22 a
Protein (mg g^{-1} DW)	3.01 ± 0.17 ab	3.65 ± 0.29 a	3.33 ± 0.25 ab	2.64 ± 0.18 b
Lactate (mmol μg^{-1} DW)	1.28 ± 0.11 c	1.45 ± 0.07 bc	1.75 ± 0.05 ab	1.98 ± 0.16 a
Alanine (mmol μg^{-1} DW)	2.66 ± 0.23 a	2.09 ± 0.16 ab	1.73 ± 0.09 b	2.29 ± 0.24 ab

In the PCA plot for *C. estrellensis* seedlings, PC1 and PC2 explained 61.01% and 21.71% of the data variance, respectively. Both PCA and heat map analysis clearly indicated that non-inoculated drought-stressed plants was the most isolated group, with a positive relation to protein, glucose, and lactate amounts. Strikingly, *A. brasilense* inoculation led to an approximation of drought-stressed plants with those in field capacity (Fig. 2 C). In contrast, the inoculation with *B. velezensis* positively influenced succinate, malate and quinic acid amounts of drought-stressed plants (Fig. 2)

In the case of *C. estrellensis* leaves, no significant differences were detected in GS activity and in lactate, malate, succinate, and sucrose amounts among the treatments (Table Supplementary 2). Moderate drought increased leaf total amino acids, protein, glucose, and reduced quinic acid contents in relation to non-inoculated plants in field capacity (Table 4). *B. velezensis* association prevented the drought-induced increase of protein content. Both PGPB

prevented the increase of glucose content induced by moderate drought, as well as *B. velezensis* association prevented the drought-induced reduction of quinic acid levels. Moreover, *A. brasilense* association reduced by almost 50% the hydroxybutyrate amount of *C. estrellensis* leaves in relation to non-inoculated plants under moderate drought.

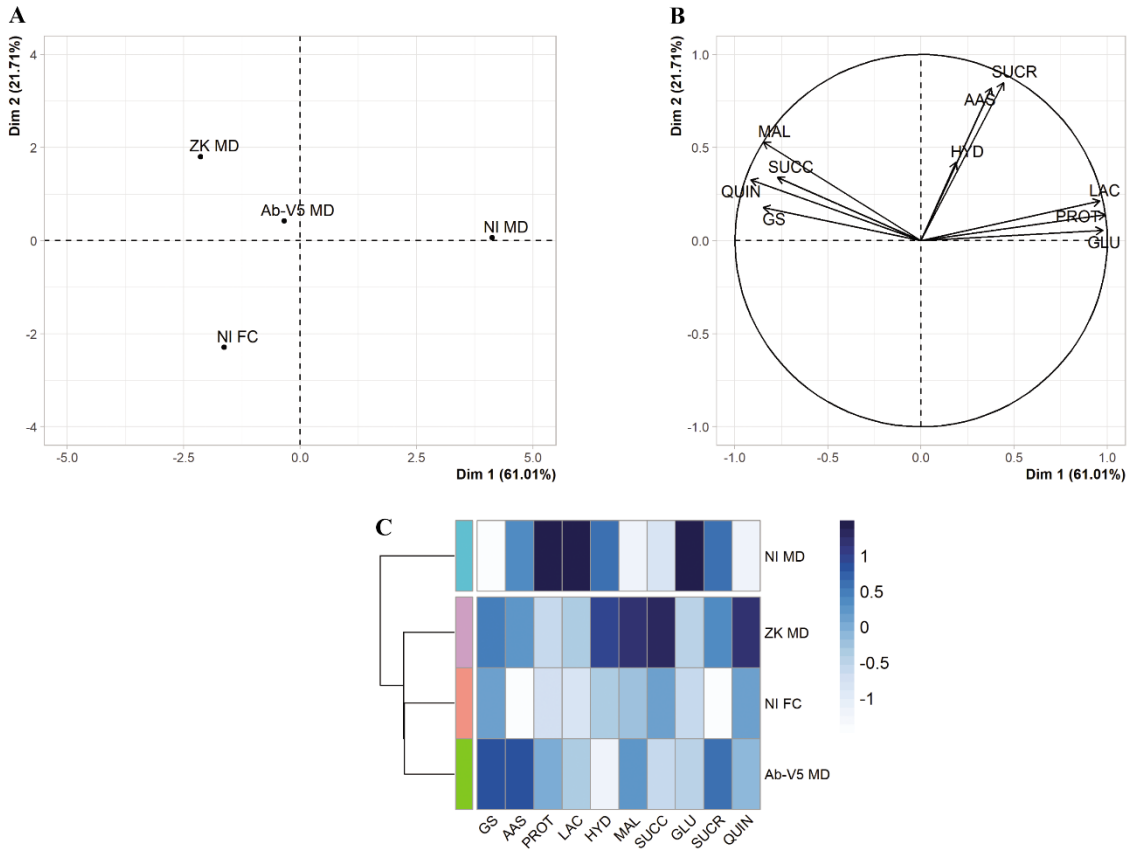

Fig. 2 Principal component analysis (PCA) and heat map of biochemical parameters of the leaves of *Cariniana estrellensis* seedlings. Treatments: NI FC - non-inoculated in field capacity. NI MD - non-inoculated under moderate drought. Ab-V5 MD - inoculated with *Azospirillum brasilense* under moderate drought. ZK MD - inoculated with *Bacillus velezensis* under moderate drought. A: Treatment diagram. B: Correlation between the biochemical parameters. C: Heat map analysis. Biochemical parameters: *GS* glutamine synthetase activity, *AAS* amino acids, *PROT* protein, *LAC* lactate, *HYD* hydroxybutyrate, *MAL* malate, *SUCC* succinate, *GLU* glucose, *SUCR* sucrose, *QUIN* quinic acid.

Table 4. Biochemical parameters of *Cariniana estrellensis* leaves. The values are means ± SE (n = 4). The same letters indicate no difference according to Fisher's LSD test ($p < 0.05$). NI FC: non-inoculated in field capacity. NI MD: non-inoculated under moderate drought. Ab-V5 MD: inoculated with *Azospirillum brasilense* under moderate drought. ZK MD: inoculated with *Bacillus velezensis* under moderate drought. DW: leaf dry weight.

Biochemical parameters	NI FC	NI MD	Ab-V5 MD	ZK MD
Amino acids (µmol g^{-1} DW)	3.79 ± 1.12 b	9.17 ± 0.23 a	10.27 ± 0.63 a	8.76 ± 0.84 a
Protein (mg g^{-1} DW)	3.51 ± 0.33 b	5.26 ± 0.35 a	4.09 ± 0.49 ab	3.63 ± 0.46 b
Glucose (mmol μg^{-1} DW)	61.38 ± 13.70 b	245.45 ± 12.76 a	74.66 ± 7.56 b	65.83 ± 16.65 b
Hydroxybutyrate (mmol μg^{-1} DW)	15.93 ± 1.20 ab	21.17 ± 1.51 a	11.62 ± 3.44 b	22.86 ± 3.56 a
Quinic acid (µmol μg^{-1} DW)	53.02 ± 4.65 ab	37.08 ± 5.45 c	49.30 ± 2.44 bc	65.10 ± 5.99 a

3.4 Multivariate analysis

To evaluate a general response in metabolism, a principal component analysis was made with the data from Tiepo *et al.,* (2020) and the data from the present study for both plant species. It was clear that the drought and PGPB treatments influenced on the plants' metabolism, leading to a clear separation of the groups (Fig. 3 A and C).

For *C. pachystachya* PC1 and PC2 explained 46.88% and 28.50% of the data variance, respectively. The four treatments were splitted in the PCA plot, indicating distinct influences on the plant metabolism according to the seedling growth condition (Fig. 3 A). The main parameters positively influenced by the PGPB treatment were succinate, ascorbate peroxidase activity, root:shoot ratio, root dry weight, amino acids, trigonelline, lactate and total dry weight. And the negative influence was observed in superoxide dismutase activity, malate, epicatechin, total phenolics, flavonoids, sucrose and glucose (Fig. 3 B).

Whereas for *C. estrellensis* PC1 and PC2 explained 41.60% and 39.66% of the data variance, respectively. In this case both PGPB treatments were plotted closer than the other treatments (Fig. 3 C). The main parameters influenced positively by PGPB were carboxylation efficiency, epicatechin, rutin, synapic acid, gallic acid, trigonelline, malate, chlorogenic acid, quinic acid and GS activity. And the negative influence was observed in superoxide dismutase activity, glucose, lactate, protein, leaf area, specific leaf area, leaf area ratio and root dry weight (Fig. 3 D).

Another fact clearly observed in the multivariate analysis (Fig. 3) was that the plant species had different comportments in relation to the PGPB treatments, in this sense, *C estrellensis* looks to respond similarly to both bacteria, whereas *C. pachystachya* had different metabolism responses for each bacteria inoculation.

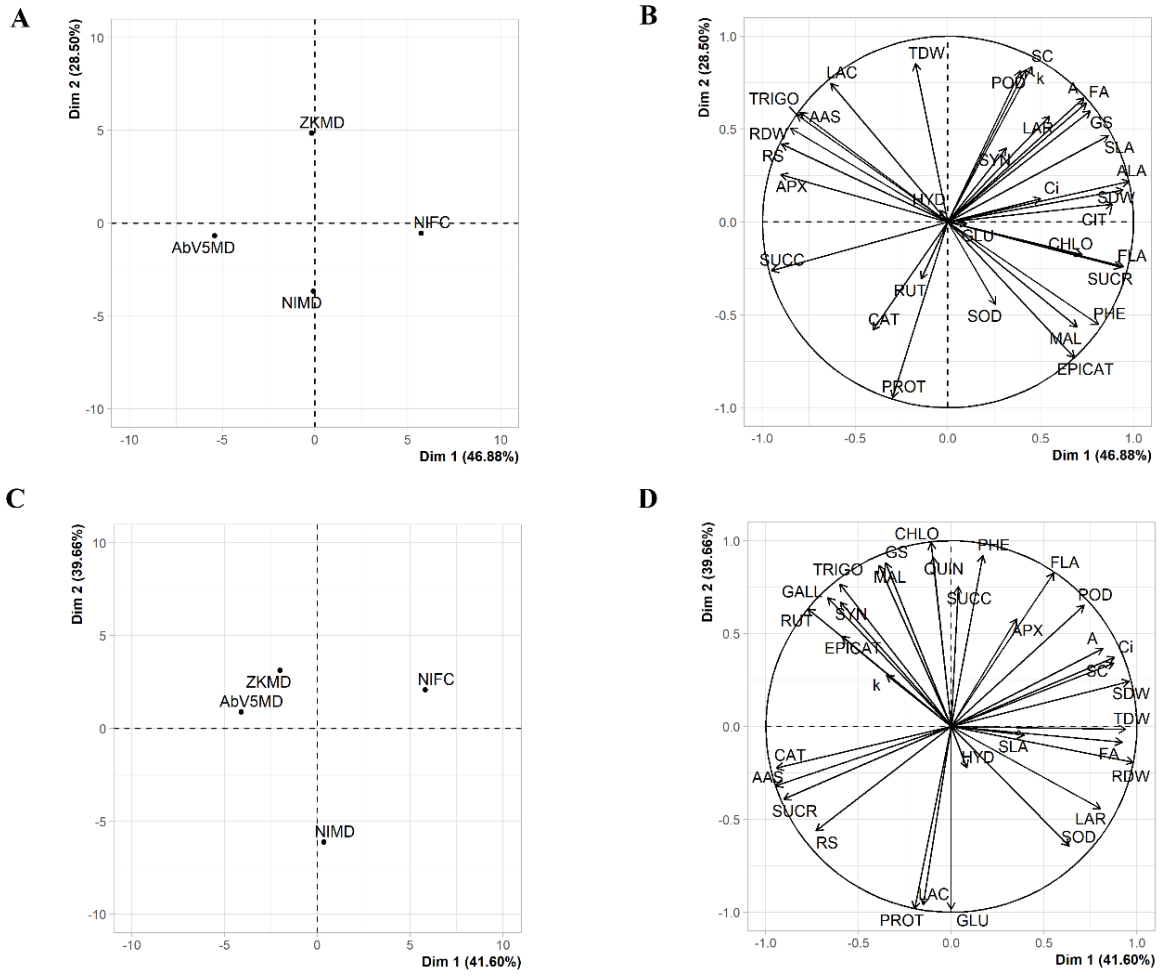

Fig. 3 Principal component analysis (PCA) of *Cecropia pachystachya* (A: Treatment diagram, B: Correlation between parameters) and *Cariniana estrellensis* (C: Treatment diagram, D: Correlation between parameters). Treatments: NI FC - non-inoculated in field capacity. NI MD - non-inoculated under moderate drought. Ab-V5 MD - inoculated with *Azospirillum brasilense* under moderate drought. ZK MD - inoculated with *Bacillus velezensis* under moderate drought. Parameters: *PHE* total phenolics, *FLA* flavonoids, *CHLO* chlorogenic acid, *QUIN* quinic acid, *SYN* synapic acid, *CAT* catechin, *EPICAT* epicatechin, *RUT* rutin, *TRIGO* trigonelline, *APX* ascorbate peroxidase activity, *SOD* superoxide dismutase activity, *POD* peroxidase activity, *MAL* malate, *SUCC* succinate, *CIT* citrate, *GLU* glucose,

SUCR sucrose, *GS* glutamine synthetase activity, *AAS* amino acids, *PROT* protein, *LAC* lactate, *HYD* hydroxybutyrate, *ALA* alanine, *RDW* root dry weight, *SDW* shoot dry weight, *TDW* total dry weight, *RS* root:shoot ratio, *SLA* specific leaf area, *LAR* leaf area ratio, *FA* leaf area, *A* net photosynthesis, *SC* stomatal conductance, *Ci* intercellular CO_2 concentration, *k* carboxylation efficiency.

4. Discussion

To cope with drought, plants need to develop or improve specifics tolerances mechanisms; so, the synthesis and amount of soluble sugars and Carbon allocation are closely related to physiological traits as respiration, photosynthesis and growth rates. In this sense, the improved leaf area in seedlings of *C. pachystachya* associated with *B. velezensis* may have influenced the observed enhanced in g_s and, consequently, *A* and the *k* rates. The C_i did not differed between treatments, indicating that CO_2 was directed to Rubisco carboxylation activity. The resulting compounds were probably used for the synthesis of molecules allocated in the growth because *C. pachystachya* seedlings associated with *B. velezensis* had increased root dry weight (Tiepo *et al.*, 2020) and also total dry weight (Table 2).

Bacillus was already characterized for exopolysaccharides production, which can lead to an increase in macroaggregates in soil, the increase in the C-rich polymers close to the roots surface could positively influenced the plant water status and stomatal conductance, protecting the plants from the negative effects of drought (Khan *et al.*, 2016, 2018). In *C. estrellensis* associated with *A. brasilense*, despite the lower leaf area and *Ci* (Table 1), an enhancement of *k* was observed in those seedlings, suggesting that the increased Rubisco carboxylation supported the synthesis of non-enzymatic antioxidant compounds related to drought tolerance as a trade-off trait (Tiepo *et al.*, 2020).

One of the drought tolerance mechanisms observed in several plant species is related to soluble sugar accumulation. Such mechanisms seem to be expressed in *C. estrellensis*, in which a positive influence of the moderate drought in soluble sugars was observed, especially in glucose (Fig. 2 and Table 4). Besides its role in cellular respiration pathway, soluble sugars are among the most frequent drought-responsive metabolites (Taïbi *et al.*, 2017; Merček *et al.*, 2019), and glucose could act as osmoprotectant and may be important to maintain more negative leaf osmotic potential (Charlton *et al.*, 2008).

Soluble sugar accumulation in drought-stressed Neotropical trees was already associated to starch degradation (Tiepo *et al.,* 2018), or may be due a reduction in the sugar transfer from mesophyll cells to phloem (Ashrafi *et al.,* 2018). In any case, glucose accumulation play an important role in the osmotic adjustment under drought, and the lower amounts of glucose in plants associated with PGPB under drought (Fig. 2 and Table 4) indicates a lower need for osmoregulation on the inoculated plants. In this sense, PGPB-inoculated plants, as observed for *C. estrellensis*, could direct the carbon fixed towards non-enzymatic antioxidant pathways as suggested by the multivariate analysis presented in the current study (Fig. 3 C and D), in agreement with previous reported by Tiepo *et al.,* (2020).

The endogenous balance between the levels of soluble sugars and the levels of organic acids from TCA cycle is altered in plant tissues under drought; and the variation of TCA cycle intermediates indicates a prominent role in drought tolerance (Ashrafi *et al.,* 2018; Khan *et. al.,* 2018). An evidence of a direct flux between soluble sugars and TCA cycle intermediates was already reported in *Cicer arietinum* L. (Khan *et al.,* 2019), such as observed in the present study for *C. pachystachya* and in *C. estrellensis* inoculated with PGPB (Fig. 1 C and Fig. 2 C).

In this sense, it was possible to observe that the drought treatment and PGPB inoculation influenced on different ways in the Neotropical seedlings' TCA cycle intermediaries (Fig. 1 and Fig. 2). For instance, synthesis pathways of the hydroxybutyrate and succinic acid are competitive, with the first being induced as a NADPH sink mechanism, contributing to the redox balance and to decrease ROS amount (Allan *et al.,* 2008; Allan *et al.,* 2009; Sweetlove *et al.,* 2010; Araújo *et al.,* 2011). Under moderate drought, *C. estrellensis* inoculated with *A. brasilense* showed hydroxybutyrate levels like those seedlings maintained in field capacity (Table 4 and Fig. 2), and the same trend was observed for *C. pachystachya* (Table S2 and Fig. 1), which indicated that this bacterium alleviated the drought stress in the Neotropical trees species.

The fluctuation of each TCA cycle intermediate may or not be correlated to other intermediates. It happens because the TCA cycle is related to other biochemical pathways, such as synthesis/degradation of amino acids, ammonium assimilation, purines metabolism, oxalate cycle and biosynthesis of secondary metabolites (Sweetlove *et al.,* 2010; Ashrafi *et al.,* 2018). The fluctuation on TCA cycle intermediates can be also observed in the malic acid, once the positive influence of succinic acid by *A. brasilense* did not directly influence the malic acid

amount in *C. pachystachya* (Fig. 1). In this sense, the positive influence on the succinic acid may be due the lack of reducing power (FAD) for the conversion of succinic acid to fumaric acid (Araújo *et al.* 2011). *B. velezensis* induced a different response in *C. pachystachya* (Fig, 1 and Table 3), reducing the amounts of organic acids in leaves except for the lactate, which was the highest between the treatments. This suggests that the pyruvate have been drained out from the TCA cycle as a reducing power sink to avoid oxidative damage (Bowne *et al.*, 2012).

Whereas the large amount of lactate was related to abiotic stress (drought, salinity and oxidative stresses) and its accumulation may be toxic, so it needs to be converted to pyruvate through lactate dehydrogenase activity and destined for the TCA cycle to generate metabolic energy (Xu *et al.*, 2016), this pathway may have been induced in *C. estrellensis* by PGPB, improving the oxidative stress tolerance (Jain *et al.*, 2018; Jain *et al.*, 2020), which is related to the enhanced antioxidant system reported in *C. estrellensis* associated with PGPB under drought (Tiepo *et al.*, 2020). In this sense, the reported results indicate that the PGPB improved the elimination of cytotoxic products in Neotropical trees under abiotic stress.

Other response related to the TCA cycle and photosynthesis was the positive influence on malate induced by *B. velezensis* in *C. estrellensis*, which suggests an important role in response to drought (Fig. 2 C), since this organic acid is related to pH balancing, to the osmotic adjustment in guard cells, to the leaf transpiration and also to the dissipation of the excess of NADPH (Charlton *et al.*, 2008; Marček *et al.*, 2019). In plant tissues, an important feature is the synergism among chloroplast/cytosol/mitochondria under stress to maintain cell redox status and avoid oxidative damage, so malate dehydrogenase NAD or NADP dependent are closely involved to it (Wang *et al.*, 2016).

Pathways, such as those related to malate shuttle between mitochondria and chloroplast, may prevent over reduction of photosynthetic electron transport components (Charlton *et al.*, 2008; Wang *et al.*, 2016). It occurs because a NADPH-dependent malate dehydrogenase acts on the dissipation of the NADPH through reduction of the oxaloacetate to malate in chloroplasts. Which improve the dissipation of the excess reductive power and reduces the synthesis of the reactive species of oxygen (ROS) in chloroplasts under drought, and consequently alleviated oxidative damage (Marček *et al.*, 2019; Zhao *et al.*, 2020). Besides, as reported by Tiepo *et al.* (2020) (Fig. 3 C e D), this PGPB induced an improved amount of non-enzymatic antioxidants in *C. estrellensis* seedlings, which seems to be a trait of a drought tolerance influenced by *B. velezensis* to avoid oxidative damage.

Besides being used in TCA cycle and growth, the fructose and glucose may be used still in the pentose phosphate pathway for synthesis of the erythrose 4-phosphate, which may be destined to shikimic acid pathway (Santos-Sánchez *et al.,* 2019). Therefore, as already observed by Tiepo *et al.* (2020) and on our multivariate analysis (Fig. 3), PGPB influenced the phenolic synthesis pathway, and, as shown by metabolomic in the present study, *C. estrellensis* has an expressive amount of quinic acid which was positively influenced by association with *B. velezensis* (Fig. 2 C and Table 4), and chlorogenic acid was positively influenced by *A. brasilense* in *C. pachystachya* (Fig. 1 C).

Importantly, chlorogenic acid is an ester of caffeic acid with the quinic acid; those compounds have already been related to an improved antioxidant defense and tolerance to abiotic stresses in *Vaccinium corymbosum* L. (Santos-Sánchez *et al.*, 2019), in *C. pachystachya* and in *C. estrellensis* associated with PGPB (Fig. 3) (Tiepo *et al.,* 2020). Similarly to the results observed in the present study, in which the association with PGPB positively influenced secondary compounds synthesis. Those compounds synthesis have important role in defense mechanisms, once these molecules are necessary for the adaptation of plants to the changes in the environment (Mierziak *et al.*, 2014; Santos-Sánchez *et al.,* 2019; Tiepo *et al.*, 2020).

Through metabolomic analysis it was also possible to found chlorogenic acid, luteolin and apigenin derivatives in *C. pachystachya* (Fig. S1 and Fig. S2), while in *C. estrellensis* kaempferol, p-coumaric and quercetin were found (Tiepo *et al.*, 2020b, no prelo). In this sense, the metabolomic made clear that each Neotropical tree has its own specific composition of metabolites and, importantly, the main difference between the species is on the secondary metabolites, which is an expression of the individuality of species (Santos-Sánchez *et al.*, 2019; Tiepo *et al.*, 2020).

Internal N concentration (amino acids and proteins) and activity of an enzyme (GS) involved on the N assimilation were also influenced by PGPB association and drought treatment in both plant species. The expression and activity of the enzyme are dependent of the substrate and flux of inorganic N into organic compounds (Meng *et al.,* 2016), and these responses can be seen on the induction of the GS activity by PGPB inoculation (Table 3, Fig. 1 C and Fig. 2 C), especially in *C. pachystachya*, the enhancement of GS activity induced by *B. velezensis* was accompanied by an increment on the amino acids amount.

The amino acids amount may be also enhanced by the protein breakdown induced by stressing conditions (Hildebrandt *et al.*, 2015), as observed there was a reduction in protein

content in *C. pachystachya* and in *C. estrellensis* seedlings inoculated with *B. velezensis* (Table 3 and 4) and a tendency of a reduction in the inoculation with *A. brasilense*, in both cases this reduction was accompanied by an increase in amino acids amount. Beyond the role of amino acids in protein constitution, they influence some physiological traits of plants, such as plant growth, intracellular pH control, generation of redox power and resistance to stresses (Hildebrandt *et al.*, 2015). Protein breakdown and amino acids catabolism may be important in stress tolerance, once the degradation of amino acids can provide an energetic connection between carbon and nitrogen metabolism, which provide the necessary energy in certain organs allowing plants to cope with stress conditions (Hildebrandt *et al.*, 2015), which in the present study it was clearly improved by PGPB inoculation.

Another important fact is that the amino acids catabolism usually is a response from a carbohydrate limitation (Hildebrandt *et al.*, 2015). In the present study, the lower carbon fixation in *C. estrellensis* seedlings under drought, and the protein breakdown coupled with amino acids catabolism may be seen as a response to maintain the respiration during drought stress, which was clearly induced by *B. velezensis* inoculation in *C. estrellensis* seedlings. In this case, less carbon skeletons were destined to growth, as a trade-off trace of drought tolerance induced by PGPB (Table 2 and biometrical data from Tiepo *et al.*, 2020).

Under stress conditions plants may also improve some amino acids biosynthesis with specific beneficial function in stress tolerance (Hildebrandt 2018). This pathway, in the light, is strongly dependent on the remobilization of TCA carbon skeleton (Sweetlove *et al.*, 2010); which may have negatively influenced the organic acids content in *C. pachystachya* associated with PGPB (Fig. 1 C). Therefore, the increased accumulation of amino acids was already reported in a drought tolerant cultivar of *Cicer arietinum* L.; in this way, the increased amount of some amino acids is considered a drought tolerance trait through enhancement in osmotic adjustments and antioxidant metabolism; which results in a negative influence in the protein and TCA cycle intermediaries (Fig. 1). (Khan *et al.*, 2018).

Other kinds of amino acids may be related to the energy generation, also in *C. pachystachya* it was observed less alanine in plants associated with *A. brasilense*, the result may indicate an improved catabolism of this amino acid to maintain the metabolic energy production, once the complete oxidation of the Alanine generate 4 NADH, 1 $FADH_2$ and 12,5 ATPs in TCA cycle (Hildebrandt *et al.*, 2015).

Another fact reported in *Arabidopsis* it was that a tolerance response to drought stress favors the destination of the amino acids to the secondary metabolites and alkaloids synthesis (Hildebrandt 2018). The same pattern was observed in the *C. pachystachya* and in *C. estrellensis* seedlings, and the inoculation with PGPB improved this response. As observed in the present study, the amino acids amount was positively influenced by PGPB association, and as reported by Tiepo *et al.* (2020), these treatments enhanced the secondary metabolites synthesis and the amount of Trigonelline (alkaloid) (Fig. 3). The higher amount of secondary compounds was related to drought tolerance, once phenolic compounds have an important role in avoid drought-induced oxidative damage (Agati *et al.,* 2012; Gómez-Caravaca *et al.,* 2014) and the Trigonelline was already related to the synthesis of osmoprotectant compounds, once it can has a similar role that the glycinebetaine (Charlton *et al.,* 2008; Ashihara 2008). In this sense, amino acids are tightly linked to the Carbon and Nitrogen cycle on drought tolerance, once some of them may be used in energy generation through TCA cycle intermediary's synthesis and other kind of them may be used in osmoregulation (Hildebrandt *et al.,* 2015; Hildebrandt 2018).

When evaluating all the plants' parameters through the multivariate analysis it was clear that the drought treatment and PGPB association influence plants species in different ways, which generated distinct group patterns (Fig. 3). Our results indicate that drought and PGPB inoculation had influence in carbon and nitrogen biochemical pathways in cyclic and non-cyclic flux modes on the Neotropical trees used in the present study. Similarly, in *C. pachystachya* and in *C. estrellensis* the groups' position showed that the association with PGPB induced responses that make the groups been located on the opposite to the non-inoculated drought treatment, which is a strong indication that *A. brasilense* and *B. velezensis* led to changes in the drought tolerance metabolism in the Neotropical tress used in the present study.

In the Neotropical trees the PGPB association induced responses in primary and in secondary metabolism, and also in the antioxidant defense mechanisms; generating consequences in biometrical parameters and improving drought tolerance (Fig. 3) (Tiepo *et al.,* 2020). Finally, the present study showed that there is not a single mechanism involved in the tolerance promoted by PGPB, but an interaction of several pathways in each specific case of inoculation which cause a final effect of ample spectrum in the plant (Fig. S3). In this sense, the association with PGPB appears as an important biotechnological tool to improve drought

tolerance in Neotropical trees and consequently enhance the forest restoration programs success.

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Author Contribution

All authors conceived and designed the experiments. ANT, IDC, GOM, AKC, MFH performed the experiments. ANT, IDC and GOM analyzed the metabolomic data. ANT drafted the manuscript. All authors revised and approved the manuscript.

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Supporting Information

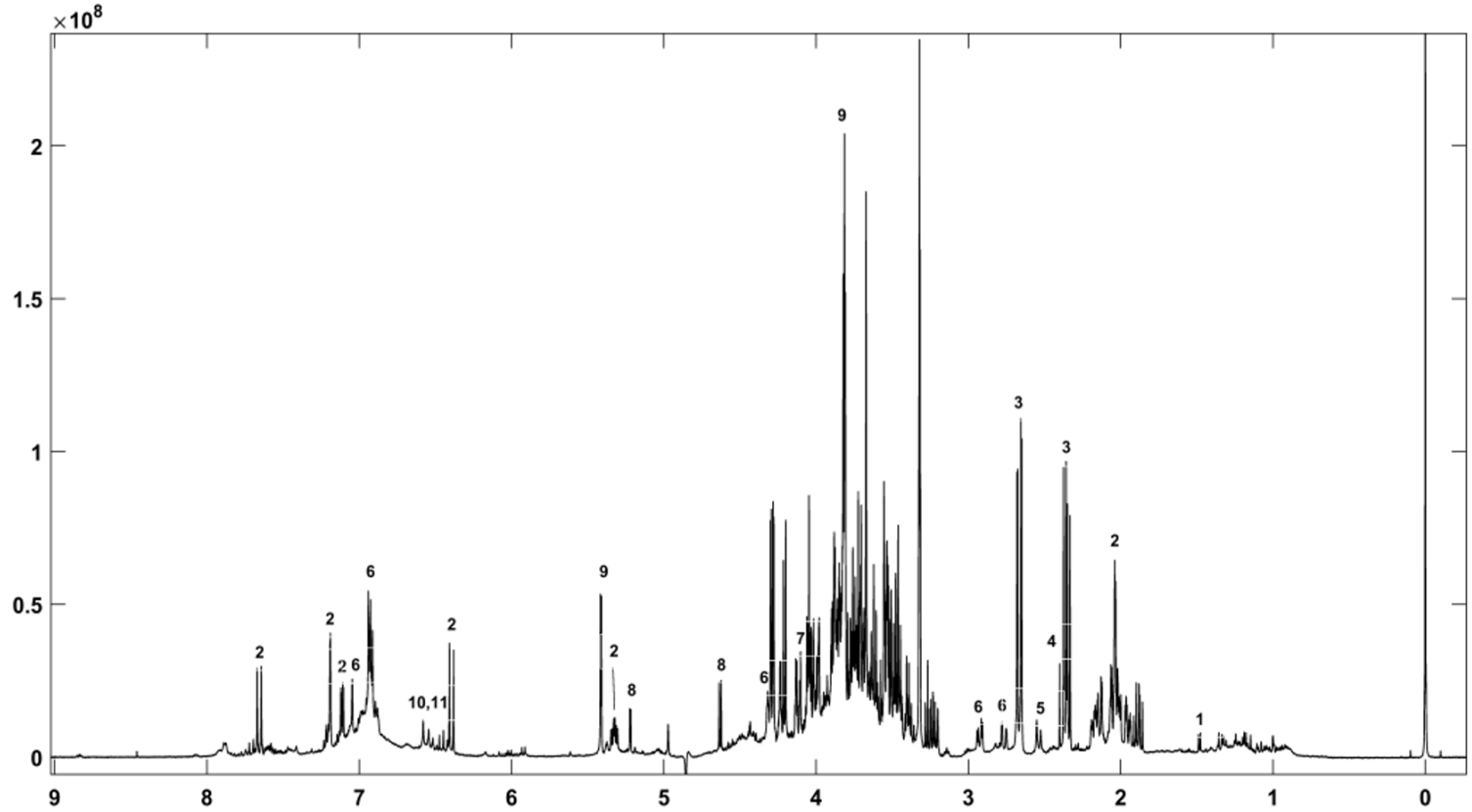

Fig. S1 [1]H NMR spectra from *Cecropia pachystachya* leaf hydroalcoholic extract. 1: Alanine; 2: Chlorogenic acid; 3: Malate; 4: Succinate; 5: Citrate; 6: Catechin; 7: Fructose; 8: Glucose; 9: Sucrose; 10: Luteonin 6-C-glycoside; 11: Luteonin 8-C-glycoside.

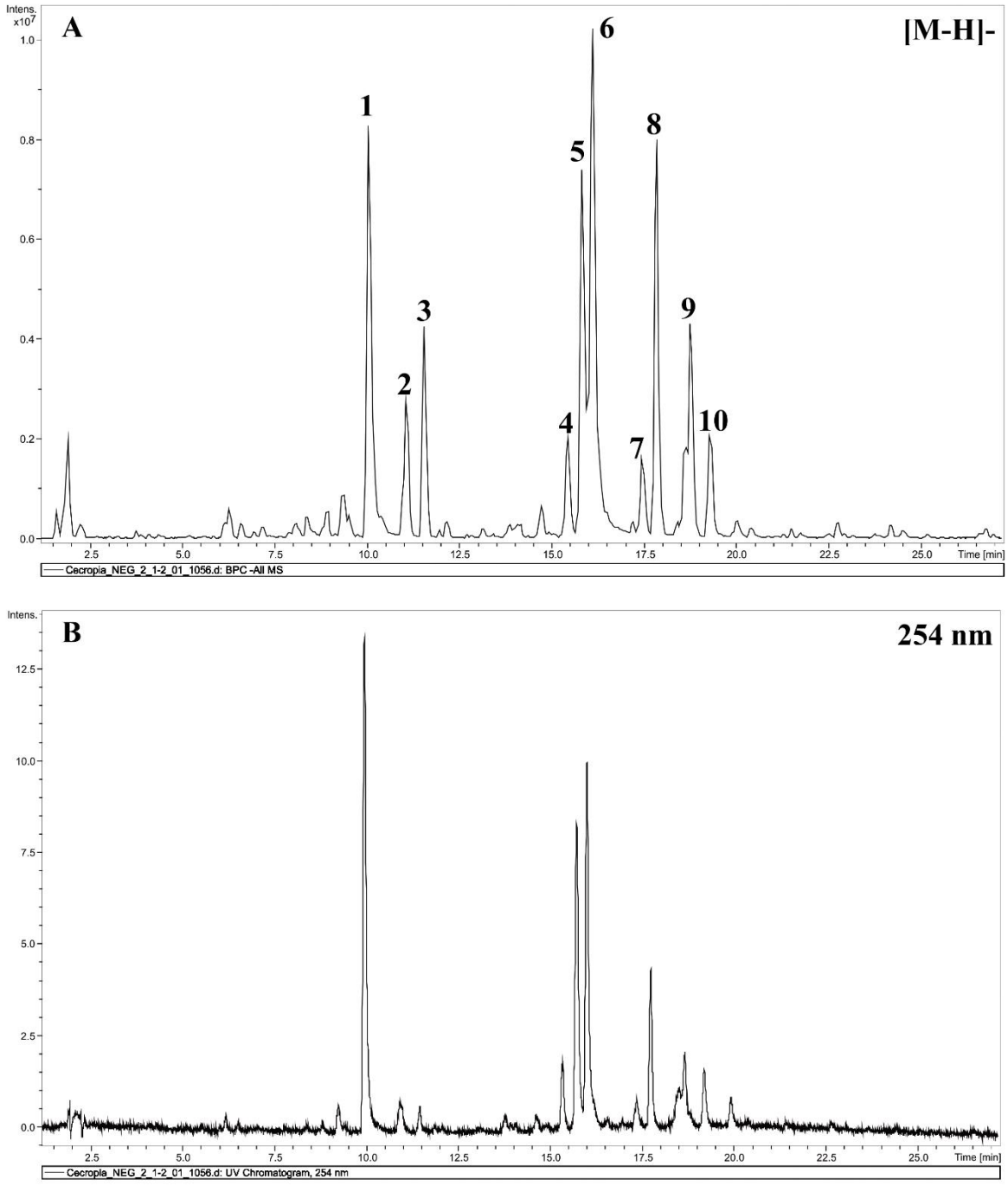

Fig. S2 LC-DAD-UV chromatogram from *Cecropia pachystachya* leaf hydroalcoholic extract. A: Base peak chromatogram (-). B: UV chromatogram at 245 nm. 1: Chlorogenic acid; 2: not identified; 3: Catechin; 4: not identified; 5: Luteolin 6-C-glycoside; 6: Luteolin 8-C-glycoside; 7: not identified; 8: Apigenin-glycosyl-arabinoside; 9: Apigenin 6-C-glycoside; 10: not identified.

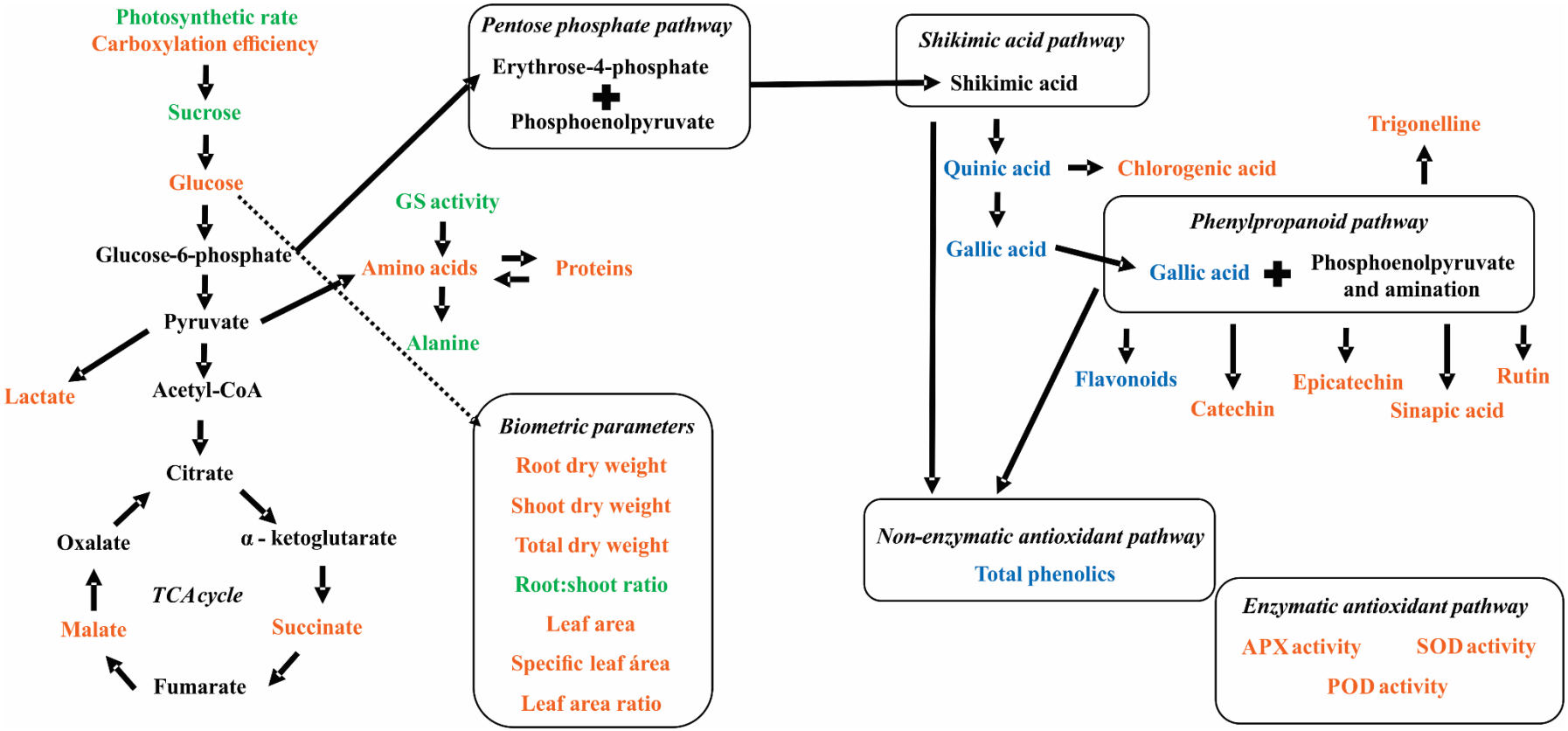

Fig. S3 Biochemical pathways of drought tolerance mediated by PGPB in *Cecropia pachystachya* (green words) or in *Cariniana estrellensis* (blue words). Compounds in orange were influenced by PGPB in both plant species. Compounds in black were not influenced or measured. The arrows indicate the destination of compounds among the pathways.

Table S1. Biochemical parameters in *Cecropia pachystachya*. The values are means ± SE (n = 4). The data were not different according ANOVA. NI FC: non-inoculated in field capacity. NI MD: non-inoculated under moderate drought. Ab-V5 MD: inoculated with *Azospirillum brasilense* under moderate drought. ZK MD: inoculated with *Bacillus velezensis* under moderate drought. DW: leaf dry weight.

Biochemical parameters	NI FC	NI MD	Ab-V5 MD	ZK MD
Hydroxybutyrate (mmol ug^{-1} DW)	0.44 ± 0.08	0.61 ± 0.04	0.44 ± 0.035	0.58 ± 0.08
Sucrose (mmol μg^{-1} DW)	67.82 ± 2.88	60.03 ± 7.72	55.85 ± 3.07	57.48 ± 3.43
Glucose (mmol μg^{-1} DW)	45.11 ± 9.99	58.67 ± 4.42	42.53 ± 11.59	54.80 ± 4.63
Citrate (mmol ug^{-1} DW)	11.08 ± 2.92	5.02 ± 1.02	4.87 ± 0.79	6.27 ± 0.37
Succinate (mmol ug^{-1} DW)	2.21 ± 0.21	2.75 ± 0.09	2.92 ± 0.27	2.53 ± 0.50
Malate (mmol ug^{-1} DW)	128.04 ± 21.51	139.06 ± 22.51	85.37 ± 9.62	99.82 ± 9.84
Chlorogenic acid (mmol μg^{-1} DW)	25.20 ± 4.49	28.63 ± 2.87	30.47 ± 3.36	28.67 ± 7.43

Table S2. Biochemical parameters in *Cariniana estrellensis*. The values are means ± SE (n = 4). The data were not different according ANOVA. NI FC: non-inoculated in field capacity. NI MD: non-inoculated under moderate drought. Ab-V5 MD: inoculated with *Azospirillum brasilense* under moderate drought. ZK MD: inoculated with *Bacillus velezensis* under moderate drought. DW: leaf dry weight.

Biochemical parameters	NI FC	NI MD	Ab-V5 MD	ZK MD
GS activity (μmol $g^{-1}min^{-1}$ DW)	83.35 ± 10.98	92.93 ± 10.78	135.15 ± 17.85	99.05 ± 17.20
Lactate (mmol ug^{-1} DW)	2.23 ± 0.13	3.16 ± 0.24	2.41 ± 0.09	2.40 ± 0.36
Sucrose (mmol μg^{-1} DW)	256.37 ± 16.70	308.65 ± 39.85	309.37 ± 15.41	303.40 ± 22.93
Succinate (mmol ug^{-1} DW)	2.26 ± 0.27	2.07 ± 0.43	2.12 ± 0.16	2.47 ± 0.24
Malate (mmol ug^{-1} DW)	24.29 ± 2.99	20.67 ± 6.00	25.88 ± 2.74	29.57 ± 4.85

CAPÍTULO 3

Phenolic compounds from leaves of *Cariniana estrellensis* (Raddi) Kuntze (Lecythidaceae): a Brazilian Atlantic Forest tree

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Phenolic compounds from leaves of *Cariniana estrellensis* (Raddi) Kuntze (Lecythidaceae): a Brazilian Atlantic Forest tree

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Abbreviations:

NMR: Nuclear Magnetic Ressonance

Abstract

Cariniana estrellensis seedlings have been used in forest restoration programs in the Atlantic Forest biome, and despite its economic and ecological relevance, it was never reported a general method for rapid identification of primary and secondary metabolites of *C. estrellensis* leaves. In this work, we explore the feasibility of using rapid analysis by 1D and 2D nuclear magnetic resonance and liquid chromatography coupled to diode array and mass detector techniques. The main secondary metabolites identified were quinic acid, hydroxycinnamic acids (*trans p*-coumaric acid, *cis p*-coumaric acid, and *trans* ferulic acid) and flavonoids (kaempferol-di-glucoside and quercetin glucosyl-rhamnoside). These compounds are particularly useful as chemotaxonomic markers for the genus *Cariniana* and for the family Lecythidaceae, and may be also important for pharmacological uses and for the seedlings' survival in reforestation programs.

Key-words: Flavonoids; Kaempferol; Phenolic compounds; Quercetin; Quinic acid, Reforestation

1. Introduction

The Brazilian Atlantic Forest is one of the 25 global biodiversity *hotspots* and comprises the second biggest tropical rain forest from the american continent. In the last decade, deforestation rates have been increasing in brazilian forest, which negatively affects the environmental homeostasis (Myers et al., 2000) and boosts the rates of local species extinction (Albino et al., 2019). These aspects enhance the need of restoration in these sites, once it is essential for the maintenance of the flora and fauna biodiversity.

Lecythidaceae family is spread in Neotropical sites, and it includes the genus *Cariniana* Casar (Ribeiro et al., 2020), which comprises nine species (Roskov et al., 2019). Non-pioneer trees, such as *Cariniana*, have a special role on epiphytes survival, carbon sequestration and accumulation, and on the input of soil organic matter by root exudation and litterfall (Soares et al., 2020; Shimamoto et al., 2014; Reis and Fontoura 2009; Hobbie et al., 2007).

The wood of *Cariniana* is widely used for manufacturing of furniture, casks for cachaça ageing process (Souza et al, 2014; Silva et al, 2012) and bark for folk medicine. Infusion of *Cariniana decandra* Ducke 1925 bark is recommended for cancer, high fever (Jovel, Cabanillas & Towers, 1996) and skin infections (Roumy et al, 2020). Bark from *Cariniana rubra* Gardner ex Miers 1874 is recommended for kidneys (Bieski et al, 2015), while maceration and decoction of *Cariniana estrellensis* (Raddi) Kuntze 1898 is used for general infection, depurative and ulcer (Ribeiro et al, 2017). *Cariniana brasiliensis* Casar presented, in vitro, 90% of mushroom´s tyrosinase inhibition (Baurin, 2002) and antifungal functions were reported for bark from *C. rubra* (Lima Neto et al., 2015).

Moreover, non-pioneer trees used in Brazilian reforestation programs, such as *C. estrellensis*, contribute to high species richness which may allow biodiversity conservation (Tiepo et al., 2020; Tiepo et al., 2018; Shimamoto et al., 2014; Reis and Fontoura 2009). Also, the presence of *C. estrellensis* in Atlantic Forest is essential for the maintenance of the environmental homeostasis, once its flowers are pollinated by bees and its seeds are taken up by fauna species, such as monkeys (Guidugli et al., 2009; Leite, 2007; Oliveira-filho and Galetti, 1996). In addition, the use of native trees in reforestation programs can supply the demand for native species wood, reducing predatory exploration, once *C. estrellensis* wood is intensively explored by industry (Silva et al., 2012), and increase the economic potential

through the use of phytochemical as bioactive compounds to the cosmetic, pharmaceutical and food industry.

Although traditional use of *Cariniana* species is described, there are few reports about the chemical composition of the leaves and bark of species of this genus. In *Cariniana domestica* (Mart.) Miers bark extracts lupeol, amyrin, sitosterol, stigmasterol, gallic acid, chlorogenic acid, rutin, quercetin, and kaempferol were identified (Janovic et al., 2012 a, Janovic et al., 2012 b). For *C. estrellensis*, it was already reported that the plant growth-promoting bacteria association induced an increase of chlorogenic acid, gallic acid, rutin and synapic acid in leaves of drought-stressed seedlings. (Tiepo et al., 2020).

Despite the economic and ecological relevance, and ethnobotanical studies of *Cariniana* species, it was never reported a general method and a study for rapid identification of primary and secondary metabolites of *C. estrellensis* leaves for metabolomic studies. A prerequisite for chemotaxonomy, ecological and metabolomic studies is to know the qualitative and quantitative composition as well the occurrence of metabolite groups in the evaluated plant species. Proton experiment is a most useful nuclear magnetic resonance (NMR) experiment in metabolomic analysis due its important structural and quantitative information. NMR and chromatography methods combined with mass spectrometry and ultraviolet spectroscopy are the most important high-throughput analytical techniques for metabolomics screening.

Thus, we applied 1D and 2D nuclear magnetic resonance and liquid chromatography coupled to diode array and mass detector for a rapid metabolic fingerprinting of *C. estrellensis* hydroalcoholic leaf extract to support future studies in ecology and metabolomic.

2. Results and Discussion

Glucose, lactate, malic acid, succinic acid, citric acid and hydroxybutyric acid were assigned by Chenomx NMR suite (Fig. 1), while secondary metabolites were identified by arrangement of the spectroscopic and spectrometric data such as chemical shift and coupling constant by ^{1}H NMR and retention time, diode array detection, mass spectrum (negative and positive mode) and MS/MS provide by LC-DAD-MS/MS. This strategy provided the putative annotation of twenty-two metabolites from *C. estrellensis* leaf. Of these, nineteen secondary metabolites had the structure confirmed at least two analytical tools as ^{1}H NMR, MS, MS/MS and UV data.

The main classes of secondary metabolites identified in the hydroalcoholic extract were cyclic polyol, hydroxycinnamic acid, and flavonoid (Table 1, Fig. 1 and Fig. S1). Quinic acid was identified by the multiplet δ 1.90 m in the ^{1}H NMR. The hydroxycinnamic acid signals were detected in the region between 5.60 – 7.70 ppm by the coupling of olefinic hydrogens with *Z* configuration (*J* = 16 Hz) and *E* configuration (*J* = 12 Hz), which were confirmed in the COSY ^{1}H-^{1}H spectra. Then, the main hydroxycinnamic acids identified were *trans p*-coumaric acid (δ 7.72 d, 15.99 Hz; δ 6.46 d, 16.04 Hz), *cis p*-coumaric acid (δ 5.97 d, 12.54 Hz; δ 7.03 d, 12.91 Hz), and *trans* ferulic acid (δ 6.48 d, 16.03 Hz; δ 7.70 d, 15.94 Hz).

Kaempferol-di-*O*-glucoside was identified mainly by the doublet in 8.05 ppm due to the coupling of H-3',4' and the overlapping doublets δ 6.99 referring to H-2',6', confirming the presence of an AA'BB', and the signals δ 6.92 and 7.02 corresponding to H-6 and H-8, which confirmed the flavonoid unit. Through the analysis by LC-DAD-MS/MS, it was possible to identify that the kaempferol-di-*O*-glucoside eluted in 18.1 minutes, with the spectrum on the UV λ_{max} 265/346 nm and *m/z* 611 $[M+H]^{+}$. MS/MS spectrum from the ion product showed ion fragments *m/z* 287 in the positive mode, characteristic of the O-flavonol pattern due to base peak corresponding to the aglycone product ion. The mass loss of 324 corresponds to two glucose units, which confirms the presence of two sugar moieties connected to kaempferol. The search for *m/z* 287 fragments in the extracted ion chromatogram allowed the identification of five kaempferol derivatives; four from which were not detected in the ^{1}H NMR spectra, but could be assigned by MS, MS/MS, and UV spectra and are described in Table 1 and in the Fig. S1 B.

Quercetin-*O*-glucosyl-rhamnoside was identified in the ^{1}H NMR spectrum with signals characteristic of a tri-substituted system in ring C due to the double doublets δ 7.20 (8.62 Hz and 1.96 Hz), doublets δ 6.88 (8.63 Hz), and δ 7.32 (1.80 Hz), which correspond to H-6', 5' and 2 ', respectively. The H-6 and H-8 were detected as broad singlet at 6.94 and 7.07 ppm. The glycosidic unit assignment was also performed according to the LC-DAD-MS/MS, in which the quercetin glucosyl-rhamnoside eluted in 16.3 minutes, with UV spectrum λ_{max} 256/354 nm and *m/z* 627 $[M+H]^{+}$. MS/MS spectrum showed ion fragments of *m/z* 303 in the positive mode, which corresponds to the aglycone unit and the mass loss of 324 corresponds to two glucose units, thus confirming the presence of a di-glycosylated quercetin derivative. The search for *m/z* 303 fragments in the extracted ion chromatogram identified five glycosylated derivatives of quercetin in low concentration, which were not detected in the ^{1}H NMR spectra, but are also described in Table 1 and in Fig. S1 C. A set of multiplets 2.60, 2.70, 2.95, and 6.45

ppm could not be identified in the ^{1}H NMR spectrum. Three peaks detected in the total ion chromatogram could be not identified and were therefore described as unknown compounds. (Table 1 and Fig. 1).

Phytochemical investigations have been reported for species from the family Lecythidaceae, which indicated the presence of polyphenols, such as flavonoids (naringin, rutin, luteolin and kaempferol derivatives) and cinnamic acid derivatives (gallic acid, ferulic acid), alkaloids, and triterpenes in leaves (Silva et al., 2017; Hussin et al., 2009; Ferreira et al., 2014). In *C. estrellensis* the presence of trigonelline, chlorogenic acid, gallic acid, rutin, sinapic acid, epicatechin and catechin was recently reported in drought-stressed seedlings (Tiepo et al., 2020).

Secondary compounds have been considered a reliable taxonomic indicator for plants, and it is an important tool used in taxonomic elucidation (Nguyen et al., 2014). Similar compounds to those identified in the present study have been already found in other genera from the Lecythidaceae family. For example, gallic acid, ferulic acid, and kaempferol were detected in leaves of *Barringtonia racemosa* (L.) Spreng., whereas kaempferol 3-*O*-glucoside, quercetin 3-*O*-glucoside, kaempferol 3-*O*-rutinoside, and quercetin 3-*O*-rutinoside were found in leaves of *Barringtonia asiatica* (L.) Kurz (Hussin et al., 2009; Iwashina and Kokubugata, 2016) and quercetin-3-*O*-rutinoside and kaempferol-3-*O*-rutinoside were identified in *Lecythis pisonis* leaves (Ferreira et al., 2014).

Based on the above-mentioned metabolites, *O*-glycoside flavonols are chemotaxonomic markers of the Lecythidaceae family. Phenolic compounds stand out because they are widely distributed and have many ecological and pharmacological functions (Lima Neto et al., 2015). The metabolites identified in the present study may have an important role for the plant survival in reforestation programs, once most of them are polyphenols that are involved in tolerance to environmental stresses (Mierziak et al., 2014). Many phenolic compounds have antioxidant properties and can be used to prevent free-radical-induced deleterious effects, as reported in leaf extracts from *Rosa canina* L., *Rosa rubiginosa* L. and *Alchemilla mollis* (Buser) Rothm (Sytar et al., 2018). Ferulic acid, *p*-coumaric acid, and kaempferol, that were also found in the present study, were related to antioxidant activity in *Origanum vulgare* L., *Lavandula angustifolia* Mill (1768) and *Melissa officinalis* L. (Spiridon et al., 2011).

Our results show that leaves of *C. estrellensis* are rich in phenolic compounds with antioxidant properties already described in literature as well as a source of new compounds still

not explored. Besides that, the present study allowed us to know better the leaves phytochemical profile from *C. estrellensis*; which is important as a chemotaxonomy trait, ecological and metabolomic studies. Besides, the metabolite screening indicates the presence of antioxidant compounds that were not reported before for *C. estrellensis* and may have importance in pharmacological uses and in plant survival.

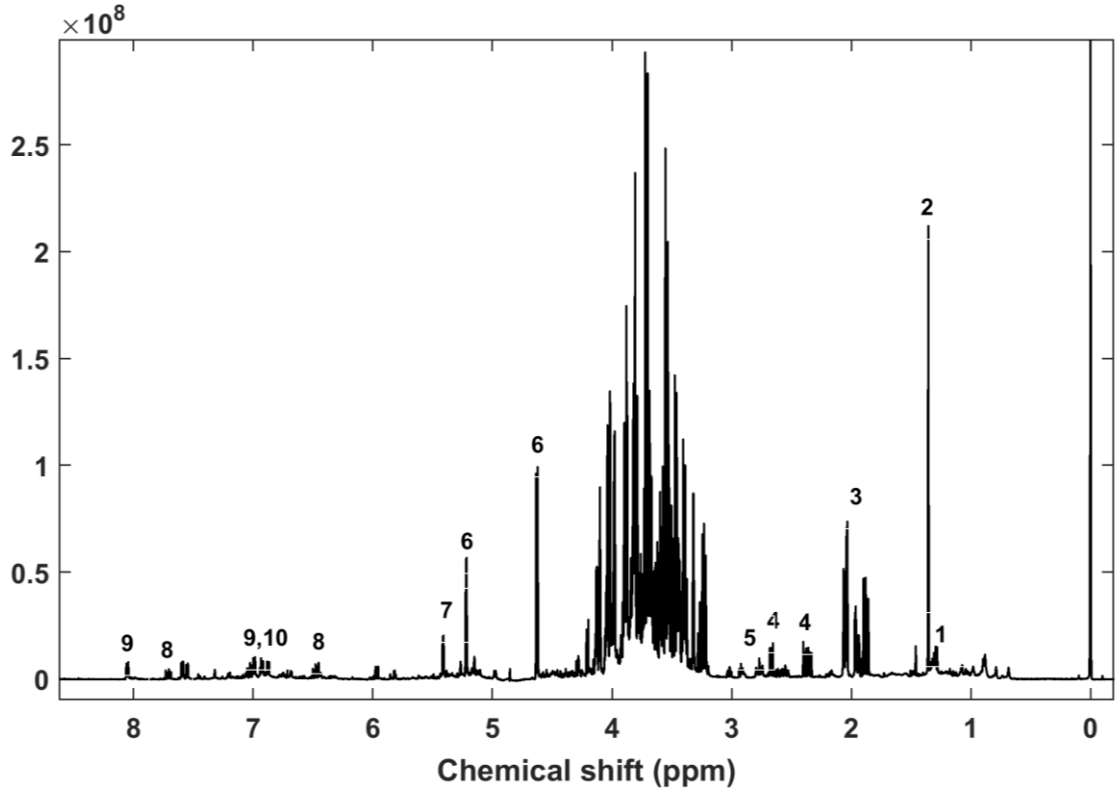

Fig. 1 [1]H NMR spectra from *Cariniana estrellensis* leaf hydroalcoholic extract. 1: Lactate; 2: Hydroxybutyrate; 3: Quinic acid; 4: Malic acid; 5 and 6: unknown compounds; 7: Glucose; 8: Hydroxycinnamic acids; 9: Kaempferol-*O*-di-glucoside; 10: Quercetin-*O*-di-glucoside.

Table 1: Metabolites identified by LC-DAD-MS/MS in the *Cariniana estrellensis* leaf extract.

RT (min)	Metabolite	[M-H]	[M+H]	UV
2.0	Quinic acid **NMR**	191.20	-	-
15.2	*Cis*-p-coumaric acid **NMR**	163.11	-	-
15.4	*Trans*-p-coumaric acid **NMR**	163.11	-	-
16.3	Quercetin di-glucoside **NMR** (**Q1)**	625.36	627.13 (303; 465)	268/353
16.9	*Cis*-ferulic acid	193.12	-	-
17.2	*Trans*-ferulic acid **NMR**	193.14	-	-
17.8	Not identified (unknown)	615.73 (473)	617.07 (315)	-
18.1	Not identified (unknown)	615.30 (491)	617.10 (493)	-
18.3	Quercetin glucoside-rhamnoside 1(**Q2)**	609.34	611.12 (303; 465)	268/354
18.6	Kaempferol di-glucoside**NMR (Ka1)**	609.37	611.13 (287)	264/348
18.7	Quercetin glucoside-rhamnoside 2 (**Q3)**	609.4	611.13 (303; 465)	-
18.9	Quercetin-glucoside isomer 1 (**Q4)**	463.25 (301)	465.08 (303)	264/354
19.3	Quercetin glucoside isomer 2 (**Q5)**	463.27	465.08 (303)	263/353
20.0	Kaempferol glucoside-rhamnoside isomer 1 **(Ka2)**	593.37	595.14 (287)	264/347
20.8	Kaempferol di-arabinoside (**Ka3)**	449.24	551.06	265/348
20.9	Quercetin arabinoside (**Q6)**	433.24	435 (303)	262/355
21.7	Kaempferol-glucoside (**Ka4)**	447.27	449.08 (287)	265/347
21.8	Not identified (unknown)	447.26	-	-
22.9	Kaempferol arabinoside (**Ka5)**	417.30	419.09 (287)	-

NMR: Confirmed by ^{1}H and 2D NMR experiments. **RT**: retention time. **Ka**: Kaempferol; **Q**: Quercetin

3. Material and Methods

3.1 Plant material

Cariniana estrellensis (Raddi) Kuntze (Lecythidaceae) is a native species from seasonal semi-deciduous forest (a phytophysiognomy of the Brazilian Atlantic Forest) and is classified as a shade-tolerant or non-pioneer species.

The seeds used in the present study were collected in forest fragments in northern Paraná, southern Brazil. The seedlings were grown under ideal conditions of light, water and nutrients. They were maintained in plastic bags (2 L, 15 cm high and 13 cm diameter) containing a mixture (1:1) of the inert substrate and fertile soil (pH 5.8; cation exchange capacity 4.4 cmolc dm^{-3}) characterized as clayey oxisol. Five months after germination, the third completely expanded leaf was collected from ten different seedlings. In the present study, triplicates from these ten leaves were analyzed.

3.2 Sample preparation

The extracts were obtained from 30 mg of milled dry leaves in 1 mL of CD_3OD:D_2O (8:2 v/v). The milled dry leaves and 1 mL of mixture of deuterated solvent were mixed in vortex for one minute. Then, the tube's contents were heated at 50 °C in a water bath for 10 minutes. The samples were then centrifuged for 5 minutes at 4°C. The supernatant was kept at 4°C overnight, centrifuged and 500 uL of supernatant was transferred to a tube. Then, 10 uL of TSP-d_4-sodium salt of trimethylsilylpropionic acid (1.00 mol/L) were added to each extract and transferred to a 5 mm NMR tube. A further aliquot of supernatant (100 uL) was transferred and diluted with H_2O/CH_3OH 80:20 v/v (800 µL) for LC-UV-MS/MS analysis.

3.3 NMR and LC-DAD-MS/MS instrumentation

C. estrellensis leaf hydroalcoholic extract was analyzed by 1D and 2D NMR experiments. The spectrum was acquired at 300 K an Avance III HD spectrometer operating at 600 MHz and equipped with a 5-mm BBO probe. ^{1}H spectra were acquired using NOESY 1D with a 2.00s presaturation delay and an acquisition time of 2.69 s (64 k points), an accumulation of 256 transients, and a spectral width of 15 ppm. All FIDs were automatically Fourier transformed after the application of an exponential window function with a line broadening of 0.3 Hz. Phase and baseline corrections were carried out within the instrument software. ^{1}H NMR chemical shifts were referenced to TSP-d4 at δ 0.00. Secondary metabolites were identified through 1D and 2D NMR spectra (correlation spectroscopy - COSY), Heteronuclear Single Quantum Correlation (HSQC). In addition, LC-DAD-MS/MS was employed to support the NMR spectrum and metabolite identification.

C. estrellensis leaf hydroalcoholic extract was analyzed by LC-DAD-MS using LC-DAD-ESI system consisting of a Shimadzu 20A HPLC equipped with a LC-20AD quaternary pump, a SPD-M20A photodiode array detector, a SIL-20A thermostated autosampler and a CTO-20A column compartment, coupled to a Bruker Ion Trap, with a heated ESI source. UV spectra were acquired from 230-400 nm. Mass spectra were acquired in negative and positive modes over *m/z* range of 100-1000, in separated runs. Operating parameters were as follows: source voltage, 4.5 kV, sheath gas, 9.00 L/min dry gas, 40 psi nebulizer and dry temperature, 300 °C. Automatic MS-MS was performed on the three most abundant ions of each scan. An isolation width of *m/z* 3 was used and precursors were fragmented by CID with normalized collision energy of 60. The data analyses were performed using Data Analysis software. The chromatographic runs were performed using Kinetex® C-18 column (3.5 µm, 46 x 150 mm

i.d., Phenomenex), which was maintained at 25°C. The gradient of elution was performed with water/0.1% formic acid (A) and acetonitrile/0.1% formic acid (B) under the following conditions: 0 min, 12% B; 32 min, 25 % B; 48 min, 100% B, 40 min, 100% B. Flow rate at 0.4 mL/min and injection volume of 5 µL.

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Supporting Information

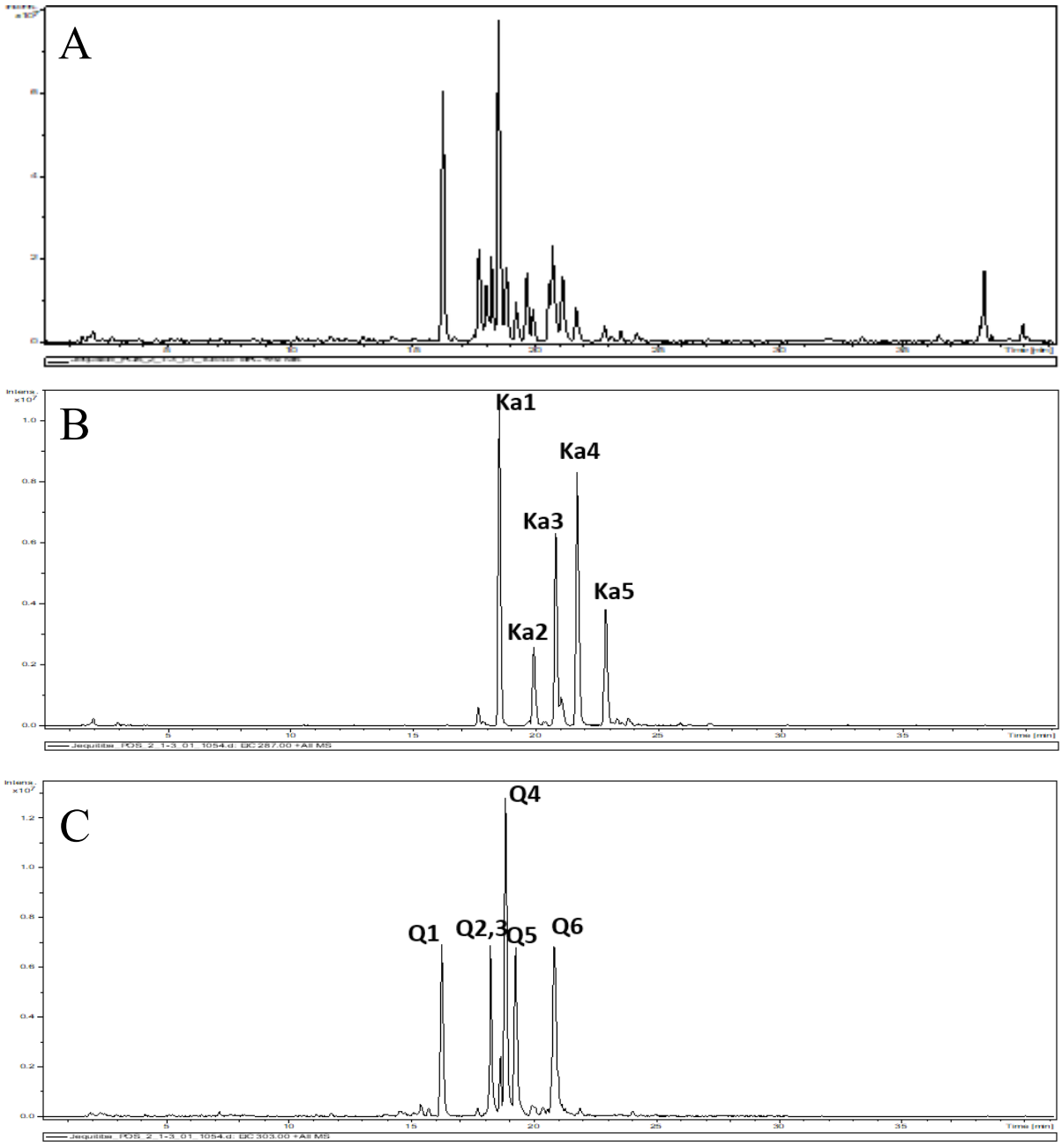

Fig. S1 LC-DAD-MS chromatogram from *Cariniana estrellensis* leaf hydroalcoholic extract. **A**: Base peak chromatogram (+). **B**: Extracted ion chromatogram (*m/z* 287) from kaempferol derivatives. **C**: Extracted ion chromatogram (*m/z* 303) from quercetin derivatives. **Q1**: Quercetin di-glucoside. **Q2**: Quercetin glucoside-rhamnoside 1. **Q3**: Quercetin glucoside-rhamnoside 2. **Q4**: Quercetin-glucoside isomer 1. **Q5**: Quercetin glucoside isomer 2. **Q6**: Quercetin arabinoside **Ka1**: Kaempferol di-glucoside. **Ka2:** Kaempferol glucoside-rhamnoside isomer 1. **Ka3**: Kaempferol di-arabinoside. **Ka4**: Kaempferol-glucoside. **Ka5**: Kaempferol arabinoside.

CONCLUSÃO GERAL

Os resultados obtidos neste estudo confirmam que a associação das BPCP com as duas espécies arbóreas neotropicais submetidas ao déficit hídrico levou a respostas de amplo espectro no metabolismo vegetal. O metabolismo antioxidante enzimático e não enzimático, e as vias bioquímicas do Carbono e do Nitrogênio foram amplamente influenciados por essa associação. Também foi determinado, por meio da metabolômica, a composição fitoquímica de ambas as espécies vegetais. Para *C. pachystachya* no capítulo 2 e para *C. estrellensisis* no capítulo 3, tornando evidente que cada uma tem a sua composição específica de metabólitos secundários, sendo esta uma expressão da individualidade de cada espécie.

A hipótese do capítulo 1 "*A inoculação com BPCP pode melhorar a resposta antioxidante de mudas de árvores neotropicais sob déficit hídrico*" foi aceita. Foi possível observar que a associação com BPCP influenciou positivamente a atividade de enzimas antioxidantes (Superóxido dismutase, Ascorbato Peroxidase e Peroxidases) e aumentou a quantidade de compostos fenólicos do metabolismo antioxidante não-enzimático de *C. pachystachya* e de *C. estrellensis* quando submetidas ao déficit hídrico.

A hipótese do capítulo 2 "*A associação com BPCP induz respostas no metabolismo do Carbono e do Nitrogênio que aumentam a tolerância à seca em árvores neotropicais*" foi aceita. Os resultados evidenciaram a influência positiva da associação com as BPCP nos intermediários do ciclo do ácido cítrico, nos compostos nitrogenados presentes no metabolismo vegetal e ainda em parâmetros fisiológicos de *C. pachystachya* e de *C. estrellensis* quando submetidas ao déficit hídrico. Além disso, as respostas fisiológicas e bioquímicas foram corroboradas pelas respostas biométricas descritas no capítulo 1 e no capítulo 2, indicando maior tolerância à seca.

Considerando o exposto, a hipótese geral deste estudo foi aceita: "*A associação entre Bactérias Promotoras do Crescimento em Plantas e espécies arbóreas nativas neotropicais induz mudanças em parâmetros fisiológicos, bioquímicos e biométricos, tornando as espécies vegetais mais tolerantes ao déficit hídrico.*"

Deste modo, a associação das BPCP com as espécies arbóreas neotropicais pode ser utilizada como uma ferramenta biotecnológica para aumentar o sucesso dos programas de reflorestamento. É importante ressaltar que o presente estudo foi realizado em casa de vegetação, e estudos futuros são necessários para validar o uso das mudas inoculadas em campo.