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REGULAÇÃO DO ACÚMULO DE MIMOSINA EM *Leucaena leucocephala* (Lam.) de Wit e *Mimosa bimucronata* (DC.) Kuntze

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SUMÁRIO

LISTA DE ABREVIATURAS

2,4-D: 2,4-dichlorophenoxyacetic acid

3H4P: 3-hydroxy-4-pyridone (3,4-DHP: 3,4-dihydroxypyridine)

ABA: abscisic acid

Arg: arginine

BABA: β-aminobutyric acid

β-ODAP: β-N-oxalyl-L-α, β-diaminopropionic acid

BIA: β-isoxazolinon-L-alanine

CAN: canavanine

DAO: diamine oxidase

DDC: decarboxylase

ETH: ethephon

FW: fresh weight

GABA: γ-aminobutyric acid

GABA-T: GABA transaminase

GAD: glutamate decarboxylase

GSM: Global System for Mobile

HPLC: High performance liquid chromatography

JA: jasmonate

JA-Ile: jasmonoyl-L-isoleucine

L-DOPA: L-3,4- dihydroxyphenylalanine

MeJA: methyl jasmonate

m-Tyr: *Meta*-tyrosine

NO: nitric oxide

NPAA: non-protein amino acid

OAS: *o*-acetylserine

OAS-TL: *o*-acetylserine-thiol-lyase

PA: polyamine

PAA: protein amino acid

PEG: polyethylene glycol

PLP: pyridoxal-5’-phosphate

PPO: polyphenol oxidase; tyrosinase

qRT-PCR: Reverse transcription polymerase chain reaction quantitative real time

RNS: reactive nitrogen species

ROS: reactive oxygen species

SA: salicylic acid

SAR: systemic acquired resistance

SNP: sodium nitroprusside

UV: ultraviolet radiation

RESUMO

Ao longo de sua evolução as plantas desenvolveram estratégias estruturais e químicas de defesa em resposta aos estresses bióticos e abióticos impostos pelo ambiente. Dentre essas, são reconhecidas moléculas quimicamente especializadas denominadas metabólitos secundários, produtos naturais ou metabólitos especializados. Aminoácidos não proteicos (ANPs) são compostos nitrogenados que constituem, além de componentes do arsenal de defesa química vegetal, uma importante fonte de reserva de carbono e nitrogênio para diversos *taxa*, especialmente aqueles pertencentes à família Fabaceae de Angiospermas. Esse grupo de moléculas quimicamente heterogêneo é assim definido por não participar da formação de estruturas proteicas funcionais, sendo frequentemente tóxicos quando erroneamente incorporados nas cadeias polipeptídicas em formação, em função da similaridade estrutural que apresentam com os aminoácidos proteicos. Sob o ponto de vista de defesa vegetal, como clássicos metabólitos especializados, ANPs são, em sua maioria, passíveis de indução por estresses de natureza biótica e/ou abiótica, como o ataque de herbívoros, exposição à radiação UV, e aplicação exógena de elicitores químicos, por exemplo. O objetivo da presente tese foi investigar o papel biológico da mimosina endógena em *Leucaena leucocephala* (Lam.) de Wit (leucena) e *Mimosa bimucronata* (DC.) Kuntze (maricá), a partir da avaliação do efeito de tratamentos relacionados ao estresse abiótico (UV-C, ácido salicílico, metil jasmonato e etileno). Mimosina é um ANP aromático, análogo da L-tirosina, com atividade tóxica para células de procariotos e eucariotos. Dentre as atividades descritas para esse ANP, destacam-se a atividade anti-mitótica ou 'bloqueadora' do ciclo celular, atividade alelopática e antioxidante. Os resultados indicaram que em leucena, a biossíntese e o acúmulo de mimosina podem ser modulados por fatores causadores de estresses, exibindo um padrão de acumulação similar ao das fitoalexinas. Em maricá, por outro lado, a indução do acúmulo dessa molécula não foi observada para os mesmos tratamentos testados para leucena, o que sugere um perfil de acumulação similar ao das fitoanticipinas. Além disso, o padrão de expressão gênica observado nas plantas de leucena estressadas com etileno sugere que o controle *steady-state* da mimosina pode ser, pelo menos em parte, regulado pela sua degradação. As respostas observadas nos testes que avaliaram a atividade de mitigação de espécies reativas de oxigênio por mimosina sugerem que essa molécula pode agir como um agente antioxidante não-enzimático em plantas de leucena em situação de estresse.

Introdução

Na condição de organismos sésseis, ao longo de sua evolução as plantas desenvolveram estratégias estruturais e químicas de defesa em resposta aos estresses bióticos e abióticos impostos pelo ambiente. Dentre essas, são reconhecidas moléculas quimicamente especializadas denominadas metabólitos secundários, produtos naturais (Kutchan *et al*. 2015) ou, mais recentemente, metabólitos especializados.

Entre as três classes mais gerais de metabólitos secundários (terpenos, compostos fenólicos e compostos nitrogenados), aminoácidos não-proteicos (ANPs) são incluídos no terceiro grupo, e constituem, além de componentes do arsenal de defesa química, uma importante fonte de reserva de carbono e nitrogênio para diversos taxa, especialmente aqueles pertencentes à família Fabaceae de Angiospermas (leguminosas, *sensu lato*).

Além dos 20 aminoácidos proteicos, estima-se que existam entre 600 e 1000 ANPs (Acamovic & Brooker 2005; Rodgers *et al*. 2015). Esse grupo de moléculas quimicamente heterogêneo é assim definido por não participar da formação de estruturas proteicas funcionais, sendo frequentemente tóxicos quando erroneamente incorporados nas cadeias polipeptídicas em formação, em função da similaridade estrutural que apresentam com os aminoácidos proteicos (Taiz & Zeiger 2010).

Conforme mencionado, a ocorrência de ANPs é comum em espécies de leguminosas, e sua distribuição pode ser restrita a alguns gêneros de plantas circunscritos nessa família botânica (*e.g.* mimosina e canavanina). Por outro lado, alguns ANPs como GABA, por exemplo, podem apresentar distribuição ubíqua no Reino Plantae, assim como ocorrer em outros tipos de organismos, como animais, por exemplo (Ramos-Ruiz *et al*. 2018).

Apesar de representarem uma fonte nutricional importante, sem tratamento prévio, o consumo de plantas que acumulam ANPs por animais é limitado. Isso ocorre, pois, em longo prazo, a ingestão prolongada de plantas (especialmente sementes) que acumulam ANPs pode representar risco à saúde, uma vez que estes comprometem o funcionamento de mecanismos basais de manutenção da homeostase celular, e podem também, em um quadro mais severo, desencadear doenças neurotóxicas degenerativas, como, por exemplo, o latirismo, causado por ácido β-N-oxalil-l-α,β-diaminopropiônico (β-ODAP) (Jiao *et al*. 2011; Kusama-Eguchi 2019).

Sob o ponto de vista de defesa vegetal, como clássicos metabólitos especializados, ANPs são, em sua maioria, passíveis de indução por estresses de natureza biótica e/ou abiótica, como o ataque de herbívoros, exposição à radiação UV, e aplicação exógena de elicitores químicos, por exemplo. No que concerne ao estudo dos efeitos da indução abiótica sobre o acúmulo de ANPs em diferentes espécies vegetais (Monocotiledôneas e Eudicotiledôneas), as moléculas mais amplamente investigadas até o momento são GABA, L-DOPA e, mais recentemente, mimosina (vide Tabela 1 do capítulo primeiro). Em termos de efeitos causados a partir da aplicação exógena de ANPs, GABA também figura como o principal aminoácido investigado, seguido de L-DOPA e canavanina (vide Tabela 2 do capítulo primeiro).

No primeiro capítulo da presente tese, estão descritas as características gerais dos principais ANPs estudados, seus possíveis papéis biológicos *in planta*, e seus efeitos quando aplicados exogenamente, bem como os estresses abióticos capazes de induzir seu(s) acúmulo(s) nos diferentes tecidos vegetais. Nos segundo e terceiro capítulos, respectivamente, são elucidados os efeitos dos tratamentos de UV-C, ácido salicílico, etileno e jasmonato (clássicos elicitores do metabolismo secundário vegetal) sobre o acúmulo de

mimosina em *Leucaena leucocephala* var. *glabrata* (Lam.) de Wit. (leucena) e *Mimosa bimucronata* (DC.) Kuntze (maricá).

Mimosina é um aminoácido aromático não-proteico, análogo da L-tirosina, com atividade tóxica para células de procariotos e eucariotos. Embora em menor concentração, mimosina foi primeiramente identificada em *Mimosa pudica*, sendo posteriormente detectada em outras espécies do gênero, como *Mimosa pigra*, por exemplo (Soedarjo & Borthakur 1998). Seu efeito tóxico é atribuído à capacidade de quelar metais, o que impede o funcionamento adequado das metalo-proteínas que dependem dos mesmos como co-fatores (Negi *et al*. 2014).

A concentração basal de mimosina em espécies de leucaena pode variar entre 1 e 12 % do peso seco do órgão (Soedarjo & Borthakur 1998). Como é comum para outros ANPs que ocorrem em espécies de leguminosas, em sementes de *Leucaena* spp. é observada uma maior concentração de mimosina quando comparada aos demais órgãos da planta (Rodrigues-Corrêa *et al*. 2019), sendo esta a fonte de extração comercial do padrão químico de mimosina vendido por empresas de reagentes de pesquisa.

Diversas atividades foram descritas para mimosina em outros organismos e/ou tipos celulares. Dentre essas, destacam-se a atividade anti-mitótica ou 'bloqueadora' do ciclo celular em células de eucariotos e procariotos. Isto ocorre porque a mimosina impede a formação da forquilha de replicação (e, portanto a síntese de DNA), interrompendo assim o avanço do ciclo de divisão celular na fase tardia G1 (Lalande 1990). Foram também descritas para mimosina, atividade alelopática observada sobre o desenvolvimento de outras espécies de leguminosas e atividade antioxidante, entre outras (Tabela 1).

A rota de biossíntese de mimosina é compartilhada em grande parte com a de cisteína, um aminoácido proteico sulfurado (Figura 1). A síntese da cisteína se dá a partir da conversão

de serina e acetil-CoA em *o*-acetilserina pela enzima SAT (serina acetiltransferase), seguida da conversão de *o*-acetilserina e ácido sulfídrico em cisteína, em uma reação catalisada pela OAS-TL (o-acetilserina tiol-liase). A síntese de mimosina por sua vez, é compartilhada com a da cisteína até esse ponto e, acredita-se que pelo menos uma das isoformas de OAS-TL catalise a conversão de *o*-acetilserina e 3-hidroxi-4-piridona em mimosina.

Tabela 1. Atividades descritas para mimosina de *Leucaena leucocephala* (Lam.) de Wit.

ATIVIDADE	ALVO AVALIADO (organismo e/ou tecido/ tipo celular)	REFERÊNCIA
Bloqueio do complexo de ativação da pré-replicação do DNA	Células de mamíferos	KUBOTA *et al*. (2014)
Alteração no ciclo ovariano e extensão da duração do corpo lúteo bovino no período pós-parto	Bovinos (*Bos taurus* x *Bos indicus*)	BOTTINI-LUZARDO *et al.* (2015)
Supressão do ciclo celular e redução da abundância bacteriana em mosquitos	*Wolbachia pipientis*; *Aedes albopictus*	FALLON (2015)
Ação inibitória da fibrose pulmonar induzida	Ratos SD	LI *et al.* (2015)
Recuperação da função do miocárdio pós-isquemia	Miocárdio de ratos (SD) machos	CROWE *et al.* (2001)
Inseticida	*Heteropsylla cubana* Crawford, 1914 e *Thrips tabaci* Lindemann, 1889	AHMED *et al*. (2016)
Alelopática	*Albizia procera, Vigna unguiculata, Cicer arietinum, Cajanus cajan*	AHMED *et al*. (2008)
Antioxidante	Sistemas modelo de oxidação lipídica (β-caroteno - ácido linolêico e lecitina)	BENJAKUL *et al.* (2013)

Até momento, versões divergentes sobre a enzima responsável pela biossíntese de mimosina (mimosina sintase) têm sido publicadas. Em 1990 Ikegami e colaboradores

identificaram uma OAS-TL responsável pela formação de cisteína como sendo também uma mimosina sintase. Mais tarde, Yafuso *et al*. (2014) realizaram a expressão heteróloga do gene que codifica para OAS-TL em *Escherichia coli*, e não foi observada a formação de mimosina, mesmo quando dadas as condições ótimas para tanto. Mais recentemente, Harun-Ur-Rashid *et al*. (2018) elucidaram a mimosina sintase como sendo uma isoforma da OAS-TL, corroborando o postulado por Ikegami e colaboradores em 1990.

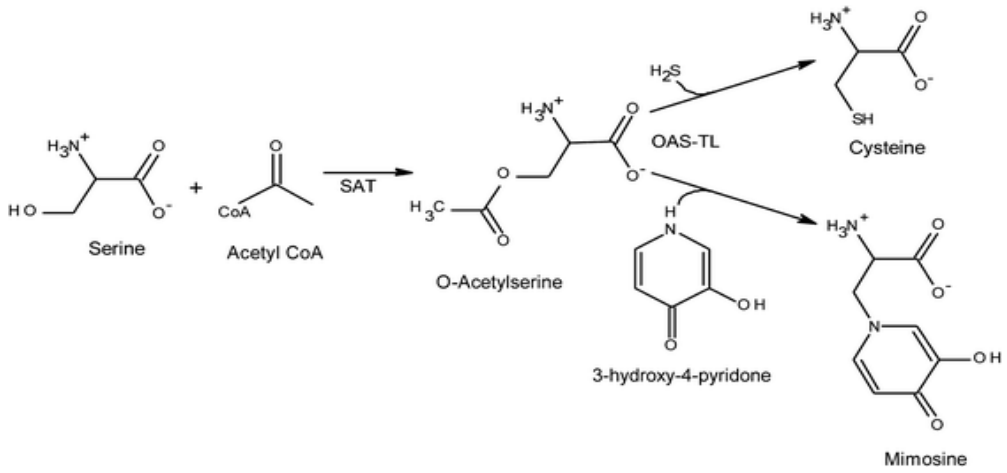

Figura 1. Rota de biossíntese da mimosina. Fonte: Ikegami *et al*. (1990).

Espécies estudadas

Leucaena leucocephala (Lam.) de Wit (leucaena, *koa haole* ou "acácia exótica" na língua Hawai'iana) é uma espécie de hábito arbóreo ou arbustivo, pertencente à família Fabaceae de Angiospermas, e caracterizada pelo acúmulo de mimosina em todos os seus órgãos. É nativa da América Central (especificamente da região sudeste do México), mas irradiou-se através de praticamente todas as zonas tropicais e subtropicais da Terra. No Brasil, leucena é amplamente distribuída e classificada como naturalizada pelo REFLORA (2019), ocorrendo em todo território Nacional. São reconhecidas no mínimo duas

subespécies de leucena ocorrentes no Brasil, *L. leucocephala* var. *leucocephala* e *L. leucocephala* var. *glabrata*, sendo a primeira a mais abundante.

Leucaena apresenta atributos morfológicos característicos das leguminosas como o fruto do tipo vagem, deiscente no período pós-maturação, folhas compostas e bipinadas. As flores são sésseis, actinomorfas e polistêmones, apresentam cálice sinsépala e corola gamopétala, e são dispostas em inflorescências do tipo glomérulo (Figura 2).

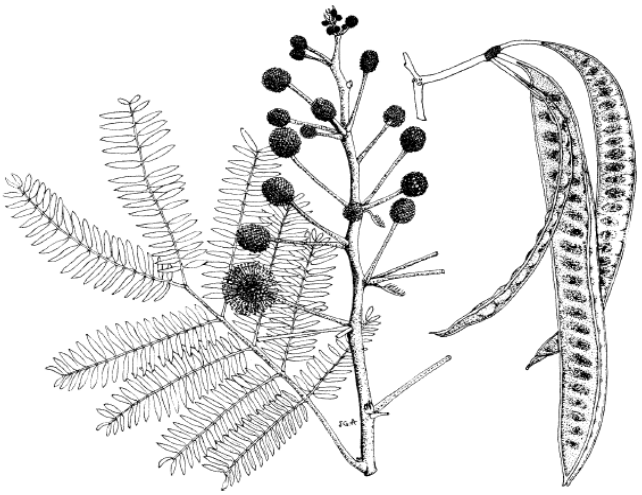

Figura 2. Órgãos vegetativos e reprodutivos de *L. leucocephala* (Lam.) de Wit. Fonte: Little Jr. & Skolmen (1989).

Com base no conhecimento etnobotânico disponível acerca dessa espécie, em diversas regiões tropicais e subtropicais, leucena é utilizada para vários fins. Extratos de diferentes órgãos de leucena apresentam atividade anti-diabética (Kuppusamy *et al*. 2014; Chowtivannakul *et al*. 2016), antioxidante (Mohammed *et al*. 2015, Chowtivannakul *et al*. 2016, Zarin *et al*. 2016), antimicrobiana (Zarin *et al*. 2016), anti-helmíntica (Soares *et al*. 2015; Jamous *et al*. 2017), bactericida (Mohammed *et al*. 2015), acaricida (Fernández-Salas *et al*. 2011), anti-tumoral (Chung *et al*. 2017), e potencializadora da resposta imune em peixes (Verma *et al*. 2018), entre outras.

Leucaena apresenta alta tolerância à seca, sendo capaz de enfrentar estações sazonais inteiras com déficit hídrico sem prejuízo permanente de seus órgãos, e de recuperar vigorosamente sua biomassa vegetativa, tão logo o regime de precipitação retome a regularidade em frequência. Acredita-se que a tolerância à seca apresentada por essa espécie ocorra em função do acúmulo de mimosina nos diferentes tecidos da planta, a qual funcionaria como um agente osmoregulador, responsável pela preservação da integridade das membranas a das macromoléculas intracelulares, em períodos de escassez de água no ambiente.

Mimosa bimucronata var. *bimucronata* (DC.) Kuntze (maricá) é uma leguminosa nativa, não endêmica do Brasil, amplamente distribuída nos domínios fitogeográficos da Caatinga, do Cerrado, e da Mata Atlântica (Simon & Proença 2000; REFLORA 2019). Como espécie pioneira (Pilatti *et al*. 2019), exerce importante papel ecológico na recuperação de áreas degradadas (Bitencourt *et al.* 2007; Silva *et al*. 2011), no estabelecimento de processos de sucessão vegetacional.

Maricá é uma espécie semi-decídua a decídua, a qual atinge até 15 m em altura (e diâmetro à altura do peito de até 40 cm) na idade adulta, com hábito arbóreo ou arbustivo (REFLORA 2019) e espinhos característicos desde os estágios iniciais de desenvolvimento (Carvalho 2004). Apresenta folhas compostas alternas e bipinadas (Figura 2), amplas inflorescências brancas, com flores reunidas em glomérulos esféricos dispostos em grandes panículas. As flores são diplostêmones, actinomorfas, hipóginas e unicarpelares (Silva *et al*. 2011).

Assim como descrito para leucena, maricá é considerado uma espécie multifuncional, sendo comumente empregada para produção de mel, como combustível (Olkoski &

Wittmann 2011), em edificações, na carpintaria, e como ‘cerca-viva’ (Marchiori 1993; Lorenzi 1998), entre outras aplicações.

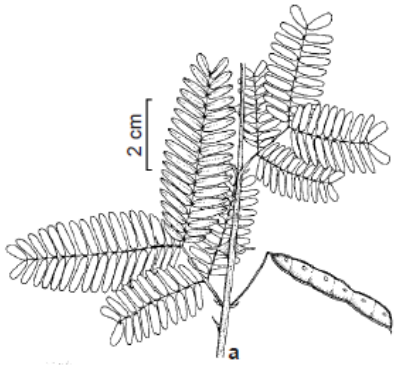

Figura 2. Folhas e fruto de *Mimosa bimucronata* (DC.) Kuntze. Fonte: Souza-Lima *et al*. (2017).

Em contraste com a amplitude de *habitats* explorados por leucena (especialmente os áridos), no Sul do Brasil, maricá ocorre preferencialmente em ambientes úmidos a alagadiços em áreas próximas às margens de rios (Patreze & Cordeiro 2004), embora possa também ocorrer em formações quase exclusivas dessa espécie nas encostas de morros (Jacobi & Ferreira 1991).

Em relação às atividades elucidadas para os extratos de maricá, foram relatados os efeitos alelopático (Jacobi & Ferreira 1991; Ferreira *et al*. 1992), diurético, natriurético e caliurético (Schlickmann *et al*. 2017).

Hipótese

Mimosina apresenta perfil dinâmico de acúmulo em *Leucaena leucocephala* e *Mimosa bimucronata* frente a estresses, associado a alterações significativas na expressão de genes relacionados ao metabolismo deste ANP, o qual contribui para mitigar o desequilíbrio oxidativo inerente a vários tipos de estresse.

Objetivo geral

O objetivo da presente tese foi investigar o papel biológico da mimosina endógena em leucena e maricá, a partir da avaliação do efeito de tratamentos relacionados a estresses ou sinalizadores de estresse.

Objetivos específicos

- Analisar a concentração constitutiva de mimosina nos diferentes órgãos de *L. leucocephala* (Lam.) de Wit (leucena) e *M. bimucronata* (DC.) Kuntze (maricá);

- Verificar se, apesar do seu alto teor constitutivo em plantas de leucena, o acúmulo de mimosina pode ser induzido com tratamentos que mimetizam diferentes estresses, a partir da avaliação do efeito de moléculas sinalizadoras (ácido salicílico, jasmonato, etileno) e da exposição à radiação UV-C na modulação do acúmulo de mimosina em leucena, bem como em maricá;

- Determinar se a expressão de genes relacionados ao metabolismo de mimosina está associada à indução por estresses fisiológicos;

- Avaliar o potencial antioxidante da mimosina em experimentos realizados *in situ*.

Abiotic stresses and non-protein amino acids in plants

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ABSTRACT

Plants have developed several strategies to overcome the broad scope of environmental challenges imposed by biotic and abiotic stresses, including chemical defenses known as secondary or specialized metabolites. Non-protein amino acids (NPAAs) comprise a large heterogeneous group of nitrogen-containing specialized metabolites with wide distribution in the Plant Kingdom, commonly found in several genera of Fabaceae. Various examples of toxic effects attributed to the NPAAs on animal, microbial and other plant cells are known and often related to their structural similarities to protein amino acids (PAAs). However, NPAAs have also been shown to play important roles *in planta* as protectant molecules against oxidative damage, besides increasing tolerance of different plant species to a variety of abiotic-induced stresses, such as drought and salinity. In this review, we discuss well-established and novel functions recently unveiled for NPAAs, besides alternative modes of action proposed for these metabolites as key mediators and effectors in responses to abiotic stresses.

KEYWORDS: GABA; BABA; β-ODAP; L-DOPA; canavanine; mimosine

I. Introduction

Throughout their evolutionary history, plants have faced diverse types of biotic and abiotic stresses. Major abiotic stressors (*e.g*. UV radiation, cold, salinity, drought, heavy metals, osmotic stress) have been shown to affect in several different ways plant homeostasis, triggering the generation of reactive oxygen species (ROS) and impairing the cell chemical balance. As a result, combined with the need arising from their sessile nature, plants have developed efficient chemical strategies to overcome environmental challenges in situ, namely the accumulation of specialized metabolites, also known as secondary metabolites or natural products.

Specialized metabolites are biosynthesized by certain plant taxa in order to cope with a broad spectrum of different environmental stimuli (Kutchan *et al.*, 2015). Among nitrogen-containing compounds, the non-protein amino acids (NPAAs) are widely distributed in the Plant Kingdom. NPAAs constitute a heterogeneous group of numerous specialized metabolites estimated between 600 to 1000 different molecules (Acamovic and Brooker, 2005; Rodgers *et al*., 2015), structurally similar to protein amino acids (PAAs). This structural proximity is related to their main mode of action as toxic compounds, since they are able to compete with for precursors (and/or enzymes co-factors) and transporters of protein amino acids in the same metabolic pathway. As a consequence, besides acting as potent chelators of metal ions, NPAAs can be erroneously incorporated into protein chains, generating non-functional or partially impaired products and interfering with biochemical pathways (Rosenthal, 1977; Rodgers *et al*., 2015).

NPAAs can represent important carbon and nitrogen reservoirs in plants (Soares *et al.*, 2014). However, their potential toxicity and anti-nutritional properties may prevent widespread use of plant biomass accumulating these products at large scale for fodder

(D'Mello, 1992; Peng *et al*., 2005; McSweeney *et al*., 2008), since they might cause important losses to livestock production. In addition, their long-term ingestion can also constitute a threat to human health, especially by promoting irreversible neurodegenerative diseases (Rodgers, 2014).

In spite of being potentially toxic to animals, great attention has been paid to NPAAs-mediated effects on prokaryotic and eukaryotic cells (including mammalian ones). The reason driving this line of research is the application of some NPAAs on pre-clinical and clinical trials, as prospective sources of new drugs against human diseases (Rosenthal, 1977; Li *et al*., 2015; Nguyen and Tawata, 2016).

There are a number of studies available on the toxic effects caused by NPAAs in herbivores and pathogens, as well as their potential applications in human health improvement (McSweeney *et al*., 2008; Huang *et al*., 2011; Dimlioğlu *et al*., 2015). However, there is a lack of information presenting a compilation of the roles of this heterogeneous group of nitrogen-containing compounds in plant defense mechanisms, as key mediators and/or effectors in response to abiotic factors. In this review, the importance of NPPAs as protectants and signaling molecules against abiotic environmental challenges faced by plants is discussed.

II. Main NPAAs found in plants

Several NPAAs can be found in plants and their list has been increasing over the years. Among them, canavanine, mimosine, GABA (γ-aminobutyric acid) and L-DOPA (L-3,4- dihydroxyphenylalanine) can be listed as some of the most studied ones because of their effects on animals. Some NPAAs are widely distributed in different kinds of organisms (*e.g*.,

GABA) and others, like mimosine, show limited distribution in plant species or families, mainly in Fabaceae (Table 1). There are also other less investigated NPAAs that have been more recently described like L-theanine from *Camellia sinensis* (L.) Kuntze (Theaceae), which probably shows relevant effects *in planta*, although it has only been investigated by its beneficial properties on human health (Siamwala *et al.*, 2013; Gong *et al*., 2018; Sharma *et al*., 2018). Moreover, other NPAAs (like pipecolic acid, derived from lysine catabolism and present both in plants and animals), have been *mainly* studied by their role as priming-molecules in plant systemic acquired resistance (SAR) against biotic elicitors (Návarová *et al*., 2012; Vogel-Adghough *et al*., 2013; Shan and He, 2018). Nevertheless, regarding their functions as mediators of abiotic stresses, new NPAAs have also been identified recently. Despite the scarce data available on its biosynthesis and mode of action, 5- hydroxynorvaline (an NPAA found in legume and grass species) accumulation in plants of maize (*Zea mays* L., Poaceae) has been shown to increase in response to drought-induced stress, treatment with phytohormones, such as MeJA (methyl jasmonate), salicylic acid (SA), and abscisic acid (ABA) (Carmo-Silva *et al*., 2009; Yan *et al.*, 2015). *Meta*-tyrosine (*m*-Tyr), a phenylalanine analog produced by fescue (*Festuca* spp., Poaceae) roots, is another recently elucidated NPAA involved in abiotic stress signaling in plants. The effect of *m*-Tyr exogenous application showed toxic activity on tomato (*Solanum lycopersicum* L., Solanaceae) by acting as an efficient ROS generator, besides impairing nitrogen metabolism in roots of this species (Table 2). The latter effect was brought about by alteration of the levels of reactive nitrogen species (RNS) and of protein nitration, as well as nitrosoglutathione reductase activity (Krasuska *et al.*, 2017; Andrzejczak *et al*., 2018).

A. GABA

GABA (γ-aminobutyric acid) is a four carbon NPAA with ubiquitous occurrence in plants, which can be also naturally found in microorganisms up to vertebrates, including humans (Ramos-Ruiz *et al*., 2018). For this reason, over the past 20 years, an expressive number of studies have been performed to investigate its effects on prokaryotic and eukaryotic organisms. Besides stress responses, experimental evidence puts GABA as a player in C and N metabolism, signal transduction, plant-microbe interactions, and pollen tube growth, although much remains to be detailed regarding its mechanisms of action and putative receptors (reviewed in Seifikalhor *et al*., 2019).

GABA is mainly biosynthesized from L-glutamate in a reaction catalyzed by the enzyme glutamate decarboxylase (GAD). In terms of regulation, however, GABA intracellular levels are, at least in part, dependent on the combined activity of GAD and GABA transaminase (GABA-T). This enzyme is responsible for catalyzing the first step of GABA catabolism, which converts GABA into succinic semialdehyde (Shelp *et al.*, 1999; Deleu *et al.*, 2013; Shimajiri *et al*., 2013). GABA-T gene regulation, however, was found to be complex and dependent on developmental stage and the organ of the plant in *Brassica napus* L. All four tested copies of *Bna*GABA-T were ubiquitously expressed (in different levels), under controlled conditions; nevertheless, under water deficit stress *Bna*GABA-T genes were up-regulated only in the youngest leaves, for example (Faës *et al.*, 2015). GABA-T loss of function in *Arabidopsis* resulted in precocious leaf senescence under stress conditions (Jalil *et al.,* 2017) whereas exogenous application of GABA significantly improved tolerance to drought stress in *Nigella sativa* (Rezaei-Chiyaneh *et al.*, 2018) (Table 2). Alternatively, GABA can also be either synthesized from polyamine (PA) degradation by conversion of

putrescine into GABA, in a reaction catalyzed by diamine oxidase (DAO), or from proline decarboxylation via Δ1-pyrroline dehydrogenase activity, under highly oxidative conditions (Bor and Turkan, 2019). Due to their key role in GABA metabolism, the gene transcript levels and the activity (or activity inhibition) of GAD and GABA-T, as well as PA and proline contents in stressed plants, have been investigated in relation to putative GABA roles in response to abiotic stresses (Deleu *et al.,* 2013; Salvatierra *et al.,* 2016; Farooq *et al.,* 2017; Yong *et al.*, 2017; Hijaz *et al.*, 2018; Zhen *et al.,* 2018). Exogenously supplied GABA to small citrus cuttings has been shown to be taken up, metabolized to succinic acid and incorporated into the Krebs cycle, indicating that the protective effects of this NPAA against stresses could be a result of energy metabolism activation through GABA shunt pathway and the tricarboxylic acid cycle (Hijaz and Killiny, 2019). GABA has also been known to relieve salt stress. Studies in *Arabidopsis* showed that its mechanism of action seems to involve activation of antioxidant defenses and H^+-ATPase, with consequent improved membrane potential maintenance. Lower Na uptake and its increased exclusion to the apoplast and vacuolar storage, as well as improved K retention, were also recorded (Su *et al.*, 2019).

Recently, the identification and functional characterization of two genes coding GABA transporters (*PeuGAT2* and *PeuGAT3*) from *Populus euphratica* (Poplar) have been performed. The overexpression of *PeuGAT3* was seen to promote physiological and anatomical changes in the tested plants, as enhanced lignin content in xylem tissues and increased proline accumulation in Poplar leaves, besides an increased thickness of xylem cells walls in *Arabidopsis* and Poplar plants. All these alterations might contribute to the *P. euphratica* tolerance to salt- and/or drought-stresses (Bai *et al*., 2019).

B. BABA

BABA is a 4-carbon isomer of GABA (Cohen *et al.*, 2016) that binds to a specific receptor in plant cell, an aspartyl-tRNA synthetase (Luna *et al*., 2014; Buswell *et al*., 2018). Unlike GABA, β-aminobutyric acid (BABA) has a rare occurrence in nature.

In plants, BABA has been shown to be involved in acquired resistance mechanisms by acting as a potent response-inducer against a wide range of types of pathogens, such as virus, bacteria, nematodes, and fungi (Jakab *et al*., 2001; Walz and Simon, 2009; Cao *et al*., 2014; Cohen *et al*., 2016). In this regard, it has recently been found that BABA-priming promotes DNA hypermethylation, inducing a transient, intergeneration resistance against *Phytophthora infestans* in potato (Kuźnicki *et al*., 2019).

BABA treatment can also cause alterations in anatomical and physiological processes in plants. It has been shown that BABA is capable of generating stress-induced morphogenic response, priming, and resistance to abiotic elicitors in *Arabidopsis*. Interestingly, all these BABA stress-induced adaptations can be inhibited by L-Glutamine (Wu *et al*., 2010). Hence, in spite of being isomers exerting similar effects in plants, as response inducers BABA and GABA can present controversial regulation mechanisms.

High BABA doses were shown to induce sterility in *Arabidopsis* by promoting higher callose deposition in ovules (Jakab *et al*., 2001). In this same species, BABA treatment enhanced low potassium stress tolerance by increasing K^+ uptake (Cao *et al*., 2008). Acquired thermotolerance can also be induced by BABA application in *Arabidopsis* in a complex interaction process involving ABA-signaling cascades (Zimmerli *et al*., 2008).

BABA can also directly induce secondary metabolism activation. For instance, in hairy root cultures of *Salvia miltiorrhiza* Bunge (Lamiaceae), BABA exogenous application

proved to be a more effective elicitor of diterpenoid biosynthesis than MeJA treatment (Ge and Wu, 2005).

One of the major defenses conferred by BABA treatment to plants seems to be drought tolerance, possibly due to its interaction with the ABA and JA signaling pathways (Macarisin *et al*., 2009; Quéro *et al*., 2015; Shaw *et al*., 2016). In *Triticum aestivum* L. (wheat), BABA increased drought-induced ABA accumulation and desiccation tolerance by decreasing water use, increasing water use efficiency, reducing ROS production, increasing antioxidant enzymes activity and reducing the oxidative damage to membrane lipids (Du *et al*., 2012). In *Solanum tuberosum* L. (potato), drought tolerance was transiently improved by BABA soil drench at a final concentration of 0.3 mM in a mechanism involving ethylene signaling and suppression of ethylene-inducible gene expression. BABA-treated plants showed lower water loss and increased tuber yields than non-treated plants. Moreover, BABA treatment delayed the expression of the drought-inducible gene (*StDS2*) in leaves and extended the period of expression of *ETR1* under drought conditions (Sós-Hegedüs *et al*., 2014). In *Linum usitatissimum* L. (flax), metabolomic and ionomic approaches showed that drought tolerance mediated by BABA application might be related to its action as osmoregulator, via modulation of the cell solute content. This action takes place by the increased accumulation of proline and of non-structural carbohydrates, associated with decreased concentration of inorganic solutes and osmotic potential in flax leaves of BABA treated plants (Quéro *et al*., 2015).

Despite its beneficial effects in abiotic stress-induced resistance in plants (Figure 2), high BABA doses can suppress plant growth (Luna *et al*., 2014). At 10 mM and 20 mM, BABA was found to be toxic for *Brassica rapa* subsp. *pekinensis* (kimchi cabbage) seedlings development. BABA interaction with ABA has shown to occur in a synergistically fashion

on the growth arrest promotion of this species (Kim *et al*., 2013). For a review on BABA history and physiological effects see Cohen *et al.* (2016).

C. Canavanine

Canavanine (CAN) (2-amino-4-guanidinooxy-butyric acid) is an alkaline NPAA structurally analog to arginine (Arg) (Udedibie and Carlini, 1998) with toxic activity to fungi, viruses, bacteria, plants and animals (Staszek *et al*., 2017). CAN naturally occurs in high concentration in seeds of leguminous (Fabaceae) species (*e.g*. *Canavalia ensiformis* L. and *Vicia disperma* DC.), and can also be found in considerable amounts in leaves of *Sutherlandia frutescens* L. R. Br., seedlings and seeds of *Medicago sativa* (L.) (alfalfa) (Fabaceae) (Rosenthal and Nkomo, 2000; Colling *et al*., 2010; Megías *et al*., 2016). In the genus *Lonchocarpus*, the presence of this NPAA is a useful taxonomic marker to differentiate the American (rich in enduracin) from the African species (rich in CAN) (Soto-Hernandéz *et al*., 2016).

Besides its reduced basicity compared to Arg, in CAN the methylene group present in the former is replaced by oxygen, which results in a more reactive molecule. Due to their structural similarity, CAN might be erroneously incorporated in newly synthesized proteins instead of Arg, generating molecules with abnormal three-dimensional conformation, sometimes referred to as canavanyl proteins (Krasuska *et al*., 2016[a]; Staszek *et al*., 2017).

CAN is mainly synthesized from canaline; however in low quantity, it might be formed from ureidohomoserine. In higher plants, green tissues (photosynthetically active) are the main sites of CAN biosynthesis, which is thought to be light-dependent. CAN catabolism is mediated by arginase activity, that catabolizes its degradation into canaline and urea. In CAN-producer legumes, the higher affinity of arginase for CAN instead of Arg

compared to the CAN-free legumes seems to be one of the key mechanisms to avoid autotoxicity (Staszek *et al*., 2017).

Unlike GABA and BABA, exogenous application of CAN has been shown to cause different deleterious effects in plants (Table 2). Although the primary mode of action of CAN has been described as protein disruption, it also interferes in ethylene and polyamine metabolisms. Moreover, CAN treatment was found to promote the inhibition of DNA synthesis and cell division, restriction in RNS and nitric oxide formation, concomitant to ROS overaccumulation (Krasuska *et al*., 2016[b]). The same authors recorded an increase in indole-3-acetic acid (IAA) in roots of tomato treated with CAN, resulting in growth inhibition. Other post-translational protein modifications, notably carbonylation, were also detected in root proteins (Krasuska *et al*., 2016[b]). For further detailed information on CAN synthesis, metabolism and mode of action, see Staszek *et al*., (2017).

D. L-DOPA

L-DOPA (L-3,4- dihydroxyphenylalanine) is a dopamine precursor, biosynthesized from L-tyrosine, found in high concentration in velvet bean seeds (*Mucuna pruriens* (L.) DC. var. *utilis*) (Fabaceae). It is well known by its effect in mammalian brain cells, as an anti-Parkinson's disease drug (Vibha *et al*., 2009; Etemadi *et al*., 2018; Singh *et al*., 2018) and by its wide bioherbicidal activity (Topal and Kocaçalişkan, 2006; Golisz *et al*., 2011).

L-DOPA acts as a potent allelochemical compound by inducing cell death and root growth inhibition of mono and eudicot species (Fujii *et al*., 1991; Matsumoto, 2011; Mushtaq *et al*., 2013; Soares *et al*., 2014) (Table 2). In plants, L-DOPA works as a precursor for melanin biosynthesis in a first reaction catalyzed by polyphenol oxidase (PPO, also named tyrosinase) or by auto-oxidation (for a review on L-DOPA biosynthesis and role in plants,

see Soares *et al.*, 2014). In spite of being present in all parts of *Mucuna pruriens* (L.), higher contents of L-DOPA were associated with mature plant stage, particularly in seeds, tender stems, and tender leaves (Singh *et al*., 2018). The same investigation showed that expression of PPO gene was elevated in mature leaves.

L-DOPA phytotoxicity (Table 2) is thought to be related to the enhancement of ROS generation (and consequent oxidative cell damage) that takes place along the enzymatic reactions required for melanogenesis (Matsumoto, 2011; Mushtaq *et al*., 2013; Soares *et al*., 2014). *Arabidopsis* microarray data indicated that allelochemical activity of L-DOPA is related to impacts on amino acid metabolism and metal homeostasis, mainly iron (Golisz *et al*., 2011). The mitigation of high levels of stress-induced L-DOPA has been demonstrated to be regulated, at least in part, by the increased expression and activity of L-DOPA decarboxylase (DDC) in plants of *Phlomis fruticosa* (Stefi *et al.*, 2019) and *Myrtus communis* L., the latter in response to high frequency non-ionizing electromagnetic fields from cell phones (Stefi *et al*., 2018). DDC converts L-DOPA into dopamine, through L-DOPA decarboxylation (Stefi *et al.*, 2019).

E. Mimosine

Mimosine [β-[N-(3-hydroxy-4-oxypyridyl)]-α-aminopropionic acid] is a toxic aromatic NPAA derived from *o*-acetylserine (OAS) and 3-hydroxy-4-pyridone (3H4P), in a reaction thought to be catalyzed by an OAS-TL (*o*-acetylserine-thiol-lyase) cysteine isoform (Harun-Ur-Rashid *et al*., 2018). Mimosine is particularly found in *Leucaena leucocephala* (Lam.) de Wit and *Mimosa* species (Jube and Borthakur, 2010), and displays a potent inhibitory activity on prokaryotic and eukaryotic cells unable to degrade it efficiently (Harun-Ur-Rashid *et al*., 2018). Mimosine toxicity is due to its propensity to form a chelate with

divalent ions, preventing them to operate as metal-enzyme co-factors, besides its high affinity for pyridoxal-5'-phosphate (PLP), which causes the inactivation of the PLP-dependent enzymes. Mimosine is degraded by mimosinase, a carbon-nitrogen lyase (Negi *et al*., 2014).

Several biological activities have been described for mimosine on different types of cells, like insecticide (Ahmed *et al.*, 2017), antimicrobial (Anitha *et al*., 2005), anticancer agent (Nguyen and Tawata, 2016), cell cycle suppressor (Fallon, 2015), inhibition of pulmonary fibrosis (Li *et al*., 2015), allelopathic compound (Ahmed *et al*., 2008), and antioxidant (Benjakul *et al*., 2013; Rodrigues-Corrêa *et al*., 2019), among others. In plants (including phytoplankton), akin to L-DOPA, one of the major effects described for mimosine is its bioherbicidal activity (Xuan *et al*., 2006; Williams and Hoagland, 2007; Xuan *et al*., 2013; Mori *et al*., 2015; Sahid *et al*., 2017), in spite of its positive effect on dinoflagellates proliferation when exogenously applied to coastal seawater samples (Yeung *et al*., 2002).

F. β-ODAP

Similar to canavanine, β-ODAP (β-N-oxalyl-L-α, β-diaminopropionic acid) is a toxic NPAA characteristic, but not exclusive, from the Fabaceae. Beta-ODAP is an analogue of the proteinogenic amino acid L-glutamic acid (Kusama-Eguchi, 2019) and occurs naturally in high amounts in leaves and seeds of *Lathyrus* spp. (especially *L. sativus* L., grass pea), although it has also been detected in significant quantities in other leguminous genera (*Crotalaria* and *Acacia* spp.) and even in some non-legume species (*e.g.*, *Panax* spp.) (Kuo *et al*., 2003; Xu *et al*., 2017). In mammalians, when *Lathyrus* seeds are long-term consumed as a staple food or as the major source of proteins, β-ODAP causes a neurotoxic, degenerative disease known as lathyrism (Jiao *et al*., 2011[a]; Kusama-Eguchi, 2019). Albeit the

participation of BIA (β-isoxazolinon-L-alanine) as one of the main β-ODAP precursor molecules is well established in callus cultures and ripening seeds of *L. sativus* L., with possible involvement of the sulfur metabolism in its formation, to date β-ODAP biosynthesis pathway has not been completely elucidated, since not all genes and enzymes involved in this process have been identified (Kuo and Lambein, 1991; Jiao *et al*., 2011[a]; Xu *et al*., 2017). Nevertheless, recent tissue-specific expression analyses and an *in silico* characterization approaches have indicated a putative cysteine synthase gene from grass pea that is possibly directly involved in β-ODAP biosynthesis (Chakraborty *et al*., 2018).

III. Abiotic stresses possibly involved in NPAAs modulation

Different kinds of stresses have been reported as some of the major abiotic stimuli responsible for inducing NPAAs biosynthesis in Monocots and Eudicots. Among them, UV-radiation-, salinity-, hypoxia-, cold-, drought-, and heavy metal-induced stresses are often listed (Seifikalhor *et al*., 2019; Rodrigues-Corrêa, 2019). A summary of some interactions between NPAAs and abiotic stresses is shown in Figure 1. [Figure 1 near here]

GABA intracellular content increases in response to high cytosolic concentrations of hydrogen, which leads to a pH-dependent GAD activation and subsequent GABA synthesis and accumulation. Cytosol acidification is commonly generated by hypoxia conditions. Hypoxia has been shown as a crucial abiotic stimulus on GABA intracellular levels regulation. In soybean, endogenous GABA accumulation was increased after germination under hypoxia condition and freeze-thawing incubation (Yang *et al*., 2016). A similar response was observed in tobacco plants submitted to root flooding. Moreover, the nicotine biosynthesis in these plants proved to be directly related to the accumulation of high contents of GABA in response to hypoxia (Zhang *et al*., 2016).

High cytosolic Ca^{2+} levels have also been identified as modulators of GABA biosynthesis in plants via calmodulin-dependent GAD activity. Modulation can occur under different stress conditions, such as salinity, heat, cold (Kinnersley and Turano, 2000), UV-A, and UV-B treatments (AL-Quraan, 2015).

Analyses of physiological and molecular changes in different parameters (such as germination and growth) in response to salt and osmotic stress in wheat plants (*Triticum aestivum* L.) revealed that GABA shunt participates in wheat adaptation to both of these stresses. Presence of increased levels of, high malondialdehyde accumulation (oxidative damage indicator) and high GAD-decarboxylase gene transcript levels in all five tested wheat cultivars were recorded (AL-Quraan *et al*., 2013). Similarly, the significant increase in GABA accumulation in plants of *Lens culinaris* Medik (Fabaceae) treated with NaCl, mannitol, sorbitol, and H_2O_2 has indicated that GABA-shunt displays a key role in stress-induced signaling, by allowing lentil plants to adapt to salt-, oxidative-, and osmotic-imbalances (AL-Quraan and Omari, 2017). At the transcriptional level, GABA exogenous application on seedlings of *Caragana intermedia* (Fabaceae) treated with NaCl, seemed to regulate H_2O_2 generation-related genes (NADPH oxidase, peroxidase, and amine oxidase), as well as ACC oxidase (1-aminocyclopropane-1-carboxylic acid oxidase, an ethylene biosynthesis-related enzyme). This was confirmed by the inhibition of H_2O_2 accumulation and supported by the reduction of NaCl-generated phenotypic damages in this legume species (Shi *et al*., 2010).

Light-mediated responses were also reported as important factors in accumulation in tomato plants (*S. lycopersicum* L.). After exposure to two different qualities of light (red or

blue) it was found that GABA content in mature post-harvested tomato fruits was higher under blue light irradiation (Dhakal and Baek, 2014).

Induction of GABA-shunt activity in *Nicotiana tabaccum* L. by medium concentrations of Zn may be related to signaling involved in triggering heavy metal stress protection; on the other hand, high concentrations of the metal could be linked to programmed cell death regulated by GABA. These observations indicated that GABA availability in tobacco cells can also be modulated by metal stresses (Daş *et al*., 2016).

An increase in GABA content was also reported as a response to the exogenous application of nitric oxide (NO), a signaling molecule involved in plant response to different stresses, in post-harvested banana fruits (*Musa* spp. cv Brazil). The endogenous GABA content increased with NO treatment, following the elevation of the PA content (through high activity of arginine decarboxylase and ornithine decarboxylase), an increase of GAD and DAO activities, and reduction of GABA-T activity (Wang *et al*., 2016).

CAN accumulation has also been shown to be induced in response to abiotic stresses. CAN levels in *calli* of *S. frutescens* is positively correlated with growth medium nitrate concentration. On the other hand, the presence of NaCl and/or PEG (polyethylene glycol) in the medium did not change significantly the CAN content, suggesting that salinity and drought stresses might not be limiting factors for its production (Colling *et al*., 2010).

Conversely, L-DOPA accumulation is not affected by nitrogen availability in faba bean (*Vicia faba* L.); however, it is strongly regulated by drought-induced stress (Etemadi *et al*., 2018). In this same species, exogenous application of the proteinogenic amino acid tyrosine was found to decrease the constitutive content of L-DOPA in the different tested plant organs (Oviedo-Silva *et al*., 2018). Plant growth regulators have also been found to induce L-DOPA biosynthesis, especially when applied to the culture medium. In suspension

cultures of *Phaseolus vulgaris* L., high levels of L-DOPA were detected over 6 days after elicitation with different concentrations of MeJA (Rahmani-Nezhad *et al*., 2018). In cultures of *Mucuna pruriens*, L-DOPA accumulation was induced upon 2,4-D (2,4-dichlorophenoxyacetic acid) exposure (Brain, 1976), whereas the opposite has also been recorded (Wichers *et al*., 1993). Salt stress has been shown to reduce overall accumulation of L-DOPA and increase the proportion released into culture medium in cell suspensions of *M. pruriens* (Wichers *et al*., 1993). In addition, taking into account the high activity of DDC as an indicator of the presence of an elevated cellular content of its substrate L-DOPA, GSM (Global System for Mobile) non-ionizing radiation might be also considered a potential L-DOPA abiotic elicitor in plants of *Myrtus communis* L. (Stefi *et al*., 2018).

In *Leucaena leucocephala* subsp. *leucocephala* (common leucaena), at the early stages of development, mimosine accumulation has been observed to be regulated by ontogenesis and environmental factors, such as light, mechanical wound (mimicking herbivore attack), besides chemical elicitors application, like SA (pathogen attack simulation) (Vestena *et al*., 2001). In addition to SA, the effect of alternative signaling molecules like jasmonate (JA), and ethephon (ETH, an ethylene-releasing compound), besides UV acute radiation was also tested on mimosine accumulation in 5-week-old seedlings of *L. leucocephala* subsp. *glabrata* (giant leucaena). Albeit no effect has been detected for SA treatment on mimosine biosynthesis induction, shoots and roots of seedlings treated with JA and ETH showed increased content of mimosine compared to the control (untreated plants) 2 and 4 days after treatment application, respectively. Mimosine accumulation was also noticed in giant leucaena shoots 3 and 6 days after UV-C exposure (Rodrigues-Corrêa *et al*., 2019). The chemical nature of the elicitor might influence on mimosine and its metabolites accumulation. Among four different forms of JA tested on the

379 metabolite profile of common leucaena, JA-Ile (jasmonoyl-L-isoleucine) was identified as
380 one of the most effective for 3,4-dihydroxypyridine production (Xu *et al*., 2018).

381 Recently, mimosine accumulation was found to be induced in seedlings of giant
382 leucaena growing under saline condition (NaCl), however, no statistical difference was seen
383 for leaflets treated with metallic salts ($FeCl_3$ and $ZnSO_4$). Differently, $CaCl_2$ treatment
384 decreased the mimosine content in treated leaflets compared to the untreated control (Honda
385 and Borthakur, 2019).

386 Leucaena plants are known for their ability to survive in stressful environmental
387 conditions like drought and wide pH range (Honda *et al*., 2019). Transcriptome, microarray
388 and qRT-PCR analyses of giant leucaena showed an up-regulation of several drought-
389 induced genes (Ishihara *et al*., 2016; Honda *et al*., 2018; Honda *et al*., 2019). Its drought
390 tolerance is thought to be at least partly related to the high accumulation of mimosine in all
391 plant tissues (Honda *et al*., 2019), which suggests regulation of mimosine metabolism
392 mediated by drought. Drought prolonged treatment decreases mimosine accumulation in
393 plants of giant leucaena (Bageel *et al*., 2019). This response has been potentially related to
394 recycling of nitrogen for other uses during stress.

395 Studies on parameters involved in β-ODAP production have shown that its
396 accumulation in seeds and leaves of grass pea is dependent on plant variety and might be
397 regulated by environmental factors (*e.g*., drought, soil availability of mineral and heavy
398 metals) and ontogeny (Jiao *et al*., 2006; Abd El-Moneim *et al*., 2010; Jiao *et al*., 2011[a]). In
399 terms of mineral nutrition, the regulatory effect on β-ODAP production depends on the
400 considered nutrient; *i. e.*, deficiency or oversupply of a particular micro or micronutrient is
401 capable of increasing or decreasing the β-ODAP content in grass pea (Table 1) (Haque *et al*.,
402 2011). For example, high β-ODAP levels were detected in grass pea growing solutions,

403 deficient in zinc, phosphorous, molybdenum, calcium and nitrogen. On the other hand,
404 individual exposure to several organic sources of nitrogen at 2 mM in nutrient solution
405 increased β-ODAP in *Lathyrus sativus* seedlings (Jiao *et al*., 2006). A similarly diverse
406 pattern of response was observed for heavy metal treatments, with increase of the amino acid
407 with Cd and Al, but decrease with the rare earth element Eu (Jiao *et al*., 2011[a]). In callus
408 culture of grass pea, salt treatment (NaCl) was found to decrease the formation of β-ODAP
409 from its precursor BIA (Haque *et al*., 2011). Low β-ODAP levels were also detected in leaves
410 of grass pea displaying high concentration of ROS (superoxide anion and hydrogen
411 peroxide), whereas low ROS leaves had higher concentrations of the amino acid (Jiao *et al*.,
412 2011[b]). Whether this reflects a genetic feature or degradation of β-ODAP under stress as a
413 means to contain oxidative damage is not clear. Osmotic stress generated by PEG and
414 mannitol show variable results depending on the nature and concentration of the osmotic
415 agent in the culture medium and/or the plant stage of development (Haque *et al*., 2011; Jiao
416 *et al*., 2011[a]). On the other hand, positive effect of drought treatment (non-induced by PEG
417 or mannitol) (Jiao *et al*., 2011[a]) on β-ODAP accumulation seems to be consistent. Application
418 of 24-epibrassinolide in well-watered and moderately water stressed plants of *L. sativus* also
419 promoted β-ODAP accumulation (Xiong *et al*., 2016). Several less investigated NPAAs have
420 also shown to have their accumulation modulated by abiotic factors.
421
422 **IV. Conclusion and prospects**
423 NPAAs play diverse important roles in the plant development processes. For instance,
424 they can either act as valuable sources of nitrogen during germination and growth, or have
425 their contents increased as a result of this process (Vestena *et al*., 2001; Kuo *et al*., 2004;
426 Martínez-Villaluenga *et al*., 2006; Wang *et al*., 2015; Huang *et al*., 2017). However, some of

these differences in profile of accumulation pre and post germination may be masked by poorly defined stages of germination. For example, it has been established that mimosine concentration decreases in imbibed seeds and, as seedlings develop, experience an increase, particularly in shoots and in the dark (Vestena *et al*., 2001). As efficient antioxidant molecules, NPAAs are able to mitigate the cell ionic imbalance generated by ROS, by quenching noxious molecules and reducing the oxidative damage in the intracellular milieu. However, depending on the cellular context and concentration, they can also promote oxidative stress by increasing as ROS and RNS. NPAAs can also act by chelating metals thereby protecting against metal-related stress or modulating metal-dependent enzymes. Potential roles as cellular compatible osmolytes have also received some support. Moreover, as part of nectar chemical composition, NPAAs exhibit ecological importance for the reproductive fitness of several species, by working as key molecules in plant-pollinator interactions by affecting the nervous system phagostimulation, and flight of pollinators (Nepi, 2014). In addition, NPAAs work as taxonomic markers in some family of plants, being especially distributed among the Fabaceae genera (Wink, 2013).

The mode of action of the NPAAs is quite diverse, involving alteration of gene expression, metal homeostasis, activity of enzymes, cell osmotic balance, modulation of secondary metabolites, protein defects by misincorporation, among others. A few NPAAs like GABA and BABA can also act as priming molecules of defense mechanisms by enabling plants to respond more rapidly and strongly to biotic and abiotic stresses, besides enhancing stress tolerance (Zimmerli *et al*., 2008; Sós-Hegedüs *et al*., 2014; Buswell *et al*., 2018; Baier *et al*., 2019). On the other hand, the exogenous application of some NPAAs on plants might cause deleterious effects such as growth inhibition and even cell death (Table 2).

It has been shown that endogenous NPAAs accumulation may be highly induced by treatments of drought, salinity, and heavy metals. Conversely, the exogenous application of NPAAs was found to increase plants tolerance to these same classes of challenges. Hence, NPAAs ability to respond to environmental stresses make them efficient priming molecules, by enabling plants to overcome more efficiently new events of biotic and abiotic challenges, as well as to explore environments potentially inhospitable to other species (Figure 2). Extensive research has been performed on the effects of NPAAs exogenous application in plants, especially for GABA. In spite of these efforts, the study of the mechanisms underlying the interaction of abiotic stresses and NPAAs biosynthesis and accumulation is still relatively incipient. Understanding the mechanisms involved in these responses might yield useful tools for developing suitable strategies aiming at improving crop species tolerance to increasingly hostile and/or degraded environments.

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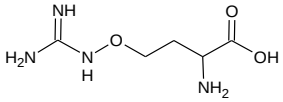
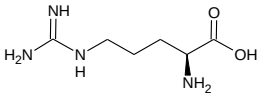
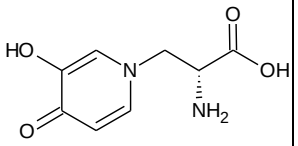
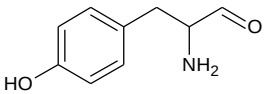
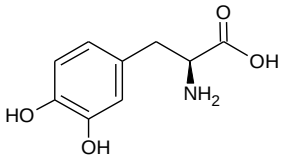
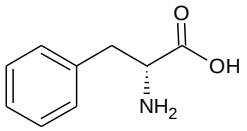
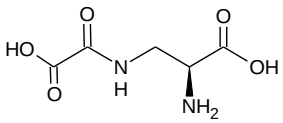
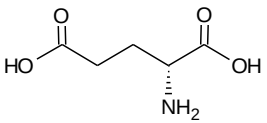
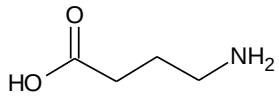
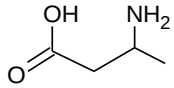
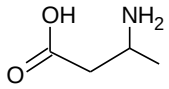
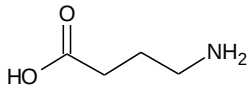
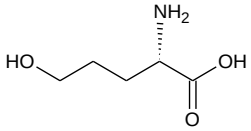
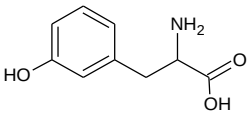
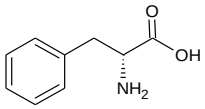
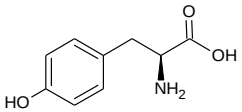
894 Table 1. Main treatments tested to induce NPAAs accumulation in plants.

NPAA	Analog AA	Precursor	Occurrence	Inducibility by abiotic elicitors	References
Canavanine 	**Arginine** 	Canaline	*Canavalia* spp., *Lonchocarpus* spp., *Colutea arborescence* L., *Sutherlandia frutescens* L. R., *Dioclea megacarpa* Rolfe, *Vicia disperma* DC., *Robinia* spp. (Fabaceae)	Nitrogen availability ↑ NaCl ≅ PEG ø	Colling *et al.* (2010)
L-Mimosine 	**L-Tyrosine** 	OAS and 3H4P	*Mimosa pudica* L. *M. pigra* L. *M. bimucronata* (DC.) Kuntze *Leucaena leucocephala* (Lam.) de Wit (Fabaceae)	Mechanical wound ↑ White light ↑ ETH ↑ JA↑ SA↑ UV-C↑ NaCl ↑ $FeCl_3$ ø $ZnSo_4$ ø $CaCl_2$ ↓ Drought ↓	Vestena *et al.* (2001) Xu *et al.* (2018) Bageel *et al.* (2019) Honda *et al.* (2019) Honda and Borthakur (2019) Rodrigues-Corrêa *et al.* (2019)
L-DOPA 	**L-phenylalanine** 	L-Tyrosine	*Mucuna pruriens* (L.) DC. var. *utilis*, *Vicia faba* L. (Fabaceae), *Phlomis fruticosa* L. (Lamiaceae), *Myrtus communis* L. (Myrtaceae)	Nitrogen availability ø Tyrosine ↓ Drought ↑ 2,4-D↑ or ↓ MeJA ↑ NaCl ↓ GSM↑	Brain (1976) Wichers *et al.* (1993) Etemadi *et al.* (2018) Oviedo-Silva *et al.* (2018) Rahmani-Nezhad *et al.* (2018) Stefi *et al.* (2018)

β-ODAP 	**L-Glutamic acid** 	BIA	*Lathyrus sativus* L. *Acacia* spp. *Crotalaria* spp. (Fabaceae) *Panax* spp. (Araliaceae)	↑ [Fe] ↑ B ↑ Drought ↑ 24-epibrassinolide ↑ Heavy metals ↑ or ↓ NaCl ↓ Zn ↓ P↓ N↓ O_2^-↓ H_2O_2↓	Jiao *et al.* (2006) Haque *et al.* (2011) Jiao *et al.* (2011[a]) Xiong *et al.* (2016)
GABA 	**BABA** 	L-glutamate	Widely distributed among different plants species	↑[H+] ↑ ↑[Ca^{2+}] ↑ H_2O_2 ↑ NO ↑ Osmotic stress (mannitol, sorbitol) ↑ Metal stress (Zn) ↑ Hypoxia ↑ Salinity (NaCl) ↑ Heat ↑ Cold ↑ Blue light↑	Kinnersley and Turano (2000) AL-Quraan *et al.* (2013) Dhakal and Baek (2014) Daş *et al*. (2016) Wang *et al.* (2016) Yang *et al*. (2016) AL-Quraan and Omari (2017)
BABA 	**GABA** 		Rare occurence in plants	Unknown	

5- hydroxynorvaline 	Unknown	Unknown	Legume (Fabaceae) and Grass (Poaceae) species	Drought ↑ MeJA↑ AS↑ ABA↑	Carmo-Silva *et al.* (2009) Yan *et al.* (2015)
***m*-Tyrosine** 	**Phenylalanine**  ***p*-tyrosine** 	Phenylalanine in *Festuca rubra* L. ssp. *commutata* or m-hydroxyphenylpyruvate (in *Euphorbia myrsinites*, Euphorbiaceae)	*Festuca* spp. (Poaceae) *Euphorbia myrsinites* (Euphorbiaceae)	Unknown	

NPAAs = non-protein amino acids; L-DOPA = L - 3,4 - dihydroxyphenylalanine; β-ODAP = β-N-oxalyl-L-α, β-diaminopropionic acid; GABA = γ-aminobutyric acid, BABA = β-aminobutyric acid; BIA = (β-isoxazolinon-L-alanine); OAS = *o*-acetylserine; 3H4P = 3-hydroxy-4-pyridone; PEG = polyethylene glycol; ETH = ethephon (ethylene-releasing compound); JA = jasmonic acid; SA = salicylic acid; UV = ultraviolet radiation; 2,4-D = (2,4-dichlorophenoxyacetic acid); MeJA = methyl jasmonate; GSM = (Global System for Mobile) non-ionizing radiation; NO = nitric oxide; ABA = abscisic acid; ↑ indicates positive effect or increased NPAA accumulation; ↓ indicates negative effect or decreased NPAA accumulation; ø indicates no effect observed on NPAA accumulation; ≅ indicates a slight increase in NPAA content.

900 Table 2. Effects of exogenous application of some NPAAs on plants.

NPAA	Tested species	General effect and/or acquired tolerance	Mode of action	References
GABA	*Cucumis melo* L. (Cucurbitaceae)	Salinity-alkalinity stress tolerance	Regulation of redox balance and chlorophyll biosynthesis	Jin *et al*., 2019
	Oryza sativa L. (Poaceae)	As(III) toxicity tolerance	Reduction of TBARS and H_2O_2 levels, elevation of PAAs levels, enhancement of the expression of PA biosynthesis related-genes	Kumar *et al*., 2019
	Cucurbita pepo L. (Cucurbitaceae)	Cold tolerance	GABA-shunt induction (increase of GAD and GABA-T), higher energy and reducing power	Palma *et al*., 2019
	Triticum aestivum L. (Poaceae)	Salt stress tolerance	Ion balance alteration (Transportation restriction of salt ions to leaves)	Wang *et al*., 2019
	Citrus sinensis (Rutaceae)	Increase of phytohormones level	Increase of endogenous GABA and PAAs levels, upregulation of genes involved in phytohormones biosynthesis and induction of GABA-T gene expression	Hijaz *et al*., 2018
	Trifolium repens L. (Fabaceae)	Salt stress alleviation during seed germination	Enhancement of starch catabolism; improvement of antioxidant enzymes activities; osmotic adjustment	Cheng *et al*., 2018
	Agrostis stolonifera (Poaceae)	Heat and drought tolerance	Upregulation of stress-protective genes (antioxidant enzymes and transcription factors responsive to drought and heat stresses)	Li *et al*., 2018
	Nigella sativa L. (Ranunculaceae)	Water deficit stress alleviation, improvement of growth and productivity	Osmoregulation and increase of antioxidant enzymes activity	Rezaei-Chiyaneh *et al*., 2018
	Cucumis melo L. (Cucurbitaceae)	Salt tolerance and alleviation of calcium nitrate-induced growth inhibition	Activity enhancement of nitrogen assimilation enzymes, regulation of transaminase activity	Zhen *et al*., 2018
	Trifolium repens L. (Fabaceae)	Alleviation of drought-induced damage in leaves	Increase of transaminase activity, acceleration of PA biosynthesis and suppression of PA catabolism, activation of proline dehydrogenase activity	Yong *et al*., 2017

Triticum aestivum L. (Poaceae)	Alleviation of drought-induced stress in grains	Increase of chlorophyll content, accumulation of total soluble phenolics, proline and glycinebetaine	Farooq *et al*., 2017
Brassica juncea L. (Brassicaceae)	Chromium stress tolerance	Modulation of the antioxidant defense and glyoxalase system	Mahmud *et al*., 2017
Oryza sativa L. (Poaceae)	Alleviation of cold-induced stress (GABA combined to glutamic acid and calcium chloride)	Membrane integrity restoration	Jia *et al*., 2017
Agrostis stolonifera L. (Poaceae)	Drought tolerance	Enhanced accumulation of protein amino acids (PAAs) and organic acid osmolytes	Li *et al*., 2017
Cassia italica Mill (Fabaceae)	Salt stress tolerance	Increase of enzymatic antioxidant activity	Alqarawi *et al*., 2016
Agrostis stolonifera L. (Poaceae)	Heat stress tolerance	Increase of accumulation of PAAs, organic acids, sugars and sugars alcohols	Li *et al*., 2016[b]
Triticum aestivum L. (Poaceae)	Salt stress tolerance	Improvement of photosynthesis and enhancement of antioxidant enzymes activity	Li *et al*., 2016[a]
Prunus sp. (Rosaceae)	Transient tolerance to root hypoxia in a sensitive genotype	Alterations in mRNA levels of some GAD isoforms, increased of GABA endogenous content, increased CO_2 assimilation rate and stomatal conductance, reduction of chlorophyll content loss and H_2O_2 levels	Salvatierra *et al*., 2016
Cucumis melo L. (Cucurbitaceae)	Alleviation of the growth inhibition $Ca(NO_3)_2$-induced	Improvement biosynthesis of PAs and endogenous GABA and prevention of PA degradation (by enhancing arginine decarboxylase (ADC), ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (SAMDC), polyamine oxidase (PAO), and DAO activities)	Hu *et al*., 2015
Lycopersicon esculentum (*Solanum lycopersicum* L.) (Solanaceae)	Chilling tolerance	Enhancement of antioxidant enzymes activity; preservation of membrane integrity	Malekzadeh *et al*., 2014

	Lolium perenne L. (Poaceae)	Mitigation of drought stress damages	Maintenance of high cell water content and of membrane stability	Krishnan *et al*., 2013
	Hordeum vulgare L. (Poaceae)	Alleviation of aluminum- and proton-induced oxidative damage	Activation of antioxidant defense responses and reduction of carbonylated proteins induced by ROS	Song *et al*., 2010
Canavanine	*S. lycopersicum* L. (Solanaceae)	Oxidative stress generation and oxidative modification of proteins	Enhancement of H_2O_2 content and superoxide radical generation, stimulation of the activity of peroxidase, polyamine oxidase and NADPH oxidase, decreased protein nitration level, increased formation of protein carbonyl groups	Krasuska *et al*., 2016[a]
	S. lycopersicum L. (Solanaceae)	Restriction of root elongation and root malformation	Slight DNA fragmentation, increasing of total RNA and protein level, alteration in NO and $ONOO^-$ localization, decreasing of total respiration rate	Krasuska *et al*., 2016[b]
	S. lycopersicum L. (Solanaceae)	Restriction on root growth	Inhibition of arginine-dependent NOS-like activity; decrease of CAT and SOD activities; decrease of transcript levels of genes encoding CAT; downregulation of *FeSOD*, *CuSOD*, and *SODP-2*; decrease of glutathione reductase transcript levels and glutathione peroxidase activity; low accumulation of *S*-nitrosoglutathione in root tips	Staszek *et al*., 2019
L-DOPA	*Lactuca sativa* L. (Asteraceae)	Cell death and root growth inhibition	Enhancement of polyphenol oxidase activity, of lipid peroxidation and ROS generation (O_2^- and H_2O_2) in root cells	Mushtaq *et al*., 2013
	Glycine max L. (Fabaceae)	Growth inhibition and root meristem damage	Reduction of photosynthetic rate, transpiration and stomatal conductance	Marchiosi *et al*., 2016
	Disphyma australe (Aiton) N.E.Br. (Aizoaceae)	Photoprotection to salinity-exposed leaves	Induction of betalain synthesis	Jain *et al*., 2015
***m*-Tyr**	*S. lycopersicum* L. (Solanaceae)	Root growth inhibition	Increased ROS generation, enhancement of	Krasuska

	nitrosoglutathione reductase activity, formation of nitrated proteins	*et al*., 2017; Andrzejczak *et al*., 2018

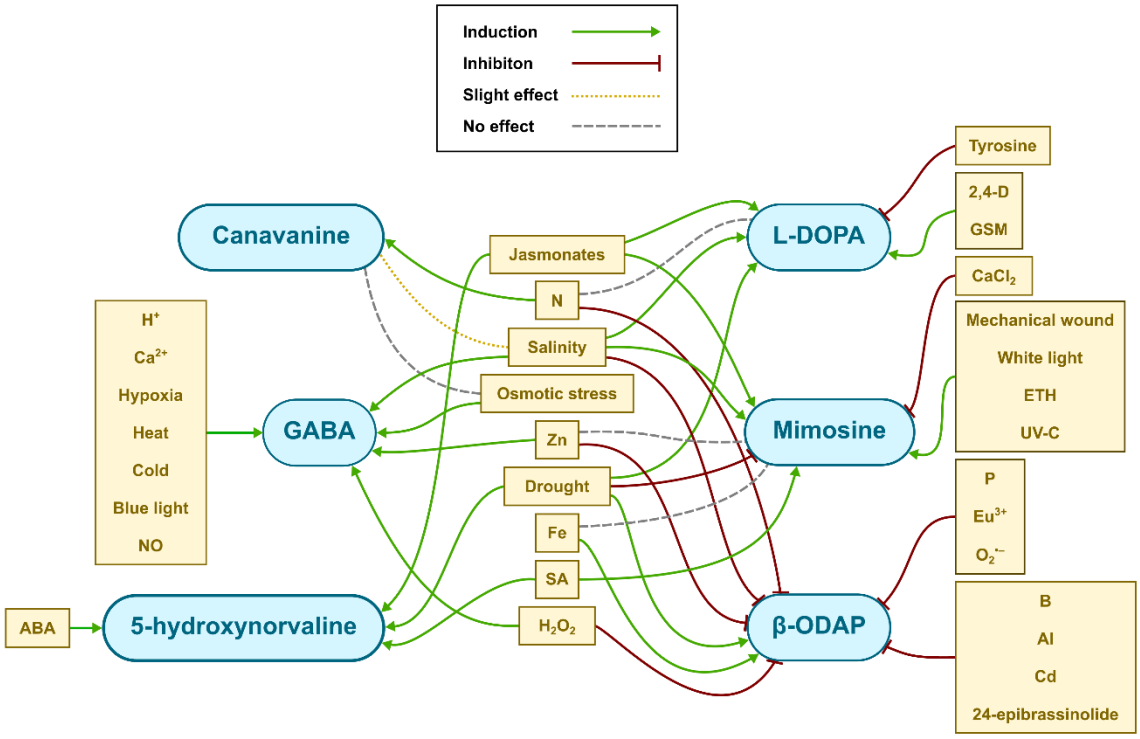


Figure 1. Some interactions between NPAAs and abiotic stresses.

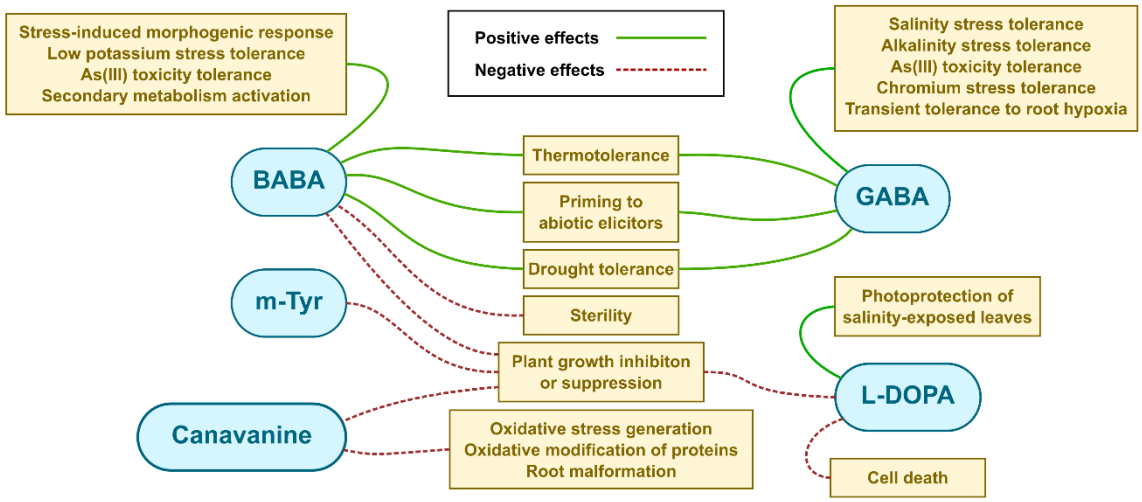


Figure 2. General effects observed in different *taxa* treated with some NPAAs.

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Research article

Mimosine accumulation in *Leucaena leucocephala* in response to stress signaling molecules and acute UV exposure

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ABSTRACT

Mimosine is a non-protein amino acid of Fabaceae, such as *Leucaena* spp. and *Mimosa* spp. Several relevant biological activities have been described for this molecule, including cell cycle blocker, anticancer, antifungal, antimicrobial, herbivore deterrent and allelopathic activities, raising increased economic interest in its production. In addition, information on mimosine dynamics *in planta* remains limited. In order to address this topic and propose strategies to increase mimosine production aiming at economic uses, the effects of several stress-related elicitors of secondary metabolism and UV acute exposure were examined on mimosine accumulation in growth room-cultivated seedlings of *Leucaena leucocephala* spp. *glabrata*. Mimosine concentration was not significantly affected by 10 ppm salicylic acid (SA) treatment, but increased in roots and shoots of seedlings treated with 84 ppm jasmonic acid (JA) and 10 ppm Ethephon (an ethylene-releasing compound), and in shoots treated with UV-C radiation. Quantification of mimosine amidohydrolase (mimosinase) gene expression showed that ethephon yielded variable effect over time, whereas JA and UV-C did not show significant impact. Considering the strong induction of mimosine accumulation by acute UV-C exposure, additional in situ ROS localization, as well as in vitro antioxidant assays were performed, suggesting that, akin to several secondary metabolites, mimosine may be involved in general oxidative stress modulation, acting as a hydrogen peroxide and superoxide anion quencher.

1. Introduction

Different plant groups synthesize a large diversity of secondary or specialized metabolites. These molecules are generally produced in response to biotic and abiotic environmental stresses. Indeed, induction of secondary metabolism usually involves stress-generating factors, which have also been explored in biotechnological processes aiming at the production of target metabolites of economic interest (Matsuura et al., 2018). Metabolic control of nitrogen-containing secondary compounds (*e.g.,* alkaloids and non-protein amino acids) has been shown to be complex and influenced by phytohormones, environmental stresses (seasonality, herbivory, pathogen attack, drought), UV radiation (Hollósy, 2002), methyl jasmonate (MeJA), salicylic acid (SA), yeast extract (Cho et al., 2008), abscisic acid (ABA), heavy metals, osmotic stress (Nascimento et al., 2013) and mechanical wounding (Porto et al., 2014).

Due to their particular trait of associating with N-fixing microorganisms, Fabaceae species (leguminous, *sensu lato*) are often protein rich, hence the relevance of several of these species as forage. Fabaceae species are also known for accumulating nitrogen containing secondary metabolites, which play important roles as ecochemical molecules and, at least for the case of non-protein amino acids, potential cell reservoirs of nitrogen (Huang et al., 2011).

High contents of mimosine, a toxic aromatic non-protein amino acid, are found in species of two leguminous genera, *Leucaena* spp. and *Mimosa* spp.. *Leucaena leucocephala* (Lam.) de Wit (leucaena, koa haole) is a fast-growing leguminous tree native from Central America (southeastern Mexico), widely distributed in tropical and subtropical zones. This species is also characterized by its high tolerance to drought, among other environmental stresses (Honda et al., 2018). Leucaena can be divided into two subspecies: (i) *L. leucocephala* subsp. *leucocephala* (common leucaena, a bushy shrub), and (ii) *L. leucocephala* subsp.

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glabrata (giant leucaena, a tree). The latter has been used as a fast growing tree for production of wood and paper pulp. The foliage of both common and giant leucaena is used as a fodder because of its high protein content and palatability to farm animals. The foliage contains up to 18% protein, 14.2% crude fiber, and 6.4% ether extract/crude fat (Soedarjo and Borthakur, 1996).

Production of nitrogen-containing secondary metabolites such as mimosine requires large amounts of carbon and nitrogen resources. Negi et al. (2014) estimated that up to 21% of the carbon-nitrogen resources may be used for production of mimosine in leucaena. Brewbaker et al. (1972) determined the mimosine content of 96 *L. leucocephala* cultivars and 8 other *Leucaena* species, collected from 38 different countries by growing them in an observational nursery in Hawaii and found that basal mimosine content varied from 1.89 to 4.77% of the dry weight.

Mimosine is biosynthesized from OAS (*o*-acetylserine) and 3H4P (3-hydroxy-4-pyridone or its tautoisomer 3-hydroxy-4-pyridine). A previous analysis suggested that mimosine synthase is an OAS-TL (*o*-acetylserine-thiol-lyase) of the cysteine biosynthesis pathway (Ikegami et al., 1990). Later, however, recombinant enzyme tests did not support an OAS-TL identity of mimosine synthase (Yafuso et al., 2014). Recent findings on mimosine biosynthesis revealed that a cytosolic cysteine-OAS-TL isoform can also catalyze the formation of mimosine under specific conditions (Harun-Ur-Rashid et al., 2018).

Mimosine toxicity is related to its ability of reducing the availability of divalent metal ions, such as Fe(II), Zn(II), Cu(II), Co(II) and Mn(II), by chelating co-factors and preventing their association with metal-dependent enzymes. Furthermore, this non-protein amino acid is capable of forming a stable complex with pyridoxal-5′-phosphate (PLP), leading to the inactivation of PLP-dependent enzymes (*e.g.*, Asp-Glu transaminase and cystathionine synthetase) (Negi et al., 2014).

Mimosine features several useful biological activities, such as allelopathic, antimicrobial, insecticide, cell cycle inhibitor agent, anticancer, phytoremediator (Nguyen and Tawata, 2016), as well as antioxidant (Benjakul et al., 2013). Despite the relatively well established biological activities of purified mimosine on other organisms or cell types, little is known about its biological role in leguminous species. However, it has been suggested that, at least in part, its activity is mainly related to defense mechanisms against some biotic and abiotic stresses and as nitrogen source during fast growth (Vestena et al., 2001).

Suda (1960) and Smith and Fowden (1966) identified enzymes involved in mimosine degradation in seedling extracts of *L. leucocephala* and *Mimosa pudica*. A mimosine-degrading enzyme named mimosinase (mimosine amidohydrolase; EC 3.5.1.61; CAS registry number: 104118-49-2) (IUBMB, 2018), a carbon-nitrogen lyase which degrades mimosine into 3H4P was later purified by Tangendjaja et al. (1986). Its biochemical characterization was described and the cDNA was isolated by Negi et al. (2014).

Although mimosinase has been described and isolated, only few studies on the role played by biotic and abiotic factors on the dynamic modulation of mimosine metabolism in leguminous species have been conducted (Vestena et al., 2001; Xu et al., 2018). In aseptic cultures of leucaena, mechanical injury of shoots promoted local mimosine accumulation (Vestena et al., 2001). In the same study, cultivation in presence of auxin or SA in culture medium also had a positive effect on mimosine accumulation. More recently, the effect of drought treatment on gene expression of leucaena was also evaluated by Honda et al. (2018). However, several potential factors regulating mimosine metabolism need to be further examined.

To date, there is a lack of information on the biological role of mimosine *in planta*, as well as on details of its metabolic dynamics. Moreover, its overt potential for pharmaceutical applications and development of new drugs, as well as the possible use as tool to address heavy metal soil contamination or plant mineral nutrition improvement, justify additional research. The objective of this study was to investigate the effect of stress signaling molecules and acute UV exposure on modulation of mimosine accumulation and metabolism in *L. leucocephala* spp. *glabrata*, in order to better understand its biological role and to identify strategies for yield improvement aiming at exploring its useful bioactivities.

2. Methods

2.1. Plant material

For the experiments carried out to evaluate the effects of elicitors on mimosine accumulation, seeds of leucaena were kindly provided by Dr. James Brewbaker and harvested at CTAHR's (College of Tropical Agriculture and Human Resources of the University of Hawai'i at Manoa), Waimanalo Research Station at O'ahu, Hawai'i. This plant material was originated from the accession K636 of *Leucaena leucocephala* ssp. *glabrata* (Brewbaker, 2008).

2.2. Induced mimosine content in 5-week-old giant leucaena

2.2.1. Seed germination

In order to overcome seed coat dormancy, seeds were submitted to a chemical scarification with sulfuric acid 95–98% for 20 min and repeatedly rinsed in distilled water to remove any residual trace of this reagent. Then, seeds were distributed in 25.4 cm × 50.8 cm plastic trays containing 1:1 v/v of vermiculite and commercial soil watered until reaching substrate field capacity. Three weeks after seed imbibition, seedlings displaying similar size and shape (*e.g.*, number of compound leaves and leaflets) were transplanted to individual pots (250 mL) in number of three plants per container.

During the experimental period (except in the UV-C radiation treatment) all tested seedlings were kept in a growth chamber and submitted to controlled conditions of temperature (circa 25 °C) and irradiance (approximately 100 μmol photons $m^{-2}\cdot s^{-1}$) with a photoperiod of 16 h light and 8 h dark.

2.2.2. Treatments

2.2.2.1. JA, Ethephon, and SA. Five-week-old giant leucaena seedlings were treated with different solutions, as described in Table 1. Ideal concentrations were defined in preliminary experiments under the same conditions indicated above. At the beginning of the experiments 30 plants were sprayed with 84 ppm JA, 10 ppm SA, 10 or 100 ppm Ethephon or Milli-Q® water (control) until the point of imminent runoff. Plant pots were kept closed inside transparent plastic bags for 24 h to avoid solution volatilization. Fifteen plants arranged in 5 sets of 3 (5 biological replicates) were harvested 48 h and 96 h after being treated.

Table 1
Treatments used to modulate mimosine biosynthesis in giant leucaena.

ELICITOR	CONCENTRATION UV FLUENCE	EXPOSURE TIME	RATIONALE FOR USE
Salicylic acid (SA)	10 ppm	24 h	Pathogen signaling molecule (Shah, 2003)
Jasmonic acid (JA)	84 ppm	24 h	Chemical elicitor of plant secondary metabolism (Dar et al., 2015)
Ethephon	10 ppm	24 h	Ethylene releasing-compound (Kim et al., 2016); elicitor of plant secondary metabolism (Wang et al., 2016)
UV-C radiation	3 $J.cm^{-2}$	10 min or 15 min	Elicitor of plant secondary metabolism (Kara, 2013; Neelamegam and Sutha, 2015)

After collection, shoots were separated from roots, immediately frozen in liquid nitrogen and stored at – 80 °C prior to HPLC analyses.

2.2.2.2. UV-C. Thirty seedlings of giant leucaena were exposed to UV-C radiation (3 J.cm^{-2}) for 10 or 15 min and kept in a growth chamber under controlled conditions as described above until the end of the experiments. Fifteen plants arranged in groups of 3 were harvested at 96 h and 120 h after UV-C exposure and processed as previously described.

2.2.3. Mimosine extraction

Mimosine extraction was based on a modified version of the protocol published by Lalitha and Kulothungan (2006), as follows: a known weight of fresh tissue (shoots or roots) of giant leucaena was first added to Milli-Q® boiling water in a proportion of 1:10 (g of plant per mL of solvent) in test tubes. Tubes were covered with foil to avoid solution evaporation and placed on a hot stirrer at 100 °C for 10 min. A proportional volume of 0.1 M HCl was added to the cooled suspensions and homogenized using mortar and pestle. The plant extracts were filtered through cotton and centrifuged twice for 7 min in a bench top refrigerated centrifuge at 4 °C and 13,200 rpm. Before being analyzed, the extracts were diluted 1:3 with *o*–phosphoric acid (*OPA*).

2.2.4. Mimosine detection

HPLC analyses were carried out as described by Negi and Borthakur (2016). Pure mimosine (*L*-mimosine from koa haole seeds, Sigma-Aldrich, CAS number 500-44-7) was used as standard. Separation and quantification of mimosine was done with a C18 column (Phenomenex C18; 5 µm; 4.6 × 250 mm) under an isocratic solvent system of 0.02 M *OPA* with a linear flow rate of 1 mL·min^{-1}. Mimosine detection was done at 280 nm by photodiode array detection (200–400 nm), showing retention time of 2.77 ± 0.042 min. Quantification was done using the method of external standard curve. Further confirmation of mimosine identity was performed by co-chromatography with standard and peak purity check. Chromatograms were analyzed using the Waters Empower 3 software.

2.3. Quantitative real-time PCR analysis of mimosinase gene expression

Fifteen, 8-week-old giant leucaena plants arranged in 4 sets of 3 (4 biological replicates) were treated with either water (control) or 10 ppm Ethephon, 84 ppm JA acid, or 15 min of UV-C radiation exposure following the methods described above. Following treatment, leucaena plants were harvested at 48 and 96 h, or 72 and 144 h (UV-C treated plants only) after treatments. Total RNA of samples was extracted and purified from roots and shoots of giant leucaena by means of a modified method using Qiagen RNeasy Plant Kit (Valencia, CA, USA) and Fruit-mate (Takara, Japan), according to the protocol described by Ishihara et al. (2016a). The assessment of RNA quality and quantity was carried out at 230, 260 and 280 nm by using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, DE, USA). In order to avoid genomic DNA contamination, RNA samples were treated with TURBO DNAfree Kit (Invitrogen, Carlsbad, CA). Two micrograms of DNase-treated RNA were used to synthesize the first-strand cDNA using M-MLV Reverse Transcriptase (Promega, WI, USA).

Quantitative real-time (qPCR) analysis was carried out to examine possible differential expression of the mimosinase gene (GenBank accession number AB298597.1) in seedlings treated with 84 ppm JA, 10 mM Ethephon or 15 min of UV-C exposure. Shoots and roots were harvested 24 h before the time of mimosine concentration peak for each treatment previously observed, as assessed by HPLC assays. The 10 µL qPCR reaction consisted of 5 µL of PowerUpTM SYBR® Green Master Mix (Applied Biosystems, Foster City, CA), 1 µL $MgCl_2$ (50 mM), 0.3 µL forward primer (10 µM), 0.3 µL reverse primer (10 µM), and 1 µL cDNA first-strand. In the experimental validation through qPCR, reaction conditions and melting curve analysis of the amplicon were performed following the protocol published by Ishihara et al. (2016b) for the same leucaena variety. qPCR analysis was conducted using StepOne™ Real-Time PCR System (Applied Biosystems). Measurements were performed using 4 biological and 3 technical replicates. Relative expression was calculated with the $2^{-\Delta\Delta ct}$ method using *OAS-TL* as reference gene, since its expression showed a consistently stable profile comparable to that of *UBQ-5* and *ELF1α* expressions. Mimosinase primer sequences used for these analyses were (FWD) 5′- GAA AGG CAG GAA TCA CAG TGA AGA G – 3'; (REV) 5′ GGA GAC TCT AGC CAC ACC AAC TTA – 3'.

2.4. Antioxidant assays

2.4.1. Mimosine effect on hydrogen peroxide (H_2O_2) accumulation

As a follow up to the induction of mimosine accumulation profiles under stress signals and conditions, tests were conducted to verify mimosine antioxidant capacity. In situ histological localization of hydrogen peroxide (H_2O_2) accumulation was evaluated on foliar disks of *Phaseolus vulgaris* L., according to the protocol described by Shi et al. (2010). Briefly, the plant foliar tissue was exposed to 1 mg·mL^{-1} diaminobenzidine (DAB) solution in 10 mM KH_2PO_4 (control) in presence or absence of 10 mM mimosine (equivalent to the average mimosine concentration induced by UV-C radiation in giant leucaena), or 10 mM ascorbic acid (positive antioxidant control). Oxidative response was identified by the formation of a brown polymer on the injured leaf areas, indicating the presence of H_2O_2, and registered in a Leica M165 FC stereomicroscope (Leica Microsystems).

2.4.2. Mimosine quenching of superoxide radicals

Generation of superoxide radical and subsequent analysis was performed by a modified protocol based on Zhishen et al. (1999). Nitro blue tetrazolium (NBT) reduction was used to measure superoxide anions quenching activity. Shortly, a 50 mM KH_2PO_4 pH 7.8 solution containing 6 µM riboflavin, 100 mM methionine, 1 mM NBT, in presence or absence of 5 mM mimosine was exposed to white light (22 J·cm^{-2}) for 25 min, on a white light transilluminator. Five micromolar rutin was used as positive control (Matsuura et al., 2016). The absorbance was read at 560 nm before and after light exposure in a SpectraMax® M2 Microplate Reader (Molecular Devices, LLC).

2.5. Statistical analyses

For HPLC and superoxide anions data, simple analyses of variance (ANOVA) followed by Tukey, or Welch ANOVA followed by Dunnett's C test were used as appropriate for data distribution characteristics. In qPCR analysis, results were analyzed by *t*-test. In all cases, at least four biological triplicates were used and experiments were repeated twice independently. All data were analyzed using the statistical package SPSS 20.0 for Windows (SPSS Inc., USA). In all cases a $p \leq 0.05$ was used.

3. Results and discussion

3.1. Increased mimosine concentrations in giant leucaena treated with chemical elicitors

Leucaena produces high amounts of mimosine that accumulate in all parts of the plants including leaves, stem, flowers, pods, seeds, roots and root nodules (Soedarjo and Borthakur, 1998). The highest concentrations of mimosine can be found in the growing shoot tips and seeds (Wong and Devendra, 1983). It is not known why leucaena produces such high amounts of mimosine. Negi et al. (2014) estimated that leucaena plants would be able to grow 21% larger if the nutrient resources spent on mimosine production were diverted for biomass increase. In a previous analysis performed to quantify the basal concentration of mimosine present in adult plants of common leucaena, the highest constitutive amount of mimosine per gram of fresh weight in

the analyzed organs was found in post-anthesis flowers (894.48 μg), followed by green pods (826.87 μg), leaves (673.58 μg) and green flower buds (512.47 μg), which showed significantly less mimosine concentration compared to the other reproductive structures (Supplementary Fig. 1). Since mature seeds have very low moisture content (Wencomo et al., 2017), its mimosine concentration was estimated as 3382.53 $\mu g \cdot g^{-1}$ of dry weight. Additionally, it was also observed that the basal mimosine distribution in shoots of field-grown adult plants of leucaena is dependent on the variety type (Supplementary Table 1).

Phytohormones, such as salicylic acid and jasmonic acid, are known to be produced by plants in response to various abiotic and biotic stresses. These phytohormones trigger adaptive responses to stress by regulating major plant metabolic processes, such as photosynthesis, nitrogen metabolism, defense systems, and plant-water relations, thereby providing protection (for review see Khan et al., 2015).

Secondary or specialized metabolite production and accumulation are also known to be controlled by biotic and abiotic stresses (Matsuura et al., 2018). In this study, exposure of 5-week-old giant leucaena seedlings to JA or Ethephon treatments significantly enhanced mimosine accumulation in shoots and roots in at least one of the two time points tested (48 and 96 h), albeit in a different way (Fig. 1). The highest concentrations of mimosine in shoots were found in seedlings treated with JA 84 ppm (434.41 $\mu g \cdot g^{-1}$) and Ethephon 100 ppm (384.12 $\mu g \cdot g^{-1}$), two days after application of the respective phytohormones. Nevertheless, after four days, shoots yielded the highest concentration of mimosine (approximately 460 $\mu g \cdot g^{-1}$) upon treatment with 10 or 100 ppm Ethephon (Fig. 1A). In roots, after two and four days, JA 84 ppm and Ethephon 10 ppm resulted in highest mimosine accumulation, 184.88 $\mu g \cdot g^{-1}$ and 158.01 $\mu g \cdot g^{-1}$, respectively (Fig. 1B). These observations show that mimosine accumulation response to specific elicitors may vary over time after exposure.

Although all treatments were applied exclusively on shoots of giant leucaena seedlings, roots of some of them were also able to respond to the different elicitors. Overall, shoots displayed higher basal and induced mimosine concentration compared to roots (Fig. 1), which agrees with previous observations in 1 to 3-week-old aseptic seedlings of common leucaena (Vestena et al., 2001). However, as previously mentioned, significant post-induction increase of mimosine concentration in roots and shoots simultaneously was only observed for JA and Ethephon 10 ppm on day 02 and 04, respectively (Fig. 1).

It is well established that perceived regulatory signals or elicitors generate a transduction network mediated by secondary messengers resulting in changes in gene expression profiles that afford adaptive responses to environmental stimuli. These modulation events are often mediated by transcription factors (TFs), which directly bind to specific gene promoters or act by forming complexes with repressor proteins labeling them to degradation, subsequently releasing other TFs to proceed with the gene expression program. This is the case of the action mechanism of JA and its active form, jasmonoyl isoleucine, for example (Kazan, 2015; Wasternack and Strnad, 2016).

JA, ethylene and SA are known as important stress regulatory signals in plants. JA, however, is thought to be the most effective signal for induction of plant secondary metabolism (Wasternack and Strnad, 2016) thereby contributing to mitigation of damage caused by several stresses (Dar et al., 2015). JA is mainly derived from linolenic acid

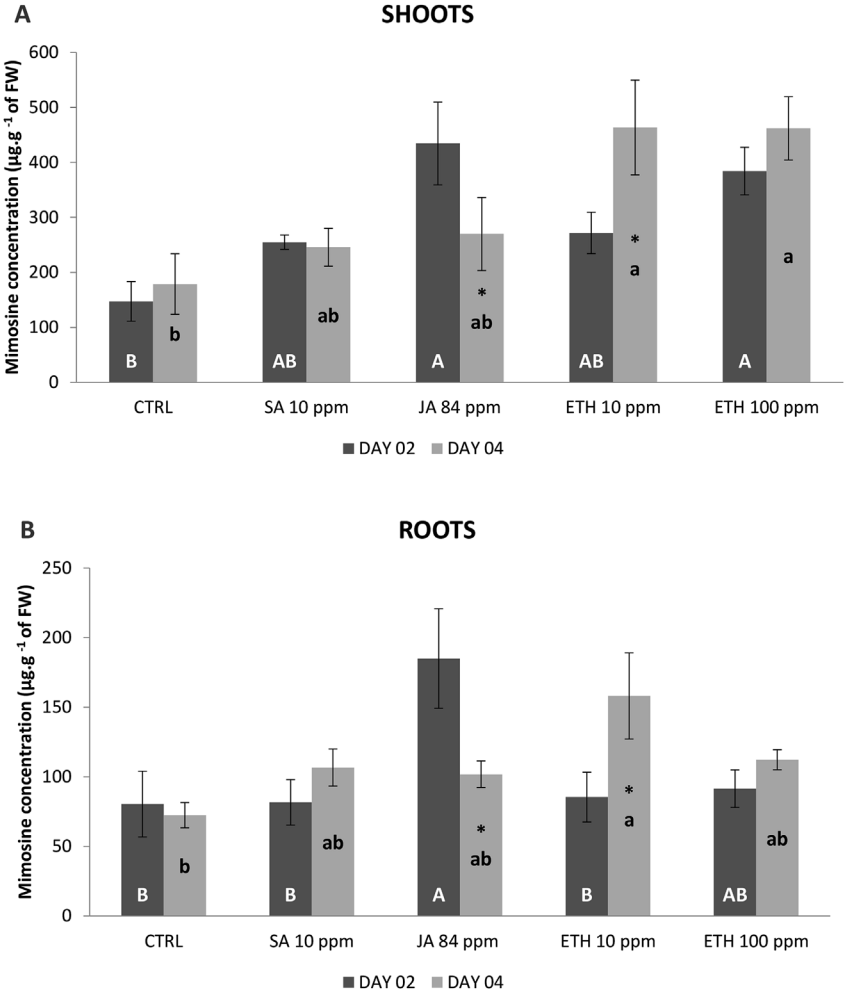

Fig. 1. Mimosine concentration in shoots (**A**) and roots (**B**) of 5-week-old giant leucaena seedlings treated with different elicitors. CTRL = Milli-Q water; SA = Salicylic Acid; JA = Jasmonic Acid; ETH = Ethephon. Bars sharing a letter of same case do not differ by Tukey test ($P \leq 0.05$). Capital letters (A, B) compare treatments on day two and lowercase letters (a, b) compare treatments on day four. *Indicates significant statistical difference between day two and day four in the same treatment by *t*-test ($P \leq 0.05$). The error bars represent standard error of five replicates (each mean was calculated with 15 individual seedlings organized in 5 groups of three).

(Wasternack and Strnad, 2016), playing important roles in different processes of plant growth and development, such as plant defense mechanisms against herbivory, pathogen attack, fungal elicitation and some abiotic factors such as osmotic, temperature and salt stresses (Dar et al., 2015).

JA and its methyl ester MeJA have several different effects on leguminous species. MeJA exogenous application has increased isoflavonoid content in cell suspension cultures of *Pueraria candollei* var. *candollei* and *P. candollei* var. *mirifica* (Korsangruang et al., 2010), as well as the production of the triterpenoid glycyrrhizin in *Glycyrrhiza glabra* roots. Enhanced production of the triterpenoid, however, was partly at the expense of root growth (Shabani et al., 2009). MeJA application on shoots was observed to suppress root nodulation and lateral root formation in *Lotus japonicus* (Nakagawa and Kawaguchi, 2006). In grapevine, a non-leguminous species, proteinogenic amino acids did not show an expressive increase under MeJA treatment (Gutiérrez-Gamboa et al., 2017).

The effects of the application of four different jasmonate forms (JA, MeJA, jasmonoyl-L-isoleucine (JA-Ile) and 6-ethyl indanoyl glycine conjugate (2-[(6-ethyl-1-oxo-indane-4-carbonyl)-amino]-acetic acid methyl ester - CGM) on leucaena metabolite profile has recently been reported by Xu et al. (2018). JA-Ile form was most effective, although no major alteration was observed on monitored metabolite abundances. Alanine, threonine and 3,4-dihydroxypyridine (3,4 DHP, a metabolite derived from mimosine degradation) (Nguyen and Tawata, 2016), among others, were the major metabolites elicited by JA-Ile. In contrast to the results described here, mimosine concentration did not change significantly. These divergent results on mimosine accumulation may be due to a number of factors, including mode of application, jasmonate form used (JA-Ile x JA), and *L. leucocephala* subspecies (common x giant leucaena).

Ethylene is also a phytohormone involved in plant response mechanisms to different types of challenges, such as mechanical damage and insect attack, among others. The integration mechanism between JA and ethylene signaling pathways is not completely understood; however, it has been shown that they may work cooperatively in abiotic stress tolerance (Kazan, 2015). MeJA can induce ethylene production (Zhao et al., 2004), and when applied simultaneously, these molecules seem to work in a synergic way, by enhancing the magnitude of the plant response to external stimuli (Liu et al., 2016).

Treatment with SA was able to significantly increase mimosine accumulation in 12-week-old plants of common leucaena (Supplementary Fig. 2). However, no significant effect of SA treatment on mimosine concentration was seen in 5-week-old seedlings of giant leucaena (Fig. 1), suggesting some degree of genotype and/or age dependency in elicitation by this phytohormone. On the other hand, several treatments, including 90 ppm MeJA, 10 and 100 ppm 2-chloroethylphosphonic acid (CEPA, an ethylene-releasing compound) significantly increased mimosine accumulation (Supplementary Fig. 2), in agreement with the data obtained for giant leucaena. The lack of systemic effects of externally applied SA on mimosine accumulation was also observed when the phytohormone was supplied in the culture medium of aseptically-grown seedlings, in which case only roots had higher content of mimosine (Vestena et al., 2001). This could be due to transport limitations or to low methyl salicylate production from applied SA, since the former is recognized as the main systemic signaling form (Vlot et al., 2009).

3.2. Increased mimosine concentrations in giant leucaena exposed to UV-C radiation

UV-C treatment promoted increased concentration of the amino acid in shoots but not in roots of giant leucaena (Fig. 2). Increased accumulation of mimosine in shoots was also observed in 12-week-old seedlings of common leucaena exposed to UV-C radiation for 10 and 15 min (Supplementary Fig. 3). Similar to the SA treatment, in giant leucaena UV-C radiation did not induce mimosine biosynthesis in roots regardless of time after exposure. The absence of mimosine induction in roots by SA and UV indicates that these effectors do not cause a systemic response. Moreover, roots are shielded from irradiance by the presence of substrate.

UV radiation effects on different aspects of plant metabolism and development have been described. However, compared to UV-B (environmentally relevant type of UV radiation) assays, there are less reports related to the UV-C effects on secondary metabolites biosynthesis and accumulation (Cetin, 2014), especially in leguminous (Fabaceae) plants. They generally concern primary metabolism aspects, such as growth and development. For instance, seedlings of *Phaseolus vulgaris* L. (Fabaceae) exposed to low intensity UV-C radiation have displayed decreased chlorophyll content and reduced height after 14 days of exposure (Kara, 2013). Negative effects on growth parameters and nitrogen metabolism were also observed in *Vigna radiata* L. (Fabaceae) after UV-B radiation treatment, in addition to adverse effects on JA, SA and antioxidant compounds accumulation (Choudhary and Agrawal, 2014a). The same authors reported increased accumulation of flavonoids, SA and JA, besides negative effects on growth, biomass, yield, nitrogen fixation and accumulation in 2 cultivars of *Pisum sativum* L. (Fabaceae) under elevated UV-B treatment (Choudhary and Agrawal, 2014b). Despite the negative UV influence on growth reported for the previously mentioned leguminous, UV-C radiation on groundnut plants (*Arachis hypogaea* L.; Fabaceae) increased seedling vigor and biomass and had no adverse effect on germination or other development parameters (Neelamegam and Sutha, 2015).

Besides its impact on growth and primary metabolism, UV exposure can cause important changes in secondary metabolism depending on intensity and time of exposure (Matsuura et al., 2013). UV-B and UV-C pre-treatments of *Artemisia annua* (Asteraceae) seedlings yielded increased biosynthesis of artemisinin, a drug which displays anti-malarial properties and activity against some others infectious diseases (*e.g.* schistosomiasis, leishmaniasis and hepatitis B), and several kinds of tumors (Rai et al., 2011). The accumulation of nicotine in *Nicotiana rustica* plants (Solanaceae) was also increased by UV-C treatment (Tiburcio et al., 1985). Similar inducing effects on production of several secondary metabolites were observed in callus cultures of *Vitis vinifera* L. Öküzgözü (grapevine, Vitaceae) treated with a UV-C source for 5 or 10 min (Cetin, 2014).

Regarding amino acid biosynthesis in response to UV radiation, Martínez-Lüscher et al. (2014) have found that, in spite of not causing changes in total amino acid content, UV-B radiation exposure can affect their profile in grape berries. Proteinogenic amino acids have been known to be important targets of the deleterious effects of UV radiation (Hollósy, 2002). On the other hand, in the present study, acute UV-C treatment was found to increase mimosine accumulation in shoots by over twofold (Fig. 2), which may suggest a possible participation of this molecule as part of the antioxidant defense system in *L. leucocephala*. This possibility is further supported by the induction of the amino acid accumulation by JA and Ethephon, involved in abiotic and biotic stress responses, which are generally associated with oxidative imbalance and are signaling components in high UV stress (Matsuura et al., 2013).

3.3. Mimosinase gene expression

In order to determine if increases in mimosine content upon exposure to JA, CEPA or UV-C radiation were related to changes in transcription of mimosine metabolism-related genes, RT-qPCR analysis was carried out. The complete pathway for mimosine biosynthesis has not yet been determined, although the final step has been characterized. Based on transcription analysis (Ishihara et al., 2016a), leucaena appears to encode for multiple cysteine synthases, one or more of which may be able to catalyze mimosine synthesis. In addition, a leucaena gene encoding a mimosinase (an enzyme responsible for mimosine degradation) has been identified and characterized (Negi et al., 2014).

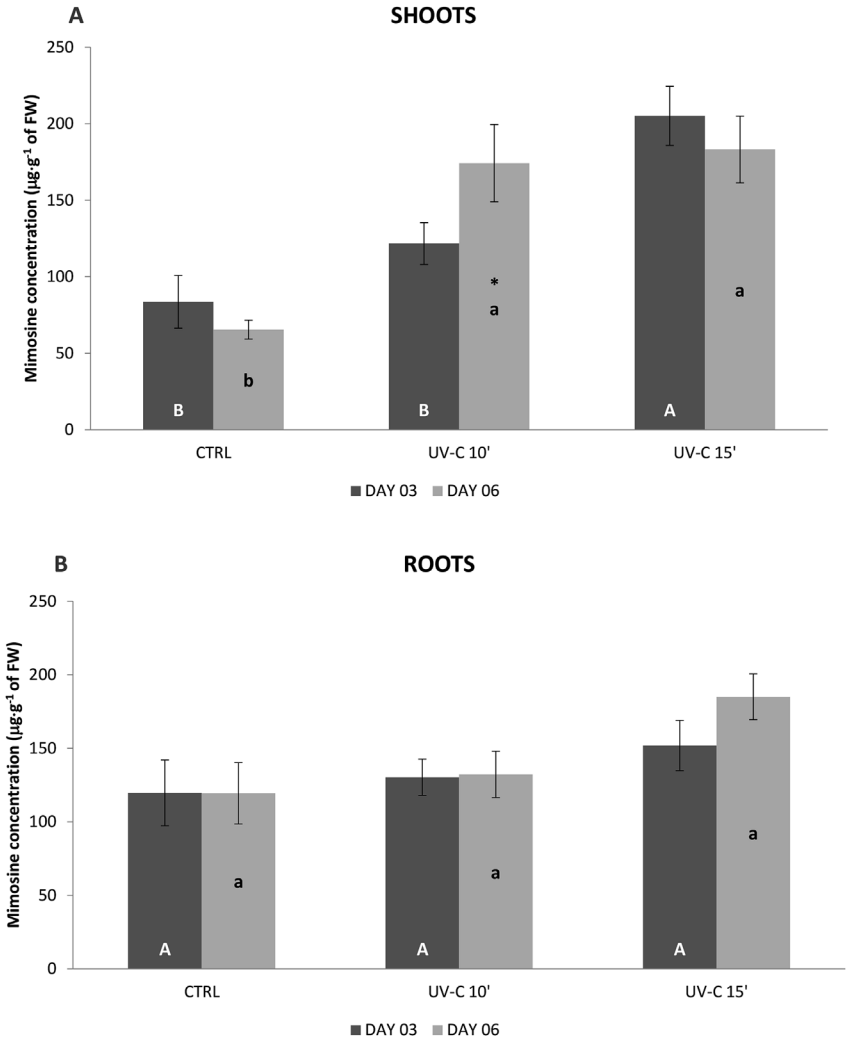

Fig. 2. Mimosine concentration in shoots (**A**) and roots (**B**) of 5-week-old giant leucaena seedlings exposed to UV-C light. CTRL = visible light (100 µmol photons m^{-2} s^{-1}); UV-C 10′ and UV-C 15' = UV-C exposure time (10 and 15 min respectively). Bars sharing a letter of same case do not differ by Tukey test ($P \leq 0.05$). Capital letters (A, B) compare treatments on day three and lowercase letters (a, b) compare treatments on day six. *Indicates significant statistical difference between day three and day six in the same treatment by *t*-test ($P \leq 0.05$). The error bars represent standard error of five replicates (each mean was calculated with 15 individual seedlings organized in 5 groups of three).

In addition to mimosinase gene expression, several gene isoforms belonging to the cysteine pathway [cysteine synthase (*CYS SYN*), serine acetyltransferase (*SAT*) and *β*-cyanoalanine synthase (*CAS*) Table 2 - supplementary material] were also tested in this study (data not shown). However, expressions of these genes did not vary in giant leucaena throughout the experiments, suggesting that the increased content of mimosine observed in the treated plants might not be related to the expression of these genes, but presumably to increased enzyme activity and/or release from conjugates, such as mimoside, a mimosine *β-D*-glucoside (Murakoshi et al., 1972).

Considering the time variation of mimosine accumulation observed in this work, mimosinase gene expression in shoots and roots was evaluated 24 h before the increase of mimosine concentration in giant leucaena seedlings (*i.e.*, 24 h and 72 h after the chemical elicitors treatments; and 48 h and 120 h after UV-C exposure).

Ethylene signaling has been shown to up-regulate expression of several genes related to secondary metabolism pathways, as is the case of phenolic compounds (Liu et al., 2016) and terpenoid indole alkaloids (Wang et al., 2016). Among all elicitors tested in the present work, Ethephon was the only one able to significantly change mimosinase gene expression. Leucaena plants treated with Ethephon showed significant increases in mimosine concentration at both day 2 and 4 following treatment, which coincided with low-level expression of mimosinase. Up-regulation of mimosinase gene expression was detected 24 h before the increase of mimosine concentration in shoots treated with 10 ppm of Ethephon (Fig. 3A), but not after JA or UV-C treatments (Fig. 3C-D and 3E-F, respectively). Nevertheless, 72 h after treatment application (24 h before the highest mimosine content measured in shoots), down regulation of mimosinase gene was seen in both shoots and roots treated with 10 ppm of Ethephon (Fig. 3B). These data indicate that mimosine content in leucaena plants is at least partly regulated by mimosinase expression in Ethephon exposed plants. On the other hand, the fact that mimosinase mRNA was not significantly affected by JA and UV-C treatments, despite their stimulating effects on mimosine biosynthesis in giant leucaena, may indicate that other levels of regulation are at play or that the chosen harvesting time window was unable to detect relevant changes.

3.4. *In situ and in vitro antioxidant assays*

Considering the stimulation of mimosine accumulation by Ethephon, JA and UV, all of which are often associated or known to cause oxidative imbalance, the antioxidant capacity of mimosine was evaluated. Mimosine has been shown to have antioxidant activities on cultured cancer cells (Parmar et al., 2015). In the present study, it was hypothesized that mimosine could confer radical scavenging properties, which would contribute to plant protection from possible damage caused by reactive oxygen species generated during stress (Supplementary Fig. 4).

Foliar disks of *P. vulgaris* L. were treated with 10 mM mimosine for 15 min. Treated disks showed less hydrogen peroxide accumulation induced by wounding in contrast to untreated ones, being comparable to those treated with ascorbic acid (a known hydrogen peroxide neutralizer) (Fig. 4A). These observations support a possible antioxidant

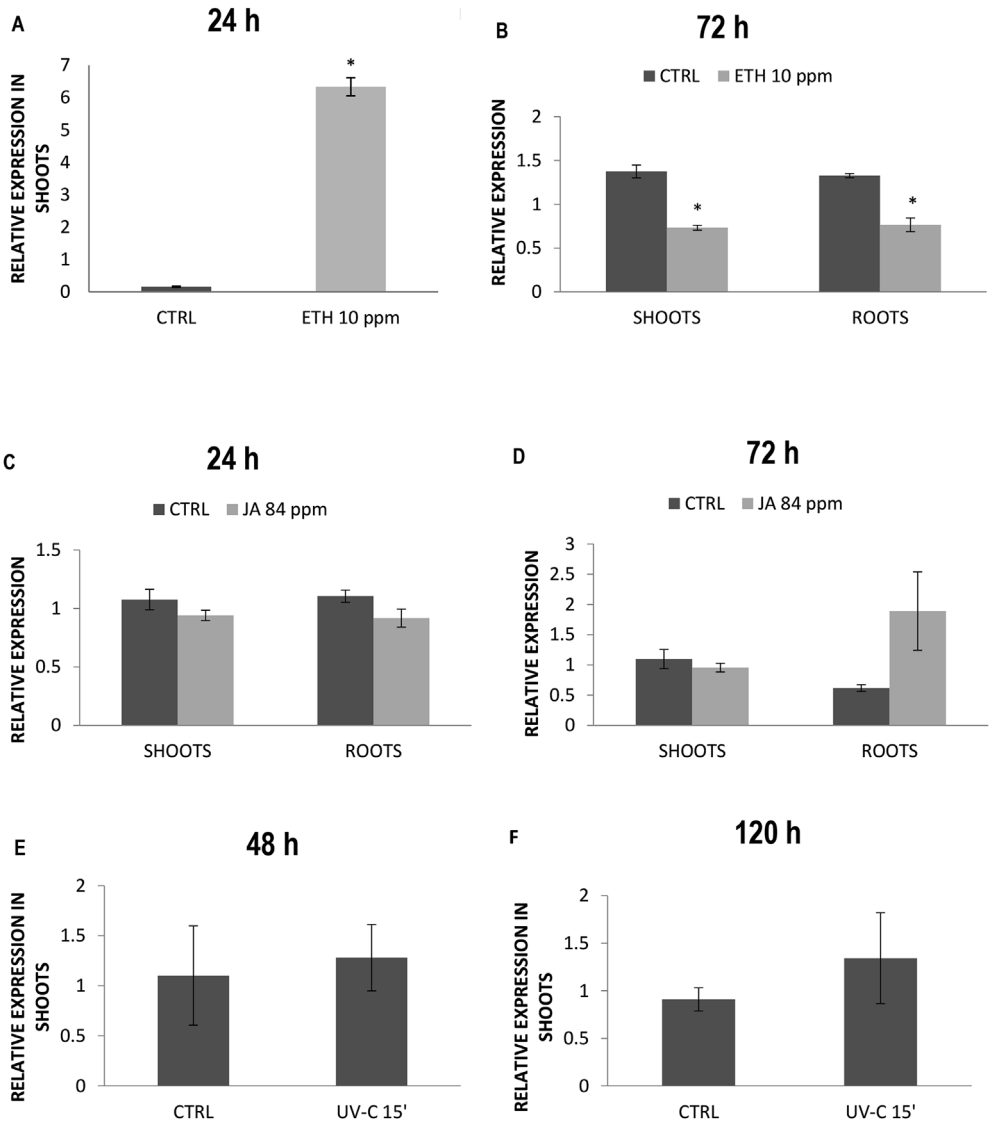

Fig. 3. Relative expression of the mimosinase gene in shoots (**A**, **E** and **F**) and shoots and roots (**B**, **C** and **D**) of giant leucaena 24 h (**A** and **C**), 48 h (**E**), 72 h (**B** and **D**) and 120 h (**F**) after treatment with stress signaling molecules or UV-C exposure. ETH = Ethephon, JA = Jasmonic Acid. *Indicates significant statistical difference between control and treatment by *t*-test ($P \leq 0.05$). The error bars represent standard error of four replicates.

role of mimosine as an in situ hydrogen peroxide scavenger.

Mimosine was also able to quench superoxide anions generated by light exposure. Mimosine exhibited equivalent antioxidant effect compared to rutin (Fig. 4B), a well-established effective superoxide anion quencher (Matsuura et al., 2016) The radical scavenging activity of mimosine may be due to the 3-OH group of the pyridine ring of mimosine (Fig. 5). The pK_a of the 3-OH of mimosine has been estimated to be 8.8 (M. Honda, unpublished results). At physiological pH, this OH group is expected to remain in a protonated state and therefore may scavenge a radical by donating a proton and an electron. In this process, mimosine itself is converted to a stable radical form, which is perhaps less toxic and less reactive than the reactive oxygen species generated during oxidative stress. It is likely that the less toxic radical mimosine produced may react with another radical or molecule, and become converted to a non-reactive indole molecule.

In vivo antioxidant activity of mimosine has been previously evaluated by means of its exogenous application on selenium-deficient seedlings of *Vigna radiata.* In spite of its allelopathic properties (Ahmed et al., 2008), the results showed mitigation of mitochondrial oxidative stress by treatment with 0.1 mM mimosine (Lalitha and Kulothungan, 2007). DPPH radical scavenging activity was also reported for aqueous seed extracts of leucaena rich in mimosine and phenolic compounds in *in vitro* assays (Benjakul et al., 2014). Mimosine antioxidant activity shown in the present work is in good agreement with data reported for other non-protein amino acids, such as *L*-DOPA (Dhanani et al., 2015) and GABA (Malekzadeh et al., 2014), for instance.

4. Conclusion

Taken together, results show that mimosine biosynthesis and accumulation can be modulated by stress-related factors, despite its relatively high constitutive content in leucaena plants. The pattern of gene expression in stressed plants suggests mimosine steady-state control may be regulated by its degradation, in possible connection with dynamic changes in carbon and nitrogen metabolism of stressed plants. Mimosine quenching activity against hydrogen peroxide and superoxide anions in the in situ staining and in vitro assays, respectively, showed that this non-protein amino acid can act as non-enzymatic antioxidant agent. Increase in mimosine content in response to elicitors mimicking environmental challenges, in addition to its antiherbivore and antimicrobial properties, may be related to its activity as protective molecule against oxidative damage, in line with other classes of plant

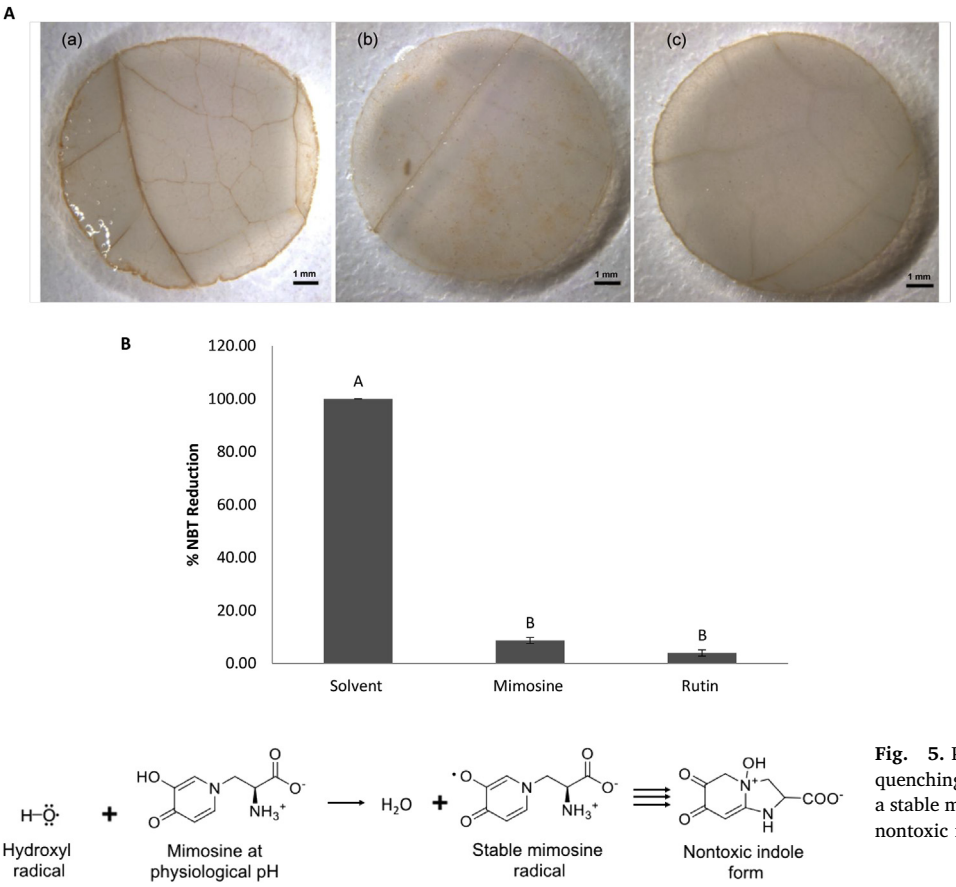

Fig. 4. A. In situ antioxidant assay. Foliar disks of *Phaseolus vulgaris* L. treated with (**a**) No antioxidant added (negative control); (**b**) 10 mM Mimosine; (**c**) 10 mM ascorbic acid (positive control). The oxidative damage can be seen by the formation of a brown polymer in leaf veins and injured areas. **B.** In vitro superoxide scavenging assay carried out with mimosine. Different letters indicate significant difference by Tukey test ($P \leq 0.05$). The error bars represent standard error of four replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 5. Predicted mimosine radical formed following quenching of hydroxyl radical. Mimosine is first converted to a stable mimosine radical, which may be then converted to a nontoxic indole form.

secondary metabolites.

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CRediT authorship contribution statement

Kelly Cristine da Silva Rodrigues-Corrêa: Investigation, Validation, Writing – original draft. **Michael D.H. Honda:** Investigation, Validation. **Dulal Borthakur:** Supervision, Writing – review & editing, Funding acquisition. **Arthur Germano Fett-Neto:** Supervision, Funding acquisition, Writing – review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2018.11.018.

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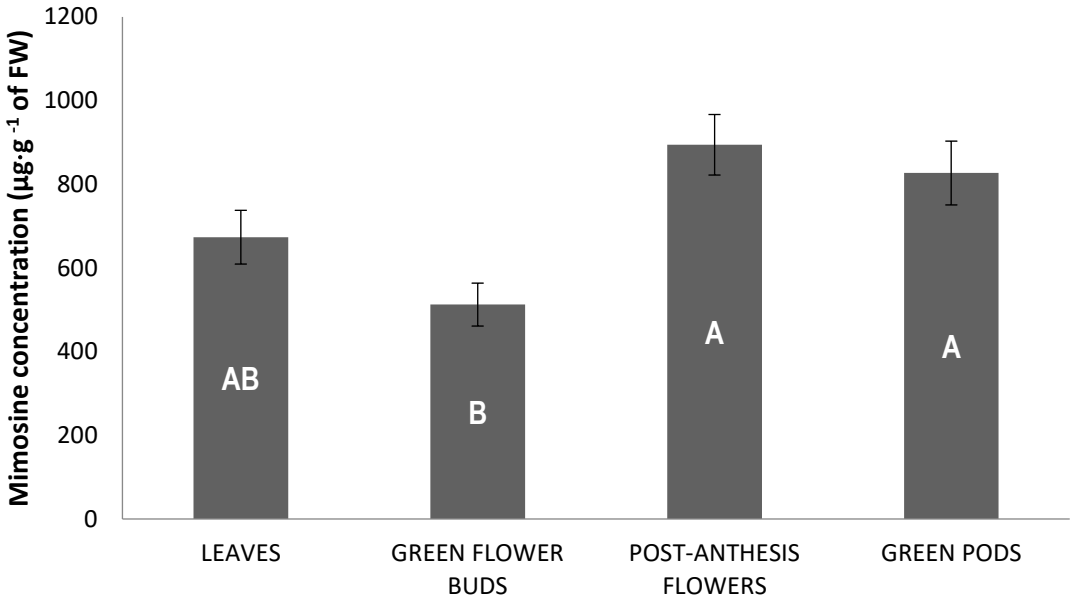
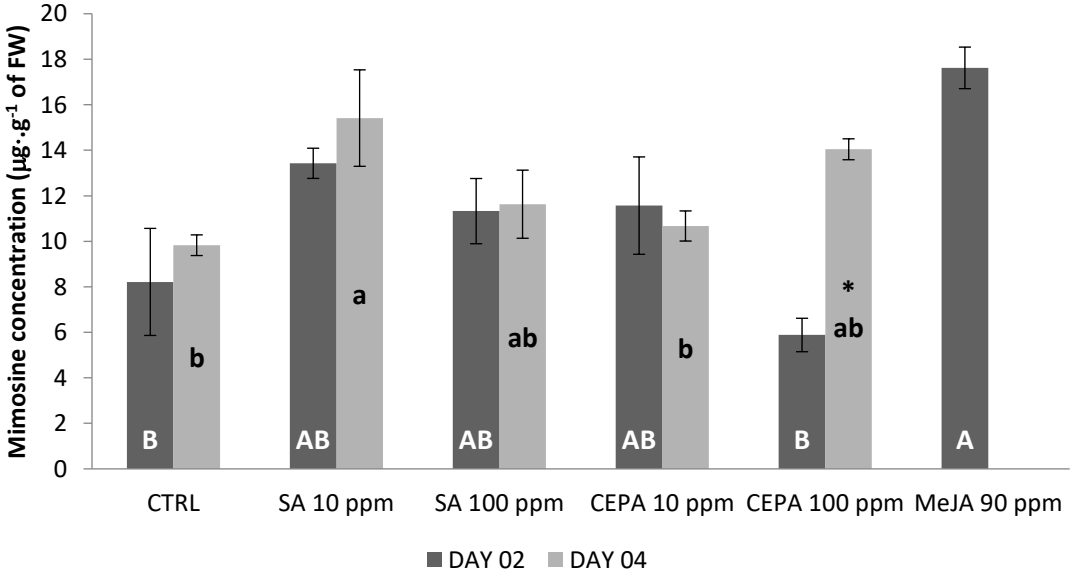
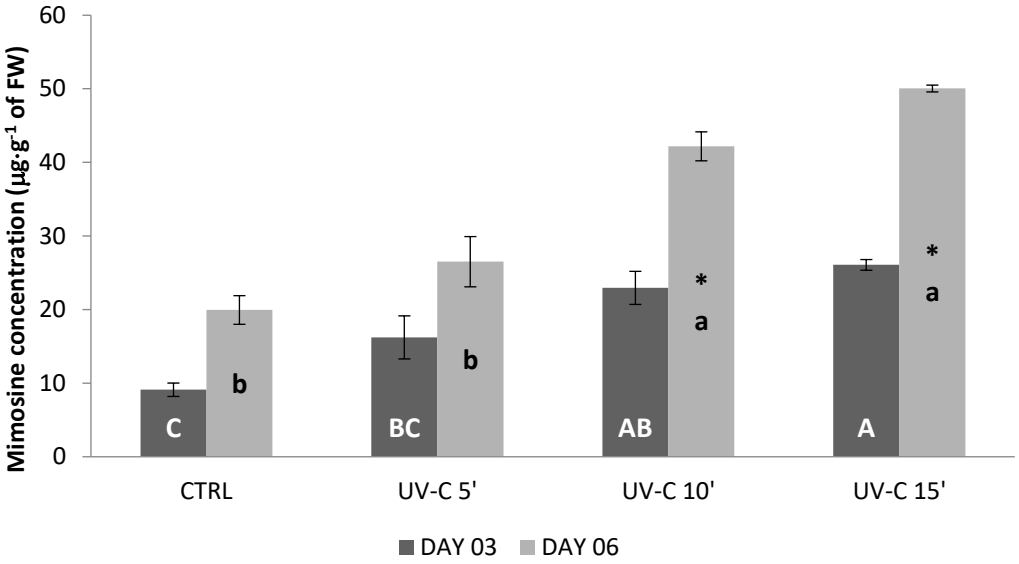


Supplementary Fig. 1. Basal mimosine concentration in adult trees of common leucaena (*L. leucocephala* var. *leucocephala*). Samples were collected from 10 field grown trees at Manoa Valley, Honolulu, Hawai'i on June 25th, 2017. Bars sharing a letter do not differ by Tukey test ($P \leq 0.05$). The error bars represent the standard error.

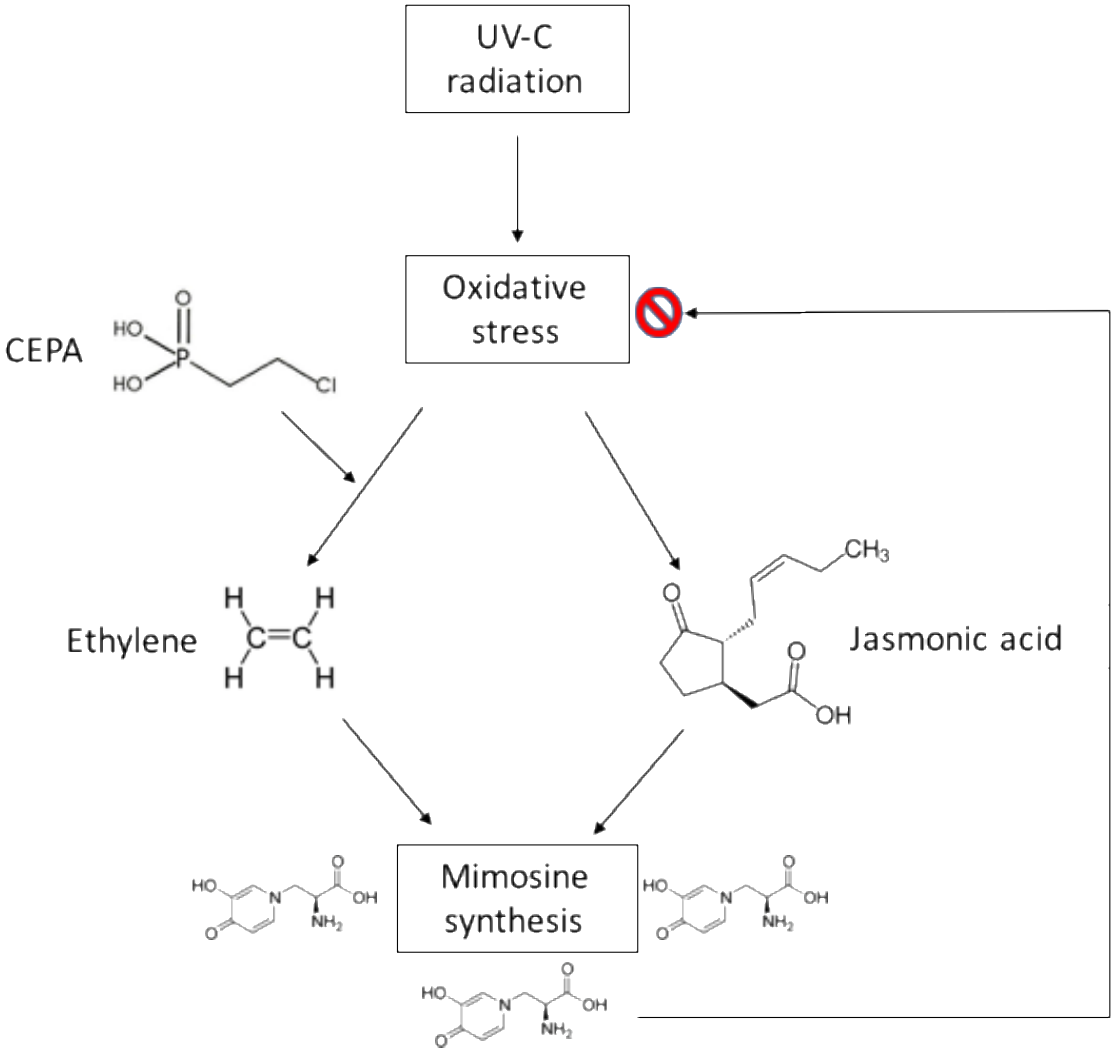


Supplementary Fig. 2. Bar diagram showing mimosine concentration in shoots of 12-week-old common leucaena seedlings treated with different elicitors. CTRL = Milli-Q water; SA = Salicylic Acid; MeJA = Methyl Jasmonate; CEPA = *2*-Chloroethylphosphonic acid (an ethylene releasing compound). Bars sharing a letter of same case do not differ by Tukey test ($P \leq 0.05$). Capital letters (A, B) compare treatments on day two and lower-case letters (a, b) compare treatments on day four. *Indicates significant statistical difference

between day two and day four in the same treatment by *t*-test ($P \leq 0.05$). The error bars represent standard error of five replicates (each mean was calculated with 15 individual seedlings organized in 5 groups of three).



Supplementary Fig. 3. Bar diagram showing the effects of UV-C radiation exposure for 5, 10, and 15 min on mimosine accumulation in shoots of 12-week-old seedlings of common leucaena. Bars sharing a letter of same case do not differ by Tukey test ($P \leq 0.05$). Capital letters (A, B, C) compare treatments on day three and lower-case letters (a, b) compare treatments on day six. *Indicates significant statistical difference between day three and day six in the same treatment by *t*-test ($P \leq 0.05$). The error bars represent standard error of five replicates (each mean was calculated with 15 individual seedlings organized in 5 groups of three).



Supplementary Fig. 4 Model depicting induction of mimosine synthesis in leucaena following application of stress elicitors, such as CEPA and jasmonic acid, or exposure to UV-C radiation. The additional mimosine synthesized may serve to alleviate oxidative stress induced by UV-C radiation.

Supplementary Table 1. Mimosine contents in leaves of common and giant leucaena.

Leucaena type	Mimosine content (% FW)	Mimosine content (% DW)	Dry matter content (% FW)	Water content (% FW)
Common (1)	0.50 ± 0.09	2.45 ± 0.51	20.11 ± 0.54	79.89 ± 0.54
Common (2)	0.43 ± 0.06	2.14 ± 0.37	19.98 ± 0.50	80.02 ± 0.50
K636 (1)	0.70 ± 0.14	3.56 ± 0.77	19.08 ± 0.52	80.92 ± 0.52
K636 (2)	0.42 0.05	2.05 ± 0.33	20.08± 0.93	79.92± 0.93
KX2 (1)	1.22 ± 0.11	6.08 ± 0.82	19.39 ± 1.23	80.61 ± 1.23
KX2 (2)	1.34 ± 0.10	6.23 ± 0.56	20.29 ± 1.14	79.71 ± 1.14
KX3 (1)	0.44 ± 0.06	2.21 ± 0.30	19.45 ± 0.73	80.55 ± 0.73
KX3 (2)	0.54 ± 0.05	2.73 ± 0.23	19.30 ± 0.38	80.70 ± 0.38
KX4 (1)	0.86 ± 0.11	4.71 ± 0.65	17.53 ± 0.84	82.47 ± 0.84
KX4 (2)	0.89 ± 0.11	4.76 ± 0.65	18.0 ± 0.72	82.0 ± 0.72
KX5 (1)	0.99 ± 0.12	4.89 ± 0.48	19.07 ±0.60	80.93 ± 0.60
KX5 (2)	1.15 ± 0.15	5.48 ±0.80	19.92 ± 0.53	80.08 ± 0.53

Common leucaena variety koa haole grows widely on the island of O'ahu. K636 is widely grown variety of giant leucaena. KX2, KX3, KX4 and KX5 are giant leucaena varieties developed through interspecies hybridization (Brewbaker 2016). (1) and (2) indicate plants from two separate locations within the University of Hawaii Waimanalo Research Center. The values are shown as mean ± standard error obtained from at least three biological replicates.

Supplementary Table 2. GenBank accession numbers of the tested cysteine pathway genes isoforms.

Gene name	GenBank accession
OAS-TL (o-acetylserine-thiol-lyase)	GDRZ01032940
	GDRZ01061620
	GDRZ01153117
	GDSA01187555
	GDSA01196891
	GDSA01214467
Cys syn (cysteine synthase)	GDRZ01015860
	GDRZ01050898
	GDRZ01086813
	GDRZ01193515
	GDRZ01202579
	GDSA01180863
	GDSA01215622
SAT (serine acetyltransferase)	GDRZ01187456
	GDRZ01189631
CAS (β-cyanoalanine synthase)	GDRZ01054066
	GDRZ01175418
	GDSA01118400

SHORT COMMUNICATION

Mimosine occurrence and accumulation in *Mimosa bimucronata* var. *bimucronata* (DC.) Kuntze

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ABSTRACT

Mimosine is a non-protein aromatic amino acid present in plants of *Leucaena* spp. and *Mimosa* spp. *Mimosa bimucronata* var. *bimucronata* (DC.) Kuntze (maricá) is a native tree from Brazil which occurs as a pioneer species on plant succession processes. In the current study, the presence of mimosine in *M. bimucronata* was verified by HPLC analyses. Moreover, mimosine accumulation upon exposure to UV-C and chemical elicitors of specialized metabolism (salicylic acid - SA, methyl jasmonate - MeJA, sodium nitroprusside - SNP and ethephon - ETH), most of which also known as promoters of the amino acid production in leucaena plants, was evaluated. The results showed a lower concentration of constitutive mimosine present in both maricá seedlings and mature trees when compared to leucaena plants. In spite of a trend towards increased mimosine accumulation observed in MeJA and ETH treatments, no statistical differences were found with the various stressors used to induce its biosynthesis in maricá seedlings. Data suggest that mimosine in *M. bimucronata* is probably a phytoanticipin-like metabolite or its accumulation is driven by other types of stresses.

Keywords: Mimosine, *Mimosa bimucronata*, stress

Introduction

Mimosa bimucronata, commonly known as maricá, is a native tree from Brazil (REFLORA 2019), ecologically important in plant succession and in processes of degraded land recovery (Bitencourt et al., 2007; Silva et al., 2011), occurring as a pioneer species (Pilatti et al., 2019). Maricá is a deciduous or semi-deciduous plant which reaches up to 15 m in height and 40 cm of diameter at breast height (DBH), displays shrub or tree habit and bears typical sharp thorns (Carvalho, 2004). This species belongs to Fabaceae, one of the most economically important families of flowering plants due to its high diversity and occurrence in different types of habitats (Gomes et al., 2018). As well as several others *Mimosa* spp., maricá is usually referred to as a multipurpose tree (Olkoski and Wittmann, 2011), employed for alternative medicinal uses (Champanerkar et al., 2010; Silva et al., 2011), honey production, constructions and remodeling of landscape architecture (living fences), for instance (Marchiori, 1993; Lorenzi, 1998).

In southern Brazil, maricá is widely distributed and typically found either in wetland areas close to river banks (Patreze and Cordeiro, 2004) or composing large and almost pure landscape formations on hillsides (Jacobi and Ferreira, 1991). In dense populations, this species, like several *Mimosa* spp. (Simon and Proença, 2000), is considered an important and highly invasive weed by preventing cattle to reach pasturesand water bodies as a result of its thorny branches (Lorenzi, 2008; Kestring et al., 2009). Its dominant and nearly exclusive pattern of distribution in those areas has led Jacobi and Ferreira (1991) to test its allelopathic potential on cultivated species. Indeed, extracts of leaves and ripe fruits (but not the green ones) of maricá showed phytotoxic effects on germination and initial radical growth of most of the target species tested.

Several investigations have been performed on maricá floristics (Silva et al., 2011), distribution (Simon and Proença, 2000), wood anatomy (Marchiori, 1993), cytogenetic parameters (Olkoski and Wittmann, 2011) and allelopathic potential (Jacobi and Ferreira, 1991; Ferreira et al., 1992). However, excluding two recent publications on maricá constitutive chemical composition (Schlickmann et al., 2017; Pilatti et al., 2019) which identified phenolic compounds (methyl gallate and water-soluble tannins) as its major compounds, little is known regarding this subject. In other *Mimosa* species (*e.g*. *M. pudica* and *M. pigra*), mimosine has been identified (Soedarjo and Borthakur, 1998) as one of the major specialized metabolites present in the different organs of the plant (Champanerkar et al., 2010). The presence of this molecule was also reported for *M. bimucronata* in a thin layer chromatography-based preliminary study performed by Ferreira et al. (1992), showing co-chromatography of a leaf extract component with authentic mimosine. The authors attributed the allelopathic effect of maricá to the accumulation of this metabolite in its leaves.

Mimosine is an aromatic non-protein amino acid initially found in plants of *Mimosa pudica* and later in *Leucaena leucocephala* (Lam.) de Wit (Soedarjo and Borthakur, 1998), a leguminous tree which biosynthesizes large amounts of this nitrogen-containing compound (Rodrigues-Corrêa et al., 2019). It is believed that the accumulation of high contents of mimosine in *L. leucocephala* tissues confers, among other traits, defense against herbivores and pathogens (Vestena et al., 2001), tolerance to drought (Negi et al., 2014), as well as general oxidative stress protection (Rodrigues-Corrêa et al., 2019). Interestingly, drought is the opposite environmental and physiological condition to that observed in the wet habitats occupied by native populations of *M. bimucronata* in Brazil (Patreze and Cordeiro, 2004; Kestring et al., 2009) and *Mimosa pudica* Linn in India (Champanerkar et al., 2010).

Nonetheless, flooding is also associated with oxidative stress, particularly as water levels change (Fukao et al., 2019).

In *Leucaena leucocephala* var. *leucocephala* (common leucaena) and *Leucaena leucocephala* var. *glabrata* (giant leucaena) mimosine accumulation has been shown to be both constitutive and inducible by stress-related phytohormones, such as jasmonic acid (JA), Ethephon (ETH: an ethylene- releasing compound), salicylic acid (SA - only common leucaena) (Vestena et al., 2001), as well as by UV-C radiation (Xu et al., 2018; Rodrigues-Corrêa et al., 2019). On the other hand, there is a lack of information regarding mimosine content and elicitation effects in *Mimosa* spp. plants.

The aim of this study was to examine the presence of mimosine in *Mimosa bimucronata* and examine the effects of stresses and stress-signaling molecules on its accumulation in leaves.

Material and Methods

Plant material

For all experiments, the plant material was collected at Morro Santana, campus do Vale of UFRGS (Federal University of Rio Grande do Sul), Porto Alegre, RS, Brazil (30°04'S, 51°08'W). Authorization for access to genetic material was obtained from SISGEN-Brazil (license number A845493). Constitutive mimosine content in adult plants of *M. bimucronata* var. *bimucronata* (DC.) Kuntze was determined in plant material (leaves, green flower buds, post-anthesis flowers and green pods) harvested in January 2017 (summer). A voucher herbarium specimen (ICN 187953) was deposited in the ICN – UFRGS herbarium (Herbário do Instituto de Biociências of UFRGS).

For mimosine elicitation experiments, legumes (fruits) of maricá were collected in the end of June 2017 (winter). Seeds were then removed from the dry fruits and kept in the dark until sowing and seedling development for use in the assays.

Seed germination

To break the coat-imposed seed dormancy, after surface sterilization, dry seeds of maricá were acid scarified by immersion in H_2SO_4 (95 – 98 %) for 2 min (see Corrêa et al., 2008) and repeatedly washed in distilled water to remove any residue of the acid. Then, seeds were distributed in 50 mL individual plastic tubes (dibble-tubes) (3.0 cm diameter x 12.0 cm depth) filled up with 1:1 (v/v) of commercial top soil and vermiculite. Tubes were watered every 2 days to avoid substrate dryness and were kept in a growth room under controlled conditions of light (*circa* 75 μmol $m^{-2} \cdot s^{-1}$ photosynthetically active radiation; photoperiod of 16 h light and 8 h dark) and temperature (24±2°C).

Treatments

In order to verify inducibility of mimosine accumulation in *M. bimucronata*, fifty 12-week-old maricá seedlings (per treatment) exhibiting similar features were selected and sprayed (saturated) with solutions of different chemical stressors (plant specialized metabolism elicitors), as follows (for further details see Rodrigues-Corrêa et al., 2019): 10 and 50 mM SA (pathogen-signaling molecule; Shah, 2003), 0.07 and 0.35 mM 2-chloroethylphosphonic acid (ETH, ethylene releasing-compound; Kim et al., 2016; Wang et al., 2016), 100 and 200 mM MeJA (Dar et al., 2015), 10 and 50 mM SNP (a nitric oxide donor; Perotti et al., 2015). Alternatively, maricá seedlings were also supplemented with UV-C radiation (13 minutes; 10.5 kJ cm^2) (elicitor of plant specialized metabolism; Kara, 2013).

After 2 and 4 days of exposure to the chemical treatments, and 3 and 6 days of UV-C supplementation, maricá shoots were harvested, immediately frozen in liquid nitrogen and stored at – 80 °C until mimosine extraction and HPLC analyses.

Mimosine extraction and detection

Mimosine extraction was conducted according to the modified protocol described by Rodrigues-Corrêa et al. (2019) for *L. leucocephala*. HPLC (Thermo Scientific Surveyor) analyses (mimosine detection and quantification) were performed following previously published procedures (Negi et al., 2014). A C18 column (ACE C18; 5 μm; 4.6×250 mm) and isocratic solvent system of 0.02M *o*-phosphoric acid with a linear flow rate of 1 mL · min $^{-1}$ were used to separate and quantify the amino acid. Mimosine detection was performed at 280 nm by photodiode array detection (200–400 nm) and retention time (2.29±0.024 min). Mimosine quantification was done by means of the method of external standard curve. Additional confirmation of mimosine identity was performed by co-chromatography with standard (Acros Organics authentic mimosine 99 % used as reference) and peak purity check. The analyses of the chromatograms were done with the ChromQuest software.

Results and Discussion

Constitutive accumulation of mimosine in *M. bimucronata*

Mimosine was detected in all analyzed samples, positively meeting all identification criteria. In agreement with what has been found for other *Mimosa* spp. (Soedarjo and Borthakur, 1998), compared to *L. leucocephala* adult plants (Rodrigues-Corrêa, 2019), mimosine content was lower in *M. bimucronata*. Of the adult plant tissues analyzed, the

highest content of mimosine in maricá (per gram of fresh weight - FW) was found in post-anthesis flowers (36.644 µg *versus* 894.48 µg in common leucaena, followed by leaves (28.838 µg x 673.58 µg), green flower buds (28.094 µg x 512.47 µg), and green pods (19.002 µg x 826.87 µg) (Fig. 1).The same pattern is observed for seedlings when both species are compared. In this study, untreated 12-week-old maricá seedlings (control at day 2) showed a shoot content of mimosine of 23.029±0.07 $\mu g \cdot g^{-1}$ of (FW). Five-week-old untreated giant leucaena seedlings, cultivated in similar conditions, exhibited between 83.640 and 178.736 $\mu g \cdot g^{-1}$ of FW (Rodrigues-Corrêa et al., 2019). In the same way, mimosine concentration percentage in dry matter of *Mimosa pigra*, was found to be rather low (0.02 % in nodules and roots, and 0.07 % in leaves) (Soedarjo and Borthakur, 1998).

In this investigation, the lowest constitutive mimosine content was found in green pods (Fig. 1). This result may partly explain the absence of phytotoxic effect observed for green pods on germination and growth of crop target plants tested by Jacobi and Ferreira (1991), compared to the other maricá parts analyzed.

Elicitation of mimosine biosynthesis in *M. bimucronata*

Chemical stressors

Secondary metabolites (or natural products) are structural- and chemically specialized compounds, derived from primary metabolism. These molecules are mainly biosynthesized as part of a complex defense mechanism, in response to biotic and abiotic stresses, such as pathogens, herbivores, water status, metal toxicity, and UV radiation, for example (Matsuura et al., 2018). Ethephon, SA, SNP, MeJA have been extensively used as chemical elicitors of specialized metabolism (Wang et al., 2016; Vestena et al., 2001; Perotti

et al., 2015; Zhang and Memelink, 2009; Xu et al., 2018). These phytohormonal signals can simulate environmental challenges and modulate plant homeostasis, often leading to alterations in gene expression (Shinozaki et al., 2015). Except SNP, all treatments tested in the present study showed positive effect on mimosine accumulation in common or giant leucaena (Vestena et al., 2001; Rodrigues-Corrêa, 2019; Rodrigues-Corrêa unpublished data). However, in spite of the trend of increasing the mimosine content observed in seedlings treated with 0.07 mM Ethephon (at day 2) and 100 mM MeJA (at day 4), no statistical difference was confirmed for these treatments when compared to the control.

On the other hand, a within treatment difference on mimosine induction was seen between day 2 and 4 in seedlings treated with 100 mM MeJA (Fig. 2). In a lower concentration (0.4 mM), jasmonic acid (JA)promoted a near threefold increase in mimosine accumulation of giant leucaena seedlings after 2 days of application.

UV-C radiation

Albeit UV-C radiation is not biologically active in natural environments, it has been widely used under controlled experimental conditions to generate acute responses of plant specialized metabolism within a shorter period of time compared to that required to with UV-B radiation (Kara, 2013; Cetin 2014). This fast response is due to the higher energy of UV-C photons that act as potent reactive oxygen species (ROS) generators, causing extensive damage to the cells either at the physiological level or on DNA structure (Gregianini et al., 2003; Matsuura et al., 2013).

Although divergent responses can be observed in plants exposed to UV-C radiation, the deleterious processes are usually reported on primary metabolism (decreasing of chlorophyll content and plant height, *e.g.*) (Kara, 2013). In the present study, no statistical

differences were observed in the mimosine concentration in maricá seedlings supplemented with UV-C radiation. However, a decreasing in its content was found for both control and treatment at day 6 post-treatment (Fig. 03). Taking into account the lower constitutive concentration of mimosine observed in maricá compared to the leucaena plants, besides its relative thermolability (Nguyen and Tawata, 2016), it seems to be plausible to consider the effect of the temperature inside the UV-C and the white light (control) chambers as an additional abiotic factor contributing to the decrease of mimosine accumulation in both group of plants.

Besides mimosine identification, the presence of 3,4-dihydroxypyridine (3,4-DHP or 3-hydroxy-4-pyridone - 3H4P), a mimosine degradation product (Negi et al., 2014; Nguyen and Tawata, 2016), was also reported for maricá leaf extracts analyzed by TLC by Ferreira et al., (1992). In our chromatograms, we detected a second large peak after that of mimosine (2.29±0.024) and similar to that identified by Negi et al. (2014), as 3H4P (data not shown). Comparing the chromatogram profiles obtained from seedlings elicited with chemical stressors and those supplemented with UV-C, the largest area for this peak was found (in all samples) in the latter treatment at day 6. It might indicate that the constitutive and/or the initially UV-C-induced mimosine was degraded into 3H4P to cope with the cellular damage caused by this treatment, associated with an increased temperature inside the chambers. Nevertheless, it was not possible to determine 3H4P concentration (or confirm its identity) in maricá plants, since there is no commercial standard (pure 3H4P) available for purchase to be used as a reference in calculations. Establishment of improved protocols for obtaining in house 3H4P reference substance by acid hydrolysis is ongoing.

Conclusion

On the basis of the overall absence of effect of the treatments tested here on mimosine concentration, it is possible to suggest that its accumulation profile is similar to that of phytoanticipins, unlike what is observed for the same amino acid production in leucaena, which shows features of inducibility resembling phytoalexin-like metabolites. Alternatively, a putative inducible pool of mimosine in maricá might be involved in other types of stress, such as extended drought periods. If involved in protection against oxidative stress as described for leucaena, mimosine in maricá may act predominantly by physical quenching of ROS, as indicated by the lack of overt chemical degradation. Nevertheless, further investigations are needed to assess these hypotheses.

To sum up, mimosine biosynthesis was not modulated by the treatments evaluated as in *L. leucocephala* (Lam.) de Wit. To the best of our knowledge this is the first work that analytically identifies and quantifies mimosine accumulation in *M. bimucronata*.

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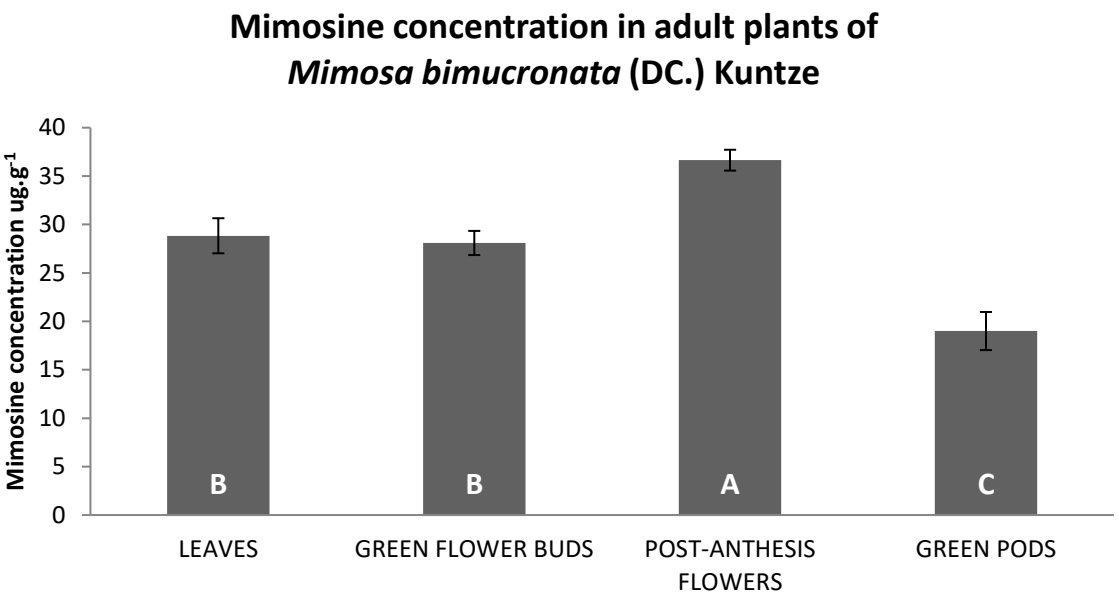

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347 Figure 1. Constitutive concentration of mimosine in different plant organs of *Mimosa*
348 *bimucronata*. Bars sharing the same letter do not differ statistically by Tukey test ($P \leq 0.05$).
349 The error bars denote standard error of 10 replicates.

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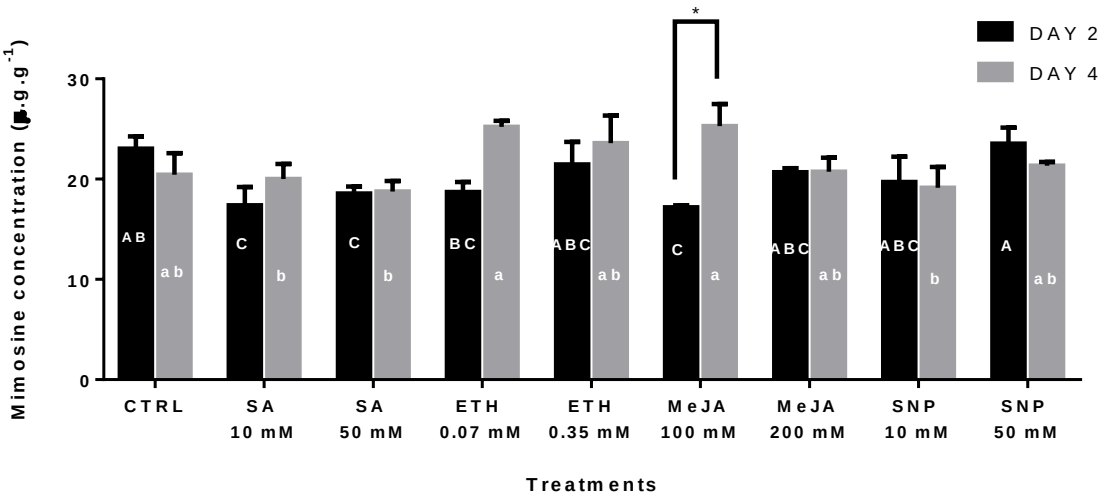

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359 Figure 2. Mimosine concentration in shoots of 12-week-old seedlings of *Mimosa*
360 *bimucronata* treated with different signaling molecules. **SA** = Salicylic Acid; **ETH** =
361 Ethephon; **MeJA** = Methyl Jasmonate; **SNP** = Sodium Nitroprusside. Uppercase and
362 lowercase letters indicate statistical differences among treatments in days 2 and 4,
363 respectively. Bars sharing a letter of the same case do not differ statistically by Tukey test
364 ($P \leq 0.05$). * Indicates statistical difference in the same treatment between day 2 and 4 by *t*-
365 test ($P \leq 0.05$). The error bars denote standard error of 5 replicates (25 individual seedlings
366 arranged in 5 groups of 5).

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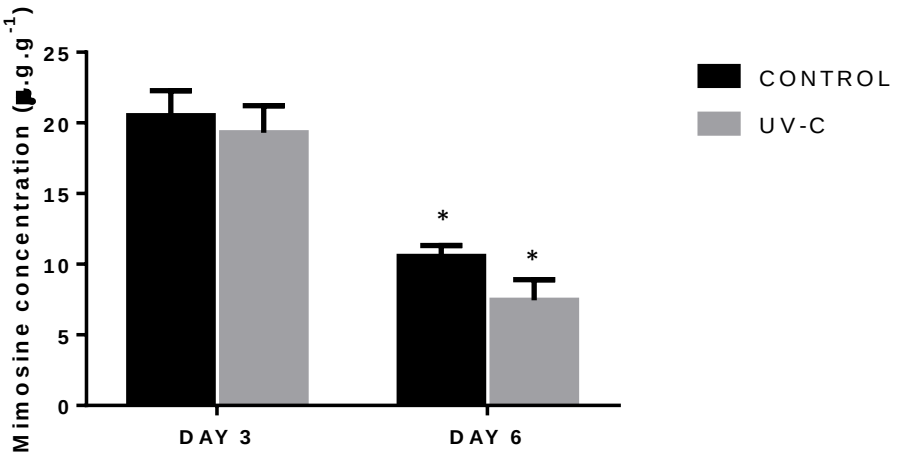

Figure 3. Mimosine concentration in shoots of 12-week-old seedlings of *Mimosa bimucronata* supplemented with UV-C radiation. * Indicates statistical difference in the same treatment between day 3 and 6 by *t*-test ($P \leq 0.05$). The error bars denote standard error of 5 replicates (25 individual seedlings arranged in 5 groups of 5).

Considerações finais

- Experimentos que avaliam os efeitos da aplicação exógena de ANPs em diferentes espécies vegetais têm sido realizados principalmente com GABA. Dentre os principais efeitos conferidos pela aplicação dessa molécula em espécies de mono e eudicotiledôneas são relatados a tolerância à seca, à salinidade e às temperaturas extremas;

- Como metabólitos especializados clássicos, os ANPs podem ter sua concentração basal endógena aumentada em resposta à indução mediada por uma vasta gama de tratamentos com moléculas sinalizadoras de estresse e fontes alternativas de estressores. De um modo geral, observa-se o acúmulo das diferentes classes de ANPs em resposta à radiação UV, elicitores químicos que mimetizam ataques por patógenos, dano mecânico, agentes osmóticos, metais pesados, entre outros;

- Especificamente em leucena, a resposta observada em relação aos diferentes tratamentos testados indica que, apesar do seu alto teor constitutivo nessa espécie, a biossíntese e o acúmulo de mimosina podem ser modulados por fatores causadores de estresses, exibindo - nessa espécie - um padrão de acumulação similar à fitoalexinas. Em maricá, por outro lado, aumento de acúmulo dessa molécula não foi observado para os mesmos tratamentos testados para leucena, o que sugere um perfil de acumulação similar ao das fitoanticipinas.

- O padrão de expressão gênica observado nas plantas de leucena estressadas com etileno sugere que o controle steady-state da mimosina pode ser, pelo menos em parte, regulado pela sua degradação;

- As respostas observadas nos testes que avaliaram a atividade de mitigação de espécies reativas de oxigênio por mimosina sugerem que essa molécula pode agir como um agente antioxidante não-enzimático em plantas de leucena em situação de estresse.

Perspectivas

- Confirmação, em espectrômetro de massas e/ou ressonância nuclear magnética, da natureza química da ‘mimosina’ presente em maricá;

- Avaliação do efeito de concentrações mais elevadas e em diferentes períodos de aplicação das moléculas sinalizadoras testadas sobre o acúmulo de mimosina em leucena e maricá;

- Ampliar a investigação dos padrões de expressão gênica dos genes que codificam para mimosinase (em maricá), mimosina sintase (em ambas as espécies testadas), bem como o perfil de precursores e catabólitos de mimosina em resposta aos tratamentos mencionados.

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Resin tapping transcriptome in adult slash pine (*Pinus elliottii* var. *elliottii*)

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Pinus elliotti
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ABSTRACT

To better understand the bases of resin production, a major source of terpenes for industry, the transcriptome of adult *Pinus elliottii* var. *elliottii* (slash pine) trees under field commercial resinosis was obtained. Samples were collected from cambium after 5 and 15 days of treatment application, which included tapping followed by application of commercial resin stimulant paste or control wounding without paste. Overall mean number of reads of all 16 libraries (2 treatments x 2 times x 4 replicated trees) was 34,582,048. Of these, 89% were mapped against the reference sequence, with a mismatch of 0.58%. Using the Blast2Go, 570 candidate genes were detected based on sequence annotation. By comparing the expression profile between paste and control, 310 differentially expressed genes (DEGs) were identified at 5 days, and 190 at 15 days with a significant fold change of $\log_2 > 1.2$. Regarding changes in time comparisons within each treatment, 210 and 105 DEGs were identified within control and paste treatment, respectively. Genes with different expression patterns in the times and treatments examined included ethylene responsive transcription factors, geranylgeranyl diphosphate synthase, diterpene synthase, cytochrome P450 and ABC transporters, all of which may play important roles in resin production. RT-qPCR analysis correlated well with the data obtained by RNAseq. Resin composition changed over time. This is the first transcriptomic investigation of resinosis of the main species used in the bioresin industry and of molecular analyses of resinosis under field operations, with implications for stand management, stimulant paste development, genotype selection and breeding for high resinosis.

1. Introduction

The adaptive success of conifers is largely due to the development of a defense system based on the synthesis and secretion of terpenes in all major organs and different tissues (Miller et al., 2005; Hall et al., 2013; Warren et al., 2015). Conifer resin is a viscous fluid composed of a complex mixture of terpenoids, such as monoterpenes, sesquiterpenes, and diterpenes (Zulak and Bohlmann, 2010). These terpenoids are secreted from severed resin ducts when the tree is under biotic attack (Ralph et al., 2006; Lange, 2015; Geisler et al., 2016), acting as protectants (Schiebe et al., 2012; Liu et al., 2015).

Biosynthesis of terpenes in conifers starts from isomerization of two isoprenoid (C5) units, dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP). These molecules can be biosynthesized via two separate routes in plants, the methyl-erythritol 4-phosphate and mevalonate pathways. IPP is synthesized and isomerized to DMAPP by isopentenyl diphosphate isomerase, then prenyl transferases catalyze the condensation of these two C5-units to geranyl diphosphate (Pazouki and Niinemets, 2016). Their elongation to prenyl diphosphates with addition of IPP molecules leads to monoterpenes (C10), sesquiterpenes (C15) and diterpenes (C20), which are the substrates for terpene synthases (TPS) (Keeling and Bohlmann, 2006b).

TPSs are part of a large family of mechanistically related enzymes involved in both primary and secondary metabolism (Keeling and Bohlmann, 2006b). The events of evolutionary diversification and expansion of plant TPSs appear to have originated from gene duplications, domain losses, and sub- or neofunctionalizations, with subsequent divergence of an ancestral TPS gene of primary metabolism (Hall et al., 2013). Modification of TPS products changes their physical properties and may alter their biological activities (Chen et al., 2011). TPSs of high sequence identity may have different functions even in closely related species. Low sequence identity of TPSs in phylogenetically distant species does not preclude the possibility of independent evolution of the same or related function of these enzymes (Zerbe and Bohlmann, 2015).

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Review

Sustainable production of bioactive alkaloids in *Psychotria* L. of southern Brazil: propagation and elicitation strategies

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ABSTRACT

Psychotria is the largest genus in Rubiaceae. South American species of the genus are promising sources of natural products, mostly due to bioactive monoterpene indole alkaloids they accumulate. These alkaloids can have analgesic, antimutagenic, and antioxidant activities in different experimental models, among other pharmacological properties of interest. Propagation of genotypes with relevant pharmaceutical interest is important for obtaining natural products in a sustainable and standardized fashion. Besides the clonal propagation of elite individuals, the alkaloid content of *Psychotria* spp. can also be increased by applying moderate stressors or stress-signaling molecules. This review explores advances in research on methods for plant propagation and elicitation techniques for obtaining bioactive alkaloids from *Psychotria* spp. of the South Region of Brazil.

Keywords: abiotic stress, alkaloids, elicitation, monoterpenes, plant propagation, *Psychotria*, southern Brazil, sustainability

Introduction

Psychotria belongs to Rubiaceae, one of the major families of flowering plants having economic interest. The family includes coffee, a few significant poisonous plants to livestock, besides several important ornamental and medicinal species (Souza & Lorenzi 2012). *Psychotria* has captured researchers' attention mostly because of its medicinal properties.

Psychotria colorata is an Amazonian species that produces polyindolinic alkaloids with analgesic activity (Matsuura *et al*. 2013). The promising results obtained with *P. colorata* motivated the investigation of southern Brazilian *Psychotria* species and the discovery of new bioactive alkaloids (Porto *et al*. 2009). Moreover, leads on *in planta* alkaloid functions were also topic of experimental evaluation.

One of the key elements that needs to be addressed early on during the process of developing new bioactive molecules from plants is the capacity to generate catalytically active biomass to support extraction and steady supply. There are a number of ways through which these goals may be reached, including greenhouse rooting of cuttings (mini-cutting

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Biomass yield of resin in adult *Pinus elliottii* Engelm. trees is differentially regulated by environmental factors and biochemical effectors

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ABSTRACT

Biomass of pine resin finds several applications in the chemical, pharmaceutical, biofuel and food industries. Resin exudation after injury is a key defense response in Pinaceae since this complex mixture of terpenes has insecticidal, antimicrobial and wound repair properties. Resin yield is increased by effectors applied on the wound area, including phytohormones and metal cofactors of terpene synthases. The interaction of resinosis mechanism effectors is not fully understood, particularly in adult forest setups under natural environmental variations. The aim of this work was to determine how resin exudation by wounded trunks of adult *P. elliottii* responded to combined chemical effectors involved in different regulatory pathways of resinosis (metal cofactors of terpene synthases, benzoic acid and plant growth regulators) and whether seasonal and tree distribution variations affected these responses. Symmetrically planted and scattered trees regenerated from the seed bank had similar resin biomass yields, suggesting that the homogeneity in development and spatial arrangement were not significant factors in resin yield. This new finding is of practical importance with the used tapping system since costs of implanting forests by regeneration can be advantageous compared to planting. In addition, it was shown for the first time that the salicylic acid precursor benzoic acid and the auxin naphthalene acetic acid promoted resin exudation when individually applied to wound sites. Both these adjuvants are two orders of magnitude less costly compared to the conventionally used ethylene precursors, besides facing less environmental and health restrictions for use. Most adjuvant-treated trees showed higher resin flow in the second year, indicating mechanisms of response build up. Overall, temperature was more important than rainfall as environmental parameter affecting resin biosynthesis, which was higher in the warmer months of spring and summer. The combination of resinosis stimulant effectors from different signaling pathways showed no significant synergistic or additive effect, suggesting possible converging signaling pathways and/or limitation of common intermediate transducing molecules.

1. Introduction

Pines occupy highly diverse environments, over a range of temperatures, water and nutrient availabilities, irradiance levels and photoperiods, being able to effectively face attacks from diverse herbivore and pathogen guilds. The success of conifers is linked to their complex terpene biochemistry hosted by specialized secretory cells. The terpenoid resin synthesized by *Pinus* spp. is one of the main mechanisms of defense of these trees, particularly against bark beetles and the fungi they carry (Fett-Neto and Rodrigues-Corrêa, 2012). Pine resin biomass is essentially composed of a monoterpene and sesquiterpene-rich turpentine and diterpenoid-rich rosin fraction, both finding numerous industrial applications as non-wood forest products (Rodrigues-Corrêa et al., 2012).

Molecules capable of modulating different signaling pathways have been identified as resin yield stimulators, including sulfuric acid (extends wound damage), 2-chloroethylphosphonic acid (CEPA, a synthetic ethylene precursor), paraquat (free radical generator), yeast extract (mimics attack by pathogens), salicylic acid (pathogen signaling molecule), auxin (promotes ethylene biosynthesis and resin canal differentiation), jasmonic acid (signals mechanical damage and promotes secondary metabolism) and metal ions, such as potassium, iron and manganese (cofactors of terpene synthases in conifers) and copper (a component of ethylene receptors) (Clements, 1970; Conrath et al., 2002; Fett-Neto and Rodrigues-Corrêa, 2012; Hudgins and Franceschi, 2004; Lewinsohn et al., 1994; Martin et al., 2002; Popp et al., 1995;

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Research Paper

Dual allelopathic effects of subtropical slash pine (*Pinus elliottii* Engelm.) needles: Leads for using a large biomass reservoir

Kelly Cristine da Silva Rodrigues-Corrêa[a], Gelson Halmenschlager[a], Joséli Schwambach[b], Fernanda de Costa[a], Emili Mezzomo-Trevizan[a], Arthur Germano Fett-Neto[a,*]

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ARTICLE INFO

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ABSTRACT

Pinus elliottii Engelm. (slash pine) is distributed along the maritime coast of Southern Brazil, where it shows invasive pattern and typical allelopathic features. Large quantities of needle litter are produced by pine trees, a biomass that is little explored in areas where this species is alien. Little is known about the dynamics of needle and litter phytochemical interactions, particularly in subtropical environments. To elucidate the full range of needle and litter allelopathic potential, the effects of litter (superficial and deep) and seasonally harvested fresh slash pine needles stored for different times were evaluated against lettuce, tomato and cucumber seeds and seedlings. Increasing concentrations (0%, 1%, 2%, 4%, and 8% w/v) of hot and cold aqueous extracts of needles and litter affected in different ways target plant development. Growth and germination inhibition were directly related to the highest extract concentrations (regardless of the season and mainly in hot water extracts) of needles. On the other hand, stimulatory effects of litter extracts on lettuce growth were observed. Growth and germination of cucumber and tomato were not affected by pine litter as substrate when compared to rice husk. The presumable high polarity and thermal stability of slash pine leaf biomass allelochemicals and their transient toxic effect or growth promoting impact suggest potential applications of this largely available biomass both as a biological herbicide and growth substrate in plant propagation.

1. Introduction

Native from the Northern Hemisphere, *Pinus* is one of the most widely distributed genera throughout different climate regions of the globe, growing either as native or alien species, even in extreme habitats (Rodrigues-Corrêa and Fett-Neto, 2012). Despite the high economic value currently attributed to pine wood and oleoresin (Rodrigues-Corrêa et al., 2012) there is increasing concern about the aggressive potential of invasiveness displayed by *Pinus* species, especially those cultivated out of their native range of distribution (Richardson et al., 2008; Rolon et al., 2011). These species are dispersed by wind and there is notably low plant diversity observed in most understories of pine plantations (Kato-Noguchi et al., 2009). This latter feature has been considered an important trait of allelopathic interference.

The term “allelopathy” was coined by Molisch in 1937 as a chemical reciprocal interaction established among plants (including microorganisms) sharing the same site by means of the release of secondary metabolites, named allelochemicals (Rice, 1984). For the most part, these metabolites are derived from the shikimic acid or isoprenoid pathway and their biosynthesis can be modulated by biotic and abiotic stresses (Nascimento and Fett-Neto, 2010), including seasonal-related changes (Sartor et al., 2013). Allelopathy studies may range from sterile assays (Aryakia et al., 2015) to soil (Corrêa et al., 2008; Sharma et al., 2016) and field tests, being a complex biological phenomenon to ascertain in several circumstances due to issues of solubility, release mechanisms and stability of bioactive compounds (Scognamiglio et al., 2013). Often the use of complementary methods provides more informative data.

The allelopathic effects of soil leachates, green needles and litter extracts of *Pinus* spp. on germination and seedling growth aspects of wild and crop species have been evaluated in natural and cultivated pine stands and have proven to be stimulatory or inhibitory (Lodhi and Killingbeck, 1982; Kil and Yim, 1983; Nektarios et al., 2005; Akkaya et al., 2006; Machado, 2007; Alrababah et al., 2009; Sartor et al., 2009; Kato-Noguchi et al., 2011; Rolon et al., 2011; Valera-Burgos et al., 2012) exhibiting, in some cases, autotoxicity (Garnett et al., 2004; Fernandez et al., 2008; Zhu et al., 2009; Monnier et al., 2011). Studies on potential dual allelopathic effects of *Pinus elliottii* Engelm. (slash

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Reference Genes for qPCR Analysis in Resin-Tapped Adult Slash Pine As a Tool to Address the Molecular Basis of Commercial Resinosis

Júlio C. de Lima[1†], Fernanda de Costa[1†], Thanise N. Füller[1], Kelly C. da Silva Rodrigues-Corrêa[2], Magnus R. Kerber[1], Mariano S. Lima[1], Janette P. Fett[1] and Arthur G. Fett-Neto[1]*

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Pine oleoresin is a major source of terpenes, consisting of turpentine (mono- and sesquiterpenes) and rosin (diterpenes) fractions. Higher oleoresin yields are of economic interest, since oleoresin derivatives make up a valuable source of materials for chemical industries. Oleoresin can be extracted from living trees, often by the bark streak method, in which bark removal is done periodically, followed by application of stimulant paste containing sulfuric acid and other chemicals on the freshly wounded exposed surface. To better understand the molecular basis of chemically-stimulated and wound induced oleoresin production, we evaluated the stability of 11 putative reference genes for the purpose of normalization in studying *Pinus elliottii* gene expression during oleoresinosis. Samples for RNA extraction were collected from field-grown adult trees under tapping operations using stimulant pastes with different compositions and at various time points after paste application. Statistical methods established by *geNorm*, *NormFinder,* and *BestKeeper* softwares were consistent in pointing as adequate reference genes *HISTO3* and *UBI*. To confirm expression stability of the candidate reference genes, expression profiles of putative *P. elliottii* orthologs of resin biosynthesis-related genes encoding *Pinus contorta* β-pinene synthase [*PcTPS-(−)β-pin1*], *P. contorta* levopimaradiene/abietadiene synthase (*PcLAS1*), *Pinus taeda* α-pinene synthase [*PtTPS-(+)αpin*], and *P. taeda* α-farnesene synthase (*PtαFS*) were examined following stimulant paste application. Increased oleoresin yields observed in stimulated treatments using phytohormone-based pastes were consistent with higher expression of pinene synthases. Overall, the expression of all genes examined matched the expected profiles of oleoresin-related transcript changes reported for previously examined conifers.

Keywords: resin, *Pinus*, gene expression, normalizer genes, terpene synthase

Chapter 2

Stimulant Paste Preparation and Bark Streak Tapping Technique for Pine Oleoresin Extraction

Thanise Nogueira Füller*, Júlio César de Lima*, Fernanda de Costa, Kelly C.S. Rodrigues-Corrêa, and Arthur G. Fett-Neto

Abstract

Tapping technique comprises the extraction of pine oleoresin, a non-wood forest product consisting of a complex mixture of mono, sesqui, and diterpenes biosynthesized and exuded as a defense response to wounding. Oleoresin is used to produce gum rosin, turpentine, and their multiple derivatives. Oleoresin yield and quality are objects of interest in pine tree biotechnology, both in terms of environmental and genetic control. Monitoring these parameters in individual trees grown in the field provides a means to examine the control of terpene production in resin canals, as well as the identification of genetic-based differences in resinosis. A typical method of tapping involves the removal of bark and application of a chemical stimulant on the wounded area. Here we describe the methods for preparing the resin-stimulant paste with different adjuvants, as well as the bark streaking process in adult pine trees.

Key words Oleoresin, Pine, Tapping, Chemical stimulant, Wounding

1 Introduction

Several conifer species produce oleoresin, a complex mixture of isoprenoid compounds relevant for defense against herbivores and pathogens. Two major fractions can be recognized in oleoresin: (a) turpentine, the volatile fraction containing mono- and sesquiterpenes, and (b) rosin, the nonvolatile diterpene fraction. Oleoresin is a forest commodity of global interest, finding applications in diverse industry sectors. Rosin is used in adhesives, printing ink manufacture, and paper sizing. Turpentine can be used either as a solvent for paints and varnishes, or as a raw material for fractionation of high-value chemicals used in the pharmaceutical, agrochemical, and food industry [1–3].

During the extraction activity, resin is obtained from the tree in a similar way as rubber tree tapping, which generally involves the

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Chapter 3

A Modified Protocol for High-Quality RNA Extraction from Oleoresin-Producing Adult Pines

Júlio César de Lima*, Thanise Nogueira Füller*, Fernanda de Costa, Kelly C.S. Rodrigues-Corrêa, and Arthur G. Fett-Neto

Abstract

RNA extraction resulting in good yields and quality is a fundamental step for the analyses of transcriptomes through high-throughput sequencing technologies, microarray, and also northern blots, RT-PCR, and RTqPCR. Even though many specific protocols designed for plants with high content of secondary metabolites have been developed, these are often expensive, time consuming, and not suitable for a wide range of tissues. Here we present a modification of the method previously described using the commercially available Concert™ Plant RNA Reagent (Invitrogen) buffer for field-grown adult pine trees with high oleoresin content.

Key words RNA, Pines, Concert plant RNA reagent, Stem RNA extraction, Oleoresin, Conifers

1 Introduction

Several conifer species, especially within the Pinaceae, have tissues with high concentrations of phenolics, terpenes, and polysaccharides [1]. Many specific protocols that are appropriate for plants rich in secondary metabolites have been developed, but the extraction of high-quality RNA from these tissues using commercial kits is often difficult and usually not applicable to woody tissues [2–6]. One of the major issues during RNA extraction concerns the presence of phenolic compounds, which oxidize and form quinones. Aromatic compounds bind RNA, which interferes in downstream steps and applications [3, 7]. Another point of concern is the harvest of plant samples in the experimental field, which constitutes another obstacle in the efforts to avoid degradation of RNA [8]. These problems often result in RNAs of low quality and insufficient amounts, especially for methodologies that normally require

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RESEARCH PAPER

Control of resin production in *Araucaria angustifolia*, an ancient South American conifer

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Keywords

Araucaria; ethylene; jasmonic acid; nitric oxide; resin; salicylic acid; terpenes.

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ABSTRACT

***Araucaria angustifolia* is an ancient slow-growing conifer that characterises parts of the Southern Atlantic Forest biome, currently listed as a critically endangered species. The species also produces bark resin, although the factors controlling its resinosis are largely unknown. To better understand this defence-related process, we examined the resin exudation response of *A. angustifolia* upon treatment with well-known chemical stimulators used in fast-growing conifers producing both bark and wood resin, such as *Pinus elliottii*. The initial hypothesis was that *A. angustifolia* would display significant differences in the regulation of resinosis. The effect of Ethrel® (ET – ethylene precursor), salicylic acid (SA), jasmonic acid (JA), sulphuric acid (SuA) and sodium nitroprusside (SNP – nitric oxide donor) on resin yield and composition in young plants of *A. angustifolia* was examined. In at least one of the concentrations tested, and frequently in more than one, an aqueous glycerol solution applied on fresh wound sites of the stem with one or more of the adjuvants examined promoted an increase in resin yield, as well as monoterpene concentration (α-pinene, β-pinene, camphene and limonene). Higher yields and longer exudation periods were observed with JA and ET, another feature shared with *Pinus* resinosis. The results suggest that resinosis control is similar in *Araucaria* and *Pinus*. In addition, *A. angustifolia* resin may be a relevant source of valuable terpene chemicals, whose production may be increased by using stimulating pastes containing the identified adjuvants.**

INTRODUCTION

Many conifer species produce terpenoid-based resins that have long been studied for their industrial importance and role in defence against attack by herbivores and pathogens. The two most important resin-producing families of conifers are Pinaceae and Araucariaceae (Langenheim 1996). The viscous resin secretion is generally composed of a complex mixture of terpenoids, consisting of roughly equal parts of volatile mono- (C_{10}) and sesquiterpene (C_{15}; turpentine) fractions and non-volatile diterpenic (C_{20}; rosin) components (Rodrigues-Corrêa *et al.* 2013). Terpenes act in a complex and multilayered defence response, providing toxicity against bark beetles and fungi, bark wound sealing, disruption of insect development and attraction of herbivore predators (Phillips & Croteau 1999).

Most conifers rely on some combination of preformed and inducible resin defences (Trapp & Croteau 2001; Zulak & Bohlmann 2010). Resin defences are controlled by environmental and genetic factors to various extents, depending on species (Roberds *et al.* 2003; Sampedro *et al.* 2010; Moreira *et al.* 2013). Resin traits have been reported as highly variable, having moderate heritability, indicating that breeding efforts towards super-resinous forests are promising (Tadasse *et al.* 2001; Roberds *et al.* 2003). Several chemicals are known as stimulants of resin production. Commercial extraction of resin from pine trees uses periodic bark streaking and application of resin stimulant pastes to the wound.

Resin-stimulant paste based on sulphuric acid (SuA) is widely used for the commercial production of pine resin. Current stimulant pastes usually have two chemically active components, SuA to magnify the wounding and an ethylene precursor (2-chloroethylphosphonic acid, CEPA or Ethrel® – ET) to stimulate resin flow (Rodrigues *et al.* 2011; Rodrigues-Corrêa & Fett-Neto 2013). Jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJa), are among the most widely used chemical elicitors of plant secondary metabolism. It has been shown that the exogenous application of MeJa or herbivore attack induce chemical and anatomical defence responses in conifers, such as the formation of traumatic resin ducts and resin accumulation in stems, along with increased biosynthesis of terpenes and phenolics (Franceschi *et al.* 2002; Martin *et al.* 2002; Heijari *et al.* 2005; Zeneli *et al.* 2006; Moreira *et al.* 2008; Gould *et al.* 2009). JA commercial use, however, is limited by its high cost.

The effects of exogenous salicylic acid (SA) on conifer terpene production have also been studied. In *Pinus elliottii*, application of 10 $mol \cdot m^{-3}$ of SA induced resin production in wound panels, but in *Pseudotsuga menziesii* and *Sequoiadendron giganteum* it had no apparent effect on resin accumulation (Hudgins & Franceschi 2004; Rodrigues & Fett-Neto 2009). Nitric oxide (NO) has also emerged as an