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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

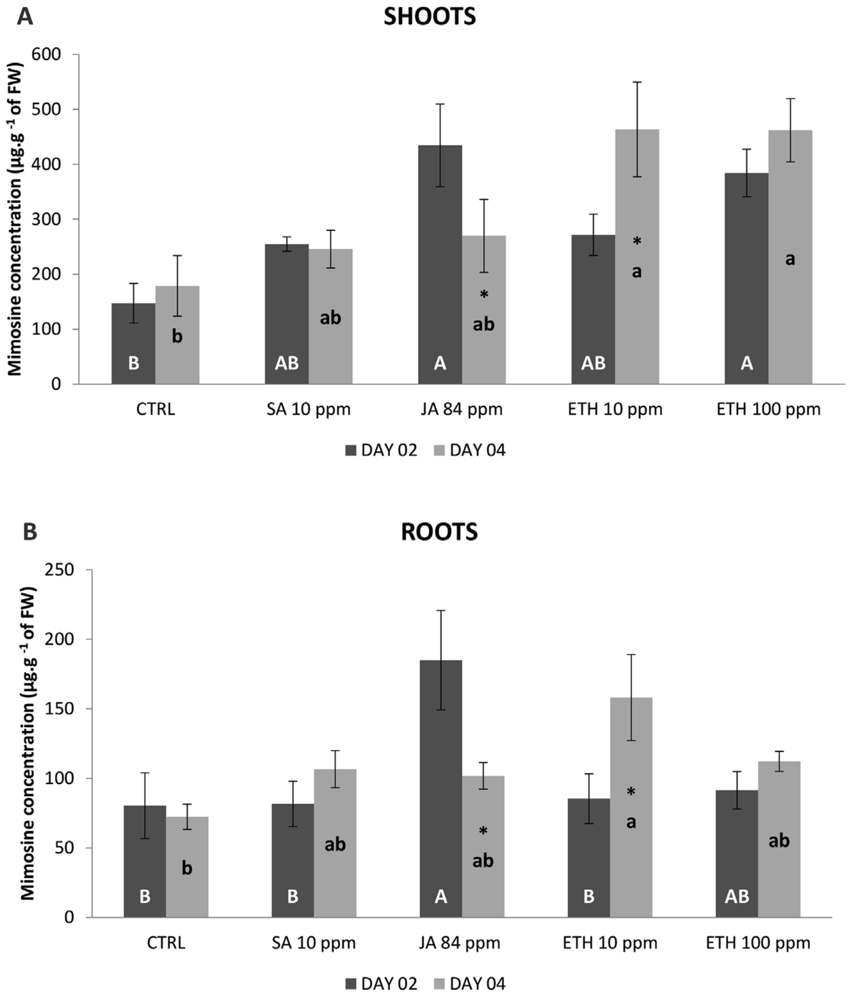

Fig. 1. Mimosine concentration in shoots (**A**) and roots (**B**) of 5-week-old giant leucaena seedlings treated with different elicitors. CTRL = Milli-Q water; SA = Salicylic Acid; JA = Jasmonic Acid; ETH = Ethephon. Bars sharing a letter of same case do not differ by Tukey test ($P \leq 0.05$). Capital 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).

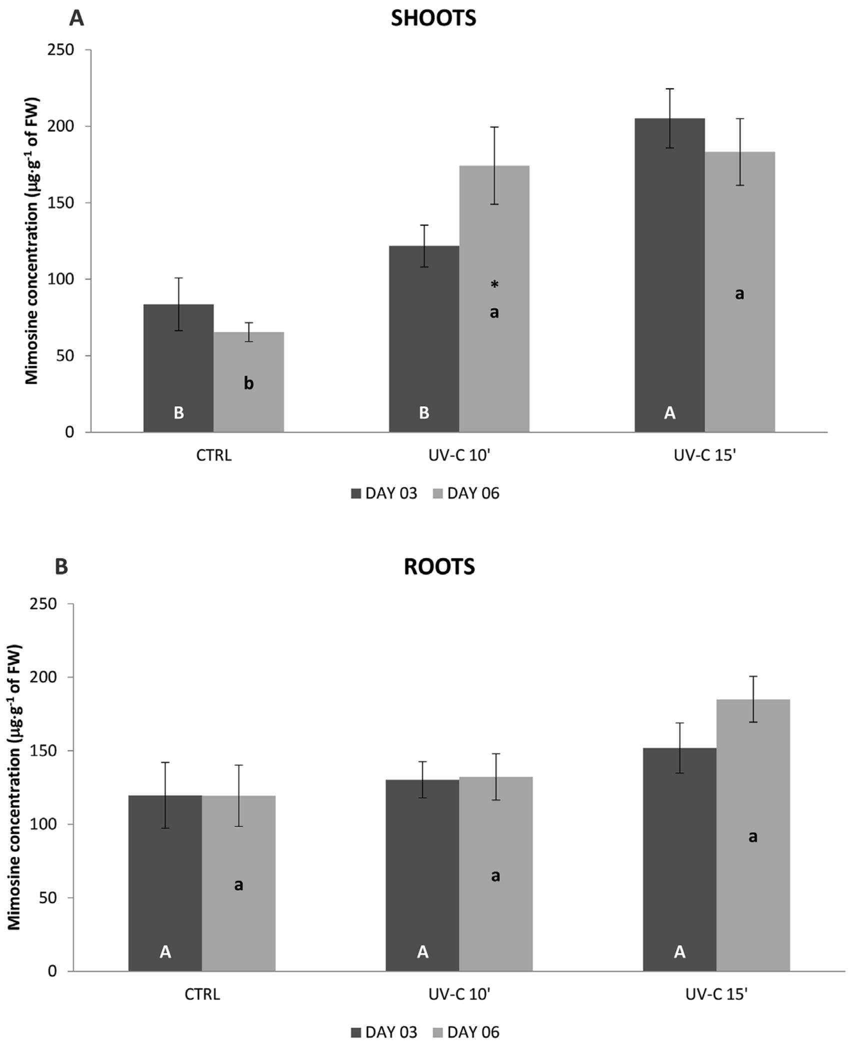

Fig. 2. Mimosine concentration in shoots (**A**) and roots (**B**) of 5-week-old giant leucaena seedlings exposed to UV-C light. CTRL = visible light (100 µmol photons m^{-2} s^{-1}); UV-C 10′ and UV-C 15′ = UV-C exposure time (10 and 15 min respectively). Bars sharing a letter of same case do not differ 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

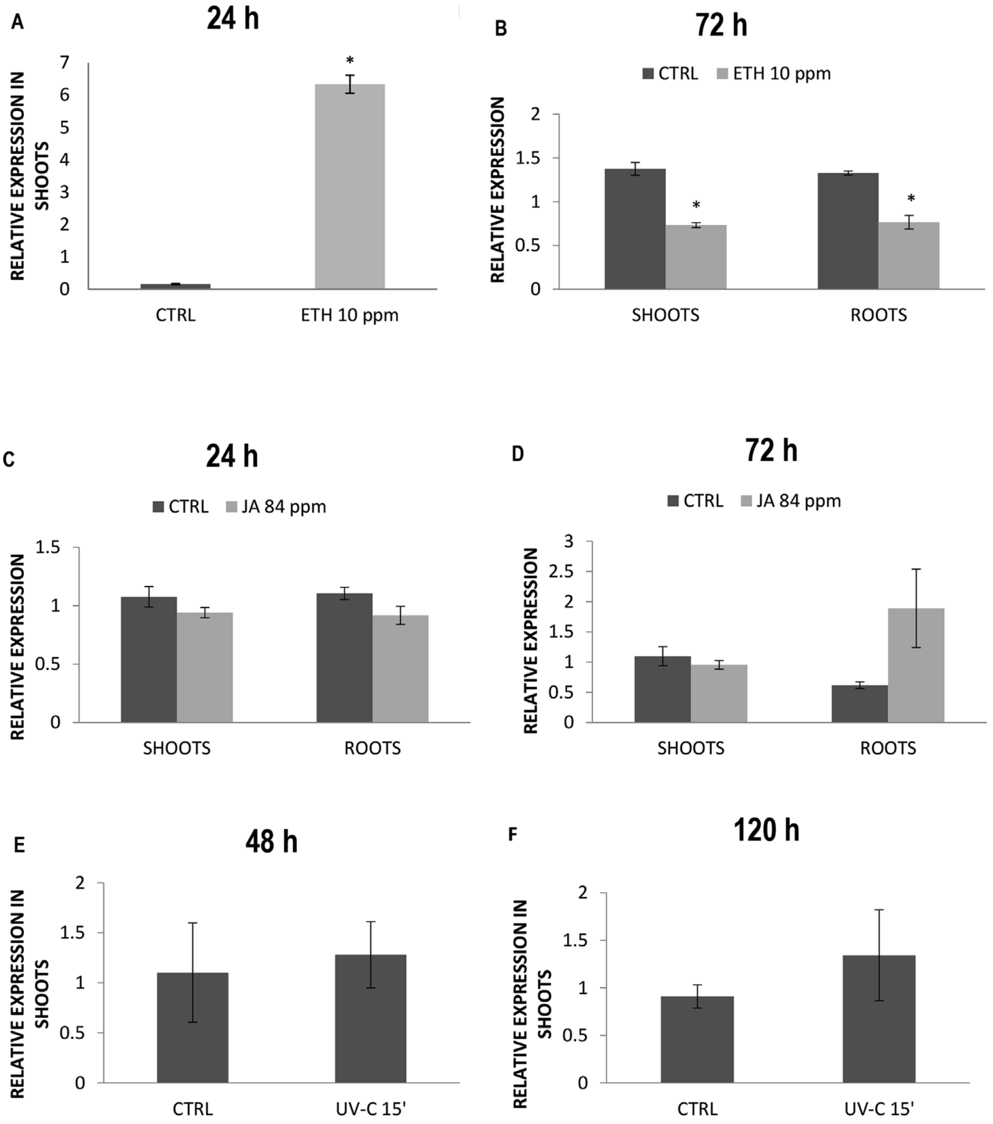

Fig. 3. Relative expression of the mimosinase gene in shoots (**A**, **E** and **F**) and shoots and roots (**B**, **C** and **D**) of giant leucaena 24 h (**A** and **C**), 48 h (**E**), 72 h (**B** and **D**) and 120 h (**F**) after treatment with stress signaling molecules or UV-C exposure. ETH = 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

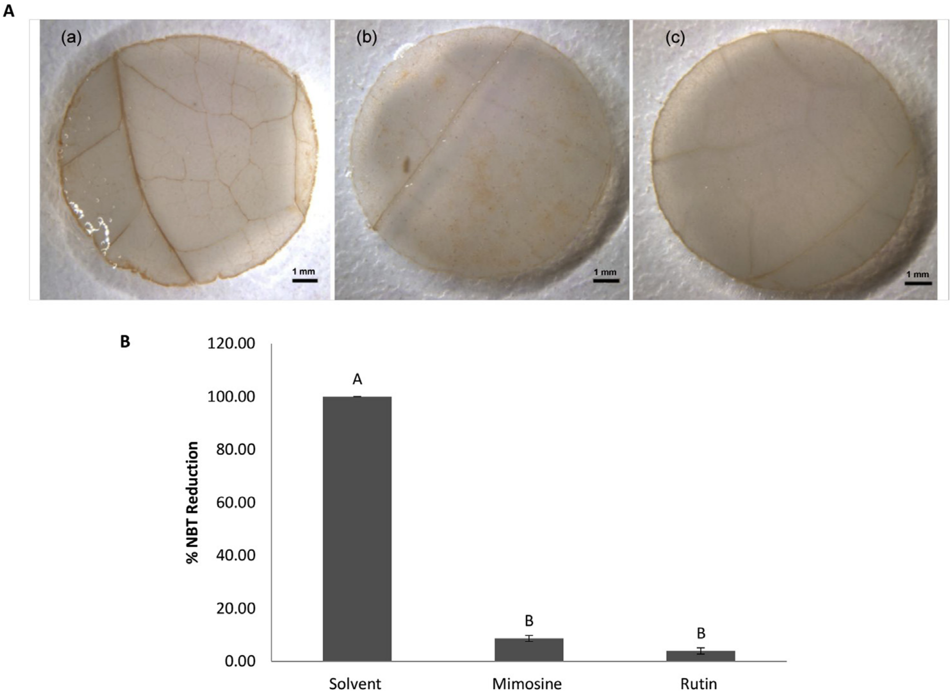

Fig. 4. A. In situ antioxidant assay. Foliar disks of *Phaseolus vulgaris* L. treated with (**a**) No antioxidant added (negative control); (**b**) 10 mM Mimosine; (**c**) 10 mM ascorbic acid (positive control). The oxidative damage can be seen by the formation of a brown polymer in leaf veins and injured 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.)

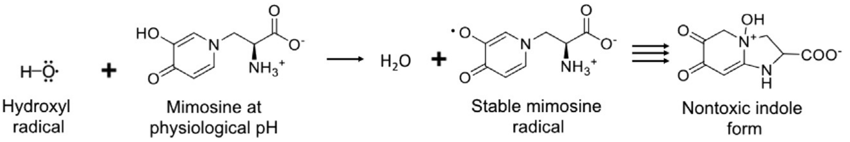

Fig. 5. Predicted mimosine radical formed following quenching of hydroxyl radical. Mimosine is first converted to a stable mimosine radical, which may be then converted to a nontoxic indole form.

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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