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Research Paper

Mimosine concentration in giant leucaena (*Leucaena leucocephala* subsp. *glabrata*) fluctuates with age and plant part

*La concentración de mimosina en la leucaena gigante (*Leucaena leucocephala *subsp.* glabrata*) fluctúa con la edad y la parte de la planta*

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Abstract

Giant leucaena is a multipurpose tree-legume found throughout the tropics and sub-tropics. Its foliage is used as animal fodder due to high protein and fiber. Giant leucaena has several other advantageous traits such as fast-growth, high yields and strong tolerance to environmental stresses. Despite having these desirable qualities, leucaena tissues contain an undesirable attribute, a toxic non-protein amino acid, mimosine, found in all parts of the plant including the foliage. The goal of this research was to determine mimosine concentrations in various tissues and life stages of giant leucaena plants to inform use of leucaena foliage as a fodder. Mimosine was extracted from different parts of giant leucaena at different ages and quantified using HPLC analysis. qRT-PCR was used to determine the relative expression of mimosine synthase in leucaena tissues. Mimosine was present in all parts of the leaf, stem and root of giant leucaena, and concentrations changed depending on the age of the plant. Green seeds had the highest expression level of mimosine synthase. Mimosine is ubiquitous and abundant in leucaena tissues with younger and immature plants and tissues containing more mimosine than older mature plants and tissues.

Keywords: Fodder, mimosine synthase, non-protein amino acid, secondary metabolites, tree-legume.

Resumen

La leucaena gigante es una leguminosa multipropósito que se encuentra en los trópicos y subtrópicos. Su follaje se utiliza como forraje para animales debido a su alto contenido de proteínas y fibra. La leucaena gigante presenta otras características ventajosas, como un rápido crecimiento, altos rendimientos y una fuerte tolerancia a las tensiones ambientales. A pesar de tener estas cualidades deseables, los tejidos de la leucaena contienen un atributo indeseable, un aminoácido no proteico tóxico llamado mimosina, presente en todas las partes de la planta, incluido el follaje. El objetivo de esta investigación fue determinar las concentraciones de mimosina en diferentes tejidos y etapas de vida de las plantas de leucaena gigante para el uso informado del follaje de leucaena como forraje. La mimosina se extrajo de diferentes partes de la leucaena gigante en diferentes edades y se cuantificó mediante análisis de HPLC. Se utilizó qRT-PCR para determinar la expresión relativa de la mimosina sintasa en los tejidos de leucaena. La mimosina estaba presente en todas las partes de la hoja, tallo y raíz de la leucaena gigante, y las concentraciones variaban según la edad de la planta. Las semilla verdes tuvieron el nivel más alto de mimosina sintasa. La mimosina está presente en forma abundante en todos los tejidos de leucaena, pero los mayores contenidos de mimosina se presentan en las plantas y tejidos más jóvenes que en las plantas y tejidos más maduros.

Palabras clave: Forraje, mimosina sintasa, aminoácido no proteico, metabolitos secundarios, leguminosa.

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Introduction

The tree-legume giant leucaena (*Leucaena leucocephala* (Lam.) de Wit subsp. *glabrata* (Rose) Zárate) is a widely used animal fodder in the tropics and sub-tropics due to its fast growth, high forage yield and favorable nutritional characteristics, including high amounts of protein and fiber (Shelton and Brewbaker 1994; Brewbaker 2016). Although it grows normally as a medium-sized tree, it can be maintained as a bushy shrub by repeatedly harvesting its foliage several times a year (Honda et al. 2022). When grown for fodder under favorable conditions, giant leucaena can produce as much as 80–99 t green forage/ha/yr, which is equivalent to 24–30 t dry matter (DM)/ha/yr (Shelton and Brewbaker 1994).

Giant leucaena is considered to be an ideal fodder legume for the tropics because (i) it is able to grow in dry areas, marginal lands, and places with eroded slopes on fertile soils; (ii) it has a deep root system and is tolerant to drought and can be grown as a rain-fed fodder with little or no irrigation; (iii) as a perennial fodder, it does not require repeated planting each season and can be maintained with relatively minimal efforts and resources; (iv) it is a nitrogen-fixing tree legume that fixes high amounts of N (196-268 kg N/ha) in nodule-forming symbiosis with *Rhizobium* (Sanginga et al. 1989) (v) it is naturally resistant to infection by microbial pathogens and insect pests (Bageel et al. 2020); and (vi) it has tolerance to environmental stresses such as acidic and alkaline soils, drought, salinity, eroded slopes, and UV light (Bageel et al. 2020; Honda et al. 2018; Ishihara et al. 2018; Rodrigues Correa et al. 2019). However, despite these beneficial attributes, giant leucaena contains large amounts of an undesirable compound, mimosine, a non-protein aromatic amino acid found in all parts of the plant (Honda and Borthakur 2021). Its concentration in the foliage can be 2–5% of the plant dry weight (DW) (Soedarjo and Borthakur 1996). Some studies have shown that its concentrations can be as high as 12-20% DW in the growing shoot tips (Honda and Borthakur 2019). The toxicity of mimosine stems from its ability to bind metallic cations like iron, copper and zinc, and pyridoxal 5'-phosphate (PLP). Iron, copper, zinc and PLP are important enzyme cofactors in folate, nucleic acid, chlorophyll and amino acid synthesis and metabolism (Negi et al. 2014). Some enzymes inhibited by mimosine include tyrosinase, tyrosine decarboxylase, DOPA decarboxylase, RNA reductase, cystathionine synthase, cystathionase, and Asp-Glu transaminase (Negi et al. 2013; 2014). The side-effects of consuming large amounts of mimosine include fetal defects, infertility, goiter, thyroid problems and hair loss (Crounse et al.1962; Hamilton et al. 1968; Dewreede and Wayman 1970). Two enzymes that degrade mimosine effectively are mimosinase, which is found in leucaena foliage, and rhizomimosinase, which is produced in *Rhizobium* strains that form nitrogen-fixing root nodules on leucaena. Both enzymes are C-N lyases that degrade mimosine into pyruvate, ammonia and 3-hydroxy-4-pyridone (3H4P) (Negi et al. 2013; 2014). Exposure to the degradation product 3H4P, its tautomer 3,4-dihydroxypyridine, and its isomer 2,3-dihydroxypyridine (2,3DHP) can also cause toxic side-effects, which include goiter, dermal, kidney and liver problems (Hegarty et al. 1979; Jones 1979; Jones and Hegarty 1984). Despite the presence of mimosine, giant leucaena fodder is considered a good protein supplement to low quality forages and has been shown to improve animal performance, fermentation and digestibility efficiency, and dietary intake (Orden et al. 2000; Khy et al. 2012). However, because of the toxic effects of mimosine, giant leucaena fodder is generally fed to animals as a protein supplement along with grass or hay at 20–30% of total diet (Jones and Hegarty 1984). Cattle and goats can tolerate leucaena foliage containing up to 0.18 g of mimosine/kg body weight without showing any harmful side effects (Bageel and Borthakur 2022). Similarly, sheep can tolerate leucaena foliage equivalent to 0.14 g of mimosine/kg body weight (Sethi and Kulkarni 1995). Recently, studies have reported that animal diets comprised of 100% leucaena did not cause long-term toxicity problems (Dahlanuddin et al. 2019; Ruiz et al. 2019).

Leucaena invests a large amount of energy and resources into mimosine synthesis. Negi et al. (2014) predicted that leucaena plants would have grown 20% larger if the same amount of energy and resources were diverted to plant growth and development. Mimosine is abundant and ubiquitous in giant leucaena and because of the importance of giant leucaena as an animal fodder, it is important to study the fluctuations of mimosine within giant leucaena based on age and tissue type. The goal of the present study was to determine how mimosine concentrations of giant leucaena change in different stages of growth and tissue types.

Materials and Methods

Germination and growth of giant leucaena seedlings

Mature seeds of giant leucaena cultivar 'K636' (K636) were collected from the University of Hawaii Waimanalo Research Station, Waimanalo, Hawaii. Seeds were scarified with concentrated sulfuric acid at room temperature for 25 min. After scarification, seeds were rinsed with sterile deionized water 5 times and placed in either petri dishes containing filter paper and water or 50.8 cm x 25.4 cm plastic trays containing a vermiculite-soil mixture. The seeds placed in vermiculite-soil were allowed to germinate and then grown in a plant growth chamber at 25 °C ± 2 °C with a 16/8 h light/dark photoperiod and an irradiance of 100 µmol/s/m, and an average humidity between 60–65%. Plants were watered twice a week throughout the experiment until harvesting. Seeds germinated in petri dishes were incubated in the dark at 28 °C and filter paper and water were replaced daily until harvesting. Plants used in this study were grown in pots in a growth chamber and organized in a completely randomized block design with 4 to 6 replications. The actual mimosine concentration of plants grown in a growth chamber are expected to be different from plants grown in the field. However, fluctuations in the mimosine concentration based on life stage are expected to be similar.

Mimosine extraction and quantification

Mimosine was extracted from different parts of giant leucaena by submerging 1 g of plant material in 30 mL of 0.1 N HCl and shaking overnight at room temperature. After incubation, acid extracts were centrifuged for 15 min at 16,000 x g to pellet and remove plant debris. The mimosine concentration of the leaf acid extracts were assayed by HPLC using a Waters 2695 separations module (Waters, Millford MA, USA) a Phenomenex C18 column (Phenomenex, Torrance, CA, USA) (5µ; 4.6 × 250 mm), and a UV detection photodiode array. An isocratic carrier solvent of 0.02 M o-phosphoric acid at a linear flow rate of 1 mL/min was used to analyze mimosine in acid extracts. For quantitative determination of mimosine, commercial mimosine (Sigma-Aldrich, St. Louis, MO, USA) was prepared in various concentrations and then quantified by HPLC following the methods described above. The areas under the curves for mimosine peaks were used to plot a standard curve, which was then used to quantify mimosine in acid extracts. Remaining plant material was rinsed and dried overnight at 65 °C and then weighed. The mimosine concentration was expressed as % of plant DW. Mimosine concentrations determined on the basis of plant DW were normalized to factor in the loss of dry matter during mimosine extraction with 0.1 N HCl.

Mimosine concentration of germinating giant leucaena seeds and mimosine secretion by germinating seeds

Seed coats were removed from mature seeds (< 6 h after scarification) and fresh green seeds (< 6 h after removal from trees) and mimosine extracted and quantified from seed coats and cotyledons separately following the methods described above. In another experiment, mimosine was extracted and determined as a percentage of the plant DW from giant leucaena mature seeds 0, 2, 4, 6, 8, and 10 d after initial scarification. At 0 d after scarification (< 6 h), mature seeds had minor swelling. At 2 d after scarification, seeds were fully swollen and considerably larger. At 4 d after scarification, the initial radicle tip could be observed from seeds. At 6 and 8 d after scarification, seeds had long and very long radicles, respectively. At 10 d after scarification, shoots could be observed growing from seeds. During these stages of germination, the seed growth medium was collected every 24 h. The amount of mimosine secreted was calculated as a percentage of mature seed DW following the methods described above. Each sample set contained at least 4 replications.

Mimosine concentration of giant leucaena during growth stages of seedlings

Mimosine was extracted and determined as a percentage of plant DW from giant leucaena seedlings at 1, 2, 3, 4, 6, 8, 12, 16, 20, 24, and 28 weeks after germination. Each sample set contained at least 6 replicates.

Mimosine concentration in leaves, stem, root, and embryonic leaves of giant leucaena seedlings

Giant leucaena seedlings were grown and then harvested at 2, 4, 8, and 12 weeks of age. A whole seedling was divided into 4 major parts as leaf, stem, root and embryonic leaf. These main parts of the leucaena seedling were separated, grouped by part, and then mimosine extracted and quantified as a percentage of DW of each part. The percentage that each main part (leaf, stem, root and embryonic leaf) contributed to the whole plant mimosine concentration was also

determined. Each experimental set contained at least 4 replicates with the plant parts collected from at least 3 seedlings per replicate.

Mimosine concentration in subparts of leaf, stem, root, green seed and mature seed

The leaves, stem, roots, green seeds and mature seeds (main parts) of giant leucaena were divided into their subparts. Leaves were divided to leaflets, rachis and petiole; stems were divided to mature stem and green stem (excluding embryonic leaves); roots were divided to primary and secondary roots; and green and mature seeds were divided to seed coat and cotyledons. The main parts were removed from 12-week-old plants and then further separated and grouped by respective subparts. Mimosine was extracted from these subparts and then determined as a percentage DW of each subpart. The percentage that each subpart contributed to the main parts were also determined. Each experimental set contained at least 4 replicates.

Expression of mimosine synthase in giant leucaena

To determine the expression levels of mimosine synthase (Ur-Rashid et al. 2018) in giant leucaena, mature seeds (no seed coat), green seeds (no seed coat), shoot tips and green seed pods (no seeds) were harvested from mature giant leucaena cultivar K636 trees from the University of Hawaii Waimanalo Research Station, Waimanalo, Hawaii. RNA was extracted from these parts using a modified CTAB method using Takara Fruit-mate™ (Takara Bio, Kusatsu, Shiga, Japan) in the extraction/lysis buffer to help increase RNA yields. After RNA extraction, RNA quality and quantity were determined using a Nanodrop spectrophotometer (Thermo Fisher, Waltham, MA, USA) and through gel electrophoresis. DNA was removed from all samples using the Turbo DNase kit (Thermo Fisher, Waltham, MA, USA) following manufacturers guidelines. cDNA was synthesized from 2 µg of total RNA using the Tetro™ cDNA synthesis kit (Meridian Bioscience, Cincinnati, OH, USA) following manufacturers guidelines. After cDNA synthesis, samples were diluted using nuclease free water. A qRT-PCR master mix was prepared in 10 µL reactions containing 5 µL of Sensifast™ SYBR® Hi-ROX kit (Meridian Bioscience, Cincinnati, OH, USA) aster mix, 0.25 µM forward primer, 0.25 µM reverse primer, 1 µL of MgCl2, 1 µL of single strand cDNA, and nuclease free water to bring the final volume to 10 µL. All qRT-PCR reactions were run on a StepOne™ Real-time PCR system (Applied Biosystems, Foster City, CA, USA) with reaction conditions set at 50 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 30 s, followed by melting curve analysis. Three biological replicates and 4 technical replicates were used for each tissue-type. The relative quantification for mimosine synthase gene expression in green and mature seeds and in shoot tips and green seed pods were determined from the cycle threshold (Ct) values, which were generated from each qRT-PCR reaction normalized against the Ct values of the internal reference gene, elongation factor-1α (ef1α). ef1α was identified as the most suitable internal control among 5 previously tested candidate reference genes (ubiquitin-5, β-actin, ef1α, 5.8S rRNA, and 18S rRNA). The relative fold change in gene expression was determined using the 2^(-delta delta CT) ($2^{-\Delta\Delta Ct}$) method.

Statistical analyses

For HPLC analyses of mimosine concentration in giant leucaena, a student's t-test, or simple analyses of variance (ANOVA) followed by Tukey post-hoc, Tukey-Kramer, or Dunnett's test were used as appropriate for data distribution characteristics. Statistical significance was determined with significant differences for $P<0.05$. qRT-PCR data were analyzed using a student's t-test ($P<0.05$).

Results

Mimosine in germinating and non-germinated seeds

Following scarification, the washed mature seeds of giant leucaena contained 3.6% mimosine at the start (day 0) of germination (Figure 1). The mimosine concentration of germinating seeds was reduced to 2.4% after 2 d of incubation. Germinating seeds released 0.66 ± 0.042% DW mimosine/d to the surroundings during the first 3 d of germination. The mimosine concentration of the germinating seeds increased from day 4 and continued to day 10 following scarification, when the mimosine concentration reached 19.8%. Apparently, mimosine is being synthesized in the germinating seeds, resulting in a 450% increase in the mimosine concentration. In ungerminated mature seeds, mimosine concentration was reduced to 1.33 ± 0.21% DW on day 10 due to leaching. Thus, following scarification, 63% seed mimosine is leached out within 10 d.

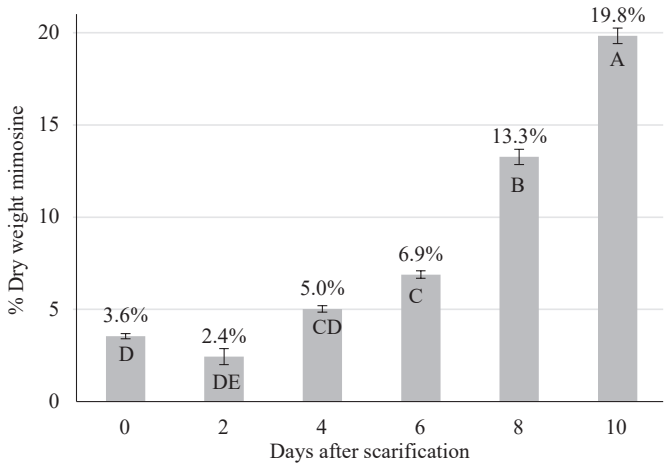

Figure 1. Mimosine concentration of giant leucaena mature seeds at 0, 2, 4, 6, 8, and 10 d after initial scarification. Error bars indicate standard error of 4 replications. Bars sharing a letter do not differ by Tukey-Kramer test (P≤ 0.05).

Mimosine concentration of giant leucaena during the first 7 months of growth

Mimosine concentration of seedlings changed significantly during early stages of growth and then stabilized after week 8 (Figure 2). At week 1, the mimosine concentration of the seedlings was 8.3% DW and at weeks 2 and 3, the mimosine concentration increased to 15.2% and 14.8% DW, respectively. Thereafter, mimosine concentrations decreased significantly to 9.4% and 4.4% DW at the 4th and 6th week, respectively. At the 8th week, total mimosine concentration of seedlings reduced further to 3.1% DW and thereafter, did not significantly change all the way through week 28. These results indicate that at a very early seedling stage giant leucaena produces high amounts of mimosine, which declines to ~3% DW after 8 weeks.

Mimosine concentration of leaf, stem, root and embryonic leaves of giant leucaena seedlings

Mimosine concentrations in plant parts were high during very early growth stages, but then decreased as the plants became older (Figure 3a). From all stages of growth tested, 2-week-old seedlings contained the highest mimosine concentrations in the leaves, stem, root and embryonic leaves. Embryonic leaves fell off between 6-10 weeks of seedling age. The amounts of mimosine present in leaves, stem and roots were also expressed as proportions of the whole plant mimosine. As the seedlings grew older, the proportions of leaf mimosine increased compared to the proportions of stem or root mimosine (Figure 3b). These results indicate that although whole plant mimosine concentration as percentage DW decreased with age, the proportion of mimosine in the leaves relative to the entire plant increased.

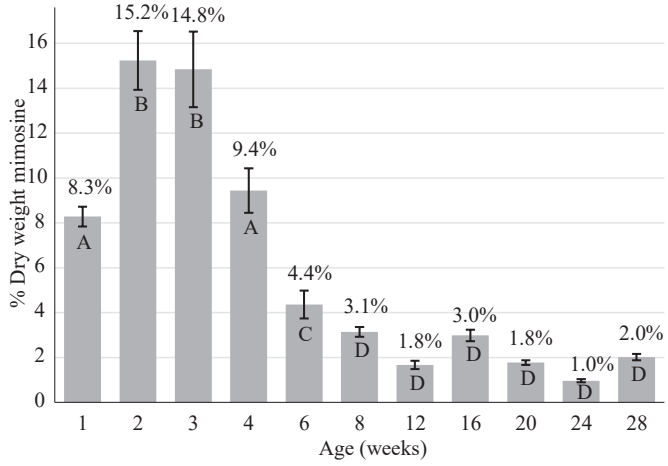

Figure 2. Mimosine concentration of giant leucaena seedlings at growth stages until 28 weeks. Error bars indicate standard error of 4 replications. Bars sharing a letter do not differ by Tukey-Kramer test (P≤0.05).

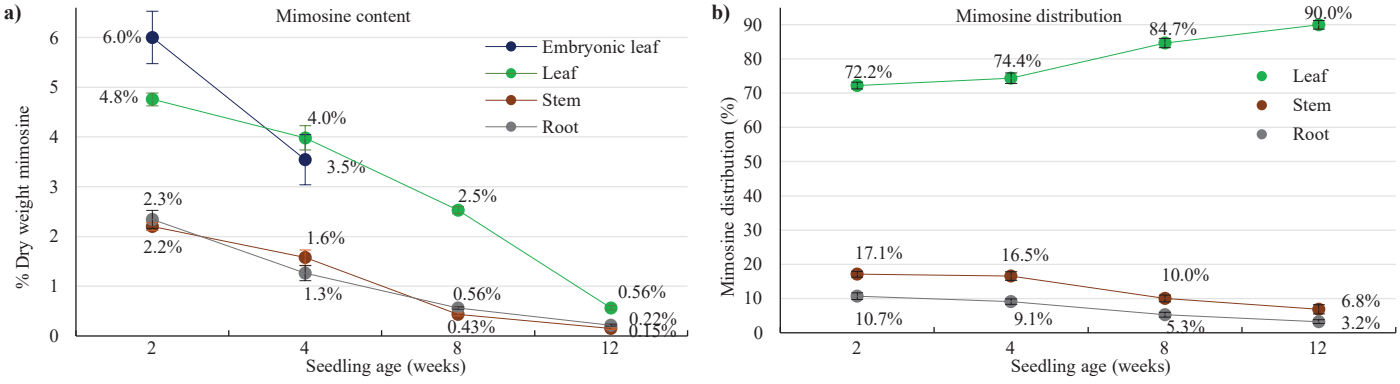

Figure 3. (a) Mimosine concentration in the leaves, stem, roots, and embryonic leaves of giant leucaena seedlings at 2, 4, 8, and 12 weeks of age; (b) Proportion of mimosine in the leaf, stem and root of giant leucaena, relative to the whole plant at 2, 4, 8, and 12 weeks of age. Embryonic leaves were excluded from the proportion. Error bars indicate standard error of 4 replications.

Mimosine distribution in different parts of the leaf

The mimosine concentration of whole leaves, including the leaflets, rachis and petiole of 8-week-old giant leucaena seedlings was 3.7% DW (Figure 4a). The mimosine concentration of leaflets was 4.1% DW, which was significantly higher than in the rachis and petiole which contained 2.5%, and 1.6% DW mimosine, respectively (Figure 4b). Among all parts of leucaena leaf, leaflets contained the highest proportion of mimosine at 79.1%, followed by the petiole and rachis at 10.7% and 10.2%, respectively (Figure 4c).

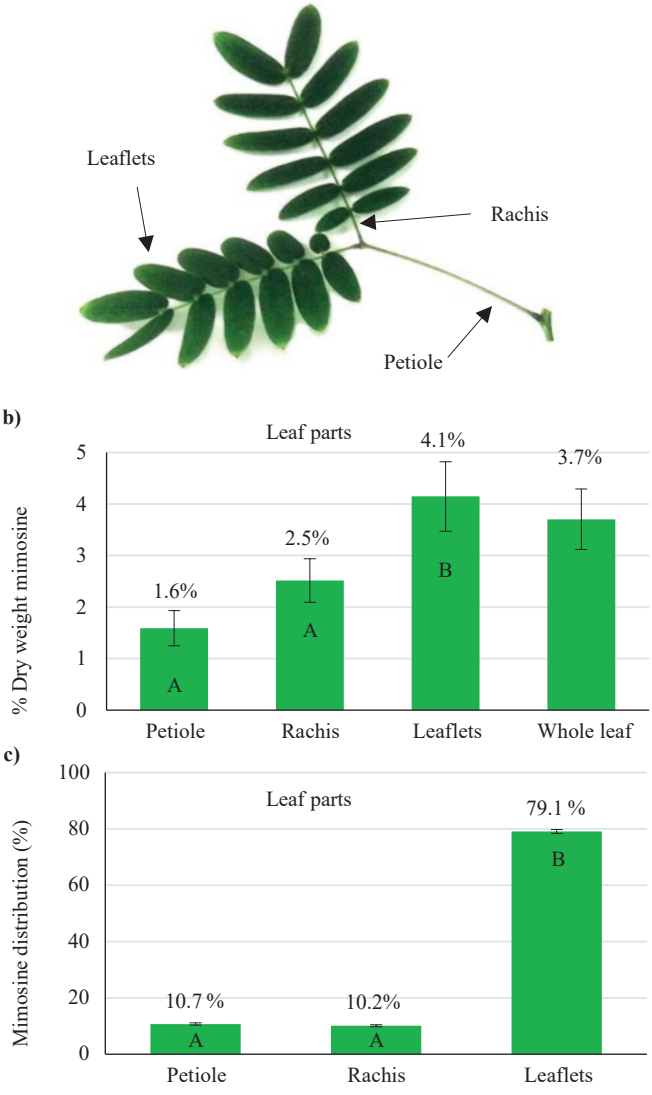

Figure 4. (a) Leaflets, rachis, and petiole of giant leucaena; (b) Mimosine concentration of leaflets, rachis, petioles, and whole leaves of giant leucaena seedlings; (c) Allocation of mimosine (% of the total mimosine of the entire leaf) within the parts of the leaf. Error bars indicate standard error of 4 replications. Bars sharing a letter do not differ by Tukey test (P≤0.05).

Mimosine distribution in different parts of the stem

Mimosine concentration of the entire stem of giant leucaena, including mature and green stem was 0.15% DW. Mimosine concentration of green and mature stems were 0.17% and 0.13% DW, respectively (Figure 5a). Mature stem and green stem contained 54.7% and 45.3% of the total stem mimosine, respectively (Figure 5b). These results indicate that although green stem has a higher mimosine concentration, the mature stem contains a higher proportion of the entire stem mimosine concentration.

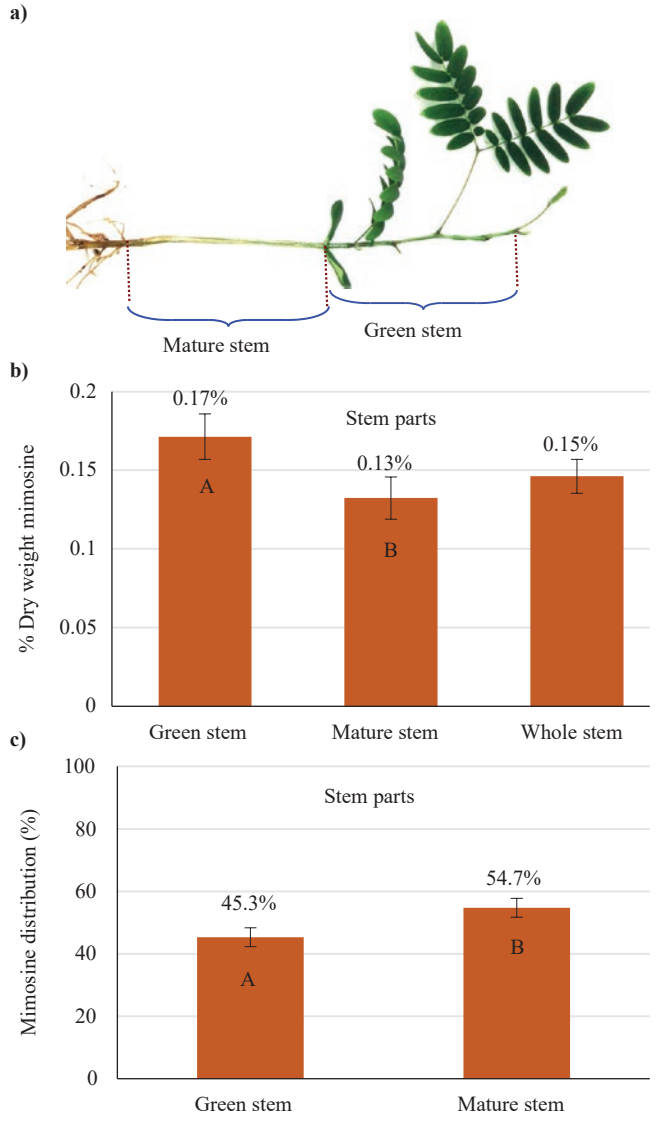

Figure 5. (a) Parts of the stem from giant leucaena; (b) Mimosine concentration of the green, mature, and whole stem of giant leucaena seedlings; (c) Allocation of mimosine (% of the total mimosine of the entire stem) within parts of the stem. Error bars indicate standard error of 4 replications. Bars sharing a letter do not differ by Tukey test (P≤0.05).

Mimosine distribution in different parts of the root

The mimosine concentration of the giant leucaena whole root system, including the primary and secondary roots was 0.25% DW (Figure 6a). The mimosine concentration of the primary root was 0.16% DW and the mimosine concentration of the secondary roots was 0.59% DW. Although the primary root had a much lower mimosine concentration, it still comprised 45.9% of the entire root system's mimosine concentration. Secondary roots of leucaena seedlings had a higher mimosine concentration than the primary root as percentage of dry weight, comprising 54.1% of the entire root mimosine (Figure 6b).

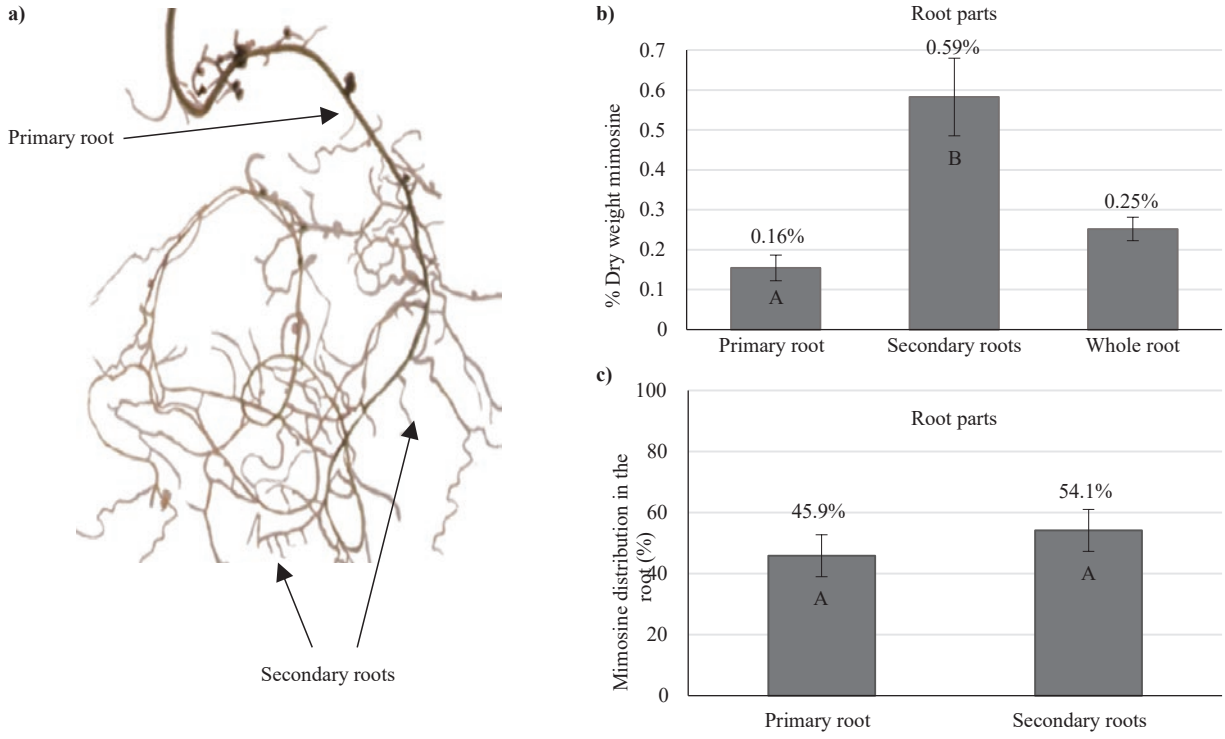

Figure 6. (a) Root parts of giant leucaena; (b) Mimosine concentration of primary, secondary and whole root system of giant leucaena seedlings; (c) Allocation of mimosine (% of the total mimosine of the entire root system) within the parts of the root. Error bars indicate standard error of 4 replications. Bars sharing a letter do not differ by Tukey test (P≤0.05).

Mimosine distribution in different parts of mature and green seeds

The mimosine concentration of whole green seeds and mature seeds were 3.8% and 3.2% DW, respectively (Figure 7a). The mimosine concentration of green and mature seed coats were 0.5% and 1.5%, respectively. The mimosine concentrations of the green and mature seed cotyledons were 21.7% and 8.9% DW, respectively. Cotyledons of both green and mature seeds contained the highest proportion of the entire seeds mimosine concentration (Figure 7b).

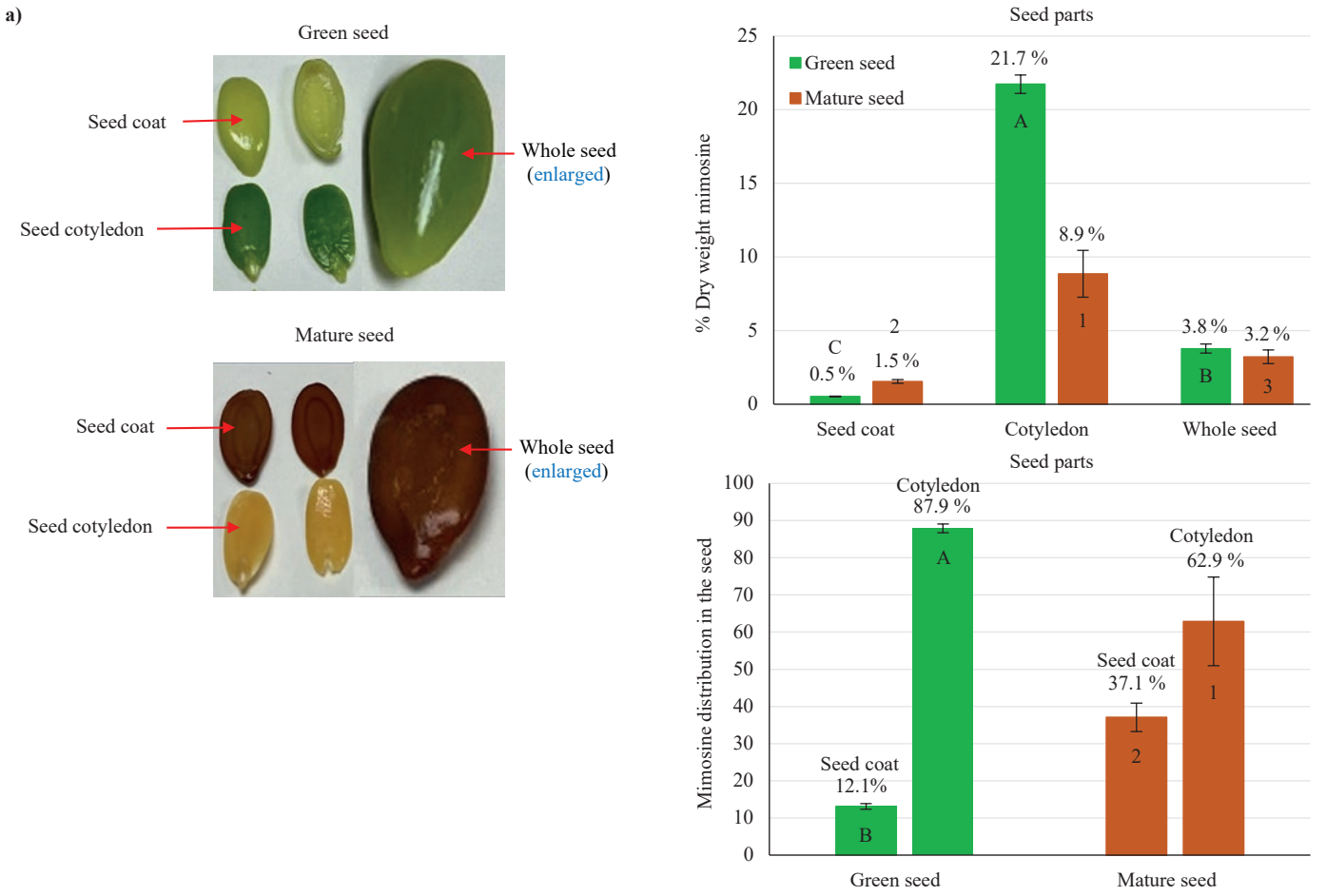

Figure 7. (a) Parts of green and mature seeds from giant leucaena; (b) Mimosine concentration of green and mature seed coats and cotyledons; (c) Allocation of mimosine (% of the total mimosine of the entire seed) within the parts of green and mature seeds. Error bars indicate standard error of 4 replications. Bars sharing a letter or number do not differ by Tukey test (P≤0.05).

Expression of mimosine synthase in green seeds, shoot tips and green seed pods

Giant leucaena green and mature seeds, green seed pods and fresh shoot tips, contain the highest amounts of mimosine. qRT-PCR analysis was used to test if there is a correlation between mimosine concentration and expression of mimosine synthase within these tissue types. Mature seeds following scarification served as the control. Green seeds had the highest relative increase in expression of mimosine synthase (5.1-fold), when compared to mature seeds (Figure 8). Shoot tips, which normally contain the highest concentrations of mimosine, were found to have 3 to 4-fold higher expression of mimosine synthase, when compared to mature seeds. Green seed pods and mature seeds appear to express mimosine synthase at similar levels. These results indicate that mimosine synthase expression does not have a large effect on the mimosine concentrations within leucaena tissues.

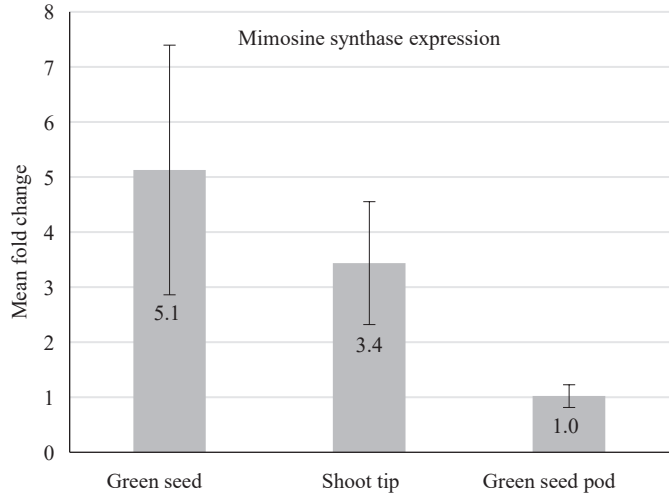

Figure 8. Mean fold change of mimosine synthase in green seeds, shoot tips and green seed pods. Mean fold change is calculated comparing the green seeds, shoot tips and green seed pods to mature seeds 0 days after initial scarification, which served as the control. Error bars indicate standard error of 3 replications.

Discussion

Mimosine is found in all parts of leucaena, including green buds, flowers, green seeds, mature seed pods, stem, root and root nodules (Soedarjo and Borthakur 1996; Rodrigues et al. 2019). Honda and Borthakur (2019; 2021) found that the mimosine concentration of giant leucaena foliage fluctuated depending on the environmental growth conditions and stresses that plants are exposed to. Mimosine in the different parts of leucaena shoots can vary from 1 to 12% with growing tips containing the highest amounts, while the old stems contain the lowest amounts (Jones 1979). In a previous study, young leucaena leaves were found to contain ~4.5% mimosine on a dry weight basis, which decreased to ~2% in 10-week-old leaves (Tangendjaja et al. 1986). Generally, green seeds, shoot tips and green seed pods contain the highest concentrations of mimosine (Soedarjo and Borthakur 1996). The results of the present study using methods developed by Da Silva Rodrigues-Honda et al. (2022) for accurate extraction and quantification of mimosine from leucaena tissues show that these tissues also had relatively high expression of mimosine synthase when compared to expression in mature seeds, which contain high concentrations of mimosine. In the present study, young leucaena seedlings contained relatively high amounts of mimosine, which decreased significantly from 15.2 % DW at week 2 to 2.0% DW at week 28. Similarly, younger parts and tissues of leucaena contained more mimosine than older or more mature parts. Germinating mature seeds also contained relatively high amounts of mimosine, which fluctuated depending on the germination stage. Cotyledons of germinating seeds synthesized mimosine and released it to the surrounding area. Mimosine synthase expression was also found to fluctuate, depending on the germination stage. The reason mimosine is high in germinating seeds and young seedlings could be because younger plants are susceptible to pest and pathogen attack and the high mimosine concentration in young and soft tissues could deter pests and microbial pathogens. Mimosine, its degradation product, 3H4P, and the degradation product isomer 2,3DHP are known to have antimicrobial, nematicidal and insecticidal properties (Anitha et al. 2005; Nguyen et al. 2015). Mimosine has also been researched for its herbicidal properties and has been shown to inhibit germination of rice seeds (Prasad and Subhashini 1994).

In the present study, mimosine was found in all major parts and subparts of the leaf, stem root, green seed and mature seed. Among the parts and subparts tested, leaves and cotyledons (embryonic leaves) contained the highest amount of mimosine. Leucaena stems and roots contained much less mimosine than leaves and seeds. Although the presence of mimosine is generally considered undesirable in leucaena foliage for use as fodder, its presence is helpful for the leucaena plant for survival under some biotic and abiotic stresses. The high mimosine concentration in the leaf and seed may serve to deter pests, herbivores and airborne and soil pathogens. Leaves are also exposed to higher levels of UV light and heat, which could induce osmotic and oxidative stress within leaf tissues (Rodrigues Correa et al. 2019). Cotyledons in the soil are exposed to different pathogens as well as varying degrees of osmotic stress. The mimosine present in the roots could be a means of releasing and widely dispersing it in the rhizosphere. Root secreted mimosine can serve as a phytosiderophore by binding and solubilizing important soil nutrients like iron, copper and zinc, making it easier for the plant to absorb from the soil, especially young seedlings that are not yet established. Secreted mimosine may also be a means to inhibit growth of soil pathogens and potential plant competitors. When seedlings are young, the high amount of mimosine present in seed cotyledons, which later become the embryonic leaves, could serve to supply the rest of the plant with mimosine. Mimosine has also been shown to be an osmolyte and antioxidant that helps plants counter secondary stresses that confers tolerance to grow successfully under osmotic and oxidative stress (Honda and Borthakur 2021). Osmotic stress is a type of secondary stress induced by a primary stress such as drought or pest and pathogen attack (Honda and Borthakur 2021).

The authors acknowledge that the gene expression portion of this study was conducted using material collected from field conditions in the state of Hawaii, USA, while the rest of this study was carried out under controlled growth conditions. The actual mimosine concentration results are expected to be different from studies conducted in other environments and regions of the world. However, this study showed that mimosine concentrations vary within giant leucaena based on age and tissue type, which can be translated to other growth environments.

In order to improve fodder quality of giant leucaena, plant breeders have tried to develop varieties with reduced mimosine (Brewbaker 2016). However, there are no mimosine-free *Leucaena* species available in nature, although there is some variation in mimosine

concentration among diploid and tetraploid leucaena species. Inter-species crosses among diploid and tetraploid species did not result in progenies with reduced mimosine (Brewbaker 2016). Jube and Borthakur (2010) developed a transgenic line of giant leucaena K636 by expressing the *pydA* gene encoding a meta-cleavage dioxygenase isolated from *Rhizobium* that forms nitrogen-fixing root nodules on leucaena. Dioxygenase encoded by *pydA* degrades 3H4P, which is an intermediate in both synthesis and degradation of mimosine. It was expected that expression of this enzyme in leucaena would reduce mimosine synthesis by degrading 3H4P, which is a precursor for mimosine biosynthesis. The mimosine concentration of the *pydA*-expressing transgenic plants was reduced by 22.5% in comparison to K636.

Giant leucaena is an allotetraploid species with 104 chromosomes and a basic chromosome number of x=26. Therefore, it is likely that the genes for mimosine biosynthesis are present in 4 copies. In mimosine-free mutants of giant leucaena, all 4 copies of a mimosine biosynthesis gene must be mutated. This may be the reason why spontaneous mimosine-free mutants are not found in nature. It should be possible to construct mimosine-free mutants of giant leucaena in the future using CRISPR, a recently developed genome editing method, in which all copies of a gene are mutated simultaneously. For the mimosine biosynthesis pathway, so far, only the final step in which mimosine synthase catalyzes reaction between O-acetylserine and 3H4P to produce mimosine is known (Yafuso et al. 2014); the biochemical steps and the enzymes or genes involved in the synthesis of the 3H4P have not yet been discovered. It is however known that the amino acid lysine is the precursor for biosynthesis of the pyridone ring (3H4P) of mimosine (Negi et al. 2021). Identification and characterization of the enzymes/genes for 3H4P biosynthesis will open the way for developing mimosine-free giant leucaena. It remains to be seen if such mimosine-free leucaena grows larger but fails to grow in alkaline soils where metallic cations such as iron and zinc are not easily available or the plant becomes susceptible to some biotic and abiotic stresses.

Giant leucaena is grown in Southeast Asia, Australia, and South America for its highly nutritious foliage that is widely used as animal fodder. The presence of mimosine in giant leucaena foliage limits its acceptability and usage as an animal fodder. Although toxic, mimosine, its degradation product 3H4P, and the 3H4P isomers can be combated in animals by inoculation with the ruminant bacterium *Synergistis jonesii*. However, if mimosine or its metabolite 2,3DHP are not present in the animal diet from leucaena foliage, the bacterial strain is lost within 6–9 months (Glatzle et al. 2019). Other mimosine-degrading bacterial strains have also been identified. Halliday et al. (2018) found that inoculation did not fully protect *Bos indicus* steers from 2,3DHP toxicity and postulated that inoculation may not be necessary. Recently, it was found that mimsoine-derived toxins can be naturally removed by animals and excreted in their urine as a mimosine-glucoronic acid conjugate (Shelton et al. 2019). Another way to combat mimosine toxicity would be to remove it post-harvest. Sundried leucaena leaves contain significantly less mimosine than untreated leaves (Agbo et al. 2017; Wee and Wang 1987). Soedarjo and Borthakur (1996) found that soaking leucaena leaves, pods and seeds in water removed up to 97% of mimosine without reducing protein amounts. Similarly, prolonged soaking of leaves in warm water (30 °C) for 48 h caused most of the mimosine to be degraded (Wee and Wang 1987). In a study conducted by Honda and Borthakur (2022), it was found that soaking leucaena leaves in an acid solution removed >90% of mimosine; however, it was also found that this method significantly reduced the gross energy of the foliage. In this same study, the authors developed a simple post-harvest processing method that significantly reduced not only mimosine, but also indigestible fibers and proanthocyanidins. Besides reducing these nutrient-limiting compounds, maceration also led to a significant increase in carbohydrates, which was thought to be in part due to the degradation of mimosine, fibers and proanthocyanidins. Ensiling was also found to lower mimosine of leucaena foliage; however, this decrease was due to degradation by endogenous enzyme release and not by fermentation (Lyon 1985).

Although mimosine in leucaena fodder can cause toxicity symptoms, some reports have stated that feeding animals a diet comprised mostly of leucaena (≥60%) was efficient and animals showed good performance (Giang et al. 2016; Halliday et al. 2018; Ruiz et al. 2019; Shelton et al. 2019). However, many studies indicate that a diet high in leucaena leads to unwanted side effects and a decline in animal productivity (Megarrity and Jones 1983; Santiago et al. 1988; Ram et al. 1994; Gupta and Atreja 1999). When utilizing leucaena fodder for food or as a protein supplement, it is important to note that the foliage, especially the active growing parts, contains a significant amount of mimosine. This can lead to unwanted side-effects that negatively affect health and animal production.

Conclusion

Mimosine is present in all parts of leucaena, and its concentrations vary with the age of the plant. Mimosine concentration is relatively high in the seeds, young leaves and shoot tips. The results of this study can be used to predict mimosine concentrations of giant leucaena plant parts as plants age and determine safe amounts for incorporating in livestock diets.

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