The Co-Occurrence of Two Pyridine Alkaloids, Mimosine and Trigonelline, in *Leucaena leucocephala*

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Leucaena leucocephala is a nitrogen-fixing tropical leguminous tree that produces two pyridine alkaloids, *i. e.* mimosine [β-(3-hydroxy-4-pyridon-1-yl)-L-alanine] and trigonelline (1-methylpyridinium-3-carboxylate). Mimosine has been detected in leaves, flowers, pods, seeds, and roots, and it is one of the principal non-protein amino acids that occurs in all organs. Asparagine was the most abundant amino acid in flowers. The mimosine content varied from 3.3 μmol/g fresh weight (FW) in developing flowers to 171 μmol/g FW in mature seeds. Trigonelline was also detected in leaves, flowers, pods, and seeds, but not roots. The trigonelline content was lower than that of mimosine in all organs. It varied from 0.12 μmol/g FW in developing seeds to 2.6 μmol/g FW in mature seeds. [2-^{14}C]Nicotinic acid supplied to the developing seeds was incorporated into trigonelline but not mimosine. This indicates that the pyridine and dihydroxypyridine structures of these two alkaloids are derived from distinct precursors. The physiological functions of mimosine and trigonelline are discussed briefly.

Key words: Leucaena leucocephala, Pyridine Metabolism, Nicotinic Acid Derivatives

Introduction

Plants produce several pyridine alkaloids, including mimosine, trigonelline, nicotine, and ricinine (Brown, 1998). *Leucaena leucocephala* is a nitrogen-fixing leguminous tree that is distributed widely in tropical and subtropical regions. It has been reported that high concentrations of mimosine [β-(3-hydroxy-4-pyridon-1-yl)-L-alanine, Fig. 1A] accumulate in leaves of *L. leucocephala* (Selmar, 2010). In a previous survey of nicotinic acid conjugate formation from [carbonyl-^{14}C]nicotinamide, it was found that *L. leucocephala* is also able to produce another pyridine alkaloid, trigonelline (1-methylpyridinium-3-carboxylate, Fig. 1B) (Ashihara *et al.*, 2012). A plant that produces both mimosine and trigonelline is therefore of interest in clarifying the biosynthetic relation between these two pyridine alkaloids unambiguously.

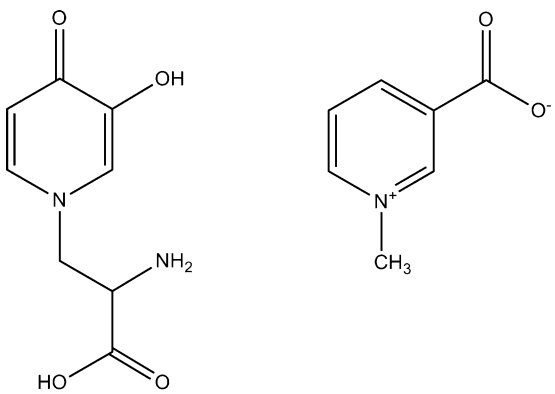

Fig. 1. Chemical structures of (A) mimosine and (B) trigonelline.

Mimosine occurs exclusively in some *Leucaena* species (Matsumoto and Sherman, 1951; Tangendjaja and Wills, 1980). Ingestion of mimosine and its dihydroxypyridinyl metabolite has resulted in toxic effects such as hair loss, goiter, reproductive disorders, epithelial damage, and ultimately death in animals (Hegarty *et al.*, 1979; Rosenthal, 1982; Kamada *et al.*, 1998; Lalitha and Rajendra Kulothungan, 2006). In plants, an allelopathic role of mimosine has been postulated (Chou and Kuo, 1986). Prasad and Subhashini (1994) reported that mimosine inhibits the germination, seedling growth, and some enzymes of *Oryza sativa*.

Conversely, trigonelline accumulates in the seeds of many plants, with an especially high content in several legumes and *Coffea* species (Tramontano *et al.*, 1986; Koshiro *et al.*, 2006; Matsui *et al.*, 2007). Small amounts of trigonelline have also been found to occur in the aerial parts of many angiosperm species (Blunden *et al.*, 2005). In legume seeds, trigonelline may be a storage form of nicotinic acid in symbiotic conditions with leguminous bacteria (Boivin *et al.*, 1990). It also acts as a bioactive substance for nyctinasty in *Aeschynomene indica* (Ueda *et al.*, 1995). A recent study suggested that trigonelline formation acts to remove excess nicotinic acid in plant cells, as a detoxification mechanism (Zheng *et al.*, 2005).

In the present study, we examined the contents of mimosine and trigonelline in various organs of *L. leucocephala*. We also compared fluctuations in their contents in leaves, flowers, pods, and seeds during growth. To investigate whether competition involving pyridine precursors takes place between the biosyntheses of mimosine and trigonelline, we examined the metabolic fate of [2-^{14}C]nicotinic acid, which is a potential precursor of both the pyridine alkaloids in developing seeds of *L. leucocephala*.

Materials and Methods

Plant materials

Samples of *Leucaena leucocephala* (Lam.) de Wit [= *Leucaena glauca* (L.) Benth] were collected on Iriomote Island, Okinawa, Japan in different seasons: leaves and flowers, March, 2012; young pods and developing seeds, June and July, 2012; mature seeds, October, 2011; and roots of the seedlings, July, 2013. These samples were stored at −80 °C prior to extraction.

Plant materials [∼100 mg fresh weight (FW)] that had been frozen in liquid nitrogen were ground using a multi-bead shocker (Yasui Kikai, Osaka, Japan). Amino acids and trigonelline were extracted with 100 mM HCl. The homogenate was centrifuged at 20,000 × *g* for 5 min. Samples of the resulting supernatant were filtered using a disposable filter unit (W-13-2; Tosoh, Tokyo, Japan). For the determination of trigonelline, aliquots of the filtered samples were used directly. For the amino acid analysis, the aliquots were diluted 5 times with 50 mM borate buffer (pH 8.0).

Determination of trigonelline and amino acids contents

Trigonelline contents were determined with an LC 10A HPLC system (Shimadzu, Kyoto, Japan), using a column (250 mm × 4.6 mm i.d.) packed with 5 μm InertSustain C18 (GL Sciences, Tokyo, Japan). Metabolites were eluted isocratically with 5% methanol including 10 mM H_3PO_4 and 5 mM sodium 1-hexanesulfonate (IPCC-06; GL Sciences), at a flow rate of 1 ml/min. The retention time of trigonelline was 7.8 min.

Amino acids, including mimosine, were separated and quantified using a gradient HPLC system with a fluorescence detector, adapted for free amino acid analysis according to Kotaniguchi *et al*. (1987). The retention time of mimosine in this system was 25.5 min. Amino acid standards were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Metabolism of [2-^{14}C]nicotinic acid

To determine the metabolic fate of the pyridine ring of nicotinic acid, we used [2-^{14}C]nicotinic acid. The experimental procedure was essentially the same as described in a previous paper (Ashihara and Deng, 2012). In summary, a seed (∼60 mg) from the pod of *L. leucocephala* was cut horizontally, and the two halves were placed in 2.0 ml of 30 mM potassium phosphate buffer (pH 5.6) containing 10 mM sucrose and 50 mM sodium ascorbate in the main compartment of a specially designed 30-ml Erlenmeyer flask with a centre well. Filter paper impregnated with 0.1 ml of 20% KOH in a small glass tube was placed in this centre well to collect $^{14}CO_2$. Each reaction was begun by the injection of 10 μl [2-^{14}C]nicotinic acid solution (37 kBq, specific activity 1.9 GBq/mmol; Moravek Biochemicals, Brea, CA, USA) into the main compartment of the flask containing the two seed slices. After 2 and 18 h of incubation at 28 °C, ^{14}C-labelled metabolites were analysed according to the methods described by Ashihara and Deng (2012), with a minor modification as fol-

lows. The metabolites were extracted from the frozen samples with ice-cold 6% perchloric acid (PCA) three times. The KOH-neutralized PCA-soluble fractions were freeze-dried and then dissolved in a small volume of 50% (v/v) ethanol. The PCA-soluble metabolites were separated by thin-layer chromatography (TLC) using microcrystalline cellulose TLC sheets (Merck, Darmstadt, Germany). The solvent systems used were: (I) *n*-butanol/acetic acid/water (4:1:2, v/v/v) and (II) isobutyric acid/ammonia/water (660:17:33) as described by Zheng and Ashihara (2004). Incorporation of radioactivity into trigonelline and mimosine was confirmed using these two solvent systems with reference compounds (Sigma-Aldrich). The distribution of ^{14}C on the TLC sheet was determined using a bioimaging analyzer (Typhoon, FLA7000; Fuji Photo Film, Tokyo, Japan). Total uptake of [2-^{14}C]nicotinic acid by the seed slices was calculated by adding the radioactivity found in the PCA-soluble, PCA-insoluble, and CO_2 fractions, and is expressed in kBq/g FW. Incorporation of radioactivity into individual metabolites separated by TLC is expressed as the percentage of the total radioactivity taken up by the seeds.

Results

Definition of the growth stages

To examine fluctuations in the metabolite content in different organs, we defined the growth stages according to the shapes and sizes of leaves, flowers, pods, seeds, and roots (Fig. 2). *Leucaena leucocephala* has pinnate compound leaves. We chose three distinct growth stages: non-flushed (L1), developing (L2), and mature leaves (L3) (Fig. 2A). The average length and fresh weight of a compound leaf in L1, L2, and L3 were, respectively, 12, 48, and 66 mm and 1.3, 56, and 140 mg. The flowers of *L. leucocephala* are head inflorescences. In this study, we selected three different sizes of whole flowers: small (F1), medium (F2), and large (F3) (Fig. 2B). The average diameter and fresh weight of flowers in stages F1, F2, and F3 were, respectively, 5, 8, and 12 mm and 62, 135, and 390 mg. For the pods and seeds, we distinguished two stages of young pods, P1 and P2; in these stages, seeds were too small to be separated from the pod (Fig. 2C). The length and fresh weight of the pods in stages P1 and P2 were, respectively, 25 and 75 mm and 37 and 180 mg. Three stages of seeds were collected from the more developed green pods (S1 and S2), and from matured brown pods (S3) (Fig. 2D). The length and fresh weight of seeds in stages S1, S2, and S3 were, respectively, 3, 10, and 7 mm and 2, 68, and 43 mg. Dehydration occurred in S3, causing a reduction in size and weight. In studying roots, we compared the pyridine alkaloid content in young (R1) and mature (R2) parts of the single roots from the ~2-year-old seedlings (Fig. 2E). The diameters of R1 and R2 roots were, respectively, 2.5 mm and 5.0 mm. We removed lateral roots which were difficult to collect intact from the soil

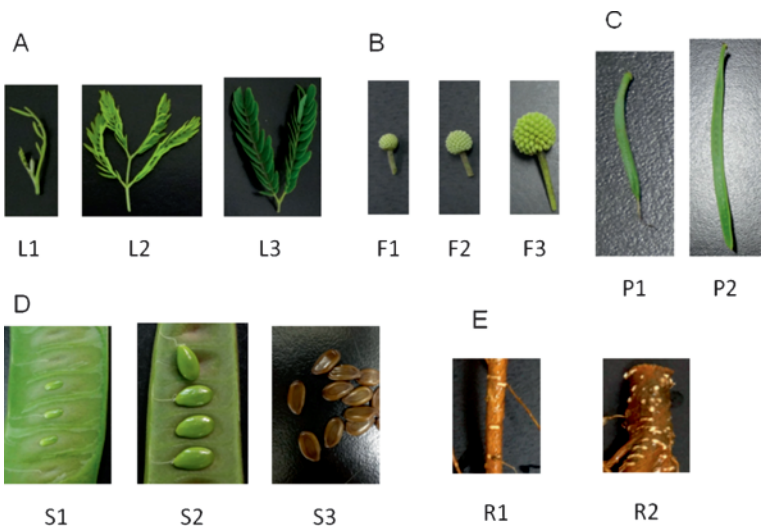

Fig. 2. Plant materials used in this study. (A) Leaves (pinnate compound leaves); (B) flowers (head inflorescences); (C) pods; (D) seeds; (E) roots of *Leucaena leucocephala*. See definitions in text.

Table I. Contents of trigonelline and mimosine in *Leucaena leucocephala*. Values and SD ($n = 3$) are expressed as μmol/g FW.

Organ	Stage	Trigonelline	Mimosine
Leaves	L1	0.68 ± 0.14	38.8 ± 2.1
	L2	0.71 ± 0.05	14.0 ± 0.7
	L3	0.36 ± 0.18	11.1 ± 1.0
Flowers	F1	1.15 ± 0.90	13.6 ± 5.6
	F2	1.39 ± 0.47	3.3 ± 2.1
	F3	0.97 ± 0.25	7.4 ± 0.1
Pods	P1	0.72 ± 0.19	16.5 ± 9.3
	P2	0.96 ± 0.47	19.6 ± 3.3
Seeds	S1	0.65 ± 0.31	19.7 ± 1.1
	S2	0.12 ± 0.02	32.1 ± 5.0
	S3	2.60 ± 0.46	171.3 ± 52.7
Roots	R1	nd	30.2 ± 1.8
	R2	nd	26.5 ± 3.0

nd, not detected.

and used ∼500-mg FW samples of the primary root for analysis.

Distribution of mimosine and trigonelline in different organs

Table I shows the contents of mimosine and trigonelline in the different organs of *L. leucocephala*, expressed as μmol/g FW. In all organs, the content of mimosine was always higher than that of trigonelline. Trigonelline was not detected in roots. The highest contents of both mimosine and trigonelline were observed in mature dry seeds (S3). The trigonelline content in *L. leucocephala* seeds (2.60 μmol/g FW) was similar to that in *Pisum sativum* (2.7 μmol/g), but much lower than that found in *Trifolium incarnatum* (58 μmol/g FW) (Matsui *et al.,* 2007). The content of mimosine in flowers (3 – 14 μmol/g FW) was lower than in leaves (11 – 39 μmol/g FW), whereas the trigonelline content in flowers (1.0 – 1.4 μmol/g FW) was higher than in leaves (0.4 – 0.7 μmol/g FW). The content of mimosine was higher in young leaves than in developed or mature leaves, while in contrast the content in seeds increased with maturation (Table I).

Amino acid profiles of leaves, flowers, and seeds

It is of interest to determine what proportion mimosine comprises of the total non-protein free amino acids in *L. leucocephala*. Profiles of free amino acids were investigated; Fig. 3 shows typical data from young leaves (L1), flowers (F1), and seeds (S1). Asparagine (Asn) and mimosine (Mim) were the most abundant free amino acids comprising more than half of the total amino acid content in all samples examined.

Changes in the content of mimosine and asparagine during growth

As mimosine and asparagine are the major amino acids in *L. leucocephala*, the patterns of the changes of

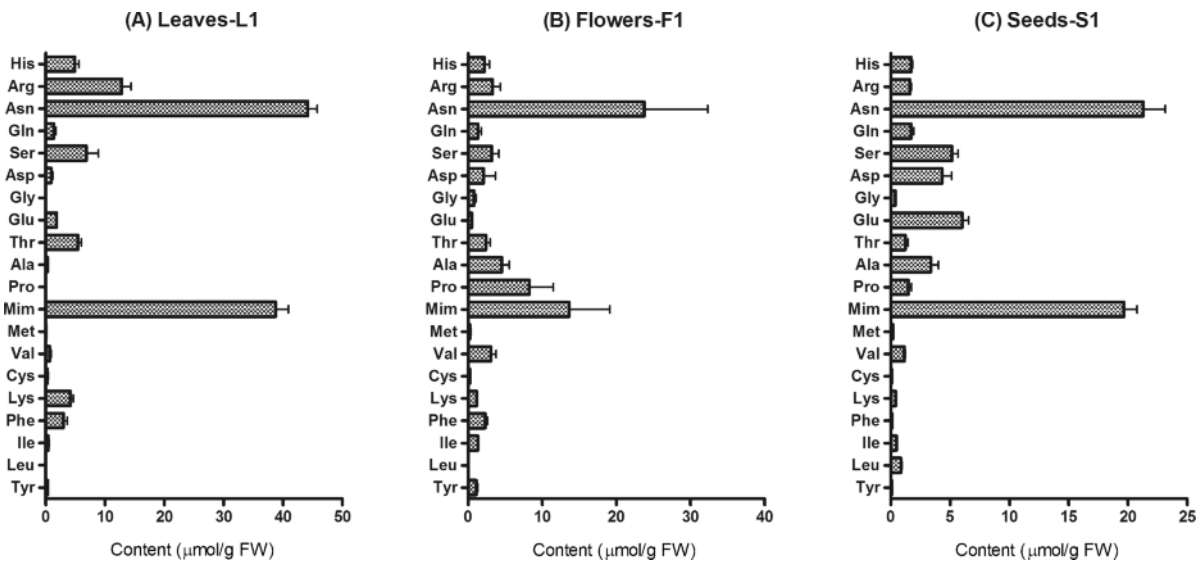

Fig. 3. Profiles of free amino acids in (A) young leaves, (B) flowers, and (C) seeds of *Leucaena leucocephala*. Average values and SD ($n = 3$) are expressed as μmol/g FW.

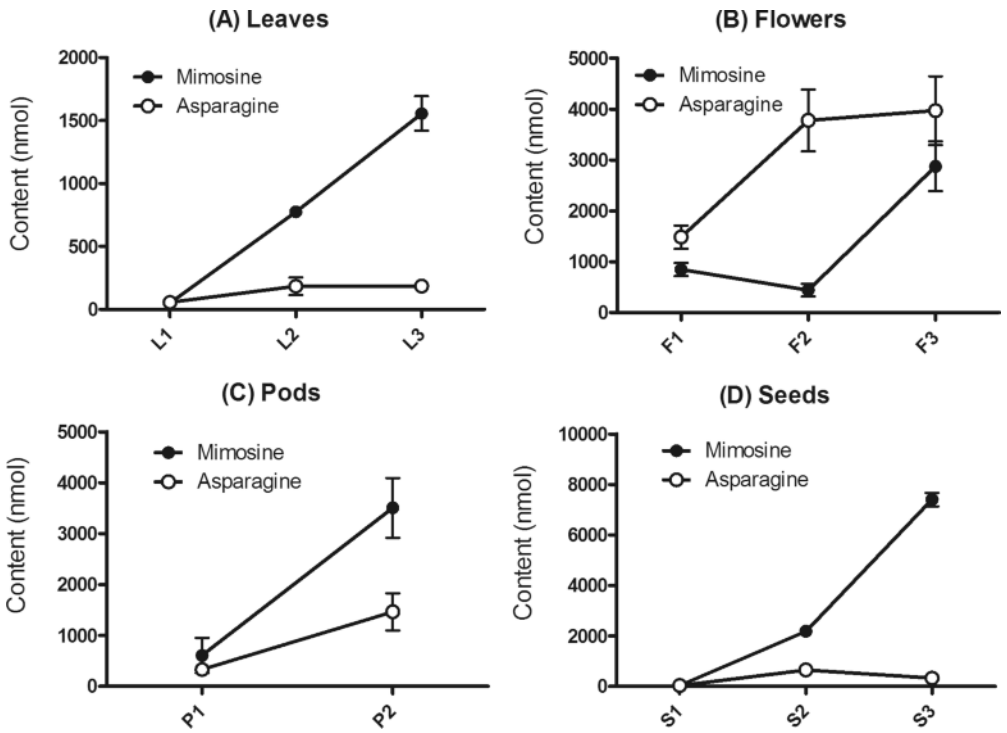

Fig. 4. Changes in mimosine and asparagine content during growth of (A) leaves, (B) flowers, (C) pods, and (D) seeds of *Leucaena leucocephala*. Average values and SD ($n = 3$) are expressed as nmol/organ.

these two compounds with growth and maturation of leaves, flowers, young pods, and seeds are illustrated in Fig. 4. Contents are expressed as nmol per organ from which it is possible to assess the changing profiles of the amino acids in each organ during growth.

The pattern of the amino acids in flowers (Fig. 4B) was different from those of the other organs (Figs. 4A, C, and D). Mimosine was the most abundant amino acid in leaves, pods, and seeds, but asparagine was the major amino acid in flowers. The relative amount of mimosine was low in young tissues, but increased with the development and maturation of leaves, pods, and seeds. In mature seeds (S3), the mimosine content was at least 20-fold higher than that of asparagine (Fig. 4D). These results suggest that mimosine is an end product accumulating in mature organs. Asparagine, in contrast, does not accumulate in parallel with the development of an organ. In seeds, the asparagine content in stage S3 is approximately half that in stage S2. Some asparagine formed and/or transported in stage S2 may therefore be converted to other compounds in seeds during maturation.

Changes in the trigonelline content during growth

Figure 5 shows the changing pattern of trigonelline during growth and maturation of leaves, flowers, pods, and seeds. As with mimosine, the trigonelline content per organ increased with growth. The pattern in leaves was slightly different from that in seeds. However, in leaves there was a marked increase in the trigonelline content between stages L1 and L2, whereas in seeds the increase was very high from S2 to S3. Trigonelline is also considered an end product and accumulates in all organs.

Metabolism of [2-^{14}C]nicotinic acid in young seeds

Several pyridine alkaloids are derived from nicotinic acid, including trigonelline, nicotine, anabasine, and ricinine (Waller *et al.*, 1966; Waller and Dermer, 1981; Shoji and Hashimoto, 2011; Zrenner and Ashihara, 2011). Mimosine is synthesized from 3,4-dihydroxypyridine and *O*-acetylserine by the action of mimosine synthase (EC 2.5.1.52), which is an isoform of cysteine synthase (Murakoshi *et al.*, 1984; Ikegami

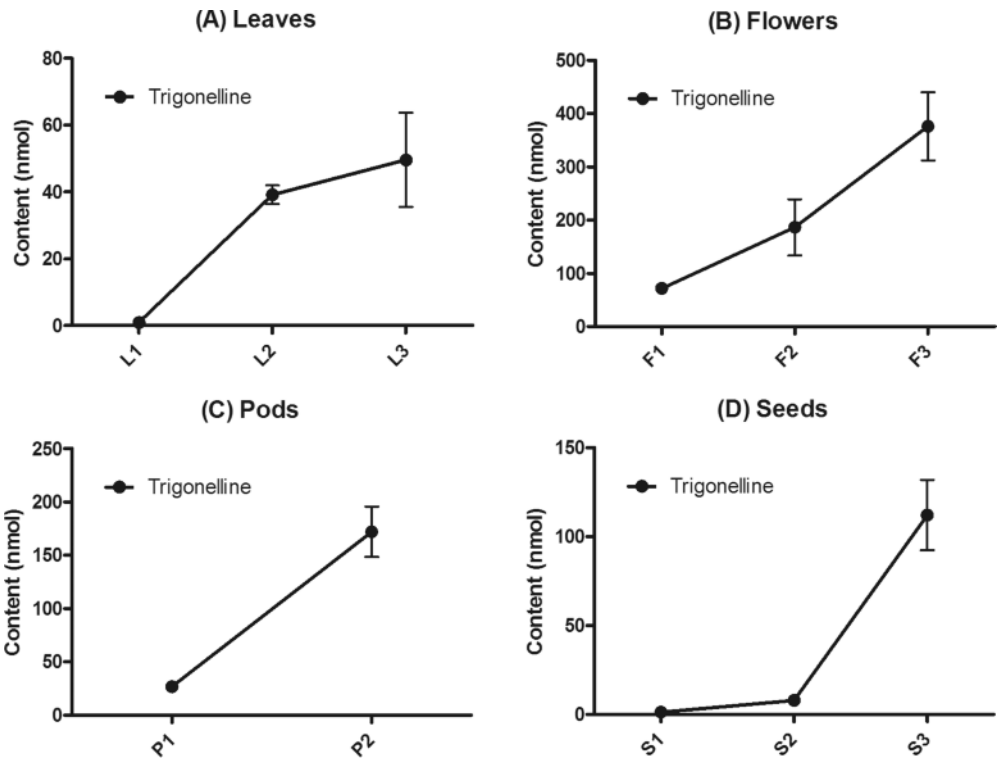

Fig. 5. Changes in trigonelline content during growth of (A) leaves, (B) flowers, (C) pods, and (D) seeds of *Leucaena leucocephala*. Average values and SD ($n = 3$) are expressed as nmol/organ.

Table II. Metabolic fate of [2-^{14}C]nicotinic acid in *Leucaena leucocephala* seeds (stage S2).

^{14}C-Metabolite	Incorporation of radioactivity			
	2 h		18 h	
Trigonelline	0.17 ± 0.03	(8.5 %)	9.77 ± 1.15	(28.3 %)
Mimosine	nd	(–)	nd	(–)
NAD/NADP	0.91 ± 0.03	(45.7 %)	10.39 ± 2.87	(30.1 %)
Nicotinic acid	0.62 ± 0.01	(31.2 %)	5.92 ± 0.45	(17.2 %)
Total uptake	1.99 ± 0.1	(100 %)	34.51 ± 2.17	(100 %)

Incorporation of radioactivity is expressed as kBq/g FW and SD ($n = 3$) and percentage of total uptake (in parentheses). nd, not detected.

et al., 1990). The radioactivity of [2-^{14}C]lysine was incorporated into mimosine in the leaflets and petioles of *L. leucocephala* (Hylin, 1964), so the pyridine ring of mimosine appeared to be derived from lysine, although the biosynthetic pathway of 3,4-dihydroxypyridine has not yet been determined in plants. In, contrast, trigonelline biosynthesis in plants is well established. Nicotinic acid is formed as a catabolite of NAD (Ashihara, 2008; Zrenner and Ashihara, 2011), and trigonelline is synthesized from nicotinic acid in a reaction catalyzed by trigonelline synthase (EC 2.1.1.7) (Upmeier *et al.*, 1988; Chen and Wood, 2004).

To examine the role of nicotinic acid in the pyridine alkaloid biosynthesis in *L. leucocephala*, the metabolic fate of [2-^{14}C]nicotinic acid was investigated in developing seeds (stage S2) (Table II). Two hours after administration, 46 % of the radioactivity from [2-^{14}C]nicotinic acid was found in the NAD and NADP fraction, and more than 8 % was in trigonelline; but no radioactivity was found in mimosine. When incubation was continued for 16 h, 28 % of the radioactivity taken up by the seed segments was incorporated into trigonelline, while there was no radioactivity in mimosine. These results suggest that the pyridine

ring of mimosine is not derived from nicotinic acid. This hypothesis is also supported by experiments on the effect of nicotinamide on pyridine alkaloids levels. When leaf segments of *L. leucocephala* were treated with 1 mM nicotinamide, there was a 3.5-fold increase in the trigonelline content (Ashihara and Watanabe, 2014), but no such increase in the mimosine content (Ashihara, unpublished result). Exogenously supplied nicotinamide appears to be converted to nicotinic acid by nicotinamidase (EC 3.5.1.19) in leaves, following which trigonelline is formed.

Discussion

The results demonstrate that *Leucaena leucocephala* produces two pyridine alkaloids, mimosine and trigonelline. The mimosine content is always higher than that of trigonelline in all organs. The present work revealed that the dihydroxypyridine ring of mimosine is not derived from nicotinic acid, so that there appears to be no competition for substrate availability between the biosyntheses of these two pyridine alkaloids. Mimosine in *L. leucocephala* may act as an allelochemical which the plant produces to defend itself from competing plants or herbivores (Chou and Kuo, 1986). The available evidence suggests that trigonelline might be formed from the excess amounts of nicotinic acid released from the pyridine nucleotide cycle. As nicotinic acid, but not trigonelline, inhibits the growth of roots of mungbean seedlings and the proliferation of lettuce cells derived from protoplasts (Zheng *et al*., 2005; Sasamoto and Ashihara, 2014), conversion of nicotinic acid to trigonelline appears to be a detoxification mechanism. Previous studies have indicated that all plants have the ability to produce trigonelline or nicotinic acid *N*-glucoside (Barz, 1985; Ashihara *et al*., 2012). Nicotinic acid *N*-glucoside formation is restricted mostly to ferns and selected orders of angiosperms, whereas other plants produce trigonelline. Parallel formation of both trigonelline and nicotinic acid *N*-glucoside does generally not occur, but some exceptions have been found. In all plant species, regardless of whether trigonelline or nicotinic acid *N*-glucoside is produced, formation of these conjugates appears to be related to the detoxification of excess quantities of nicotinic acid.

The nutritive value of pyridine alkaloids is also relevant in legume plants. Genes for mimosine degradation have been found in the *L. leucocephala* symbiont *Rhizobium* sp. (Borthakur *et al.*, 2003; Awaya *et al.*, 2005). Mimosine therefore appears to be degraded, and the catabolites are utilized as nutrients in root nodules. Similarly, trigonelline may act as a storage form of nicotinic acid under symbiotic conditions, since it has been reported that catabolism of trigonelline is found in legume roots with leguminous bacteria, such as *Rhizobium meliloti* (Boivin *et al*., 1990). If trigonelline is converted to nicotinic acid, it is utilized for NAD synthesis by the salvage pathway of pyridine nucleotide synthesis (Zrenner and Ashihara, 2011). Other catabolites are also used as nitrogen and carbon sources.

In conclusion, *L. leucocephala* produces two pyridine alkaloids via distinct biosynthetic pathways. In addition to their ecological and/or detoxification roles, mimosine and trigonelline may play a role in nutritional nitrogen storage in symbiotic relations involving this legume tree.

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