CAFFEINE BIOSYNTHESIS AND PURINE METABOLISM IN LEAVES OF *MASCAROCOFFEA* SPECIES

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Caffeine, a purine alkaloid, was not detected in leaves of two *Mascarocoffea* species, *Coffea millotii* and *Coffea perrieri*. Trigonelline, a pyridine alkaloid, occurred in these species, but the levels (3−4 μmol g^{-1} fresh weight) were much lower than that of Robusta coffee (*Coffea canephora)* (36 μmol g^{-1} fresh weight). Feeding experiments with [8-^{14}C]adenine indicated that purine alkaloid biosynthesis was terminated at 7-methylxanthine formation and as a consequence theobromine and caffeine were not produced in *Coffea millotii* and *Coffea perrieri*. The adenine salvage activity was lower, but its degradation activity was higher in leaves of these *Mascarocoffea* species than those in *Coffea canephora*. The metabolic fate of the purine nucleosides, [8-^{14}C]inosine, [8-^{14}C]guanosine and [8-^{14}C]xanthosine was investigated in leaves of *Coffea millotii*. The biosynthesis of 7-methylxanthine, but not theobromine or caffeine, from these precursors was detected. Large amounts of these purine nucleosides were catabolized via allantoin. Limited amounts of [8-^{14}C]inosine and [8-^{14}C]guanosine were salvaged and utilized for RNA synthesis, however, no [8-^{14}C]xanthosine salvage was observed. Little or no ^{14}C-metabolites were observed when [8-^{14}C]theobromine and [8-^{14}C]caffeine were applied to leaf disks of *Coffea millotii*. From the results obtained in this study, possible metabolic pathways of purines in *Mascarocoffea* species are discussed.

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Introduction

Coffee plants accumulate two types of alkaloids, caffeine and trigonelline which are derived from purine and pyridine nucleotides, respectively.[1-4] Caffeine-free species in the genus *Coffea* have been screened by several investigators and reviewed by Hamon et al.[5] Most wild coffee species belonging to the *Mascarocoffea* section grown in Madagascar and neighboring islands are caffeine free, although the occurrence of theobromine and caffeine has been reported in a few *Mascarocoffea* species.[5-8]

In Arabica coffee (*Coffea arabica*) and Robusta coffee (*C. canephora)* plants which are used for coffee beverages, rapid biosynthesis of caffeine have been demonstrated in young leaf and fruit tissues, but the degradation of caffeine is very slow, as a result, caffeine is accumulated in mature leaves and seeds.[9] The activity of biosynthesis and degradation of caffeine in leaves of low-caffeine-containing *Coffea* species, *C. salvatrix*, *C. eugenioides*, and *C. bengalensis* has been investigated by Ashihara and Crozier.[10] The biosynthesis of caffeine is less pronounced in leaves of these *Coffea* species than in *C. arabica*. Degradation of caffeine was slow in both of high- and low caffeine coffee species, except in *C. eugenioides* which rapidly catabolises caffeine.[10, 11]

In the present studies, we used two *Mascarocoffea* spices, *C. millotii* (Fig. 1) and *C. perrieri* and the level of caffeine and trigonelline and the activity of caffeine biosynthesis are compared with those in caffeine-accumulating *C. canephora*. As expected, caffeine was found only in *C. canephora*. In contrast, trigonelline occurred in all three *Coffea* samples examined. Since purine and purine alkaloid metabolism have not yet been investigated in *Mascarocoffea* species, we examined by the feeding experiments with ^{14}C-labelled adenine, inosine, guanosine, xanthosine, theobromine and caffeine. Possible pathways of purine metabolism in *Mascarocoffea* species is discussed.

Figure 1. A tree (A) and fruits (B) of *Coffea millotii*.

Materials and methods

Plant materials

Leaves of *Coffea millotii* (A950), *C. perrieri* (A305) and *C. canephora* (K28) used in this study were obtained at the FOFIFA Experimental Stations, Kianjavato, Madagascar.

Determination of purine alkaloids and trigonelline

Endogenous levels of purine alkaloids and trigonelline were determined according to Zheng and Ashihara[12]. Samples were ground with 80% methanol in a mortar and pestle. After incubation at 60°C for 30 min, the homogenates were centrifuged and the supernatant was collected. After complete evaporation of the methanol, the extracts were dissolved in distilled water. The aliquots (10–50 μL) of water-soluble fraction were used for determination by HPLC after centrifugation.

HPLC was carried out with a Shimadzu CLASS-VP HPLC system, Type LC 10A (Shimadzu Corporation, Kyoto, Japan) on a ferrule-less column (250 mm x 4.6 mm i.d.) packed with a 5 μm ODS Hypersil support (Thermo Electron Corporation, Waltham, MA, USA), eluting a flow rate of 1mL min^{-1} with a 35 min, 0–70 % gradient of methanol in 50 mM sodium acetate, pH 5.0 (0–10 min: 0 % methanol; 10–20 min: 0–10 % linear gradient; 20–35 min: 10–70 % linear gradient). The absorbance at 190–370 nm was monitored using a Shimadzu Diode Array Detector, type SPD-M10A. The purine alkaloid and trigonelline contents were estimated from the absorption of 270 nm of standards obtained from Sigma-Aldrich Co., St. Louis, MO, USA.

^{14}C-Feeding experiments

All radiochemicals used were obtained from Moravek Biochemicals Inc, Brea, CA, USA. The labeled compounds were administered essentially as described in previous reports.[11, 13, 14] In brief, leaves were sterilized with 1 % sodium hypochlorite solution and then washed with sterilized distilled water, and leaf disks (~4 mm x 4 mm) were prepared aseptically. Samples (~100 mg f.w.), and 2.0 mL of 30 mM potassium phosphate buffer (pH 5.6) containing 10 mM sucrose and 1 % sodium ascorbate, were placed in the main compartment of a 30 mL Erlenmeyer flask fitted with a glass tube containing a piece of filter paper impregnated with 0.1 mL of 20 % KOH in the centre well. Each reaction was started by addition of ^{14}C-labelled purine compounds.

The flasks were incubated in an oscillating water bath at 27°C. After incubation, the glass tube was removed from the center well and placed in a 50 mL Erlenmeyer flask containing 10 mL of distilled water. At the same time, the leaf disks were separated from the incubation medium by filtering through a tea strainer. The samples were washed with distilled water and then frozen with liquid N_2 and stored at -80° C. The $KH^{14}CO_3$ that had been absorbed by the filter paper was allowed to diffuse overnight into the distilled water, and 0.5 mL aliquots of the resulting solution were used for the determination of radioactivity incorporated into CO_2.

Metabolites from ^{14}C-labelled purine bases and nucleosides were extracted successively with 4 mL of cold 6 % perchloric acid solution. Lipids in the insoluble materials were removed with a mixture of ethanol and diethyl ether (1:1, v/v) at 50°C for 15 min. The precipitate was washed with the same mixture, then with distilled water. RNA in the insoluble fraction was hydrolysed with 0.3 M KOH at 37°C for 18 h, and RNA hydrolysates was obtained.[14]

The perchloric acid-soluble metabolites and the RNA hydrolysates were neutralized with KOH and radioactive metabolites were separated by TLC using cellulose plates and the solvent systems shown in a previous paper.[14] Radioactivity of liquid samples and on the TLC plates was determined using a multipurpose scintillation counter (Beckman, LS 6500) and a bio-imaging analyzer (Type FLA-2000, Fuji Photo Film Co. Ltd.), respectively.

Results and discussion

Endogenous levels of caffeine and trigonelline

Table 1 shows the leaf sizes and fresh weights of two species of *Mascarocoffea* and *Coffea canephora*. We used well-developed young leaves in this study. Accumulation of caffeine was found only in *C. canephora* while caffeine could not be detected in *C. millotii* and *C. perrieri*. In contrast, trigonelline was found in the leaves of all samples. The level of trigonelline in *C. perrieri* and *C. millotii* leaves were respectively 8- and 12-fold lower than the level in *C. canephora* leaves.

Comparison of purine alkaloid biosynthesis

Among exogenously administered purine precursors, it has been known that adenine is the most effective precursor for the biosynthesis of caffeine.[15] Therefore, we first examined the metabolic fate of [8-^{14}C]adenine in two *Mascarocoffea* species, *C. millotii* and *C. perrieri* and compared with the metabolism in *C. canephora*. As shown in Table 2, a limited amount of radioactivity from [8-^{14}C]adenine accumulated as methylxanthines (purine alkaloids). Although radioactivity was found in 7-methylxanthine in all three species, incorporation into theobromine and caffeine was found only in *C. canephora*. In *C. canephora*, more radioactivity was found in 7-methylxanthine and theobromine than in caffeine. This is probably due to the fact that the precursors could not be transformed into caffeine during the incubation period, although caffeine is the end-product.

In *C. arabica* and *C. canephora*, three *N*-methyltransferases for caffeine biosynthesis occur. The first enzyme, 7-methylxanthosine synthase (EC 2.1.1.158) catalyzes the conversion of xanthosine to 7-methylxanthosine (step 11 in Fig. 2).[16] The product, 7-methlyxanthosine is hydrolysed to 7-methylxanthine (step 12) by *N*-methynucleosidase (EC 3.2.2.25).[17] Thus, all three species possess the machinery up to 7-methylxanthine.

Table 1. Leaf sizes (length and width) and purine alkaloid contents in three different *Coffea* species used in this research. nd, not detected.

Species	FOFIFA accession number	Leaf size (l x w, mm)	Leaf weight (f.w., g)	Caffeine (µmol g f.w.$^{-1}$)	Trigonelline (µmol g f.w.$^{-1}$)
C. millotii	A950	105 x 57	1.05 ± 0.07	nd	2.89 ± 0.03
C. perrieri	A305	127 x 33	0.68 ± 0.01	nd	4.37 ± 0.29
C. canephora	K28	229 x 98	2.52 ± 0.06	25.3 ± 0.79	36.0 ± 2.1

Table 2. Comparison of adenine metabolism in leaf segments of *Coffea millotii*, *C. perrieri*, and *C. canephora.*

Metabolites	*C. millotii*	*C. perrieri*	*C. canephora*
Nucleotides	23.8 ± 2.1	34.7 ± 0.73	12.1 ± 3.8
RNA	14.6 ± 0.27	16.5 ± 1.6	61.0 ± 6.4
Nucleosides and bases			
Adenine	1.52 ± 0.09	6.27 ± 1.28	8.46 ± 0.90
Hypoxanthine	2.53 ± 0.24	nd	nd
Xanthosine	7.09 ± 0.93	7.95 ± 0.70	3.35 ± 1.99
Xanthine	12.4± 0.20	5.68 ± 1.30	2.73 ± 1.68
Purine alkaloids			
7-Methyl-xanthine	1.13 ± 0.61	4.18 ± 0.43	2.45 ± 0.22
Theobromine	nd	nd	2.49 ± 1.53
Theophylline	nd	nd	0.26 ± 0.01
Caffeine	nd	nd	0.31 ± 0.03
Degradation products			
Ureides (allantoin and allantoic acid)	21.1 ± 0.67	20.8± 1.0	4.14 ± 2.11
Carbon dioxide	15.9 ± 0.94	4.02 ± 0.93	2.72 ± 2.03
Total uptake	39.4 ± 1.7	20.8 ± 0.58	8.95 ± 4.05

[8-^{14}C]Adenine (10 µM, specific activity 1.9 GBq mol^{-1}) was administered for 18 h. The mean incorporation of radioactivity into individual metabolites is expressed as the percentage of total radioactivity taken up by the segments ± SD. Total uptake is expressed as kBq 100 mg f.w.$^{-1}$, nd, not detected

However, it seems likely that *Mascarocoffea* species lack the enzyme(s) which catalyze the conversion of 7-methylxanthine to caffeine (steps 13 and 14). In *C. arabica* and *C. canephora*, plural *N*-methyltransferases, namely dual functional caffeine synthase (EC 2.1.1.160) and theobromine synthase (EC 2.1.1.159) catalyze this step[18, 19]. In *C. canephora*, in addition to theobromine, very small amounts (<0.3 %) of radioactivity were detected in theophylline (Table 2). It might be produced by a minor pathway of purine alkaloid biosynthesis as has been observed in tea leaves.[14]

Comparison of adenine salvage and degradation

In addition to purine alkaloids, the radioactivity from [8-^{14}C]adenine was distributed in various metabolites (Table 2). Since only small amounts of [8-^{14}C]adenine (2−8 % of total radioactivity) were detected in the leaf disks, exogenously supplied [8-^{14}C]adenine was not retained as adenine, but rapidly metabolized to other compounds. Substantial amounts of radioactivity from [8-^{14}C]adenine was incorporated into the salvage products, namely, nucleotides and RNA. However, compared with *C. canephora* (73 % of total radioactivity), the rate of adenine salvage (38−51 %) was low in *Mascarocoffea* species.

As shown in other plants, adenine was converted to AMP by adenine phosphoribosyltransferase (EC 2.4.2.7, step 1 in Fig. 2) and then entered the adenine nucleotide pool comprising AMP, ADP and ATP. Some ATP was utilized for RNA synthesis.[10] In addition to the methylxanthines shown above, radioactivity was also found in xanthosine, hypoxanthine and xanthine. In *C. millotii* and *C. perrieri*, significant amounts of radioactivity (25−37 %) were incorporated in the purine degradation products, ureides (allantoin and allantoic acid) and CO_2.

Unlike in bacteria and animals, no adenosine deaminase or adenine deaminase is present in plants,[20, 21] deamination of adenine molecules is performed by AMP deaminase (step 5).[22, 23] The product, IMP, is converted to inosine and hypoxanthine (steps 8 and 9). Hypoxanthine is oxidized yielding xanthine (step 15) and then catabolised by the purine degradation pathway (steps 17−22). Compared to *Mascarocoffea* species, the catabolic activity of [8-^{14}C]adenine is much lower (7 % of total radioactivity) in *C. canephora*.

Purine nucleoside metabolism in *C. millotii*

Metabolic fate of [8-^{14}C]inosine, [8-^{14}C]guanosine and [8-^{14}C]xanthosine applied to the leaf disks of *C. millotii* is presented in Table 3. As the same as [8-^{14}C]adenine, radioactivity from these precursors was incorporated into 7-methylxanthine, but not into theobromine and caffeine. The radioactivity from [8-^{14}C]inosine and [8-^{14}C]guanosine (16 % and 23 % of total radioactivity, respectively) was incorporated into free nucleotides and RNA, but [8-^{14}C]xanthosine was not salvaged. Inosine and guanosine appear to be converted to IMP and GMP by inosine-guanosine kinase (EC 2.7.1.73) and/or nucleoside phosphotransferase (EC 2.7.1.77) and converted to GTP (steps 25 and 6−7 or step 24) and then incorporated into RNA (step 2−4).

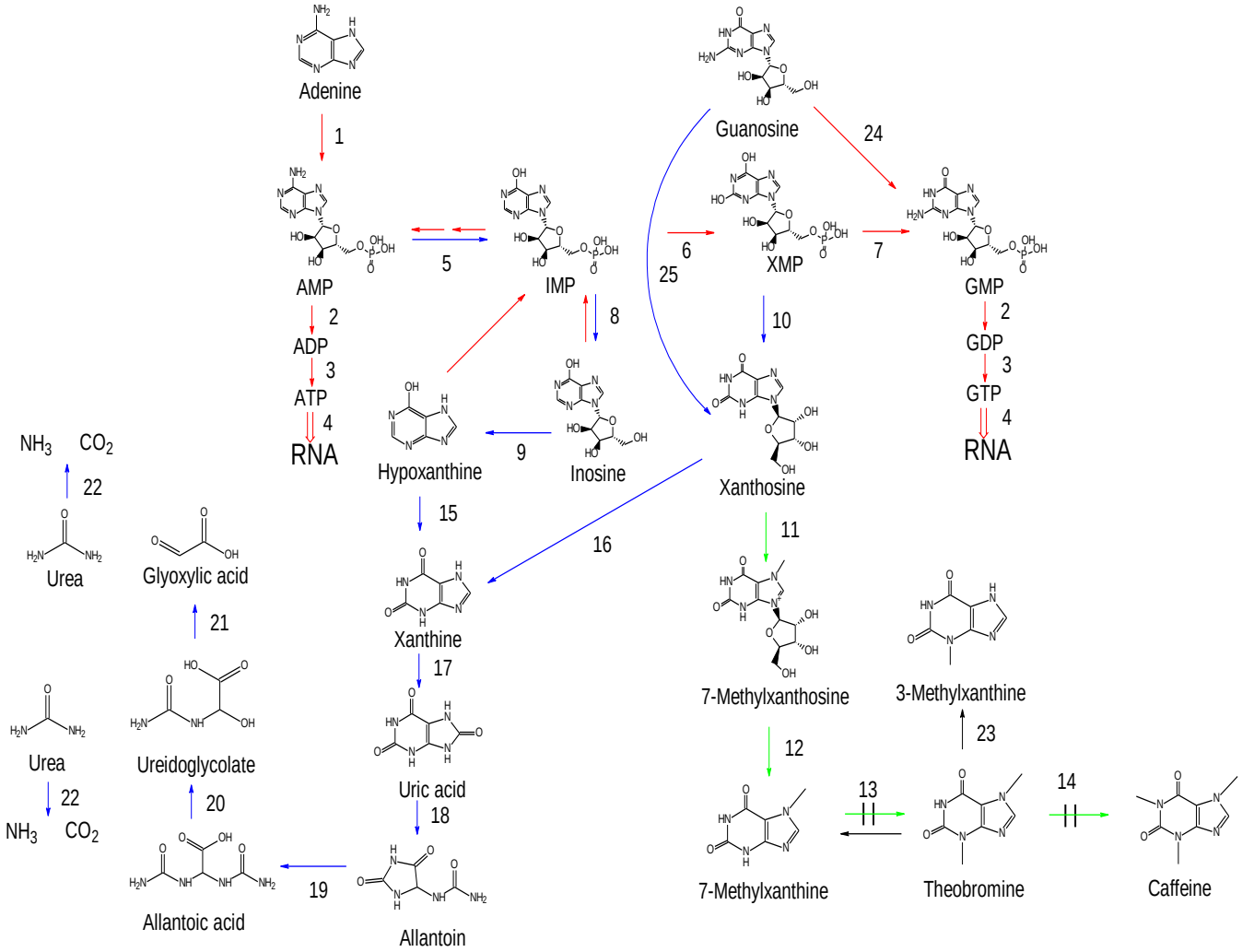

Figure 2. Possible metabolic routes of adenine, inosine, guanosine and xanthosine in *Coffea canephora* and *Mascarocoffea* species. → (red), salvage pathways; → (green), purine alkaloid biosynthesis; → (blue), degradation pathway.

Substantial amounts of inosine (68%), guanosine (55%) and xanthosine (84%) were degraded to allantoin, allantoic acid and CO_2. There are several possible routes of the formation of xanthine, a key starting metabolite for purine catabolism. From the results obtained from other plant sources,[14, 24] the following routes are the most likely: inosine → hypoxanthine → xanthine (steps 9 and 15); guanosine → xanthosine → xanthine (steps 25 and 16); xanthosine → xanthine (step 16). Although the metabolic profile of these purine nucleosides has not reported in Coffea plants, the results are essentially the same as the profile reported to operate in other plants.[14, 24-26]

Purine alkaloid metabolism

Metabolic fate of exogenously supplied [8-^{14}C]theobromine and [8-^{14}C]caffeine in the disks of *C. millotii* is presented in Table 4. In contrast to purine bases and nucleosides, these methylxanthines were not actively metabolized; 93% of theobromine and 99% of caffeine were retained unmetabolized. In the case of [8-^{14}C]theobromine, small amounts of radioactivity was detected in 3-methylxanthine (3.5%), 7-methylxanthine (1.9%), xanthine (0.6%) and CO_2 (1.4%). Thus, *C. millotii* leaves seem to have machinery, i.e., enzymes of theobromine catabolism, although its activity is low. Theobromine is a direct precursor of caffeine biosynthesis in *C. arabica* and *C. canephora*, but no conversion of theobromine to caffeine was detected in *C. millotii*.

Comparison of purine metabolism in caffeine reduced *Coffea* plants

Biosynthesis and degradation of caffeine occur in low caffeine *Coffea* plants including native and transgenic plants.[9] In most cases, the activity of *N*-methyltransferase is reduced. There are at least two distinct *N*-methyltransferases, 7-methylxanthosine synthase and dual functional caffeine synthase which catalyzes the last two steps, namely, 7-methylxanthine → theobromine → caffeine (steps 13 and 14 in Fig. 2).[9] The reduction of activity of *N*-methyltransferase activity was suggested in all cases. In addition, a few species including *C. eugenioides* and *C. dewevrei* catabolize caffeine and this may also cause reduction of the caffeine level.[10, 27-29]

In the present study, we found that the purine alkaloid synthesis was stopped at 7-methylxanthine, thus dual functional caffeine synthase activity appeared to be missing in *Mascarocoffea* species.

Table 3. Metabolism of inosine, guanosine and xanthosine in *Coffea mellotii* leaf segments.

Metabolites	[8-^{14}C]-labelled		
	Inosine	**Guanosine**	**Xanthosine**
Nucleotides	7.16 ± 4.15	6.33 ± 1.05	nd
RNA	9.15 ± 0.74	16.8 ± 0.29	nd
Nucleosides and bases			
Inosine	4.17 ± 0.36	nd	nd
Hypoxanthine	2.45 ± 0.14	nd	nd
Guanosine and guanine	nd	11.3± 0.63	nd
Xanthosine	8.43 ± 0.92	9.16 ± 0.86	10.5 ± 0.93
Xanthine	nd	nd	4.03 ± 0.20
Purine alkaloids			
7-Methylxanthine	0.86 ± 0.07	0.67 ± 0.08	1.39 ± 0.71
3-Methylxanthine	nd	nd	nd
Theobromine	nd	nd	nd
Caffeine	nd	nd	nd
Degradation products			
Ureides	20.8 ± 1.3	15.9 ± 1.71	20.7 ± 2.3
Carbon dioxide	46.9 ± 3.5	38.7 ± 0.68	63.4 ± 1.9
Unidentified	nd	1.03 ± 0.15	nd
Total uptake	25.4 ± 0.5	24.6 ± 1.2	4.73 ± 0.10

[8-^{14}C]Inosine (10 µM, specific activity 1.9 GBq mmol^{-1}), [8-^{14}C]guanosine (10 µM, specific activity 1.9 GBq mmol^{-1}) and [8-^{14}C]xanthosine (9 µM, specific activity 2.1 GBq mmol^{-1}) were administered for 18 h. The mean incorporation of radioactivity into individual metabolites is expressed as the percentage of total radioactivity taken up by the segments ± SD. Total uptake is expressed as kBq 100 mg f.w.$^{-1}$, nd, not detected.

In contrast, Silvarolla et al.[30] reported that theobromine was accumulated in a naturally decaffeinated *C. arabica* plant from Ethiopia designated "AC". They found that the leaves accumulated radioactivity in theobromine when [^{14}C]adenine was fed to the "AC" plants, with none being incorporated into caffeine. In contrast, no difference was found in degradation of [^{14}C]caffeine between in the "AC" plants and caffeine accumulating normal *C. arabica* plants. These results indicate that the low caffeine content in the "AC" plants is due to the lack of the activity of *N*-methyltransferase which catalyzes the last step in the caffeine biosynthesis pathway (step 14 in Fig. 2). Although no data was shown, they mentioned that no caffeine synthase activity was detected in the leaves, and concluded that the caffeine synthase gene had mutated in the AC plants. In this case, theobromine synthase which is distinct from a dual-functional caffeine synthase may be functional.

The reduction of caffeine synthase has also been suggested in low-caffeine hybrid coffee by ^{14}C-feeding experiments, the hybrid named GCAs which are new tetraploid interspecific hybrids developed in Madagascar from *C. eugenioides*, *C. canephora* and *C. arabica*. Selected GCA contained low caffeine (<0.4 % dry wt.) and no detectable theobromine in seeds. Low caffeine accumulation in GCA plants is due mainly to the low caffeine biosynthesis activity, possibly due to extremely weak *N*-methyltransferase reactions. No significant catabolic activity of caffeine was found in the GCA, in common with *C. arabica*.

Table 4. Metabolism of theobromine and caffeine in *Coffea mellotii* leaf segments.

Metabolites	[8-^{14}C]Theobromine	[8-^{14}C]Caffeine
Nucleotides	nd	nd
RNA	nd	nd
Nucleosides and bases		
Xanthine	0.60 ± 0.05	nd
Purine alkaloids		
7-Methylxanthine	1.89 ± 0.28	nd
3-Methylxanthine	3.54 ± 0.72	nd
Theobromine	92.5 ± 1.4	nd
Caffeine	nd	98.6 ± 0.01
Degradation products		
Ureides	nd	nd
Carbon dioxide	1.38 ± 0.25	0.43 ± 0.12
Total Uptake	13.2 ± 2.3	1.45 ± 0.21

[8-^{14}C]Theobromine (9 µM, specific activity 2.0 GBq mmol^{-1}) and [8-^{14}C] caffeine (10 µM, specific activity 2.0 GBq mmol^{-1}) were administered for 18 h. The mean incorporation of radioactivity into individual metabolites is expressed as the percentage of total radioactivity taken up by the segments ± SD. Total uptake is expressed as kBq 100 mg f.w.$^{-1}$, nd, not detected.

Similar results were also demonstrated in anti-sense and RNA interference transgenic plants of *C. canephora* in which the expression of an *N*-methyltransferase gene was suppressed. Compared with wild-type control plants, total purine alkaloid biosynthesis from adenine and conversion of theobromine to caffeine were both reduced in the transgenic plants. In the transgenic plants, metabolism of [8-^{14}C]adenine shifted from purine alkaloid synthesis to purine catabolism or salvage for nucleotides.

From these results, we can speculate that in most caffeine-free *Coffea* plants including *Mascarocoffea* species, caffeine synthase-related genes are varied and, as a result, there is reduced the enzyme activity *in planta*. In these plants, the metabolic flow of purine metabolites to the purine alkaloids synthesis appeared to be shifted to the purine catabolism.

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