Studies on the Metabolic Control of Caffeine Turnover in Developing Endosperms and Leaves of *Coffea arabica* and *Coffea dewevrei*

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Leaves and immature fruits of *Coffea dewevrei* and *Coffea arabica* var. Mundo Novo and var. Laurina were fed with [8-^{3}H]caffeine and the products analyzed by reversed-phase high-performance liquid chromatography with ultraviolet and radioactivity detection. In fruits, only endosperms were analyzed. In addition, the activities of two methyltransferases involved in the biosynthesis of caffeine were studied in cell-free preparations from leaves and endosperms. The results provide evidence that the low caffeine content in leaves and endosperms of immature fruits of *C. dewevrei* may be due to slow biosynthesis and fast degradation. The high caffeine content in leaves and endosperms of *C. arabica* seems to be a consequence of a rapid rate of production coupled with a slow degradation rate.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) is the major alkaloid in coffee, and variations in caffeine content are found within the same tree, with the highest levels in seeds, flowers, and leaves (Raju and Gopal, 1979). Variability is also observed among varieties of the same species and among different species of coffee (Charrier, 1978; Mazzafera and Carvalho, 1992).

Studies on the synthesis and degradation of caffeine in coffee have been carried out, predominantly, with fruits of *Coffea arabica* [for a review see, Suzuki et al. (1992)], and although the metabolic steps are known (Figure 1), the control of the level of caffeine in coffee tissues is not fully understood.

Adenine and guanine, derived from the nucleotide pool, are the primary precursors of caffeine in coffee (Suzuki and Waller, 1984b). Thus, its level, in some tissues, might be related to general metabolic activity (Waller and Nowacki, 1978), and tissues such as young leaves, flowers, and immature fruits should be expected to contain more caffeine than mature or old tissues (Clifford and Kazi, 1987; Frischknecht et al., 1986; Raju and Gopal, 1979).

Roberts and Waller (1979) and Suzuki and Waller (1984a,b) demonstrated that immature fruits had a higher capacity for caffeine degradation, even though they contained more caffeine than mature fruits. At the same time, cell-free preparations from immature fruits had higher activities of methyltransferases involved in the biosynthesis of caffeine than preparations from older fruits (Roberts and Waller, 1979). These investigations described differences found in fruits of *C. arabica* at different stages of development but did not deal with differences between species or different tissues of the same tree.

In a previous paper, we showed that endosperms of immature fruits of different coffee species markedly differed in their catabolism of caffeine (Mazzafera et al., 1991). Endosperms from *Coffea dewevrei*, containing 0.25% caffeine, had a much higher degradation rate than those from *C. arabica* var. Mundo Novo, having 1.3% caffeine. The caffeine contents in endosperms of mature fruits of these species were 1.2% and 1.1%, respectively. It was suggested, therefore, that the turnover of caffeine is high in endosperms of immature fruits of *C. dewevrei*, decreasing with maturation. Caffeine contents in endosperms from immature and mature fruits of *C. arabica* var. Laurina were 0.8% and 0.6%, respectively, although showing the same degradation rate as *C. arabica* var. Mundo Novo (Mazzafera et al., 1991). The difference in caffeine content between these two *C. arabica* varieties might be due a higher biosynthesis rate in var. Mundo Novo.

In this paper we examine the results of *in vitro* and *in vivo* experiments, performed to study the synthesis and degradation of caffeine in leaves and endosperms of immature fruits of *C. dewevrei* and *C. arabica* var. Mundo Novo and var. Laurina. The activities of *N*-methyltransferases, catalyzing the methylation of 7-methylxanthine and theobromine (3,7-dimethylxanthine), were assayed in cell-free preparations of leaves and endosperms of immature fruits. In the *in vivo* experiments, leaves and immature fruits were fed with [8-^{3}H]caffeine before extraction and analysis of metabolites by reversed-phase high-performance liquid chromatography (RP-HPLC), with ultraviolet and radioactivity detection.

MATERIALS AND METHODS

Radiochemicals. [8-^{3}H]Caffeine (22.2 Ci/mmol) and *S*-adenosyl[^{3}H-CH_3]methionine (15 Ci/mmol) were purchased from Amersham International (Sweden).

***In Vivo* Experiments.** *Plant Material.* Immature fruits of *C. dewevrei* (30 weeks old) and of *C. arabica* var. Mundo Novo and var. Laurina (25 weeks old) were collected at the Experimental Station of Instituto Agronômico, Campinas, SP, Brazil, between August 1991 and January 1992. Leaves of the third pair were collected in November 1991. Fruits and leaves were collected from the same coffee trees used by Mazzafera et al. (1991). The *C. dewevrei* tree was 50 years old, and both *C. arabica* trees were 8 years old.

Radioisotopic Feeding Experiments. Radioisotopes were fed to fruits essentially as described by Mazzafera et al. (1991). Each fruit received 2 μCi of [8-^{3}H]caffeine or 2 μCi of [8-^{3}H]caffeine plus 50 μg of theophylline (1,3-dimethylxanthine). Theophylline was dissolved in 50 mM sodium phosphate, pH 6.0, and 0.65 mM

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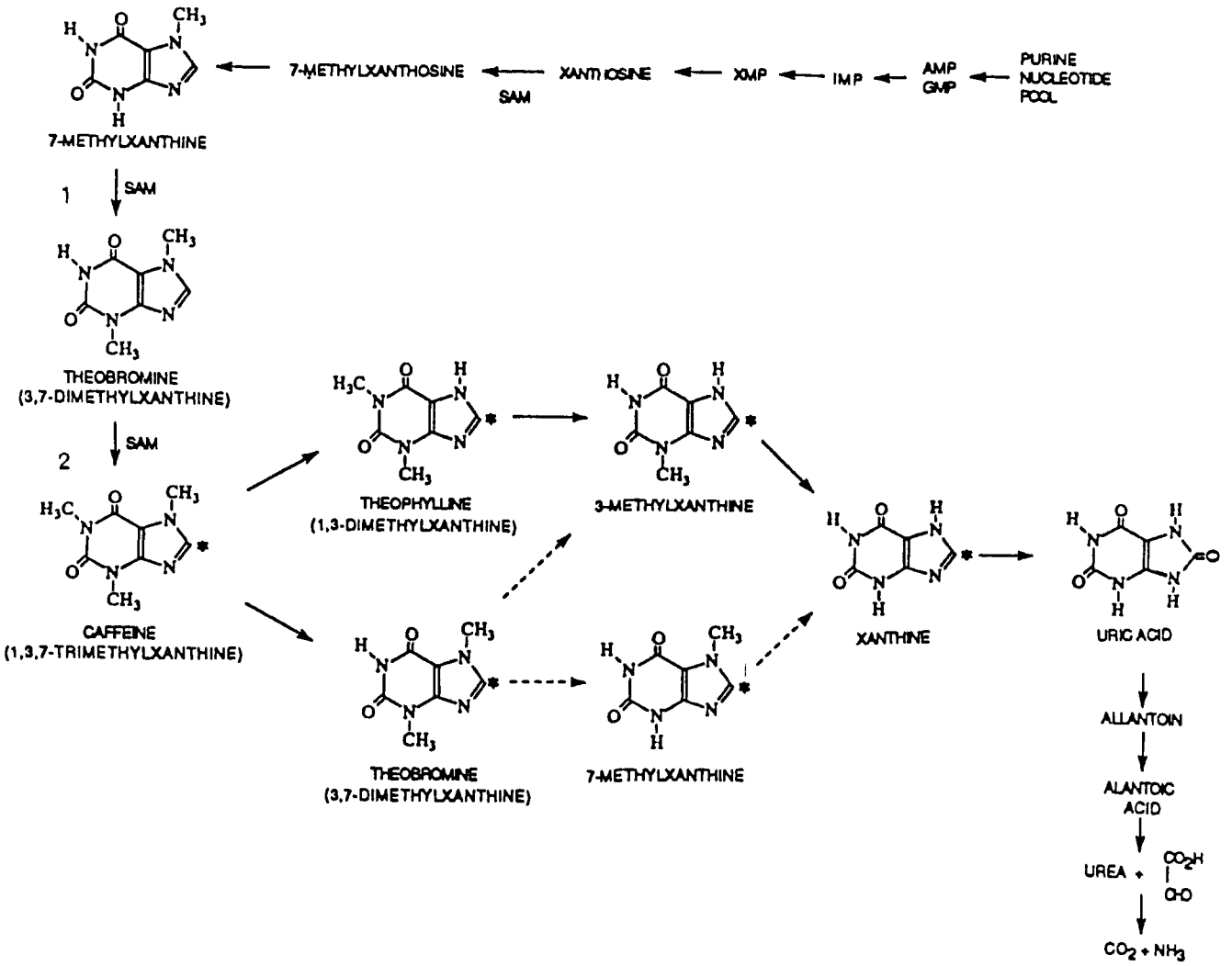

Figure 1. Biosynthesis and biodegradation pathways of caffeine in coffee: 1, 3-*N*-methyltransferase; 2, 1-*N*-methyltransferase; *, ^{3}H-label in the caffeine molecule.

ascorbic acid. After 12 h, the fruits were cut and the white opaque endosperms collected, dried at 80 °C, and finely ground in a mortar. Endosperms from at least 35 fruits were used in each treatment.

For radioisotopic feeding of leaves, whole branches were collected and washed thoroughly with tap water. After quick blotting with absorbent tissue, leaves of the second and third pairs were detached and the petiole was immersed in vials containing 1.5 mL of 50 mM sodium phosphate, pH 6.0, 0.65 mM ascorbic acid, and 1.5 μCi of [8-^{3}H]caffeine with or without 800 μg of theophylline. During the incubation period, any solution absorbed by the leaves was replaced by buffer. After 24 h under constant light (300 μmol m^{-2} s^{-1}) at 25 ± 2 °C, the petioles were quickly washed under tap water, and the leaves were dried at 80 °C and finely ground. The volumes of the remaining solutions in the vials were determined, and aliquots were removed to measure the uptake of [8-^{3}H]caffeine and theophylline. [8-^{3}H]-Caffeine was measured by radiometry in a scintillation counter and theophylline by RP-HPLC at 280 nm. The column used was a TSK ODS-120T (Pharmacia; 5 μm, 250 mm × 4.6 mm i.d.), and chromatography was developed isocratically with 35% MeOH in distilled H_2O at a flow of 1 mL/min. Ten leaves were used in each treatment.

Extraction of Caffeine and Metabolites. Leaves and endosperms were extracted with 6.3 mM H_2SO_4 according to the method of Petermann and Baumann (1983) and the extracts clarified by addition of heavy MgO. After centrifugation, the supernatants were freeze-dried, resuspended in 2 mL of distilled H_2O, and passed through C_{18} Sep-Pak cartridges (Millipore, Waters). The first eluate (xanthine was poorly retained) and subsequent washings with 2 × 2 mL of 50% MeOH were combined, freeze-dried, resuspended in 0.4 mL of distilled H_2O, and filtered into Ultrafree-MC Eppendorf tubes (Millipore, Waters). These extracts were used for the determination of endogenous methylxanthines, [8-^{3}H]caffeine, and metabolites by RP-HPLC. Two replicates of extraction and further analysis in RP-HPLC were made for each treatment.

High-Performance Liquid Chromatography. A C_{18} guard column and an ODS Hypersil column (5 μm, 250 mm × 4.6 mm i.d.) (Capital HPLC Specialist, U.K.) were used with a gradient of 30 min of 0–45% MeOH in 0.5% acetic acid, pH 5.0 (with 5 M NaOH), and a flow rate of 1 mL/min. The elution was followed at 280 nm, and when labeled samples were analyzed, the eluate emerging from the absorbance monitor was mixed with scintillation fluid and monitored by a radioactivity monitor, as described by Mazzafera et al. (1991).

***In Vitro* Experiments.** *Plant Material.* Fruits used here were collected simultaneously with those used in the *in vivo* experiments. They were washed with tap and distilled water before being cut into halves. The endosperms were removed and immediately immersed in 100 mM sodium phosphate, pH 7.0, and 6.5 mM ascorbic acid at 4 °C. The buffer was drained, and the endosperms were frozen at −20 °C, freeze-dried, finely ground in a mortar, and stored at 4 °C until use. Leaves of the second pair were collected from 12-month-old seedlings grown under greenhouse conditions (18/6 h, 24/21 °C) in Umeå, Sweden, in July 1992. They were frozen in liquid nitrogen, freeze-dried, finely ground, and stored at 4 °C until use.

Cell-Free Preparations and Enzyme Assays. Leaves from two plants composed one replicate, and four such replicates were used for each coffee species or variety. As fruits were collected from individual trees, four independent extractions and measurements of activities were made. Freeze-dried leaves and endosperms were extracted with a buffer modified from that of

Wingsle et al. (1991) containing 50 mM sodium phosphate, pH 7.5, 5 mM DTT, 5 mM EDTA, 6.5 mM ascorbic acid, and 4% poly(vinylpyrrolidone) (M_r 360 000) in a proportion of 0.1 g/mL. After 15 min on ice, 0.25 mL of cold saturated aqueous $(NH_4)_2SO_4$ solution was added per milliliter of extract. The gummy precipitate was eliminated by centrifugation at 31000*g* for 30 min at 4 °C. The supernatant was filtered through Sephadex PD-10 columns (Pharmacia) and eluted with 50 mM sodium phosphate, pH 7.5. The filtrate was used for measurements of *N*-methyltransferase activity. These were carried out for 30 min at 28 °C in Eppendorf tubes in reaction mixtures containing 50 mM sodium phosphate, pH 7.5, 0.75 mM substrate (theobromine or 7-methylxanthine), 3 mM $MgCl_2$, ca. 100 μg of protein, and 0.1 μCi of *S*-adenosyl[^{3}H-CH_3]methionine, as the methyl donor, in a final volume of 100 μL. Our previous trials had shown that, under the conditions described above, enzyme activities were linear up to 13 min, with highest activities being attained at 15 min, and a longer incubation period (30 min) gave similar final activities. Good proportionality was observed up to 120 μg of protein added to the reaction mixture. The reaction was started with the addition of *S*-adenosyl[^{3}H-CH_3]methionine and stopped with 10 μL of 6 M HCl. After addition of 1 mL of chloroform, the Eppendorf tubes were vortexed for 2 min and briefly centrifuged for separation of the phases. RP-HPLC showed that when theobromine was used as substrate, *S*-adenosyl[^{3}H-CH_3]-methionine was retained in the aqueous phase and the product [^{3}H-CH_3]caffeine transferred to the chloroform phase. The organic phase was dried in scintillation vials, and the radioactivity was counted after addition of scintillation fluid. When 7-methylxanthine was used as substrate, the reaction was stopped with 100 μL of acetone, −20 °C, and the tube was centrifuged to eliminate precipitated proteins. Aliquots from the supernatant were dried under a stream of nitrogen, resuspended in distilled water, and analyzed by RP-HPLC, as described for the metabolites of [8-^{3}H]caffeine for the *in vivo* experiments. In the assay for 3-*N*-methyltransferase only theobromine could be detected in the chromatograms.

Protein Determination. Protein was assayed according to the method of Bradford (1976) using a prepared Bio-Rad reagent.

RESULTS AND DISCUSSION

After anthesis, fruit maturation in *C. arabica* takes ca. 32 weeks and in *C. dewevrei* ca. 50 weeks. To obtain fruits at a similar stage of development, fruits of *C. arabica* and *C. dewevrei* were collected at 25 and 30 weeks after anthesis [cf. Mazzafera (1990)]. At the time of collection the dry matter contents of fruits of *C. arabica* var. Mundo Novo and var. Laurina and *C. dewevrei* were 21.1%, 19.5%, and 20%, respectively, and endosperms represented 3.4%, 3.1%, and 2.8% of the total dry matter.

Estimates of endogenous contents of caffeine and related methylxanthines (Table 1) are in agreement with data reported in the literature (Fobé and Carvalho, 1965; Mazzafera et al., 1991; Raju and Gopal, 1979). Caffeine was the alkaloid present in higher amounts in endosperms and leaves of both species. Endosperms of *C. dewevrei* also showed a much higher theobromine content.

In the radioisotopic feeding experiments the uptake of caffeine and theophylline by leaves was determined. Both *C. arabica* varieties absorbed ca. 65% of [8-^{3}H]caffeine, and 80% was absorbed by *C. dewevrei*. When supplied together, ca. 30% of theophylline and ca. 35% of [8-^{3}H]-caffeine were absorbed by both *C. arabica* varieties and 65% and 66%, respectively, by *C. dewevrei*. An increase of theophylline content was detected in leaves and endosperms; however, the endogenous contents of caffeine and theobromine were not affected (Table 1).

As previously observed (Mazzafera et al., 1991), for all treatments there was a low recovery of the applied radioactivity (Table 2). For fruits this would be partially explained because only endosperms were analyzed. Nevertheless, extraction with [8-^{3}H]caffeine blanks also gave low yields (45–50% recovery), indicating losses due to adsorption during the extraction and purification procedures.

Table 1. Content of Methylxanthines in Endosperms of Immature Fruit and Leaves of *C. arabica* Var. Mundo Novo and Laurina and *C. dewevrei*, As Determined by RP-HPLC and UV Detection

		mg/g dry weight[a]		
treatment	species	theobromine	theophylline	caffeine
		Endosperms		
^{3}H-caf	M. Novo	0.040	0.003	12.6
	Laurina	0.042	0.001	6.7
	C. dew.	0.367	0.007	4.7
^{3}H-caf + Thp	M. Novo	0.033	0.027	11.7
	Laurina	0.039	0.048	7.7
	C. dew.	0.334	0.051	4.3
		Leaves		
^{3}H-caf	M. Novo	0.103	ND	9.3
	Laurina	0.035	ND	7.3
	C. dew.	0.020	0.005	0.2
^{3}H-caf + Thp	M. Novo	0.087	0.197	8.6
	Laurina	0.040	0.209	7.1
	C. dew.	0.027	0.098	0.2

[a] Data are means of two replicates and in no case was the coefficient of variation higher than 8%. ^{3}H-caf, [8-^{3}H]caffeine; Thp, theophylline; ^{3}H-caf + Thp, [8-^{3}H]caffeine plus theophylline; M. Novo, Mundo Novo; *C. dew.*, *C. dewevrei*; ND, not detected.

In agreement with earlier studies (Mazzafera et al., 1991), fruits of var. Mundo Novo and var. Laurina did not metabolize caffeine as rapidly as *C. dewevrei* fruits, and the same was observed in leaves (Table 2). In keeping with data obtained with *C. arabica* varieties, less radioactivity was recovered from endosperms and leaves of *C. dewevrei*, and products of caffeine catabolism accumulated radioactivity in this species. Two polar compounds (**I** and **II**), detected previously by Mazzafera et al. (1991) in fruits, were also detected in leaves, and their radioactivities are shown in Table 2. The identities of these compounds are under investigation since they can be catabolites of an alternative caffeine degradation pathway. Romero and Waller (1988) isolated from coffee fruits a compound that was identified as a product of theophylline catabolism.

In coffee fruits caffeine is mainly synthesized via theobromine and degraded via theophylline (Suzuki and Waller, 1984a,b). Although feeding experiments have shown that theophylline could be also involved in the biosynthesis of caffeine in fruits of *C. arabica*, considering theobromine as reference, only 4% of the applied theophylline was converted into caffeine. In such biosynthesis theophylline would be synthesized by the methylation of 1-methylxanthine (Suzuki and Waller, 1984a,b). However, since caffeine degradation involves two distinct N-demethylations (cf. Figure 1), the theobromine content in endosperms of *C. dewevrei* might be a result of a more active N-demethylating step rather than a consequence of higher caffeine degradation rate. As theobromine is the immediate precursor as well as one of the products of caffeine catabolism, experiments with labeled theobromine can lead to erroneous conclusions. Thus, fruits and leaves were fed with [8-^{3}H]caffeine plus theophylline with the expectation that 7-N-demethylation of caffeine to theophylline would be inhibited and caffeine catabolism converted to theobromine, via 1-N-demethylation (Table 2). It was observed, however, that inhibition of [8-^{3}H]-caffeine conversion was higher in endosperms of *C. dewevrei* for the theobromine than for the theophylline route. These findings suggest an effective conversion of caffeine into theobromine in this coffee species.

Table 2. [8-^{3}H]Caffeine Metabolites in Endosperms of Immature Fruit and Leaves of *C. arabica* Var. Mundo Novo and Laurina and *C. dewevrei*

treatment	species	recovered[a] (%)	content (% of recovered radioactivity)					
			I/II	Xan	3mx	Thb	Thp	Caf
			Endosperms					
^{3}H-caf	M. Novo[b]	23.5	0.49	ND	ND	0.12	ND	99
	Laurina	20.2	0.45	ND	ND	0.32	ND	99
	C. dew.	14.1	1.03	0.82	1.97	35.0	2.44	59
^{3}H-caf + Thp	M. Novo	20.3	0.44	ND	ND	0.26	ND	99
	Laurina	22.7	0.22	ND	ND	ND	ND	99
	C. dew.	13.2	0.85	ND	0.21	10.8	2.27	86
			Leaves					
^{3}H-caf	M. Novo	19.9	ND	ND	ND	ND	ND	100
	Laurina	13.2	ND	ND	ND	ND	ND	100
	C. dew.	4.7	10.0	ND	4.54	7.1	15.8	63
^{3}H-caf + Thp	M. Novo	34.0	ND	ND	ND	ND	ND	100
	Laurina	30.5	ND	ND	ND	ND	ND	100
	C. dew.	23.9	2.72	ND	1.93	3.5	7.2	85

[a] Data are expressed as percentage of applied radioactivity (2 μCi/fruit). [b] Data are means of two replicates, and in no case was the coefficient of variation higher than 11%. I/II, compounds I and II; Xan, xanthine; 3mx, 3-methylxanthine; Thb, theobromine; Thp, theophylline; Caf, caffeine; ^{3}H-caf, [8-^{3}H]caffeine; ^{3}H-caf + Thp, [8-^{3}H]caffeine plus theophylline; M. Novo, Mundo Novo; *C. dew.*, *C. dewevrei*; ND, not detected.

Table 3. Activities of 1-*N*-Methyltransferase and 3-*N*-Methyltransferase in Endosperms of Immature Fruit and Leaves of *C. arabica* Var. Mundo Novo and Laurina and *C. dewevrei*

species	endosperms[a]		leaves[a]	
	1-*N*	3-*N*	1-*N*	3-*N*
Mundo Novo	5.9 ± 0.3	5.1 ± 0.7	4.5 ± 0.8	7.1 ± 0.4
Laurina	6.8 ± 0.9	4.2 ± 0.8	2.6 ± 0.6	3.1 ± 0.2
C. dewevrei	0.9 ± 0.4	ND	1.4 ± 0.3	ND

[a] Data are expressed as picomoles of [8-^{3}H]theobromine produced by milligram of protein for 3-*N*-methyltransferase and picomoles of [8-^{3}H]caffeine produced by milligram of protein for 1-*N*-methyltransferase. 1-*N*, 1-*N*-methyltransferase activity; 3-*N*, 3-*N*-methyltransferase activity; ND, activity not detected; means ± SD of four replicates.

Theophylline affected [8-^{3}H]caffeine catabolism in leaves of *C. dewevrei* in a different ways (Table 2). Both theophylline and theobromine routes seemed to be inhibited to ca. 50%. Radioactivity in 3-methylxanthine also decreased aproximately 50%. A possible explanation for the effects of theophylline addition in the [8-^{3}H]-caffeine catabolism in *C. dewevrei* could be that both N-demethylating reactions are catalyzed by the same enzyme but show different affinities for the methyl groups in the caffeine molecule. This has been observed in bacteria (Dixon and Webb, 1979).

Petermann and Baumann (1983) showed that, in a specific developmental stage, young leaves of *C. dewevrei* can also degrade caffeine through methyluric acids. We could not detect endogenous methyluric acids in our material; however, since the tritium label of [8-^{3}H]caffeine is lost during oxidation to uric acid derivatives, we cannot exclude the contribution of this degradative pathway occurring in the degradation pathway of [8-^{3}H]caffeine (Figure 1).

Table 3 presents the results of the enzyme assays for the 1-*N*- and 3-*N*-methyltransferase activities in endosperms and leaves. Lowest activities were found in *C. dewevrei*. Endosperms of *C. arabica* var. Mundo Novo and var. Laurina showed similar activities of 1-*N*- and 3-*N*-methyltransferases, and excluding 3-*N*-methyltransferase activity in leaves of var. Mundo Novo, endosperms had higher activities than leaves. The activities of both *N*-methyltransferases were higher in leaves of var. Mundo Novo.

We did not study other enzymes of caffeine biosynthesis, but the results of the 1-*N*- and 3-*N*-methyltransferase assays and the data from the radioisotopic feeding experiments suggest that a high degradation rate combined with a low biosynthesis rate may explain the low content of caffeine in leaves and endosperms of *C. dewevrei* (see Table 1). On the other hand, the reverse situation occurs with *C. arabica*—a slow degradation rate combined with a high rate of synthesis. However, these data do not provide a satisfactory explanation for the differences in the caffeine contents between the varieties Laurina and Mundo Novo.

Since mature endosperms of *C. dewevrei* contain as much as 1.2% caffeine, one might expect significant alterations in the rate of biosynthesis and biodegradation of caffeine during fruit ripening. As endosperms of immature and mature fruit of *C. arabica* have similar levels of caffeine, such a pronounced alteration would not be expected during fruit ripening.

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