CAFFEINE DEGRADATION IN LEAVES AND FRUITS OF *COFFEA ARABICA* AND *COFFEA DEWEVREI*[1]

ANGELA PIERRE VITÓRIA[2] and PAULO MAZZAFERA[3]

ABSTRACT - The coffee species *Coffea dewevrei* and *Coffea arabica* have marked differences in caffeine metabolism and the control of the caffeine content during fruit ripening and leaf aging is still not clear. The aim of this work was a detailed investigation on the alkaloid degradation in young and aged leaves, and immature and mature fruits of these species. Young and aged leaves, and immature and mature fruits were fed with [2-^{14}C] caffeine. After an incubation period they were extracted for [2-^{14}C] caffeine and metabolites and analysed by reversed-phase liquid chromatography and radiocounting of collected fractions. In leaves and fruits of *Coffea dewevrei* there were higher degradation rates of caffeine. In both species, compared to young tissues, aged leaves and mature fruits presented lower capacity to degrade the alkaloid, what was shown by the low radioactivities detected in the metabolites formed in the degradation pathway. Radioactivity was detected in 7-methylxanthine, which is also a precursor in the caffeine biosynthesis. The data support the conclusion that the ratio between biosynthesis and biodegradation controls the variation of the caffeine content during fruit ripening and leaf aging in *C. arabica* and *C. dewevrei*.

Index terms: caffeine metabolism, coffee, methylxanthines.

DEGRADAÇÃO DE CAFEÍNA EM FOLHAS E FRUTOS DE *COFFEA ARABICA* E *COFFEA DEWEVREI*

RESUMO - As espécies de café, *Coffea dewevrei* e *Coffea arabica*, apresentam diferenças marcantes quanto ao metabolismo de cafeína, e ainda não é claro o controle do conteúdo desse alcalóide durante o amadurecimento dos frutos e envelhecimento das folhas. O objetivo deste trabalho foi fazer uma investigação detalhada da degradação de cafeína em folhas jovens e velhas, e em frutos imaturos e maduros dessas espécies. Folhas jovens e velhas, e frutos imaturos e maduros de *Coffea arabica* e *Coffea dewevrei* foram incubados com [2-^{14}C] cafeína. Após a incubação procedeu-se a extração de [2-^{14}C] cafeína e seus metabólitos, que foram analisados por cromatografia líquida de fase reversa e determinação da radioatividade de frações coletadas. Em folhas e frutos de *C. dewevrei* houve maior degradação de cafeína. Em ambas espécies de café, em comparação com tecidos jovens, folhas velhas e frutos maduros mostraram ter menor capacidade em degradar o alcalóide, sendo isto mostrado pelas radioatividades detectadas nos metabólitos formados na via de degradação da cafeína. Foi detectada radioatividade em 7-metilxantina, que também está presente na via biossintética de cafeína. Os dados suportam a conclusão de que a razão entre biossíntese e biodegradação controla a variação do conteúdo de cafeína observada durante o amadurecimento dos frutos e envelhecimento das folhas em *C. arabica* e *C. dewevrei*.

Termos para indexação: metabolismo da cafeína, café, metilxantinas.

INTRODUCTION

Kalberer (1965) fed *Coffea arabica* leaves with four differently labelled caffeine and detected 3 or/and 7-methylxanthine, allantoin, allantoic acid and urea as alkaloid metabolites. Later, Suzuki & Waller (1984a, 1984b) showed in fruits of the same species that caffeine was firstly converted to theo-

[1] Accepted for publication on May 29, 1998.
Granted by Fundação de Amparo à Pesquisa do Estado de São Paulo-FAPESP.

[2] Bióloga, M.Sc., Dep. Fisiologia Vegetal, IB, Universidade Estadual de Campinas, Caixa Postal 6109, CEP 13083-970 Campinas, SP. Bolsista do CNPq.

[3] Eng. Agr., Dep. Fisiologia Vegetal, IB, Universidade Estadual de Campinas. Bolsista do CNPq. E-mail: pmazza@obelix.unicamp.br

phylline (1,3-dimethylxanthine) and theobromine (3,7-dimethylxanthine), being the first dimethylxanthine the main degradation pathway. Therefore, the caffeine catabolic pathway was established as: caffeine → theobromine/theophylline → 3-/7-methylxanthine → xanthine → uric acid → allantoin → allantoic acid → glyoxilic acid + urea → CO_2 + NH_4.

Mazzafera et al. (1991) and Mazzafera (1993) investigated [8-^{3}H] caffeine degradation in immature fruits of several coffee species observing that in *C. dewevrei* the alkaloid was rapidly metabolized when compared to the other species. Enzymic studies carried out by Mazzafera et al. (1994) suggested that the ratio between biosynthesis and biodegradation might determine caffeine content in the coffee tissues. This might explain why caffeine content is high in immature fruits and young leaves of *C. arabica* and low in immature fruits and leaves of *C. dewevrei* (Mazzafera et al., 1991; Mazzafera & Magalhães, 1991).

Recently, Ashihara et al. (1996) showed that the caffeine catabolic pathway in *C. arabica* leaves was very similar to fruits (Suzuki & Waller, 1984a, 1984b) and that ageing leaves degraded less caffeine than young ones. In addition, they observed also that 7-methylxanthine could be a product from xanthine methylation, this being more pronounced in aged leaves. This monomethylxanthine had not been detected by Mazzafera et al. (1991) and Mazzafera (1993) in the same coffee species and in *C. dewevrei*.

C. dewevrei and *C. arabica* have marked differences in caffeine metabolism and control of the caffeine content during fruit ripening and leaf aging is still not clear.

The aim of this work was a detailed investigation on the alkaloid degradation in young and aged leaves, and immature and mature fruits of these species.

MATERIAL AND METHODS

C. dewevrei leaves and fruits were collected from a tree growing in a living collection at the Instituto Agronômico de Campinas, SP, Brazil. Leaves and fruits of *C. arabica* were collected from a tree growing at the experimental area of our department. Immature fruits were considered those with ivory and opaque endosperm, as described by Mendes (1941). Mature fruits were considered those with hardened endosperm. Young leaves were collected from the first leaf pair, and old leaves from the fifth and sixth leaf pairs.

For the radioisotopic feeding experiments, [2-^{14}C] caffeine was synthezised by methylation of [2-^{14}C] xanthine (specific activity 52.4 mCi.mmol^{-1}, Sigma Chemical Company, St Louis, USA) with dimethylsulfate, according to Heftmann (1971).

The fruits were fed with [2-^{14}C] caffeine using the procedure used by Mazzafera et al. (1991). The amount of radioactivity given varied according to the absorption of the solution by the fruits (Table 1). After 72 hours, of incubation the fruits were extracted for caffeine and metabolites.

Leaf disks were obtained from leaves with a cork-borer (2 cm diameter) weighed and infiltrated under vacuum with water containing the labelled caffeine and 0.1% Tween 20. After infiltration, they were quickly blotted dry and weighed again. By this way it was possible to estimate the [2-^{14}C] caffeine infiltrated. The disks were transferred to a plastic box containing a sheet of foam wet with water at the bottom. The box was kept opened until the excess of water had been evaporated from the disks. Then the box was closed and maintained under constant laboratory light (300 mE.m^{-2}.s^{-1}). After 48 hours they were extracted for caffeine and metabolites.

The same procedure was used for the extraction of caffeine and metabolites from leaves and fruits. The material was frozen with liquid nitrogen and finely ground in mortar with pestle. The powder was transferred to cap sealed tubes, 500 mg of MgO was added and the extraction was done with 10 mL of 0.0125 N aqueous H_2SO_4 in 80°C water-bath for one hour. The tubes were centrifuged at 1,200 *g* and the supernatants reserved. Two following extractions were made with 10 mL of 0.0125 N aqueous H_2SO_4 and the supernatants combined. Then they were fractionated with 3 x 20 mL of chloroform and the combined chloroform fractions were dried under reduced pressure at 25°C. The compounds solubilized in 2 mL of distilled water were filtered in 0.22 mm membrane for subsequent analysis in HPLC (high performance liquid chromatography). Most of the caffeine, theobromine and theophylline was expected to be found in these fractions.

The remaining aqueous phase were dried at 80°C and the residues solubilized in 2 mL of distilled water. C_{18} Sep-Pak cartridges (Millipore - Waters Associates) were used for cleaning up the samples. The first eluting liquid and a 2 mL further 50% aqueous methanol washing were combined, filtered in 0.22 mm membranes and used for analysis in HPLC. In these fractions it was expected to find monomethylxanthines, xanthine, uric acid, allantoin and allantoic acid.

In the samples from the chloroform phase, 10 μg of caffeine, theobromine and theophylline as internal standards were added. The compounds were separated by reversed-phase liquid chromatography using a Shimadzu HPLC system with a Spherisorb ODS2 column (5 mm, 4 mm x 250 mm). The solvent (12.5% methanol in 0.5% aqueous acetic acid) was delivered by a LC-10AS pump at a flow rate of 1 mL.min^{-1} and the compounds eluting from the column were detected in SPD-10A UV-detector operating at 280 nm. The signals from the detector were integrated in a C-R6A Chromatopac recorder. Caffeine, theobromine and theophylline eluting from the column, monitored by the UV-detector and the recorder were collected in scintillation vials. After drying at 80°C, 5 mL of scintillation fluid was added to the vials and the radioactivity determined in a LS 6000TA scintillation counter (Beckman Instruments).

In the samples from the aqueous phase, 10 μg of allantoin, allantoic acid, uric acid, xanthine, 7-methylxanthine, 3-methylxanthine, theobromine, theophylline, caffeine as internal standards were added. At beginning of the chromatography, 0.5% aqueous acetic acid was used as solvent. The solvent was delivered at a 0,5 mL.min^{-1} flow rate and the detector at 280 mm. After 20 minutes, enough time for the elution of the monomethylxanthines, allantoin, allantoic acid, uric acid and xanthine, the solvent was changed to 12.5% methanol in 0.5% aqueous acetic acid and the flow was increased to 1 mL.min^{-1} in order to detect caffeine, theobromine and theophylline. Allantoin, allantoic acid and uric acid elute very close from the HPLC, therefore, they were collected as only one fraction. The eluting compounds, monitored by the UV-detector and the recorder, were collected in scintillation vials and processed as above for the determination of radioactivity.

RESULTS AND DISCUSSION

Previous data in the literature have shown that caffeine degradation in *C. arabica* fruits and leaves proceeds slowly (Suzuki & Waller, 1984a, 1984b; Mazzafera et al., 1991; Mazzafera et al., 1994; Ashihara et al., 1996).

Suzuki & Waller (1984b) observed that caffeine degradation in ripening fruits was slow compared to immature fruits. The fruits were incubated with [2-^{14}C] caffeine and after the incubation period 93% of the radioactivity was still present in caffeine. However, fruits incubated with [8-^{14}C] theophylline showed much less radioactivity in this dimethylxanthine compared with the [2-^{14}C] caffeine incubations, indicating that the conversion of caffeine to theophylline is the limiting step in caffeine degradation. Incubations with [2-^{14}C] xanthine showed a very high metabolism to uric acid, allantoin and allantoic acid. In [2-^{14}C] caffeine incubations radioactivity was detected in both theophylline and theobromine, however less in the first dimethylxanthine, suggesting that the main route for caffeine degradation was via theophylline and subsequently to 3-methylxanthine.

The results in the Table 1 show that fruits of *C. dewevrei* metabolized caffeine more extensively than *C. arabica* fruits. Similar to the data of Suzuki & Waller (1984b), for both species radioactivity was also detected in theobromine and theophylline, with higher levels in the first dimethylxanthine, indicating theophylline as the main route for caffeine degrada-

TABLE 1. Radioactivity distribution among [2-^{14}C] caffeine metabolites in mature and immature coffee fruits incubated for 72 hours[1].

Coffee species	Radioactivity applied (cpm)	Caf (%)	Thb (%)	Thf (%)	7mx (%)	3mx (%)	Xan (%)	Ua + All + Ala (%)
C. arabica								
Immature	1,071,000	91.4	4.8	2.2	nd	nd	1.6	nd
Mature	1,250,000	92.9	6.0	1.1	nd	nd	nd	nd
C. dewevrei								
Immature	843,000	2.9	73.5	1.6	1.3	11.6	1.4	7.7
Mature	899,000	3.9	71.3	3.6	1.0	7.7	3.8	8.8

[1] Applied radioactivity refers to [2-^{14}C] caffeine; radioactivities associated with the compounds are expressed as % of recovered radioactivity; Caf: caffeine; Thb: theobromine; Thf: theophylline; Xan: xanthine; 7mx: 7-methylxanthine; 3mx: 3-methylxanthine; Ua: uric acid; All: allantoin; Ala: allantoic acid; nd: not detected.

tion. After 72 hours of incubation, immature fruits of *C. arabica* accumulated less radioactivity in theobromine and more in theophylline than mature fruits, suggesting a more intense caffeine degradation although the differences of radioactivity in this alkaloid were not pronounced. Radioactivity was detected in xanthine only in immature fruits, also denoting a higher caffeine degradation.

Similarly to Mazzafera et al. (1991) and Mazzafera (1993), a significant accumulation of radioactivity in theobromine was observed in fruits of *C. dewevrei* (Table 1). These results are not sufficient to affirm that caffeine degradation via theobromine proceeds with higher intensity than in *C. arabica*. This might be only a consequence of a more accelerate caffeine degradation, and a limited degradation of theobromine to 3-methylxanthine.

Much less radioactivity was present in caffeine in immature and mature fruits of *C. dewevrei* (Table 1). Except for theophylline, in mature fruits less radioactivity accumulated in the other methylxanthines. This, in addition to the higher radioactivity in caffeine, suggests lower degradation rate of the alkaloid in mature fruits of *C. dewevrei*.

The data of this work with *C. arabica* leaves (Table 2) are in agreement with those of Ashihara et al. (1996). These authors observed that caffeine degradation was similar in mature (third leaf pair) and aged leaves (near the base of the shoot), but lower than in young leaves (first leaf pair). Here it was observed that, compared to *C. arabica*, caffeine was more metabolized in *C. dewevrei* leaves, particularly in young ones, with expressive accumulation of radioactivity in theobromine. However, this accumulation was proportionally lower than in fruits.

In *C. arabica* fruits and leaves, consecutive methylation of 7-methylxanthine and theobromine leads to caffeine biosynthesis (Suzuki & Waller, 1984b; Ashihara et al., 1996). However, from studies with fruits (Suzuki & Waller, 1984a, 1984b) it was not clear whether during caffeine degradation, 7-methylxanthine was also a product of theobromine demethylation. Using tritiated caffeine at position 8, which is lost after xanthine conversion to uric acid Mazzafera (1993) concluded that 7-methylxanthine was not formed from theobromine in mature leaves and immature fruits of *C. dewevrei*. On the other hand, Ashihara et al. (1996) reported that in aged leaves of *C. arabica* 7-methylxanthine was newly synthesized from xanthine, but they did not give evidence that this monomethylxanthine was a product of theobromine degradation. Therefore, 7-methylxanthine detected here might be from xanthine and not from theobromine.

The role of 7-methylxanthine in caffeine degradation is still unclear. However, its re-utilisation in caffeine biosynthesis might be possible, as suggested in a recent work of Ashihara et al. (1997) with tea, where it was observed that xanthine was salvaged for caffeine biosynthesis, by formation of 3-methylxanthine.

TABLE 2. Radioactivity distribution among [2-^{14}C] caffeine metabolites in young and aged coffee leaves incubated for 48 hours[1].

Coffee species	Radioactivity applied (cpm)	Caf (%)	Thb (%)	Thf (%)	7mx (%)	3mx (%)	Xan (%)	Ua + All + Ala (%)
C. arabica								
Young	446,000	76.6	13.3	10.1	nd	nd	nd	nd
Aged	726,000	84.1	11.7	2.1	2.1	nd	nd	nd
C. dewevrei								
Young	412,000	32.1	50.4	4.1	3.0	nd	5.4	5.0
Aged	1,501,000	64.1	24.5	6.9	nd	1.6	1.2	1.7

[1] Applied radioactivity refers to [2-^{14}C] caffeine; radioactivities associated with the compounds are expressed as % of recovered radioactivity; Caf: caffeine; Thb: theobromine; Thf: theophylline; Xan: xanthine; 7mx: 7-methylxanthine; 3mx: 3-methylxanthine; Ua: uric acid; All: allantoin; Ala: allantoic acid; nd: not detected.

CONCLUSIONS

1. *Coffea dewevrei* leaves and fruits show higher caffeine degradation than *Coffea arabica* ones.

2. In both species, aged leaves and mature fruits show lower capacity to degrade caffeine.

3. 7-methylxanthine, which is also involved in the caffeine biosynthesis, is a product of the alkaloid degradation in aged leaves of *Coffea arabica* and young leaves of *Coffea dewevrei.*

4. The biosynthesis and biodegradation ratio controls the caffeine content variation during fruit ripening and leaf aging in *Coffea arabica* and *Coffea dewevrei.*

REFERENCES

ASHIHARA, H.; GILLIES, F.M.; CROZIER, A. Catabolism of caffeine and related purine alkaloids in leaves of tea (*Camellia sinensis* L.). **Plant and Cell Physiology**, v.38, n.4, p.413-419, 1997.

ASHIHARA, H.; MONTEIRO, A.M.; MORITZ, T.; GILLIES, F.M.; CROZIER, A. Catabolism of caffeine and related purine alkaloids in leaves of *Coffea arabica* L. **Planta**, v.198, n.3, p.334-339, 1996.

HEFTMANN, E. Synthesis of caffeine-2-^{14}C. **Journal of Labelled Compounds and Radiopharmaceuticals**, v.7, n.4, p.463-465, 1971.

KALBERER, P. Breakdown of caffeine in the leaves of *Coffea arabica* L. **Nature**, v.205, n.4971, p.597-598, 1965.

MAZZAFERA, P. 7-methylxanthine is not involved in caffeine catabolism in *Coffea dewevrei.* **Journal of Agricultural and Food Chemistry**, v.41, n.10, p.1541-1543, 1993.

MAZZAFERA, P.; CROZIER, A.; MAGALHÃES, A.C. Caffeine metabolism in *Coffea arabica* and other species of coffee. **Phytochemistry**, v.30, n.12, p.3913-3916, 1991.

MAZZAFERA, P.; CROZIER, A.; SANDBERG, G. Studies on the metabolic control of caffeine turnover in developing endosperms and leaves of *Coffea arabica* and *Coffea dewevrei.* **Journal of Agricultural and Food Chemistry**, v.42, n.7, p.1423-1428, 1994.

MAZZAFERA, P.; MAGALHÃES, A.C.N. Cafeína em folhas e sementes de *Coffea* e *Paracoffea.* **Revista Brasileira de Botânica**, v.14, n.2, p.157-160, 1991.

MENDES, A.J.T. Cytological observations in *Coffea.* VI. Embryo and endosperm development in *Coffea arabica* L. **American Journal of Botany**, v.28, n.9, p.784-789, 1941.

SUZUKI, T.; WALLER, G.R. Biodegradation of caffeine: formation of theophylline and theobromine from caffeine in mature *Coffea arabica* fruits. **Journal of the Science of Food and Agriclture**, v.35, n.1, p.66-70, 1984a.

SUZUKI, T.; WALLER, G.R. Biosynthesis and biodegradation of caffeine, theobromine, and theophylline in *Coffea arabica* L. fruits. **Journal of Agricultural and Food Chemistry**, v.32, n.4, p.845-848, 1984b.