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CATABOLISM OF CAFFEINE IN PLANTS AND MICROORGANISMS

Paulo Mazzafera

Departamento de Fisiologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas, CP 6109, 13083-970, Campinas, SP, Brasil

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1. ABSTRACT

Caffeine has been found in tissues of several plants. Because of its stimulating effect on the central nervous system, a great number of reports have been published on its content in beverages and foodstuffs. However, a much more restricted number of reports have dealt specifically with caffeine metabolism in plants. This review presents, in chronological manner, the contribution of these reports to the vast knowledge accumulated on caffeine catabolism in plants and microorganisms over the last 40 years. In plants, the accumulated data indicate the operation of a main catabolic pathway: caffeine → theophylline → 3-methylxantine → xanthine → uric acid → allantoin → allantoic acid → glyoxylic acid + urea → NH_3 + CO_2. Some studies have shown that, depending on the plant species, other minor routes may operate with the formation of theobromine and 7-methylxantine, which are salvaged for caffeine formation since they also appear in the biosynthetic pathway. A specific group of coffee known as liberio-excelsioides has the ability to convert caffeine to the corresponding methyluric acid, which is methylated to other uric acid derivatives. In bacteria caffeine is either degraded to theobromine or paraxanthine. Both dimethylxanthines are demethylated to 7-methylxantine which in turn is demethylated to xanthine and then enters the catabolic pathway of purines. In bacteria, theobromine, paraxanthine and 7-methylxantine may also be oxidized to their corresponding methyluric acids.

2. CAFFEINE CATABOLISM IN PLANTS

Caffeine was isolated from plants (coffee) for the first time in 1820 by Ferdinand Runge (1). In 1827 Oudry isolated a compound from tea (*Camellia sinensis*) showing similar physiological effects of caffeine, and Jobat and Mulder, in 1837, identified it as caffeine. However, only in 1832 did Pfaff and Liebig identify caffeine as a purine compound, denominated 1,3,7-trimethylxanthine (1). Caffeine was then identified in several plants and since then its physiological effects has been the main reason for this alkaloid becoming one of the most studied drugs so far.

Anderson and Gibbs (2) were the first to report a study on the caffeine metabolism. They fed coffee (*Coffea arabica*) leaves with (1-^{14}C) and (2-^{14}C) glycine, (3-^{14}C) serine, $H^{14}CHO$ (formate), $H^{14}CO_2Na$ (Na formate), $^{14}CH_3OH$ (methanol), $^{14}CO_2$ and (methyl-^{14}C) methionine and suggested that xanthine ring of caffeine was derived from glycine, formate and CO_2, and that 3-methylxanthine, theobromine (3,7-dimethylxanthine) and caffeine were formed from the methylation of xanthine. They concluded that caffeine biosynthesis followed the same route as the formation of the purine ring.

A few years later, Kalberer (3, 4) identified compounds of the catabolic pathway of caffeine. A previous study with tea also included caffeine metabolism but the alkaloid catabolites were not identified (5). Kalberer's objective was to answer a question raised in a previous study carried out by H. Wanner (cited in 3) with coffee leaves: Why does caffeine content of older *Coffea* leaves decrease after an initial increase in the young leaves? To answer this he fed coffee leaves with caffeine labeled with ^{14}C (in carbons 2 and 8 of the purine ring and in methyl groups N1 and N7) and followed the total radioactivity and production of labeled CO_2. Alcoholic extracts were co-chromatographed with expected breakdown caffeine products and those containing radioactivity were identified by autoradiography. He concluded that 1) the lower caffeine content in older leaves was due to breakdown, 2) coffee leaves were able to degrade caffeine to CO_2 and 3) 3- and/or 7-methylxanthines, allantoin, allantoic acid and urea were degradation products of the alkaloid.

Following Kalberer's work several others were carried out predominantly with coffee and tea, in order to elucidate the biosynthetic route of caffeine (6, 7). Most of them used labeled compounds thought to be precursors of caffeine formation. Some of these studies used labeled xanthine and theobromine separately and allantoin and allantoic acid were detected. Nowadays it is known that caffeine formation from xanthine is uncertain or that it is only a minor route and that theobromine is the immediate precursor of caffeine in all plants studied so far. It is also known that xanthine is an intermediate of caffeine degradation as well as a product formed during the degradation of purine nucleosides (7). In both cases uric acid and the ureides allantoin and allantoic acids are

produced. Therefore, this grid probably turned out to be an obstacle for the faster elucidation of caffeine catabolism in tea and coffee. Figure 1 shows the several possibilities for caffeine breakdown in plants, compiled from several studies which will be further commented below.

Suzuki and Waller (8) were the first to focus an investigation exclusively on caffeine degradation. *C. arabica* fruits received (8-^{14}C)caffeine and (8-^{14}C)theophylline through the petiole and the incubation proceeded for different periods of time. In those fruits fed with (8-^{14}C)caffeine radioactivity was found in theophylline, theobromine, 3- and 7-methylxanthines, ureides and urea. In the experiments with labeled theophylline (1,3-dimethylxanthine), radioactivity was detected in caffeine, 3-methylxanthine, ureides and urea. Therefore, in addition to theophylline being defined as the first product of caffeine degradation it was also shown that theobromine was involved in the biosynthesis as well as in the biodegradation of caffeine. With (8-^{14}C)caffeine feedings most of the radioactivity was recovered in caffeine. However, with (8-^{14}C)theophylline significant radioactivity was found in ureides indicating that perhaps the first demethylation would be a limiting step in caffeine breakdown.

A further study carried out by the same authors answered some questions left open in the former work and established the main caffeine biosynthetic and biodegradation route in coffee (9). They used several labeled substances ((2-^{14}C)caffeine, (8-^{14}C)theophylline, (8-^{14}C)adenine, (8-^{14}C)guanine, (2-^{14}C)xanthine and (methyl-^{14}C)methionine) fed to immature fruits and the first conclusion was that in immature fruits both biosynthetic and biodegradation reaction rates are more rapid than for mature fruits. Confirming the results of the previous work, a larger amount of radioactivity was recovered in caffeine when the fruits were fed with labeled caffeine, indicating that the first demethylating reaction would be a limiting step in degradation of the alkaloid. Due to the lower radioactivity detected in theobromine when the fruits received (8-^{14}C)caffeine, it was suggested that theobromine was mainly involved in the biosynthesis of caffeine.

Although Suzuki and Waller (9) have proposed caffeine degradation through theophylline, theobromine, 3-methylxanthine, 7-methylxanthine, xanthine, uric acid, allantoin, allantoic acid, urea, glyoxylic acid, CO_2 and NH_3, radioactivity was not detected in some of these substances. Xanthine and uric acid could not be isolated in the feeding experiments probably because the rate of this oxidative step was high, as previously suggested for tea (10). In addition, the authors could not determine wether theobromine was degraded to xanthine through 3-methylxanthine or 7-methylxanthine, since these could not be separated well by TLC or PC.

In his studies, Kalberer (3, 4) concluded that the decrease of caffeine in older leaves of coffee was due to degradation. The same seemed to occur in fruits. Suzuki and Waller (8) analyzed *C. arabica* fruits from 0-3 months to 7-8 months old. At 0-3 months the fruits were green and although not characterized by the authors they were probably at the stage known as pinhead, with approximately 3 mm diameter. The last stage was described as brown-black, meaning that the fruits were over-ripe. Caffeine, theobromine and theophylline were determined in these fruits without separation of the endosperm, perisperm and pulp. Endosperm is the economically important product of the coffee tree and it starts to develop in the fruit after 4-5 weeks of flowering. Theophylline was detected in very low amounts (2 mg/g dry weight) and only in the late stages of fruit development. Caffeine was found in much higher amounts in the first sampled fruits (9.2 mg/g dry weight) decreasing significantly in the over-ripe fruits (6.1 mg/g dry weight). The same was observed for theobromine, decreasing from 0.146 to 0.056 mg/g dry weight. These data obtained by Suzuki and Waller together those from the following study carried out with several labeled compounds (9) showed that caffeine content in the immature coffee fruits was a consequence of the higher biosynthetic rate relative to biodegradation, despite degradation being highest in these fruits. This conclusion is supported by Roberts and Waller (11) who showed that the biosynthetic activity in immature fruits as determined by *N*-methyltransferase activity was 150 times higher than yellow-red fruits.

However, the same was not observed with other species. Mazzafera *et al.* (12) reported on the catabolism of caffeine in several coffee species containing different levels of caffeine in their seeds. The endosperm of immature and mature fruits of *C. arabica* cv. Mundo Novo and cv. Laurina, *C. canephora* cv. Kouillou, *C. dewevrei* cv. Excelsa, *C. eugenioides*, *C. stenophylla*, *C. salvatrix*, *C. bengalensis* were collected and analyzed for caffeine, theobromine and theophylline. Endosperm of immature fruits had a milky aspect. Surprisingly, a significant contrast in terms of caffeine content was observed between endosperm from immature (2.5 mg/g) and mature (11.7 mg/g) fruits of *C. dewevrei*. Although not so evident, a similar situation was observed for *C. salvatrix*. Endosperm from immature and mature fruits of this species had 5.1 and 7.2 mg/g caffeine, respectively. When (8-^{3}H)caffeine was administered to immature fruits, in most of the species low total radioactivity was recovered (2.4 - 14%) indicating a high efficiency of degradation of the alkaloid. *C. arabica* and *C. canephora* were exceptions and showed high recovery rates (approximately 25%). However, among the species that had apparently degraded caffeine very efficiently, in general, more than 60% of the radioactivity was detected in caffeine. An exception was *C. dewevrei* where only 7% of the radioactivity was recovered in caffeine. Ninety percent of the radioactivity was recovered in caffeine in *C. arabica* and *C. canephora* and no radioactivity was found in theobromine and theophylline in neither species. *C. eugenioides*, *C. stenophylla*, *C. salvatrix* and *C. bengalensis* showed 10-37% of the recovered radioactivity in theobromine while 84.3% was detected in *C. dewevrei*. Except for *C. arabica* and *C. canephora*, radioactivity (2.6-17%) was observed in theophylline and 3-methylxanthine in the other species. In any case radioactivity was detected in xanthine confirming that conversion to uric acid is very rapid, as suggested previously

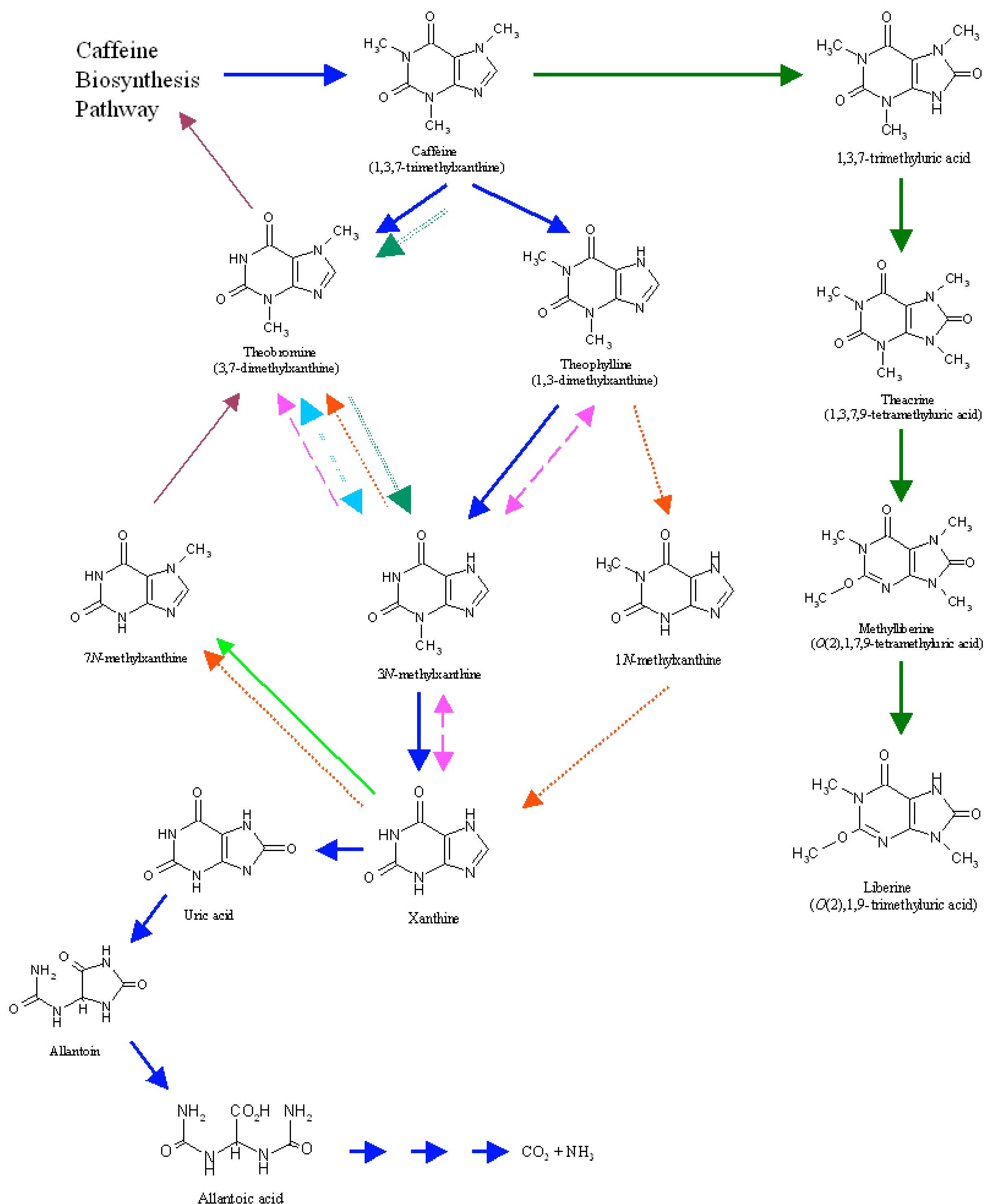

Figure 1. Caffeine catabolism in plants. Blue arrows indicate the main caffeine degradation pathway initially established by Suzuki and Waller (1984a,b) and confirmed by several reports in coffee, tea and cocoa. Other colors indicate alternative and minor pathways. ( ) *C. arabica* leaves – Ashihara et al. (1996); ( ) *C. dewevrei* leaves and fruits – Mazzafera et al. (1991, 1994); ( ) *C. eugenioides* – Ashihara and Crozier (1999); ( ) Cocoa tea leaves – Ashihara et al. (1998); ( ) tea leaves – Ashihara et al. (1997); ( ) *C. liberica* - Petermann & Baumann (1983), Citroreksoko (1979), Kucha tea – Zheng *et al.* (2002)

for coffee fruits (9). Uric acid and ureides could not be detected because of the loss of the ^{3}H-label from C-8 of xanthine. Therefore, in contrast to previous observations made with *C. arabica* (9) caffeine accumulation in *C. dewevrei* could not be explained by the proportionally higher biosynthetic rate compared with degradation. It was suggested that *C. dewevrei* has a high turnover of caffeine in immature fruits, decreasing with maturation.

Suzuki and Waller (9) detected very low radioactivity in 7-methylxanthine in experiments where coffee fruits received ^{14}C-labeled caffeine. But they could not show conclusively that 7-methylxanthine occurred in the degradation pathway of caffeine. On the other hand, Mazzafera *et al.* (12) did not find radioactivity in any species that received (8-^{3}H)caffeine. To investigate the participation of 7-methylxanthine in caffeine degradation in coffee fruits, specifically at some point between caffeine and xanthine, immature fruits and young leaves (second leaf pair from the apex) from *C. dewevrei* were fed with large amounts of (8-^{3}H)caffeine in the presence of allopurinol (13), a potent inhibitor of xanthine oxidase, which therefore blocks degradation of xanthine to uric acid (9). The period of incubation was 8 h. *C. dewevrei* was used because of its high ability to degrade caffeine. Radioactivity was detected in theobromine, theophylline, 3-methylxanthine and xanthine, but not in 7-methylxanthine, even in incubations with allopurinol. Interestingly, leaves of *C. dewevrei* proved to be as efficient as fruits for caffeine degradation.

However, in a further study with young (second leaf pair) and old (fifth to sixth leaf pairs) leaves and immature (milky endosperm) and mature (hardened endosperm) fruits from *C. dewevrei* and *C. arabica* (14), radioactivity was also detected in 7-methylxanthine when the incubations were carried out with (2-^{14}C)caffeine for 48 h (fruits) or 72 h (leaves). 7-Methylxanthine was found in both mature and immature fruits of *C. dewevrei* but not *C. arabica*. On the other hand, old leaves from *C. arabica* and young leaves of *C. dewevrei* accumulated radioactivity in 7-methylxanthine. In all cases recovered radioactivity was lower than 3% of the applied radioactivity.

Ashihara e*t al.* (15) carried out a detailed study with leaves of *C. arabica* at different developmental stages and the participation of 7-methylxanthine in the catabolism of caffeine was established. Feeding experiments with ^{14}C-labeled caffeine, theobromine, theophylline and xanthine allowed them to confirm previous reports that caffeine was mainly degraded through theophylline, 3-methylxanthine, xanthine, uric acid, allantoin, allantoic acid, urea, CO_2 and NH_3. Labeled theobromine was predominantly converted to caffeine but incubations with either labeled xanthine or theophylline, and allopurinol resulted in the detection of considerable radioactivity in 7-methylxanthine, indicating a salvage pathway for this monomethylxanthine, which is the precursor of theobromine in the biosynthetic caffeine pathway. In the abscence allopurinol, low levels of radioactivity were detected in 7-methylxanthine and only in older leaves. This probably explains the results previously reported by Suzuki and Waller (8, 9) who fed coffee fruits with labeled theophylline and recovered a little radioactivity in caffeine.

Therefore, despite the variations observed for this monomethylxanthine in leaves and fruits of *C. arabica* and *C. dewevrei*, probably in view of the genetic background and developmental stages of the tissues, it appears that in both species 7-methylxanthine is not an intermediate in the demethylating steps occurring between caffeine and xanthine. Instead, it is salvaged by xanthine methylation and re-enters caffeine biosynthesis.

Ashihara *et al.* (15) also showed that coffee tissues receiving labeled theobromine produced more labeled CO_2 than tissues incubated with labeled caffeine. They raised the possibility that during caffeine catabolism, theobromine was converted to xanthine. This is consistent with the fact that *C. dewevrei* accumulated significant radioactivity in theobromine in fruits and leaves fed with labeled caffeine (12, 13, 16).

Tea (*C. sinensis*) leaves present marked differences from coffee in terms of caffeine catabolism. Young, mature and old leaves degrade caffeine through theophylline and then to 3-methylxanthine and xanthine, which is degraded in the purine degradation pathway (17). However, it seems that xanthine may be reverted to 3-methylxanthine, which can then be methylated back to theophylline as well as used to form theobromine and thereby become re-integrated into caffeine biosynthesis. Theophylline was not methylated back to caffeine. These re-methylating steps are supported by previous studies with *N*-methyltransferase activities of tea leaf extracts (18, 19). As in coffee, caffeine biosynthesis in tea is most active in young leaves and the conversion of caffeine to theophylline occurs very slowly, enabling the tissue to accumulate caffeine up to 50 mg/g.

The metabolism of theophylline was compared in leaves of tea (*C. sinensis*), *Camellia irrawadiensis*, maté (*Ilex paraguariensis*) and *Avena sativa* and also in root segments of *Vigna mungo* and cell suspension cultures of *Catharanthus roseus* (20). While extensive uptake of labeled theophylline was observed in caffeine-containing species, this was very low in the other species and small amounts of radioactivity were recovered in 3-methylxanthine, xanthine and CO_2. On the other hand, theophylline was efficiently converted in tea and *C. irrawadiensis* into 3-methylxanthine, xanthine, uric acid, ureides and CO_2. Maté also appears to use the same degradation pathway but it was less efficient. Two tea cultivars differing in caffeine content (20 and 5 mg/g) were used in this study where it was observed that theophylline degradation was most rapid in mature and old leaves as well as in the cultivar with less caffeine, therefore, partially explaining its alkaloid content. Although the three developmental stages showed radioactivity salvaged through methylation of 3-methylxanthine to theobromine and then caffeine, as previously observed by Ashihara *et al.* (17), this radioactivity incorporation was higher in young leaves, perhaps explaining the highest accumulation of caffeine. Maté also salvaged theophylline in the same way in both young and mature leaves.

Theobromine is the major alkaloid in *C. irrawadiensis* which contains little or no caffeine. In contrast to the high and low caffeine cultivars of *C. sinensis*, mature and old leaves of *C. irrawadiensis* have less capacity to degrade labeled theophylline and little radioactivity is recovered in caffeine or theobromine, indicating a limited salvage pathway through 3-methylxanthine and theobromine for caffeine re-synthesis (20).

Degradation of caffeine was also investigated in leaves of cocoa tea (*Camellia ptilophylla*), a new tea resource in China (21). Theobromine is the main alkaloid in this tea species. Very low amounts of theophylline are detected but caffeine is absent. Young leaves are richer in alkaloids than old leaves. Feeding studies with (8-^{14}C)adenine showed that theobromine was synthesized in the same way as coffee and tea, but there was no conversion to caffeine. On the other hand, labeled caffeine was poorly taken up by the leaves and it was converted to theobromine. Labeled theobromine was slowly and directly degraded to 3-methylxanthine which in turn was converted to xanthine and on to the ureide degradation pathway.

It might seem obvious that the control of caffeine levels in plants is a function of the balance between rates of synthesis and degradation. However, this balance seems to vary depending on the plant species and the tissue developmental stage.

Suzuki and Waller (8, 9) were the first to suggested that demethylation to theophylline was the main limiting step of caffeine degradation in coffee fruits. Theophylline degradation was not limiting and as indicated by the highest recovered radioactivity in 3-methylxanthine, it was most rapid in mature fruits. The same conclusion was drawn from others studies with coffee and tea that used labeled compounds (15, 17, 20). However, degradation of caffeine was seemed to be more extensive in other coffee species (12, 13).

In order to investigate the control of caffeine metabolism in *C. dewevrei*, Mazzafera *et al.* (16) measured the methyltransferase activities involved in the biosynthetic pathway, as well as by feeding fruits and leaves with (8-^{3}H)caffeine. In addition, they studied the metabolism of caffeine in two cultivars of *C. arabica*. Mundo Novo is a commercial cultivar with 10-12 mg/g caffeine in the seeds and 8-10 mg/g in the leaves. Laurina is a *C. arabica* mutant (22, 23) containing half the caffeine found in the seeds of Mundo Novo but has approximately the same levels in the leaves (7 mg/g). The lower caffeine content of Laurina could not be explained by the methyltransferase activities and degradation rate of caffeine since little difference was found between cultivars for both the degradation and the enzyme activities. However, much lower activities were observed in fruits and leaves of *C. dewevrei* coupled with a high degradation rate. It was suggested that the low caffeine content in immature fruits of this species was due to a low rate of biosynthesis associated with a high rate of degradation. However, mature fruits of this species contain as much caffeine as *C. arabica* (12 mg/g). Therefore, it seems that during ripening there is a change in the balance between biosynthesis and biodegradation, such that when degradation is lower than synthesis, caffeine levels increase. In the same study, fruits and leaves were fed with (8-^{3}H)caffeine plus theophylline. This caused a 50% reduction of the radioactivity in theophylline and theobromine, indicating that the same enzyme could demethylate caffeine to these dimethylxanthines but presenting different affinity for the methyl group. Radioactivity in 3-methylxanthine also decreased approximately 50% with theophylline feeding.

A similar study was carried out with leaf segments from flush shoots of *C. sinensis* cultivars differing in their caffeine content (24). Feeding experiments with (8-^{14}C)adenine and (8-^{14}C)caffeine showed that there was a more rapid rate of caffeine biosynthesis in the high caffeine cultivars while the rate of degradation of both adenine nucleotides and caffeine to CO_2 was greatest in cultivars with low caffeine content. Activity of N-methyltransferase in cell-free preparations from tea shoots was highest in preparations from high caffeine containing cultivars. This suggests that the high caffeine containing cultivars have a more rapid rate of caffeine biosynthesis and a slower rate of caffeine catabolism than cultivars with low endogenous caffeine content.

Ashihara *et al.* (25) expanded the study initiated by Mazzafera *et al.* (16) with endosperm and leaves of *C. arabica* and *C. dewevrei* to other coffee species containing low caffeine in the leaves. Caffeine contents in young (the most recently emerged leaf) and mature (second and third leaf pairs) leaves of *C. salvatrix*, *C. eugenioides* and *C. bengalensis* were 0.92/0.30, 1.30/0.37 and 1.20/0.76 mg/g, respectively. *C. arabica* leaves contained 7.10/2.10 mg/g. These leaves were incubated with (8-^{14}C)-labeled adenine, caffeine and theophylline. *C. salvatrix*, *C. eugenioides* and *C. bengalensis* synthesized caffeine in a similar way to *C. arabica*, but at much lower rates, especially in *C. bengalensis*, partially explaining its low alkaloid content. Feeding labeled caffeine showed however, that the lower alkaloid content of leaves of *C. eugenioides* was due to a strong catabolic activity. The classical degradation pathway caffeine -> theophylline -> 3-methylxanthine -> xanthine was observed, although some label was incorporated into 1-methylxanthine when (8-^{14}C)theophylline was fed. In contrast to previous studies with *C. arabica*, 7*N*-methylxanthine was not formed from theophylline in *C. salvatrix*, *C. eugenioides* and *C. bengalensis* leaves. Therefore, it seems that the caffeine level in the young leaves of *C. eugenioides* is determined by a similar mechanism to that observed in immature fruits of *C. dewevrei* (16), that is, association of low biosynthesis with fast degradation. However, in old leaves of *C. eugenioides*, the low caffeine content is probably a result of lowered biosynthesis coupled with active degradation. Mature fruits of *C. dewevrei* accumulate more caffeine than immature fruits due to a lower degradation rate associated with a similar biosynthetic rate at both developmental stages (16).

Although several investigations have reported that the conversion of caffeine to theophylline is the main

limiting step in the alkaloid breakdown in plants, so far no report has described the detection of caffeine demethylase. In humans and other animals caffeine is first degraded to either paraxanthine (1, 7-dimethylxanthine), theobromine or theophylline. The demethylation process is carried out by cytochrome P450 (26-28, 29). After a short period of caffeine ingestion, these dimethylxanthines can be detected in the urine. However, they can also be further demethylated to xanthine, which is catabolised by the uric acid route. Alternatively, the methylxanthines produced by the catabolic pathway can be oxidized to the corresponding methyluric acids (27).

In plants, to our knowledge, few investigations have reported any type of demethylase activity, especially involving an alkaloid. Strong indication that a cytochrome p450 would be the responsible for nicotine demethylation to nornicotine was obtained by Chelvarajan *et al.* (30). Maximum activity was recovered from microssomes, together with indirect evidence obtained with cofactors and inhibitors that indicated nicotine breakdown was carried out by a cytochrome p450. More recently, evidence has been obtained that an oxidative reaction demethylates nicotine to nornicotine (31).

Some attempts to detect this demethylating activity in coffee were unsuccessful. Vitória (32) tried different protocols for protein extraction, including microssomes, and several additions to the reaction medium to detect caffeine demethylase activity. Labeled caffeine was used as substrate but radioactivity was not recovered in theophylline or other methylxanthine. The same procedure was attempted by Huber and Baumann (32) without success.

Indirect evidence in favor of a p450 mechanism was obtained by Huber and Baumann (33) and Mazzafera (unpublished data). They infiltrated leaf disks taken from *C. liberica* and *C. dewevrei*, respectively, with caffeine and followed degradation of the alkaloid. Leaf disks from *C. liberica* infiltrated with 5 mM caffeine showed a decrease of 50% after 48 h incubation. Leaf disks of *C. dewevrei* infiltered with 1 mM caffeine showed almost 90% of degradation after the same incubation period. Incubations with typical inhibitors of cytochrome p450-dependent monooxygenases did not have any effect in the demethylating activity. P450-dependent monooxygenase activity is dependent of oxygen. Incubations of *C. liberica* leaf disks in an atmosphere of N_2 completely inhibited caffeine demethylation. Darkness or light did not affect demethylation. Therefore, it was not possible with these results to obtain evidence of the nature of the caffeine demethylase and the first step of caffeine degradation is still a mystery.

In most of the studies on caffeine degradation using labeled compounds radioactivity is usually associated with ureides but uric acid is not detected. In addition, the low recovery of radioactivity in xanthine has been attributed to the fast rate of degradation imposed by xanthine oxidase/dehydrogenase. The control of these metabolic steps was studied in *C. arabica* and *C. dewevrei* (32, 34). Leaves of both species accumulate 0.2 - 0.4 mg/g of ureides and fruits approximately 3 times more. Incubations with (2-^{14}C) xanthine showed that young leaves degrade ureides less efficiently, since they retained more radioactivity in these compounds. No uric acid was detected in leaves. However *C. dewevrei* fruits were less efficient in xanthine degradation, since proportionally more radioactivity was recovered in ureides and uric acid compared with fruits of *C. arabica*. These data were in agreement with the observation that leaves had the highest activity of xanthine dehydrogenase while this activity was very low or not detected in fruits of *C. dewevrei*. Similar results were observed with uricase and urease. Instead of an oxidase, a dehydrogenase activity was characterized as the enzyme responsible for xanthine conversion to uric acid. Activity determinations showed that uricase was very active which explains why uric acid is usually not detected in incubations with labeled compounds. The activity of uricase in leaves varied from 1,500 to 5,300 ng of allantoin formed per mg protein per hour while xanthine dehydrogenase activity was 18 to 399 ng of uric acid formed per mg protein per hour. It appears that the radioactivity recovered in ureides is related to the levels of allantoinase and allantoate amidohydrolase. Attempts with several protocols modified from soybean studies were unsuccessful in measuring these enzymes indicating very low activities.

3. METHYLURIC ACIDS AS INTERMEDIATES IN CAFFEINE DEGRADATION

Wanner *et al.* (35) confirmed previous observations made by Johnson (36) that methylated oxypurines (methyluric acids) are components of plants and not artefacts formed during extraction. 1,3,7,9-Tetrametyluric acid was extracted from tea (36) and *Coffea* species which belong to a group presenting strong genetic affinities denominated as liberio-excelsioides (37). This group includes *C. liberica*, *C. dewevrei* and *C. excelsa*. Methyluric acids have also been found within the genera *Herrania* and *Theobroma* (38, 39).

Two other methyluric acids have been isolated from *C. liberica*, *O*(2),1,9-trimethyluric acid and *O*(2),1,7,9-tetramethyluric acid (35, 40). Baumann *et al.* (41) followed the distribution of methyluric acids in *C. liberica* plants, from seedlings to adult trees and concluded that caffeine was used for the synthesis of these compounds.

Citroreksoko (42) followed the formation of methyluric acids in fruits of *C. liberica* as well as feeding synthesized ^{14}C-labeled 1,3,7,9-tetrametyluric acid and *O*(2),1,7,9-tetramethyluric acid to the pericarp and perisperm of the fruits. In contrast to the other methyluric acids, *O*(2),1,7,9-tetramethyluric acid was present in considerable amounts (up to 7.6 mg/g) in the pericarp. It was suggested that caffeine was oxidized and methylated to 1,3,7,9-tetrametyluric acid, which was converted either to *O*(2),1,7,9-tetramethyluric acid or *O*(8),1,3,7-tetramethyluric acid by rearrangement of one methyl group. These would be demethylated to *O*(2),1,9-tetramethyluric

or *O*(8),3,7-trimethyluric acids and then oxidized to correspondent trimethylallantoic acids.

In order to study the metabolic relationship between caffeine and methyluric acids, Petermann and Baumann (43) fed leaf disks from *C. liberica*, *C. dewevrei* and *C. abeokutae*, which belong to the liberio-excelsioides group, with ^{14}C-labeled caffeine, theobromine and 1,3,7,9-tetrametyluric acid (theacrine). Analyses made in leaves during the plant development showed that young leaves had the highest concentration of theacrine, decreasing with leaf age. At the same time *O*(2),1,9-trimethyluric acid (liberine) appeared in the older leaves. In the older leaves both theacrine and liberine were very low. The experiments with labeled compounds showed that, caffeine was indeed demethylated to theobromine as well as being methylated and oxidized to theacrine. In a second moment, theacrine was converted to liberine. Eventually *O*(2),1,7,9-tetramethyluric acid (methylliberine) was detected and it was presumed to be the direct precursor of liberine. Old leaves of these species are also almost devoid of caffeine, although the fruits accumulate as much as 10 mg/g in the seeds (12, 44).

Caffeine metabolism in culture cell suspensions from *C. dewevrei*, which belongs to the liberio-excelsioides group, was investigated by Sartor and Mazzafera (45). Cultures were fed with (2-^{14}C)caffeine and the incorporation of radioactivity in methylxanthines and methyluric acids determined in the cells and in medium. Radioactivity was found in theobromine and theophylline in the medium. In the cells, it was detected in both dimethylxanthines in addition to paraxanthine (1,7-dimethylxanthine) and 3, 7-dimethyluric acid. Radioactivity was also found in these compounds when $Ba^{14}CO_3$ was incorporated to the medium. However, the incorporated radioactivity was not higher than 4% of the total radioactivity supplied. Cell cultures may change secondary metabolic pathways (46). Baumann and Frischknecht (47) did not find theacrine, liberine and methylliberine in cell culture of *C. liberica*, which is genetically close to *C. dewevrei*. On the other hand, paraxanthine, which is not found in intact coffee tissues, was identified in cotyledonary leaves of *C. arabica* (48) and in cell suspensions of *C. eugenioides* and *C. liberica* (47). 3,7-Dimethyluric acid is the oxidized form of theobromine and it has been detected as a catabolite of caffeine culture of *Pseudomonas putida*, and its formation seems to be the consequence of the high and unspecific activity of xanthine oxidase activity (49).

Theacrine formation was also investigated in *Camellia assamica* (kucha) (50). Theacrine and caffeine are the major alkaloids in this species of tea, accumulating to similar levels in expanding buds, however theacrine predominates in young and mature leaves. Feeding experiments with ^{14}C-labeled adenosine, caffeine and S-adenosyl-L-methionine showed that theacrine was synthesized from caffeine with 1,3,7-trimethyluric acid as an intermediate, as previously suggested for *C. liberica* (42).

4. CAFFEINE CATABOLISM IN BACTERIA AND OTHER MICROORGANISMS

Although high concentrations are required for bactericide action, caffeine is regarded as toxic for bacteria. Nevertheless, several microorganisms have developed the ability to grow in the presence of caffeine and survival would be related to their capacity to degrade the alkaloid. As a result, it is not rare to find fungi and bacterial strains resistant to caffeine. The use of microorganisms has been studied as an alternative to decaffeinate residues (husk) of coffee processing in order to be used to feed animals (51, 52).

Several studies were carried out to investigate the use of purines, including caffeine, as a source of energy for microorganism growth (53-58).

Dikstein *et al.* (59) and Bergmann *et al.* (60) studied the degradation of 3-methylxanthine mediated by dehydrogenase activity in *Pseudomonas fluorescens*. They did not find activity with 1-methylxanthine as substrate. However, Woolfolk (55) demonstrated dehydrogenase activity against both monomethylxanthines using a *P. fluorescens* strain with ability to grow on caffeine. He suggested that a hydrolytic enzyme degraded caffeine, with the methyl groups being removed by sequential hydrolysis. Methanol and xanthine were the final reaction products, with indications that methanol was further oxidized to CO_2.

Blecher and Lingens (61) isolated bacterial strains by enriching humus soil from Stuttgart, Germany, with caffeine and incubating at 30°C for 6 months. Then a portion of this soil was transferred to a minimum medium containing caffeine and $(NH_4)_2SO_4$, both at 1%. They also incubated soil samples from various other regions of Germany with the same enrichment procedure but only for one week. Several isolates were characterized for their ability to grow on caffeine. *Pseudomonas putida* was the only bacterium isolated from all incubations. *P. putida* ATCC strains used as controls did not grow on caffeine although all could grow on xanthine. They identified 14 catabolites incubating the *P. putida* isolates with caffeine: theobromine, paraxanthine, 7-methylxanthine, xanthine, 3,7-dimethyluric acid, 1,7-dimethyluric acid, 7-methyluric acid, uric acid, allantoin, allantoic acid, ureidoglicolic acid, glyoxylic acid, urea and formaldehyde.

Also using an enrichment procedure, Middelhoven and Bakker (54) isolated a *P. putida* strain that could grow on caffeine as the sole source of carbon and nitrogen. Middlhoven and Lommen (62) used this same strain to study caffeine degradation as influenced by oxygen. Their results suggested that caffeine degradation was probably mediated by mono-oxygenases.

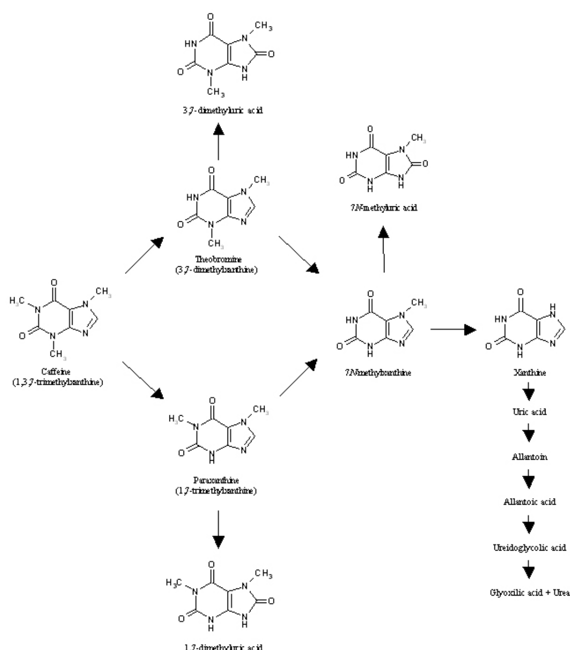

Figure 2. Caffeine catabolism in bacteria. Pseudomonas putida (Blecher & Lingens, 1977; Yamaoka-Yano & Mazzafera, 1999) and Serratia marcescens (Mazzafera et al., 1996)

More details on the enzymological aspects of caffeine degradation by *Pseudomonas putida* were reported by Hohnloser *et al.* (63). Using NADPH as a cofactor in enzyme assays, they observed that only theobromine was formed from caffeine. When paraxanthine, theobromine and 7-methylxanthine were used as substrates they did not detect any demethylase activity. According to the authors, this was an indication that there was only a single enzymatic system responsible for the sequential demethylation of caffeine. Regarding the lack of activity against theobromine and other substrates they suggested that although not detected *in vitro*, enzymatic degradation might occur *in vivo* but at very low rates. It was also concluded that the slow and poor growth of the bacteria on caffeine as the sole source of carbon was due to a limiting demethylation of caffeine as well as other methylxanthines.

Limited rates of caffeine demethylation were observed in a strain of *Serratia marcescens* isolated from soil collected under coffee trees (53). By cultivating the bacteria on different substrates as the sole source of carbon and nitrogen it was established that caffeine was converted to paraxanthine and/or theobromine, and subsequently to 7-methylxanthine and xanthine.

Sauer *et al.* (64) obtained indications that caffeine in yeast was degraded by cytochrome P450, suggesting that the catabolic pathway might be similar to animals. In humans, several cytochrome P450 isoforms are responsible for caffeine degradation (27). However, the results obtained by Schwimmer *et al.* (55), who studied the degradation of caffeine to theophylline in fungi, and by Blecher and Lingens (61), who studied degradation of caffeine to theobromine in bacteria, did not indicate participation of P450.

Gluck and Lingens (65) isolated a *P. putida* from soil by enrichment with caffeine and induced mutants of this strain with N-methyl-N'-nitro-N-nitrosoguanidine. Their aim was to isolate mutants that could accumulate other methylxanthines from caffeine degradation. The mutants were able to degrade caffeine to a mixture of theobromine and paraxanthine supporting the data previously obtained by Blecher and Lingens (61), who suggested that caffeine was degraded by two routes, either via theobromine or via paraxanthine. A similar conclusion was reached with *S. marcescens* (53).

In most of the studies on caffeine degradation by *Pseudomonas*, the bacterial strains were obtained by a procedure known as enrichment. Caffeine was added either to soil or culture medium (56, 61-63) to induce the appearance of mutants. In the case of soil enrichment, caffeine was mixed to the soil and incubated for several months. In the second case, caffeine was added in low concentrations in artificial media and the bacteria subcultured several times until mutants were obtained. After that, the bacteria were maintained in media containing caffeine as the sole source of carbon. However, bacterial strains with a great ability to degrade caffeine could be isolated directly from soil samples collected under coffee trees (66, 67). Water was added to the soil samples and after shaking for a few hours, aliquots were plated in solid medium containing caffeine as the sole source of carbon and nitrogen. A *Serratia marcescens* strain (66) and several *P. putida* strains (67) were isolated. These results argued against the role of caffeine as an allelopathic compound in coffee plantations as well as causing toxicity to the coffee plant itself (68, 69).

A strain of *P. putida* isolated by Yamaoka-Yano and Mazzafera (67) showed an impressive ability to growth in high concentrations of caffeine. Growth was observed at 25 g/L in liquid medium and at 50 g/L in solid medium. The direct isolation from soil without any enrichment is a strong indication that, due to competition for organic nutrients, bacteria growing in soil under coffee trees have developed mechanisms to degrade the caffeine released by the plants (leaves, fruits and litter). In other words, there was a natural enrichment.

Yamaoka-Yano and Mazzafera (49) studied the caffeine degradation pathway in this *P. putida* strain and in agreement with previous reports (61) they suggested the degradation pathway depicted in Figure 2. They also purified a xanthine oxidase, responsible for the conversion of methylxanthines to their respective uric acids. According to Yamaoka-Yano and Mazzafera (49) the formation of methyluric acids was due to an unspecific and high activity of xanthine oxidase in the bacterial cells.

Purification of the demethylase involved in the first step of caffeine degradation was also attempted in the same *P. putida* strain isolated by Yamaoka-Yano and

Mazzafera (67) but without success (Yamaoka-Yano and Mazzafera, unpublished). The activity was labile in partially purified extracts. The enzyme was dependent on NADH or NADPH, producing theobromine and paraxanthine from caffeine. Activity was highest for paraxanthine as substrate. It was also observed that caffeine demethylase was excreted in the culture medium. Addition of Zn to the reaction mixture inhibited caffeine degradation through theobromine, but not paraxanthine. Optimal activity was obtained with temperatures between 20 and 30°C and at pH 7.0. Since analysis of the liquid medium by SDS-PAGE revealed the presence of few proteins, proteins from two liters of culture medium were concentrated by $(NH_4)_2SO_4$ precipitation, desalted by dialysis and loaded on to an anionic exchange column (Mono Q). Demethylase activity was detected as a single peak eluted by a NaCl gradient and both theobromine and paraxanthine were detected as products in the reaction mixture. Despite attempts to preserve activity using several methodologies, the enzyme was very labile and lost activity rapidly.

The caffeine demethylase in cell free extracts prepared from *P. putida* cultured on caffeine could be better stabilized by cryoprotectants and concentration by freeze-drying (70). The enzyme appeared consist of a complex formed by two major proteins of 43.5 and 36.6 KDa.

Blecher and Lingens (61) and Glück and Lingens (65) isolated *P. putida* mutants with the ability to degrade caffeine and attempted to block its degradation in order to produce caffeine derivatives for commercial application. In contrast to these authors, Asano *et al.* (71) were successful in isolating a *P. putida* strain where caffeine degradation could be blocked by addition of Zn to the culture medium, resulting in theobromine accumulation. This dimethylxanthine was excreted into the medium, precipitating at the bottom of the flask due to its low solubility in aqueous solution. This was the first time that a caffeine derivative was selectively produced using bacteria. In a further study these authors purified a monooxygenase specific for theobromine demethylation (72). As found in previous studies on enzymes of caffeine catabolism, theobromine demethylase was very labile and purification had to be carried out very quickly. During purification three distinct activities were observed with specificity for (A) caffeine, (B) caffeine and theobromine, and (C) 7-methylxanthine and caffeine. On assaying these fractions with caffeine as substrate none of these fractions (A, B or C) were inhibited by addition of Zn in the reaction medium. On the other hand, fraction B was inhibited by Zn and no 7-methylxanthine was formed when theobromine was the substrate. The authors concluded that caffeine demethylation to 7-methylxanthine is carried out by two enzymes, caffeine demethylase and theobromine demethylase. Theobromine demethylase was purified to homogeneity and it was concluded that the native protein (250 KDa) was a hexamer composed of six identical subunits (41 kDa). The enzyme required NADPH and it was stimulated by cobalt.

Recently, a caffeine demethylase nucleotide sequence from *P. putida* was deposited in the NCBI gene bank under the accession E11325. A search with this nucleotide sequence in the EST database of the Brazilian Coffee EST Genome project did not show any similarity with any sequence.

Compared with bacteria, much less is known about caffeine degradation in fungi. *Aspergillius niger* (73) and *Penicillium roqueforti* (55) seem to have theophylline as the first degradation product. Hakil *et al.* (74) tested the ability of twenty strains of filamentous fungi to grown on caffeine as a sole source of nitrogen and only strains of the *Penicillium* and *Aspergillus* genera were able to degrade the alkaloid. Caffeine was degraded to theophylline and then to 3-methylxanthine. However, the strains also grew on theobromine, paraxanthine and theophylline as a sole source of nitrogen but different growth rates and degradation efficiencies. 3-Methylxanthine was formed from theobromine and theophylline while 1- and 7-methylxanthine were formed from paraxanthine.

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Send correspondence to: Dr Paulo Mazzafera, Departamento de Fisiologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas, CP 6109, 13083-970, Campinas, SP, Brasil, Tel: 55-19-3788-6213, Fax: 55-19-3788-6210, E-mail: pmazza@unicamp.br