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Molecular and biochemical characterization of caffeine synthase and purine alkaloid concentration in guarana fruit

Flávia Camila Schimpl [a], Eduardo Kiyota [a], Juliana Lischka Sampaio Mayer [b], José Francisco de Carvalho Gonçalves [c], José Ferreira da Silva [d], Paulo Mazzafera [a,*]

[a] Departamento de Biologia Vegetal-IB, CP 6109, Universidade Estadual de Campinas, 13083-970 Campinas, SP, Brazil
[b] Departamento de Biologia, Universidade Estadual do Centro-Oeste, Guarapuava, PR, Brazil
[c] Instituto Nacional de Pesquisas da Amazônia, Manaus, AM, Brazil
[d] Departamento de Produção Animal e Vegetal, Faculdade de Ciências Agrárias, Universidade Federal do Amazonas, Manaus, AM, Brazil

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ABSTRACT

Guarana seeds have the highest caffeine concentration among plants accumulating purine alkaloids, but in contrast with coffee and tea, practically nothing is known about caffeine metabolism in this Amazonian plant. In this study, the levels of purine alkaloids in tissues of five guarana cultivars were determined. Theobromine was the main alkaloid that accumulated in leaves, stems, inflorescences and pericarps of fruit, while caffeine accumulated in the seeds and reached levels from 3.3% to 5.8%. In all tissues analysed, the alkaloid concentration, whether theobromine or caffeine, was higher in young/immature tissues, then decreasing with plant development/maturation. Caffeine synthase activity was highest in seeds of immature fruit. A nucleotide sequence (*PcCS*) was assembled with sequences retrieved from the EST database REALGENE using sequences of caffeine synthase from coffee and tea, whose expression was also highest in seeds from immature fruit. The *PcCS* has 1083 bp and the protein sequence has greater similarity and identity with the caffeine synthase from cocoa (BTS1) and tea (TCS1). A recombinant PcCS allowed functional characterization of the enzyme as a bifunctional CS, able to catalyse the methylation of 7-methylxanthine to theobromine (3,7-dimethylxanthine), and theobromine to caffeine (1,3,7-trimethylxanthine), respectively. Among several substrates tested, PcCS showed higher affinity for theobromine, differing from all other caffeine synthases described so far, which have higher affinity for paraxanthine. When compared to previous knowledge on the protein structure of coffee caffeine synthase, the unique substrate affinity of PcCS is probably explained by the amino acid residues found in the active site of the predicted protein.

1. Introduction

Guarana is the name given to *Paullinia cupana* var. *sorbilis* Kunth, a native species to the Maués region in the Brazilian Amazon. The variety *sorbilis* is known as the Brazilian guarana, while *Paullinia cupana* var. *typica* is the Venezuelan guarana (Schimpl et al., 2013). Except for *Paullinia pinnata* L., which is also present in tropical Africa, all other species of the genus are restricted to tropical and subtropical America (Missouri Botanical Garden, 2012). In this study, guarana will designate *P. cupana* var. *sorbilis*, which is the only commercially exploited species and has been studied most intensively.

Guarana has the highest caffeine (1,3,7-trimethylxanthine) (**1**) concentration reported in plants and its seeds may contain from 2.5% to 6.5% caffeine at dry weight (Oliveira, 2010; Spoladore et al., 1987). Theobromine (3,7-dimethylxanthine) (**2**) and theophylline (1,3-dimethylxanthine) (**3**) are present in smaller quantities (see structures 1, 2 and 3 in Fig. 1). Indians and natives of the Amazon have consumed guarana for centuries as an aqueous beverage made from the powder obtained from roasted seeds, whose best known physiological effect is improved alertness because of caffeine (Henman, 1986; Schimpl et al., 2013; Schmidt, 1941).

Caffeine (**1**) has been reported in 13 plant orders, mostly eu-dicotyledonous plants. With minor variations, the main route of its synthesis is highly conserved in the plants studied so far, mainly coffee (*Coffea arabica*) and tea (*Camellia sinensis*) (Ashihara et al., 2008; Ashihara and Suzuki, 2004; Mazzafera, 2004).

Caffeine (**1**) biosynthesis has three methylation steps and xanthosine is the first compound to be methylated. It has been suggested that there are four possible routes in the origin of

* Corresponding author. Tel.: +55 19 3521 6213.
E-mail address: pmazza@unicamp.br (P. Mazzafera).

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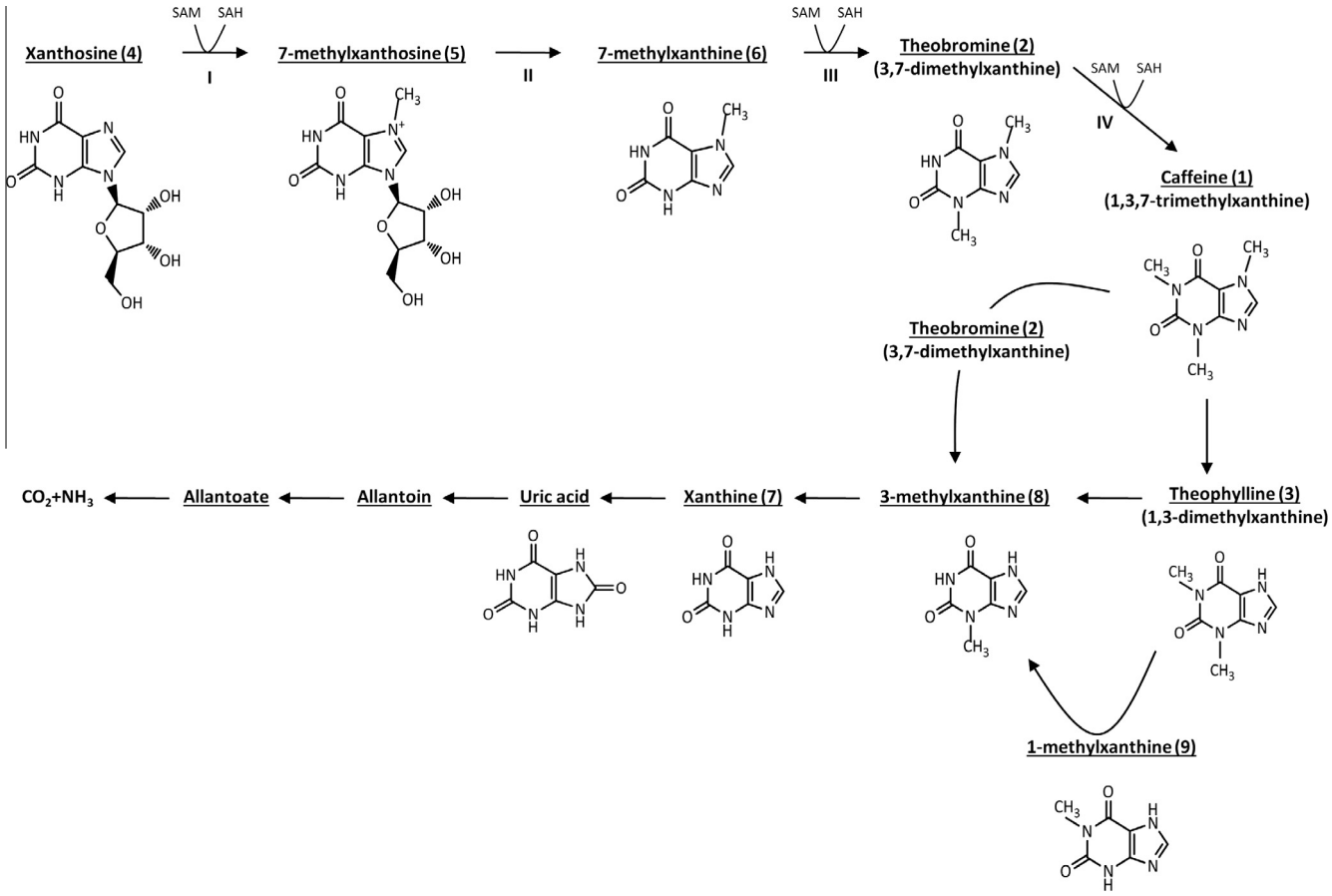

Fig. 1. Main metabolic pathway for the biosynthesis and biodegradation of caffeine. SAM = S-adenosyl-L-methionine, SAH = S-adenosyl-L-homo-cysteine. I = 7-methylxanthosine synthase (xanthosine methyltransferase), II = nucleosidase, III = theobromine synthase or caffeine synthase, IV = caffeine synthase.

xanthosine (**4**) (Ashihara, 2006; Ashihara et al., 2008). In coffee and tea, the caffeine biosynthesis pathway is: xanthosine (**4**) → 7-methylxanthosine (**5**) → 7-methylxanthine (**6**) → theobromine (3,7-dimethylxanthine) (**2**) → caffeine (1,3,7-trimethlxanthine) (**1**) (Fig. 1) (Ashihara et al., 2008; Mizuno et al., 2003a; Uefuji et al., 2003). S-adenosyl-L-methionine (SAM) is the methyl donor in caffeine (**1**) biosynthesis (Suzuki, 1972). The genes coding for the *N*-methyltransferase enzymes catalysing the three methylations have been studied mainly in coffee, and to a lesser extent in tea (Ashihara et al., 2008, 2011; Kato and Mizuno, 2004).

So far studies on guarana have mostly been restricted to characterization of caffeine (**1**) and other purine alkaloid concentration in the seeds. Recently, with the aim of increasing genetic knowledge of this plant, an EST database was made from guarana fruit at three different stages of maturation [Rede Amazônia Legal de Pesquisas Genômicas – Realgene (Ângelo et al., 2008)]. Recently, Figueiredo et al. (2011) analysed the 15,490 ESTs of the Realgene database and, among 4697 full-length clones identified, 84 clones were identified as *N*-methyltransferases and 18 were sequenced for confirmation of their identity. Phylogenetic analyses were carried out for three clones (with only 0.017% dissimilarity), which were close to a postulated caffeine synthase of *Theobroma cacao* (BTS1/BCS1). However, the recombinant protein from the cocoa sequence and other sequences resembling caffeine synthase only had 3-*N*-methyltransferase activity, i.e., theobromine synthase activity (Yoneyama et al., 2006).

In this study, the purine alkaloid concentration in different tissues of five guarana cultivars grown in Brazil was analyzed and, in one of them, caffeine synthase activity was assayed. Using the Realgene database, a nucleotide sequence displaying was assembled high similarity to caffeine synthase from cocoa, and the recombinant protein proved to be a bifunctional caffeine synthase, being able to methylate 7-methylxanthine (**8**) and theobromine (**2**). In contrast with all caffeine synthases studied so far, which have paraxanthine (**8**) as the best substrate, *P. cupana* caffeine synthase (PcCS) has theobromine as the best substrate and no activity against paraxanthine. *PcCS* had higher expression in immature seeds compared to other guarana tissues.

2. Results

2.1. Methylxanthines concentration

Theobromine (**2**) was the main alkaloid found in leaves, followed by theophylline (**3**), and higher concentrations of both alkaloids were found in young leaves. Caffeine (**1**) was detected only in intermediate and mature leaves. Theobromine (**2**) concentration ranged from 3.47% to 4.18% (Fig. 2).

Similar to leaves, apical and basal portions of stems had theobromine (**2**) as the predominant alkaloid, followed by theophylline (**3**). Caffeine (**1**) was detected only in the basal portion. Among the tissues analysed, stems were those containing the lowest methylxanthine (**8**) concentrations (Fig. 3).

Theobromine (**2**) was also the most abundant alkaloid in the inflorescences of all cultivars and, for most of them, the theophylline (**3**) concentration was higher than caffeine (**1**) (Fig. 4).

In the pericarp, theobromine (**2**) was also the predominant alkaloid but, in this tissue, caffeine (**1**) was more abundant than theophylline (**3**) (Fig. 5). In general, the alkaloid concentration

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