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Caffeine and theobromine in epicuticular wax of *Ilex paraguariensis* A. St.-Hil.

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Abstract

Caffeine and theobromine were identified and quantified in leaf epicuticular waxes of *Ilex paraguariensis* A. St.-Hil. (Aquifoliaceae). The total epicuticular leaf wax content was ca. 0.5% on average of dry leaf weight. Epicuticular caffeine and theobromine contents varied from 0.16 to 127.6 μg/mg and from 0 to 9.5 μg/mg of wax, respectively. For some selected samples, the intracellular methylxanthine concentration was also determined. A positive correlation was found between inner and epicuticular caffeine contents.

Keywords: *Ilex paraguariensis*; Aquifoliaceae; Epicuticular wax; Caffeine; Theobromine

1. Introduction

The epicuticular waxes provide a physico–chemical barrier that improves resistance to water loss and protects against invasion by fungi and other harmful organisms. Many simple chemical constituents (hydrocarbons, aldehydes, wax esters, fatty alcohols, ketones and fatty acids) have been isolated from waxes (Wollenweber, 1981). A number of cyclic compounds have also been isolated from plant cuticular lipid fractions. The pentacyclic triterpenoids such as α- and β-amyrin, ursolic and oleanolic acids are generally the most common (Wollenweber, 1981; Niemann and Baas, 1985).

Ilex paraguariensis A. St.-Hil. is a South American native perennial tree belonging to the holly family (Aquifoliaceae). It has been used historically as a source of a mildly stimulant beverage, called maté (''erva-mate'' or ''yerba-mate''), prepared by infusion of its dried leaves and twigs. The presence of caffeine and theobromine in maté leaves has been known since the 19th century while the presence of theophylline, reported in very small quantities (Mazzafera, 1994), is a matter of controversy, as other researchers could not detect this substance (Baltassat et al., 1984; Clifford and Ramirez-Martinez, 1990; Ashihara, 1993; Filip et al., 1998). In spite of numerous analyses for methylxanthines in developing and old leaves, bark, wood, and immature and mature fruits of the maté plant, there is no report concerning their occurrence in epicuticular wax. In this work, as a part of our investigations on maté chemistry (Gosmann et al., 1995; Pires et al., 1997; Schenkel et al., 1997) and on populational variability, the methylxanthine content of three different populations of native *I. paraguariensis* collected in three States from Brazil were determined in the soluble chloroform fraction of the whole dried leaves (epicuticular wax) and after this, for some selected samples, also in the residual crushed leaves. To the best of our knowledge, this is the first report concerning the occurrence of caffeine and theobromine in epicuticular wax.

2. Results and discussion

Leaves of three native populations of *I. Paraguariensis* from three States of Brazil: Mato Grosso do Sul (MS), Paraná (PR) and Rio Grande do Sul (RS), were collected during mid-summer (February) and in spring 1997 (September in MS, October in PR and

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November in RS). From each plant, the yield of epicuticular wax, and the concentration of caffeine and theobromine in the wax was analysed. The epicuticular wax was obtained by dipping entire dried leaves in $CHCl_3$ for 1 min. The obtained extracts were colourless, suggesting the cuticular origin of the measured methylxanthines. Considering the hypothesis that during the drying the epicuticular wax layer may be damaged, the dipping procedure was also carried out with some fresh leaves. Spectrophotometric scanning ranging from 700 to 200 nm confirmed the absence of chlorophylls in both extracts from dried and fresh leaves, since no maxima for chlorophylls (Mackinney, 1941) were observed. Instead, a typical absorption curve for methylxanthines was observed.

In an additional experiment to demonstrate that the methylxanthines originated from the wax and not from the leaf interior, the surfaces of some dried leaves were carefully scraped with a blade and the material was analysed by TLC and HPLC. The results from samples of scraped surfaces ranged from 5.9 to 17.0 μg of caffeine per mg of wax, and from 0.9 to 3.5 μg of theobromine per mg of wax, respectively.

2.1. Wax yields

The average yields of surface wax extracted from dried leaves of different plant populations are presented in Table 1. Considering all samples examined, the leaf wax content was ca 0.5% on average of dry leaf weight. No differences in the average wax content were found among the three populations sampled in February. Comparing different harvest times, the leaves of MS and PR populations exhibited more epicuticular wax in summer (February) than in spring (September and October respectively). On the other hand, higher yields of epicuticular wax were measured in leaves from the RS population in November, when compared to those from February.

Table 1
Leaf wax averages (% dry leaf weight) of plant populations collected from different states of Brazil in different months; different bold superscript letters indicate significant differences in the Student *t*-test to $\alpha = 0.05$[a]

State	Months	
	February	September
Mato Grosso do Sul	0.49 (±0.16)[a] (0.21–0.71)	0.38 (± 0.17)[b] (0.08–0.55)
	February	October
Paraná	0.47 (±0.04)[a] (0.41–0.54)	0.26 (± 0.12)[b] (0.14–0.46)
	February	November
Rio Grande do Sul	0.53 (± 0.09)[a] (0.41–0.64)	0.95 (± 0.30)[c] (0.55–1.42)

[a] The results are expressed as average (± standard deviation). Ranges are shown in parenthesis.

2.2. Methylxanthines in the epicuticular wax

TLC analysis of all wax samples indicated almost the same qualitative composition. While caffeine was clearly detected in all samples, quantitative differences among the samples were remarkable. Theobromine was detected in most samples, while theophylline was not detected (based on co-chromatography with authentic standards). Therefore all samples were submitted to HPLC analysis for caffeine and theobromine quantification. The results are summarized in Table 2, in which the average values for each population and the statistical conclusions are presented.

For the samples collected in February, the highest concentration of caffeine was noted for the population of the MS State, the northernmost State in this study, decreasing towards RS State, the southernmost one (Table 2). Concerning the theobromine contents in February, we observed the same tendency, but the populations were not statistically different. Comparing the caffeine contents of the spring and summer collections, we found statistical differences only for the PR population. In this case, two samples are clearly out of the range in October (data not shown). We have no explanation for this phenomenon as it can have many origins, e.g. the induction of secondary metabolism by insect or fungus attack. Such induced increase of caffeine content was reported for the stems of *Camellia sinensis* when attacked by fungus (Kumar et al., 1995).

2.3. Inner methylxanthine versus epicuticular methylxanthine content

In order to verify if the methylxanthine quantities observed in the epicuticular waxes could be correlated with the intracellular contents of these substances, we selected some samples from the February collection to analyse for residual methylxanthines in the ground leaves after removal of epicuticular wax by the dipping procedure. The correlation between concentrations of caffeine in epicuticular wax and in the extract of residual crushed leaves of these selected samples are showed in Fig. 1. We obtained a Spearmann rank correlation (Zar, 1999) equal to $r_s = 0.745$ between inner caffeine and epicuticular caffeine ($P < 0.05$). Correlation between inner theobromine and epicuticular theobromine, however, is not significant ($r_s = 0.418$, $P > 0.05$, data not shown).

Caffeine passes easily through biological membranes, presumably due to its dual, hydrophilic and lipophilic character (Baumann et al., 1995). In coffee, caffeine is probably present in the seed as a 1:1 complex with potassium chlorogenate, which is poorly soluble, thus

Table 2
Epicuticular caffeine and theobromine contents of plants collected in February, September, October and November of 1997.[a,b]

States	Caffeine		Theobromine	
	February	September	February	September
Mato Grosso do Sul	13.6±8.8[a]	8.7±7.6	1.5±1.0[a]	1.2±1.7
	(4.1–31.0)	(3.7–22.3)	(0.3–2.8)	(0.1–4.5)
Paraná	February	October	February	October
	8.2±6.3[ab]	54.7±44.2	1.6±0.7[a]	4.1±3.5
	(1.0–15.6)	(4.2–127.6)	(0.9–2.6)	(0.6–9.5)
Rio Grande do Sul	February	November	February	November
	1.7±1.9[b]	3.2±4.2	0.8±0.6[a]	1.0±1.0
	(0.2–5.5)	(0.1–9.5)	(0.0–1.7)	(0.0–2.9)

[a] The results are expressed as average ± standard deviation (μg/mg leaf wax dry weight); ranges are shown in parentheses.
[b] The populations are different based on Kruskall–Wallis test ($\alpha = 0.05$); different bold superscript letters indicate significant differences based on Nemenyi test to pairwise comparisons (only comparisons among populations and among data from February are shown).

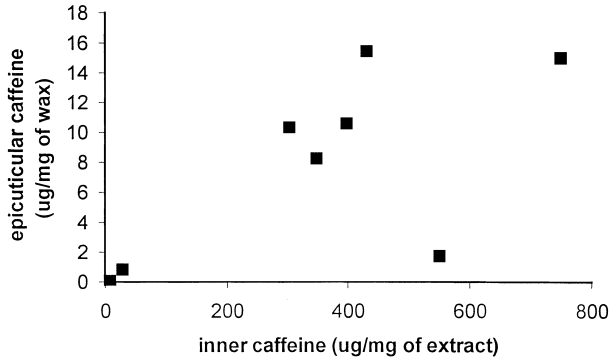

Fig. 1. Correlation between inner and epicuticular caffeine; Spearman rank correlation $r_s = 0.745$ ($P < 0.05$).

preventing it from moving freely within all tissues (Martin et al., 1987). Chlorogenic acid and other caffeoylquinic acids are phenols and these complexes are compartmentalized in the vacuoles (Baumann and Röhrig, 1989; Waldhauser and Baumann, 1996).

The observed correlation between inner and epicuticular caffeine in the *I. Paraguariensis* leaves may be explained by an equilibrium with phenolic compounds, e.g. an excess of caffeine not complexed in the vacuole may permeate to the cuticle. Alternatively, the methylxanthines might be secreted actively.

2.4. Possible roles of methylxanthines in epicuticular wax

The functions of epicuticular waxes include control of transpiration, reflection of light and regulation of plant surface microflora (Juniper and Jeffree, 1983). Investigation of other plant material has shown that considerable changes may occur in the composition of epicuticular wax during the course of leaf development (Tulloch, 1973; Niemann and Baas, 1985). Moreover, environmental conditions may affect the composition of the epicuticular wax (Giese, 1975; Shepherd et al., 1995; Rieley et al., 1995).

Although methylxanthines are frequently used as stimulants by humans, little is known about their natural function in plants. It is known, however, that many plants produce endogenous substances which can discourage insect feeding. These include specific toxins, compounds with pheromone-like activity, and bitter tasting repulsive substances (Nathanson, 1984). Phytochemicals are known to influence feeding behavior in herbivores. Particularly, caffeine is an antifeedant to larvae of *Callosobruchus maculatus*, a bruchid beetle (Janzen et al., 1977), it reduces fecundity and longevity of *Drosophila prosaltans* (Itoyama and Bicudo, 1992), and it increases susceptibility of *Manestra configurata* (Lepidoptera: Noctuidae) to *Bacillus thuringiensis* (Morris et al., 1994). This methylxanthine is also detected by the papillae of *Manduca sexta* when applied to leaves of tobacco plants and provokes an avoidance by these caterpillars, indication of a deterrent attribute (Glendinning, 1996). In tea plant stems, fungal attack can induce accumulation of caffeine, and plants with higher caffeine production are more resistant to the fungi (Kumar et al., 1995). These facts suggest that caffeine may have antifeedant functions in plants. Caffeine is one of the most bitter substances present in maté leaves (Taketa et al., 1998), and a bitter taste is an important feature in interactions between insects and plants.

The results presented here demonstrate the presence of small quantities of caffeine and theobromine in epicuticular wax deposits on the leaves of *I. paraguariensis*, although the major quantities of these methylxanthines are present inside the leaves. In this context, they could play a role in avoiding attack by phytophagous insects, a hypothesis that requires more detailed investigation.

3. Experimental

3.1. Plant material

Plants from three native populations were numbered in situ for further studies in genetics, physiology, morphology, ecological and chemical analysis. We analysed a sub-sample from each population. Plants were identified by the authors and voucher specimens were deposited at ICN (Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil). Only fully expanded leaves were collected, without herbivory damage. Leaves were first harvested in the middle of February (summer) when almost all leaves had ca 5 months of development and at spring time (September/October/November) of 1997.

Samples from the State of Mato Grosso do Sul (MS) were collected in February and September 1997 from seven individual plants. Samples from the State of Paraná (PR) were taken in February and October 1997 from six individual plants. Samples from the State of Rio Grande do Sul (RS) were taken in February and November 1997 from six individual plants.

3.2. Plant extraction

For quantitative analyses, whole leaves were dried at 40°C for 48 h. Dried leaves (1.5 g) were immersed in chloroform for 1 min to remove the wax layer. The extract was filtered and concentrated to dryness under reduced pressure to determine total surface wax. The residue was dissolved in MeOH–H_2O (25:75) prior to TLC and HPLC analysis.

To confirm the cuticular origin of the methylxanthines, the adaxial surfaces of three dried leaves were carefully scrapped with a blade, and the residues were analysed using the same chromatographic procedures.

3.3. Spectrophotometrical measurements

To analyse for the presence of chlorophylls in the wax extracts from dried and fresh leaves, aqueous Me_2CO (1:4, 10 ml) was added to 10 mg of the wax from three selected samples, and the UV/Vis spectrum from 200 to 700 nm was measured (Mackinney, 1941).

3.4. Analysis of inner leaf caffeine contents

Following dipping in chloroform, the leaves of 10 previously extracted samples were crushed and boiled for 10 min in a 20% sulphuric acid solution (Reginatto et al., 1999), then filtered. The filtrate, after being neutralized with 50% aqueous ammonium hydroxide was extracted (4×10 ml) with a chloroform–isopropanol mixture (3:1). The organic phases were combined, and concentrated, to dryness, to yield the inner methylxanthine fraction. This residue was submitted to HPLC analysis by dilution in the mobile phase.

3.5. TLC

All concentrated extracts were analysed on silica gel TLC using a mixture of $CHCl_3$–EtOH (95:5). The presence of methylxanthines was detected under UV by fluorescence quenching.

3.6. HPLC

A liquid chromatograph (WATERS, model 600E) with a Rheodyne injection valve fitted with a 20 μl injection loop, a variable ultraviolet detector (WATERS, model 486), and an integrator (WATERS, model 747) were used. Chromatographic separation was accomplished using a column NovaPak RP8 (3.9×150 mm, 4.8 μm), equipped with a RP18 precolumn (3.0× 3.9 mm, 50 μm). An isocratic system (MeOH–H_2O 25:75) was used as mobile phase at a flow rate of 1.0 ml/min at ambient temperature (25 to 30°C) (Petermann and Baumann, 1983). Detection was performed at 280 nm at 0.05 AUFS. Analytical solutions were injected in triplicate (CV < 5%) and peak areas were compared with those obtained for a standard curve.

3.7. Calibration curve for HPLC

Suitable amounts of caffeine and theobromine standard were dissolved in MeOH:H_2O (4:6). The standard solutions were injected in triplicate with peak areas measured. Linearity was evaluated by linear regression, and the precision and accuracy were determined by coefficient of variation (CV less than 3%). Their correlation coefficients were $r = 0.998$ for caffeine and $r = 0.996$ for theobromine, respectively.

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