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Caffeine in *Citrus* flowers

Josef A. Kretschmar, Thomas W. Baumann*

Institute of Plant Biology, University of Zürich, Zollikerstr. 107, 8008 Zürich, Switzerland

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Abstract

The allocation of purine alkaloids within citrus flowers was studied and found to be linked to anthesis, with 99% of the total flower caffeine confined to the androecium. The main alkaloid is caffeine accompanied by considerable (up to 30% of caffeine) concentrations of theophylline. In the anther, these purine alkaloids reach altogether a concentration of 0.9% dry wt which is close to the caffeine content of the Arabica coffee bean. The pollen alkaloid concentration is in the same range. Much lower but still marked concentrations were found in the nectar. A considerable breakdown of alkaloids during honey production is assumed. The biological significance of this particular secondary compound allocation as well as possible effects on the key pollinator, the honey-bee, are discussed. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Citrus; Rutaceae; Caffeine; Theophylline; Purine alkaloids; Flower development; Nectar; Pollen; *Apis mellifera*; Honey-bee

1. Introduction

The compilations often found in literature (e.g. Willaman & Schubert, 1961), regarding the occurrence of caffeine may give the impression that this purine alkaloid is shared by a large number of genera. However, if we rely exclusively on data re-examined by advanced analytical techniques, we arrive at the conclusion that during evolution, 'invention' of caffeine, i.e. the purine alkaloid pathway, was a relatively rare event meaning that out of ca. 10,000 angiosperm genera only seven developed, to our present knowledge, this phytochemical feature, namely *Coffea, Camellia, Theobroma, Herrania, Cola, Ilex,* and *Paullinia*. Therefore, a report by Stewart in 1985 (Stewart, 1985) was most exciting because it claimed the presence of caffeine, even though in the very low range of 6 and 50 ppm (31 and 258 nmol g^{-1} fr. wt) in leaves and flowers, respectively, of several *Citrus* species. His findings were confirmed by an Italian group (Trova, Cossa, & Gandolfo, 1994) which detected caffeine in dried citrus flowers (237 to 856 nmol g^{-1} dry wt) commercially available for preparing a tea. Moreover, the authors found caffeine for the first time also in honeys originating from the activities of honey-bees (*Apis mellifera*) visiting either frequently (unifloral honey) or sporadically ('millefiori' honey) the flowers in orange plantations. Caffeine in these honeys ranged from 2.6 to 52 nmol g^{-1}. Later, the analyses were extended to various other citrus honeys (Defilippi, Piancone, & Tibaldi, 1995; Vacca & Fenu, 1996) and possible source-flowers (Vacca, Agabbio, & Fenu, 1997) with the aim to establish a measure of quality control. However, a correlation could not yet be established. In all these studies, neither single flower organs nor nectar and pollen were examined.

In a preliminary investigation on coffee (Kretschmar & Baumann, 1998) we recognised that flower caffeine was relatively abundant (ca. 3200 nmol g^{-1} fr. wt) and preferentially, even though not very markedly, allocated in the androecium together with other purine alkaloids. Therefore, we analysed the within-the-flower distribution of these alkaloids in citruses (including *Poncirus*) considered ideal to demonstrate organ-specific allocation because of the low average content. Indeed, among the flower organs analysed the androecium had by far the highest concentration of alkaloids,

* Corresponding author. Fax: +41-1-634-8204.
E-mail address: twb@botinst.unizh.ch (T.W. Baumann)

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Table 1
Purine alkaloid content (nmol g^{-1} fr. wt) in flowers, nectars and honeys of *Citrus* spp. $n = 3$–10 (flowering units); n.d. = not detectable

	Caffeine	Theobromine	Theophylline	Paraxanthine
Flower development (*C. limon*)				
Small bud (148 mg; $n = 5$)	n.d.	n.d.	6 ± 1[a]	n.d.
Large bud (840 mg; $n = 3$)	166 ± 2[a]	2	52 ± 5	1
Full anthesis (938 mg; $n = 3$)	318 ± 3	1	27 ± 1	1
Stamens				
C. paradisi	$3'233 \pm 42$	28	173 ± 2	22
C. maxima	$1'110 \pm 13$	13	305 ± 4	5
P. trifoliata	807 ± 11	5	56 ± 1	5
C. limon	$1'415 \pm 18$	67	115 ± 2	8
Filament				
C. paradisi	$1'917 \pm 29$	18	139 ± 2	15
C. maxima	850 ± 11	8	12 ± 1	4
Anther				
C. paradisi	$8'551 \pm 169$	26	$1'491 \pm 29$	32
C. maxima	$7'900 \pm 119$	18	$2'753 \pm 41$	20
Pollen				
C. medica	$6'857 \pm 321$	n.d.	$1'921 \pm 90$	n.d.
Nectar (nmol ml^{-1})				
C. paradisi	487 ± 15	22	55 ± 2	12
C. maxima	91 ± 5	10	3 ± 1	3
C. limon	60 ± 2	n.d.	n.d.	n.d.
Honey (nmol g^{-1})				
Sicilia	31 ± 1	6	3 ± 1	3
California	2 ± 1	1	n.d.	n.d.

[a] Mean value (±the estimated experimental error, see Section 3).

with a main allocation in the anther and pollen at a very high level. Moderate, but still marked purine alkaloid concentrations were found in the nectar. Since caffeine is known to be insecticidal, the results were discussed also in the context of intoxication of honeybees.

2. Results and discussion

In a first approach the purine alkaloid content of entire flowers of *Citrus paradisi* and *C. maxima* was determined. Caffeine was the main alkaloid (21 and 77 nmol g^{-1} fr. wt, respectively) accompanied by theophylline (4 and 15 nmol g^{-1} fr. wt, respectively), while theobromine and paraxanthine occurred in traces only. Similarly, commercially available orange flower tea (*C. sinensis*) contained caffeine (182 nmol g^{-1} dry wt) and theophylline (46 nmol g^{-1} dry. wt). These caffeine values are in the range as found before (Stewart, 1985; Trova et al., 1994), but until now the presence of theophylline and other dimethylxanthines in *Citrus* has not been reported. Theophylline is a trace compound in the 'classical' caffeine plants consumed by the human. In some of the citrus flower tissues it showed considerable accumulation (Table 1).

The chemical analysis of flower development (*C. limon*) revealed, that the small, round-shaped flower bud was virtually alkaloid-free, with only a trace of theophylline, whereas the elongated bud shortly before anthesis contained well-measurable concentrations of both caffeine and theophylline, accompanied by little theobromine and paraxanthine. During anthesis the caffeine content increased by a factor of almost 2 (Table 1). Similarly (not shown), two stages (145 and 216 mg fr. wt) of flower buds of the closely related *Poncirus trifoliata* were purine alkaloid-free, whereas the freshly opened flower (375 mg fr. wt) had an overall caffeine concentration of 109 nmol g^{-1} fr. wt. A similar increase was observed during anthesis of *C. paradisi*. Therefore we may conclude that anthesis in citruses is coordinated with a rapid allocation of caffeine.

Then, the flowers of *C. paradisi*, *C. maxima*,, *C. limon* and *P. trifoliata* were separated into petals, pistils and stamens and analysed (the tiny green sepals and the flower base were found in preliminary experiments to be virtually alkaloid-free). Both, petals and pistils contained very small concentrations (mostly in the range of 2 to 10 nmol g^{-1} fr. wt, respectively) of caffeine, theobromine, or theophylline (not shown). However, the stamens (Table 1) contained the highest concentrations of caffeine and theophylline and were the exclusive site of flower paraxanthine which was hardly detectable in the related entire flowers because of dilution. The separate analysis of filament and anther revealed maximum alkaloid concentrations in the latter exceeding altogether the concentration of

Table 2
Purine alkaloid allocation within the flower of *Citrus lemon*. The flower base is virtually alkaloid-free

Organ		Caffeine			ΣPurine alkaloids		
	fr. wt (mg)	nmol g^{-1} fr. wt	amount (nmol)	% of total	nmol·g^{-1} fr. wt	Amount (nmol)	% of total
Petals (4–5)	509	3.9	2.0	0.8	15.3	7.8	2.5
Stamens (31–32)	183	1415.0	258.9	99.1	1604.5	293.6	96.5
Pistil (1)	103	2.5	0.3	0.1	29.5	3.0	1.0
Flower base (1)	119	n.d.	0	–	–	–	–
Total	914	285.8[a]	261.2	100	333.0[a]	304.4	100

[a] Overall concentration of the entire flower. In parenthesis: number of organs per one flower. For nectar see text.

10,000 nmol (=10 μmol) per g fr. wt. If related to dry wt, it results a value of ca. 0.7–0.8% caffeine and 0.9% total purine alkaloid in the anther. Hence, the purine alkaloid concentration in the anther is close to that in the Arabica coffee bean (1.2%, almost exclusively caffeine)!

Finally, pollen of (due to the absence of blooming of the other species) *Citrus medica* (citron) was analysed. This single analysis revealed a high purine alkaloid concentration (altogether almost 8800 nmol $g^{-1)}$ in the microspores (Table 1), which is in the range of that found in the anther of the closely related species. We cannot yet decide whether the anther alkaloid amount is completely confined to the pollen, or whether the anther wall contains alkaloid at concentrations similar to pollen. Also, we have not yet studied the localisation of purine alkaloids within the pollen grain. At the present we can only speculate about the significance of this conspicuous allocation. Besides protection against (unknown) pollen predators, purine alkaloids are well-studied defence compounds (reviewed in Harborne (1993)), the cytokinin-like effect of caffeine (Vitória & Mazzafera, 1997) may play a role during pollination and seed set in citrus (Hernandez Minana & Primo Millo, 1990).

Blossom honey essentially consists of nectar concentrated by the activities of specialised bees in the hive. The pollen present in the honey is quantitatively negligible but a valuable nectar 'contaminant' which is of help to trace the source flowers. It has been reported that on average 64% of the pollen found in unifloral citrus honeys is citrus pollen (White & Bryant, 1996) meaning that roughly two third of the honey originate from citrus nectar. Since the latter was shown to contain caffeine in the range of ca. 60 to 490 nmol ml^{-1} (Table 1) and undergoes a concentration process by a factor of ca. 2 during honey production, one should expect a much higher caffeine concentration in citrus honey than found in the present (2 and 31 nmol g^{-1}; Table 1) or in earlier (2.5–50 nmol g^{-1} (Trova et al., 1994)) studies. It can be calculated that about 95% of the nectar caffeine is removed or degraded by a still unknown mechanism. However, even though the nectar was sampled with caution we cannot rule out contamination by pollen.

In order to obtain an estimate of chemical defence allocation, the number and weight of the individual flower organs were determined and the alkaloid distribution calculated as exemplified for *C. lemon* in Table 2. In summary, one citrus flower contains about 260 nmol (ca. 50 μg) caffeine and 300 nmol total purine alkaloid, 99 and 96.6%, respectively, allocated to the androecium! The amount of alkaloids found in the nectar (ca. 20 μl) at the moment of flower dissection is negligible in the case of *C. lemon* (ca. 1 nmol; caffeine only), but was distinctly higher in *C. paradisi* (ca. 10 nmol) and may be considerable if extrapolated to the entire period of flowering. However, nectar secretion was not studied in detail. Diurnal fluctuations are to be expected and may account for some of the differences in pattern and concentrations of nectar purine alkaloids listed in Table 1.

Finally, we should mention that orange flower tea (Aurantii flos) is pharmaceutically recommended for treatment of sleeplessness [ÖAB, Ph. Helv. VI]. The amount of caffeine ingested by consumption of such a calming tea is below 100 μg, a dose present in homeopathic coffee preparations used against insomnia (HAB, (Baumann & Seitz, 1992)).

2.1. Caffeine and honey-bees

High concentrations of secondary compounds have been detected in microspores of both wind- (e.g. Meurer, Wray, Wiermann, & Strack, 1988) and insect- (reviewed in Detzel & Wink, 1993) pollinated flowers. In the latter case the question of intoxication of the pollinators arises. Detzel and Wink (Detzel & Wink, 1993) tested a large number of such allelochemicals on the feeding behaviour of honey-bees. Caffeine was found to act as a deterrent and its toxicity was comparatively low under no-choice conditions (LD_{50} at 0.2 %). In an earlier study (Ishay & Paniry, 1979), honey-bees, offered free choice of either the sugar solution alone or the sugar solution with the caffeine, similarly preferred the sugar solution. The concentration of the

caffeine solution (ca. 250 μM) happened to be in the range of citrus nectars (Table 1). It was readily accepted under no-choice conditions. After five days, a 300–500 % boost in oviposition by the (young) queen, an enhanced activity of the bees outside the hive, and an improved defence by bees against hornets at the hive entrance was observed. In contrast, hornets also fed with caffeine ceased foraging in the field and also failed to clear the dead (poisoned by caffeine) larvae out of the nest. On the cellular level, caffeine was shown to influence the cytosolic Ca^{2+} concentration in various organisms including honey-bees, where the caffeine-sensitive Ca^{2+} release from the endoplasmic reticulum in photoreceptors has been studied (Walz, Baumann, & Ciriacy-Wantrup, 1994).

Caffeine and related substances are known not only to exert insecticidal activity but also to synergize the effects of pesticides (Nathanson, 1984). On the one side it appears that the toxicity of caffeine in the honey-bee larvae, which to our knowledge do not suffer from the caffeine-rich pollen in citrus orchards, is relatively low. On the other side, however, one should in future evaluate bee toxicity of pesticides also in the presence of purine alkaloids, in order to account for the above-mentioned synergistic effect which may occur not only in citrus but also in coffee and tea plantations.

Finally, we should mention that recently citrus pollen was found to be toxic to the predatory mite *Euseius mesembrinus* (Yue, Childers, & Fouly, 1994) which is a facultative pollen feeder widely distributed in citrus plantations. If toxicity is due to the presence of purine alkaloids, one may suggest that mites, in contrast to honey-bees, are very susceptible to purine alkaloids, a situation which could be advantageous in the chemical control of the ectoparasitic mite *Varroa jacobsoni* associated with the honey-bee.

3. Experimental

3.1. Plant material

Citrus plants (*C. paradisi* Macf., grapefruit; *C. maxima* (Burm.) Merr., shaddock; *C. limon* (L.) Burm.f., lemon, and *C. medica* L., citron) were grown in the greenhouse. The trifoliate orange plant (*P. trifoliata* Raf.) was kept outdoors in the institute garden. Tea of orange flowers (*C. sinensis* (L.) Pers. was bought in a local store (Coop, Switzerland) as well as orange flower honeys of Sicilian (Globus, Switzerland) and Californian (Biorex AG, Ebnat-Kappel, Switzerland) origin. Nectar was sampled using a glass (hematokrit) capillary.

3.2. Purine alkaloid extraction

Fresh entire flowers ($n = 3$ to 10) or the related flower parts were pooled and extracted in 0.1 N HCl (1 ml per 50 to 150 mg fr. wt) at 50° for 30 min by sonication. One ml of the extract was applied onto a Kieselgur column (Extrelut1, Merck). Essential oil was removed by 12 ml hexane and thereafter purine alkaloids were eluted with 12 ml CH_2Cl_2. The eluate was dried under a stream of N_2 and the residue dissolved, for HPLC, in 1 ml H_2O. Orange flower tea was extracted likewise (1 ml 0.1 N HCl per 250 mg, without further drying the flowers) as well as honey (1 g), which was diluted with 0.1 N HCl (2 ml) prior to application onto the Kieselgur column. Nectar was mixed (1:1) with 8% MeOH and directly injected. Pollen was collected and processed as follows: stamens were harvested, dried at room temp. and transferred into a pre-weighed Eppendorf tube, which then was vortexed at high speed to spin off the pollen. The stamens were removed and the weight of the pollen was determined by weighing the tube again (Mettler AE 240). The pollen (2.84 mg) was suspended in 300 μl 0.1 N HCl. After 3 h at room temp. a 30-min-sonication at 40° followed. Thereafter, the suspension was filtered through a membrane filter and directly injected into HPLC. No attempt was made to determine the dry weight of the pollen.

3.3. HPLC separation of purine alkaloids

HPLC separation of purine alkaloids was carried out on a Nucleosil-100-5 C18 HD column; precolumn 4 × 8 mm; ChromCart, Macherey-Nagel). Parameters were controlled by a Hewlett-Packard liquid chromatograph equipped with a diode array detector set at 272 nm. Chromatography was carried out using the following gradient: 0–4 min with 0–7.5% MeOH and 0–2.5% AcN, 4–20 min with 7.5% MeOH and 2.5% AcN. The R_t's (min) were 7.1, 9.1, 9.6 and 16.0 for theobromine, paraxanthine, theophylline, and caffeine, respectively. The flow rate was 1.1 ml min^1 and injection vol 150 μl. Peak identification was achieved by comparing UV spectrum (library established under separating conditions) and retention time of authentic standards.

3.4. Calculation of the mean value and the 'systematic experimental error'

Since in this study we did not aim at showing the variability of purine alkaloids and their concentrations among flowers from one or several plants, the flowers were randomly collected and pooled (with n generally = 10, in a few cases of shortage lower but never less than 3). The obtained mean value has an exper-

imental error which was calculated by considering the accuracy of the fresh weight determination (±1 mg) and the quantitation by HPLC (±1%).

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References

Baumann, T. W., & Seitz, R. (1992). In R. Hänsel, K. Keller, H. Rimpler, & G. Schneider, (p. 926). In *Hagers Handbuch der Pharmazeutischen Praxis*, *Vol. 4*. Berlin: Springer-Verlag.

Defilippi, A., Piancone, G., & Tibaldi, G. (1995). *Industrie Alimentari*, *34*, 6.

Detzel, A., & Wink, M. (1993). *Chemoecology*, *4*, 8.

Harborne, J. B. (1993). *Ecological biochemistry*. London: Academic Press.

Hernandez Minana, F. M., & Primo Millo, E. (1990). *Journal of Horticultural Science*, *65*, 595.

Ishay, J. S., & Paniry, V. A. (1979). *Psychopharmacology*, *65*, 299.

Kretschmar, J. A., & Baumann, T. W. (1998). *Future trends in phytochemistry, PSE – symposium*. The Netherlands: Rolduc.

Meurer, B., Wray, V., Wiermann, R., & Strack, D. (1988). *Phytochemistry*, *27*, 839.

Nathanson, J. A. (1984). *Science*, *226*, 184.

Stewart, I. (1985). *Journal of Agricultural and Food Chemistry*, *33*, 1163.

Trova, C., Cossa, G., & Gandolfo, G. (1994). *Industrie Alimentari*, *33*, 403.

Vacca, V., & Fenu, P. (1996). *Industrie Alimentari*, *35*, 368.

Vacca, V., Agabbio, M., & Fenu, P. (1997). *Industrie Alimentari*, *36*, 611.

Vitória, A. P., & Mazzafera, P. (1997). *Biologia Plantarum*, *40*, 329.

Walz, B., Baumann, O., & Ciriacy-Wantrup, E. V. (1995). *Journal of General Physiology*, *105*, 537.

Willaman, J. J., & Schubert, B. G. (1961). *Agric. Res. Serv. US Dept. Agric. Techn. Bull.*, *1234*, 1.

White, J. W., & Bryant, V. M. J. (1996). *Journal of Agricultural and Food Chemistry*, *44*, 3423.

Yue, B., Childers, C. C., & Fouly, A. H. (1994). *International Journal of Acarology*, *20*, 103.