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FORMATION AND INTRACELLULAR ACCUMULATION OF CAFFEINE AND CHLOROGENIC ACID IN SUSPENSION CULTURES OF *COFFEA ARABICA*

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Key Word Index—*Coffea arabica*; Rubiaceae; tissue culture, caffeine; chlorogenic acid, compartmentation

Abstract—Suspension cultures of *Coffea arabica* produce considerable amounts of both caffeine and chlorogenic acid (5-CGA). Whereas most of the caffeine is synthesized, as reported earlier, at the end of the culture cycle, 5-CGA exhibits a biphasic formation curve with a first maximum at the beginning and a second at the end of the growth phase. In contrast to 5-CGA, which is exclusively located within the cells, caffeine is released into the medium. However, it could be shown that caffeine is also accumulated intracellularly to a certain extent and that this is correlated with the 5-CGA concentration in the cells Vital staining with methylene blue indicate that 5-CGA is compartmented predominantly in the vacuole.

INTRODUCTION

Suspension cultures of *Coffea arabica* produce the purine alkaloid caffeine in concentrations nearly corresponding to those found in the plant [1]. When cultured in permanent darkness, cells do not accumulate caffeine, but excrete it into the medium This equal partition of caffeine and other purine alkaloids between cell water and medium [2, 3] makes coffee especially suitable as a model cell line for bioreactor engineering studies [4] as secondary product formation can be monitored easily by direct analysis of the medium. The phenomenon of an almost free exchange raises some questions regarding cellular compartmentation of caffeine. From washout experiments with coffee leaf discs it is known that only a fraction of caffeine can be removed and it was assumed [5] that the residual caffeine is intracellularly complexed by chlorogenic acid, the 5-*O*-caffeoyl ester of quinic acid (5-CGA), having been described as forming not only in crystals [6, 7] but also in aqueous solution a 1:1 complex with this and related methylxanthines [8–10] In order to assess the physiological role of this complexation reaction we followed both chlorogenic acid and caffeine formation in suspension cultures, and consequently found evidence that intracellular caffeine accumulation, distinctly pronounced in the photoperiod, may be due to the presence of chlorogenic acid in the vacuole

RESULTS AND DISCUSSION

Production kinetics

Compared to the standard conditions (dark regime) the cell lines kept under the photoperiod produce considerably more of both caffeine and 5-CGA. The extent of stimulation depends on the specific productivity of the cell type (Table 1). A low-producing cell line is stimulated to a greater extent than a high-producing one. The maximal factor of stimulation for either compound is *ca* 5 However, the kinetics are quite different. As reported earlier [1] the accumulation of caffeine follows a sigmoidal curve signifying that most of it is produced in the late growth phase. In contrast, it has been shown for a selected cell line cultured in the dark that 5-CGA is accumulated biphasically [11] In our studies we

Table 1 Production of caffeine and 5-CGA by two different cell lines grown in darkness or under photoperiod

		Caffeine		5-CGA	
		mg/l	(% dry wt)	mg/l	(% dry wt)
Line 1/1/100	darkness	0.5	(0.004)	28	(0.2)
(low-producing)	photoperiod	2.5	(0.02)	142	(1.2)
Line 1/1/12f	darkness	96	(0.7)	155	(1.2)
(high-producing)	photoperiod	128	(1.0)	480	(3.8)

Values taken at the time of highest 5-CGA accumulation, i.e after six days (38% of cultivation cycle) for cell line 1/1/100 and (43% of cultivation cycle) for line 1/1/12f

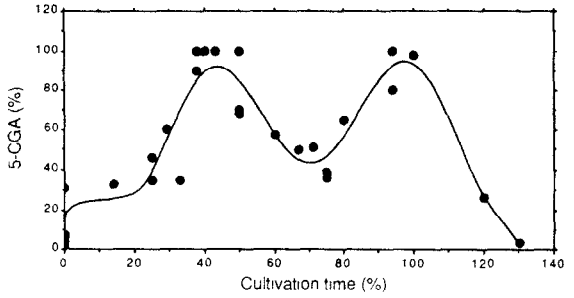

Fig. 1 Relative changes of 5-CGA contents in the course of a culture cycle Combined results from cell line 1/1/100, 1/1/12 and 1/1/12f Since the length of the culture cycle differs among the cell lines (16, 11 and 14 days respectively) the data were standardized so that 100% cultivation time corresponds to the end of the growth phase coinciding with maximum caffeine content of the culture The highest 5-CGA level reached in each experiment was set as 100%

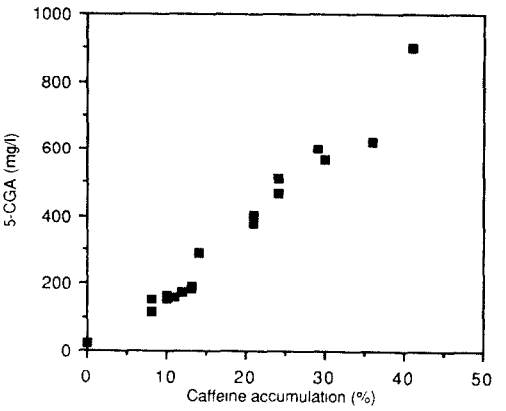

Fig 2 Intracellular caffeine accumulation as a function of the 5-CGA content of the cell culture Line 1 1/12f after 7 days of cultivation under different conditions darkness, photoperiod without and with two combined stress factors (7 5 g/l NaCl, 148 g/l PEG 6000). The '% caffeine accumulation' represents the difference between the total caffeine contents of the culture ('medium & cell probe') and of the medium ('medium probe'), respectively The latter was set as 100%

made the same observations with the various cell lines investigated under different conditions. A compilation of the results yields a 5-CGA formation curve presented in Fig 1 with two maxima, one at the beginning and the other at the end of the growth phase This biphasic production which is more pronounced under photoperiod than in the dark may reflect the fluctuations of 5-CGA contents found in intact plants under various physiological and developmental conditions [12, 13] In cultivated cells the first production phase taking place before the onset of rapid cell division may be attributed to the role of 5-CGA in the temporary storage of carbon, since up to almost 10% (dry wt) are accumulated and subsequently metabolized A four-fold increase in the supply of sucrose (120 instead of 30 g/l) causes a doubling of the production of 5-CGA Not only the first but especially the second phase of 5-CGA formation at the end of the growth cycle may be seen in the light of a physiological function of 5-CGA in the compartmentation of caffeine

Intracellular accumulation of caffeine

Caffeine determinations in the medium before and after destroying the cells (see Experimental) allows the estimation of intracellular purine alkaloid. As no chlorogenic acids could be detected in the medium at any time of cultivation and since cells in the die-off-phase are devoid of 5-CGA, we conclude that 5-CGA is localized and catabolized intracellularly. In Fig 2 the amount of 5-CGA is plotted against the relative measure of cellular caffeine accumulation obtained from the difference between 'medium & cell probe' and 'medium probe' analyses It appears that a close correlation exists between intracellular caffeine and the 5-CGA content, in other words, increasing intracellular 5-CGA concentrations are associated with increasing cellular compartmentation of caffeine presumably due to the formation of the caffeine-chlorogenic acid complex. At the highest level found for 5-CGA (900 mg/l), calculation of caffeine concentration in terms of mg/l cell volume versus mg/l medium yields an accumulation factor in the cells of 4 1

Localization of caffeine–chlorogenic acid complex

In order to locate the above-mentioned complex subcellularly we attempted to stain 5-CGA with methylene blue at low concentration, this having been described as associating with spherical intravacuolar formations containing chlorogenic acids [14] Wernicke and Kohlenbach [15] suppose that these microdroplets originate from a supersaturation of chlorogenic acids in the vacuole In all coffee cell lines studied some of the cells show dark blue stained microdroplets which tend to stick together and finally form flaky precipitates Examination of an additional mixotrophic cell line continuously growing under photoperiod revealed densely stained inclusions in almost all of the cells, some of them resembling the precipitates described above, but the majority of the cells possessing one large intravacuolar droplet When cultures stained with methylene blue are extracted and analysed for 5-CGA and methylene blue, it appears that the relative contents of these compounds are positively correlated with each other (Fig. 3). Although 5-CGA has been shown to have a relatively low affinity for methylene blue in the test tube [16], we must assume that in the vacuole this depside associates with the dye-stuff to form the precipitates described, since no other phenolic compounds could be detected, except 3,4-dicaffeoylquinic acid which may be formed sporadically Moreover it is to be expected that *in vivo* other constituents of the cell sap such as proteins or inorganic ions will contribute to the precipitation process The elucidation of the physiological significance of 5-CGA in subcellular caffeine compartmentation will require studies employing protoplast and vacuole isolation.

EXPERIMENTAL

Cell lines Three lines derived from orthotropic internodes of *C arabica* var. *caturra* were established according to ref [2] They differ in size, colour and consistency of the aggregates Line

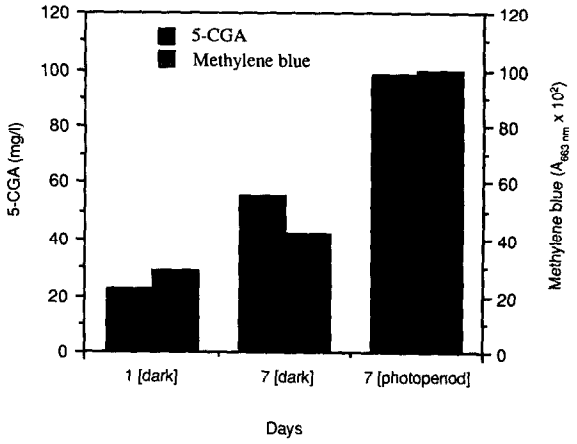

Fig. 3 5-CGA and methylene blue content. Cells of the line 1/1/12f were either at time zero or after a cultivation time of six days (darkness and photoperiod) stained by addition of methylene blue to a final concentration of 0.001%. After 24 hr under the same conditions the cells were harvested for analysis

1/1/100 has light-coloured, small and soft aggregates (0.2–1.0 mm) Line 1/1/12 is characterized by light-brown, hard aggregates of large size distribution (0.2–10.0 mm), whereas 1/1/12f differs from 1/1/12 by a narrow size distribution (1.0–4.0 mm) of the aggregates

Conditions of maintaining cell lines These were cultivated in a Murashige and Skoog medium, slightly modified as described earlier [2] and subcultured (6–9 g fr. wt and 30–40 ml medium) every 2 weeks (1/1/12f one week) Cultures in 100 ml Erlenmeyer flasks were kept in the dark at 27° on a gyratory shaker with 90 rpm.

Experimental conditions Cells (2.5 g fr wt) were suspended in 14 ml medium in a 50 ml Erlenmeyer flask covered by Al foil. They were either shaken in the dark, i.e. standard conditions (see above) or in a photoperiod of 13 hr light 11 hr dark in a growth chamber with a radiation of 490 μmol/m^{2}sec at 27°.

Growth measurement It has been shown [17] that the decrease of the conductivity of the medium is proportional to the cell dry wt increase The correlation factor was determined for each cell line by harvesting cultures at intervals during the cultivation cycle. Cells were dried to constant wt at 60° and, after filtration (Acro LC 13, 0.2 μm, Gelman Sciences), the conductivity of the medium was measured at 20° 2 ml of medium were taken for this purpose prior to analyses of secondary compounds

Extraction of caffeine and CGA One ml of the medium ('medium probe') was filtered through a 0.2 μm filter and used directly for HPLC analysis Then the rest of the culture was acidified by addition of 0.6 ml conc HCl and sonicated at 28–30°. The resulting suspension was filtered ('medium & cell probe') and subjected to HPLC

HPLC was carried out using a diode array detector and a HP ODS-Hypersil reversed phase column (100 × 2.1 mm, 5 μm particle size) The flow rate was 0.4 ml/min and the UV detector was set at 271 and 325 nm for quantitation of caffeine and CGA, respectively. Authentic standards were used for identification and on-line UV spectra between 220 and 400 nm. Samples of 25 μl were injected and chromatographed using gradient elution [18] Solvent A was 1% THF in 0.025 M citric acid monohydrate (pH 2.5) and solvent B was 1% THF in MeOH. The gradient employed was 2–10% B in A over 6 min and 10–65% B in A over 10 min. The R_ts (min) were 2.4, 6.3 and 9.7 for theobromine, caffeine and 5-CGA, respectively

Staining with methylene blue [14, 15] After 1 and 7 days of cultivation in the dark or under photoperiod, cell suspensions (line 1/1/12f) were fed with a 1% sterile-filtered soln of methylene blue to a final concn of 0.001% and maintained under the corresponding conditions for 24 hr. Intracellularly accumulated methylene blue was measured as follows Cells were sepd from the medium and rinsed thoroughly with dist H_2O Then H_2O was added to a final vol of 5 ml and, after acidification with 0.18 ml conc HCl, the suspension was sonicated The supernatants showed the typical spectrum of methylene blue with A_{max} at 663 nm which served as a relative measure of dye accumulation 5-CGA was determined separately in methylene blue treated controls

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