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PURINE ALKALOID FORMATION IN BUDS AND DEVELOPING LEAFLETS OF *COFFEA ARABICA*: EXPRESSION OF AN OPTIMAL DEFENCE STRATEGY?

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Key Word Index—*Coffea arabica*; Rubiaceae; coffee; developmental physiology; chemical defence; alkaloids; theobromine; caffeine.

Abstract—In buds and emergent leaflets of *Coffea arabica* formation of the purine alkaloids caffeine and theobromine was studied with the aim of characterizing the chemical defence strategy of a tissue with a high risk of predation. As long as the leaflets are fully covered by a resin layer and by two stipules, their alkaloid content varies between 1 and 3% dry wt. With leaflet emergence, the alkaloid formation increases and the variation decreases. Maximum content of about 4% is reached when the leaflets are fully open. In the subsequent developmental period alkaloid content decreases. A comparison between the investment in alkaloid formation with that in primary metabolic processes demonstrates that chemical defence is costly: influx of carbon atoms into caffeine is 15% of that into respiration. A defence strategy which is based on an antagonism between mechanical and chemical protection is discussed.

INTRODUCTION

In the debate concerning the significance of secondary metabolites the theory that they protect the producing plant against physical [1] and biotic [1, 2] environment has gained increasing prominence. Based on principles of evolution, general strategies for optimal chemical defence against predation were postulated [3, 4]. According to one of these strategies plants are expected to accumulate protective secondary compounds in a tissue in direct proportion to the risk of predation of that tissue [3]. Therefore tissues with a high dietary value (seeds, buds, young leaves) have a particularly high risk of predation.

We examined this hypothesis with regard to the formation of the purine alkaloids theobromine (3,7-dimethylxanthine) and caffeine (1,3,7-trimethylxanthine) in *Coffea arabica* L. Caffeine is known to have a toxic effect on insects and fungi at concentrations found in plants. Larvae of the tobacco hornworm (*Manduca sexta*) were killed when fed by a nutrient medium supplemented with 0.3% caffeine. At lower concentrations it reduces weight gain of the larvae. The effect is mainly due to the inhibition of the phosphodiesterase activity and to the concomitant increase of the intracellular cyclic AMP [5]. In *Callosobruchus chinensis* (L.) caffeine causes nearly 100% sterility at a concentration of 1.5% [6]. The fungitoxic effect was demonstrated on a number of *Aspergillus* and *Penicillium* species [7], and on four species of Saprolegniaceae [8]. In these cases a dose-dependent growth reduction was observed at concentrations between 0 and 0.4%, and 0 and 1.0% respectively.

Several reports demonstrate that purine alkaloid accumulation is generally in accordance with the optimal defence strategies. In cell cultures of *Coffea arabica* a considerable stimulation of caffeine production was achieved by the application of stress [9]. An investigation of eight different species [10] showed that the relative amount of theobromine and caffeine declines with ageing of the leaves. In *Coffea arabica* the speed of formation of caffeine decreases by a factor of more than a hundred during leaf development [11]. In the present paper we studied purine alkaloid formation in buds and emergent leaflets since during this very early period of leaf development chemical defence should be highly apparent.

RESULTS

Time course of alkaloid accumulation

In the budding process four developmental stages of leaflets were designated in which different requirements of chemical defence were expected (Fig. 1): stage B1, the leaflets are fully covered by a resin layer and by two stipules; stage B2, the leaflets have broken through the resin layer and the stipules; stage B3, the leaflets lie fully open (from stage B1 to B3 the leaflets are light green coloured and very fragile); and stage B4, area, lamina rigidity and intensity of greenness of the leaflets are increased, elongation of the corresponding internode has started.

The developmental time from stage B1 to B4 takes about 20 days. For older branches a delay in development of up to 30 days may occur between stage B1 and B2. In the present investigation, however, only buds with a continuous development were used.

Alkaloid accumulation is summarized in Fig. 2. The relative amount of theobromine and caffeine increases during budding from 2% upto a maximum of 4% dry wt at stage B3. From stage B3 to B4 theobromine content decreases by a factor of nearly three whereas caffeine content drops only slightly. At stage B1 considerable variation of alkaloid content was observed (Fig. 3). Coefficient of variation (standard deviation in % of the

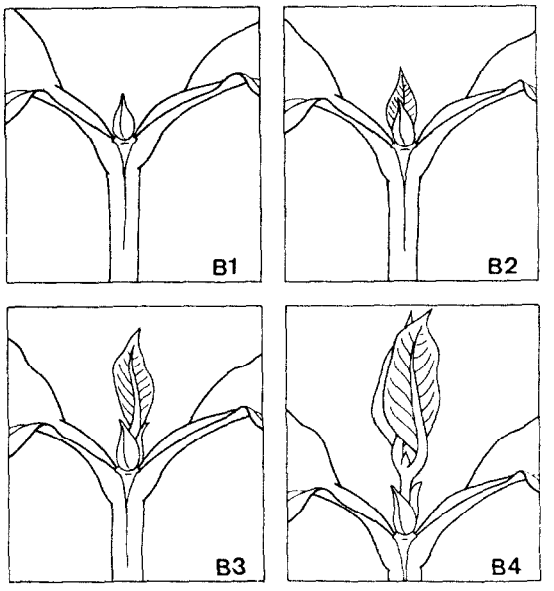

Fig. 1. Leaflets at the four developmental stages investigated.

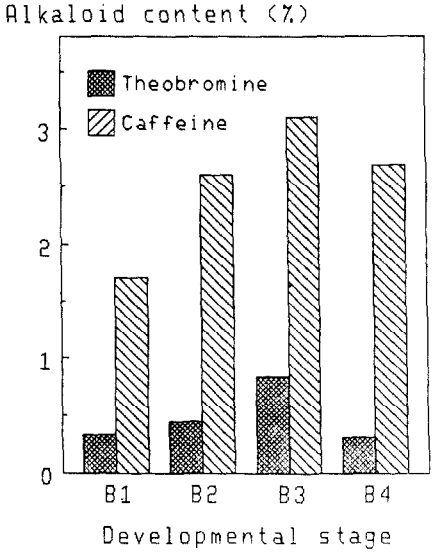

Fig. 2. Caffeine and theobromine content in leaflets during budding. Values shown are means of seven (B1), two (B2), twelve (B3) and five (B4) analyses for theobromine and thirteen (B1), five (B2), seventeen (B3) and seven (B4) analyses for caffeine.

mean) is more than 90% for theobromine and more than 60% for caffeine. On emergence of the leaflets variation decreases drastically. Coefficient of variation of caffeine is three times lower at stage B3 than at stage B1; in the case of theobromine this decrease is slightly retarded.

Purine alkaloid content of the entire bud (leaflet, stipules and resin layer)

As regards an entire bud at stage B1, relative dry wt part of the leaflets lies between 15% and 30%. Therefore the defence potential of the stipules and the resin layer has to

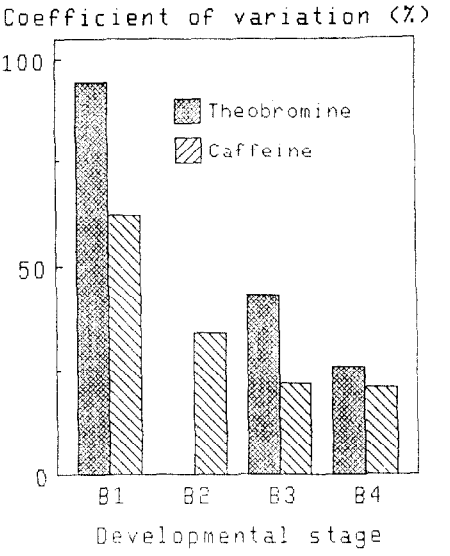

Fig. 3. Coefficient of variation of the caffeine and theobromine content. Number of analyses as indicated in Fig. 2; theobromine value for B2 was omitted because only two samples were analysed.

be taken into account as well. 'Total purine alkaloid' accumulation of seven individual buds is shown in Fig. 4 and represents the sum of theobromine and caffeine in the leaflet, the stipules and the resin layer. Other purine alkaloids such as theophylline, paraxanthine and methylated uric acids are present in *Coffea arabica* only in minute quantities [12]. The content of the leaflets as well as the content of the stipules varies within a wide range (15–70%) of total alkaloid. With one exception (bud No. 4) the relative alkaloid part of the resin layer is very low (< 3%). The average alkaloid content (about 2.0%) of the entire bud, however, is fairly constant (coefficient of variation 16%).

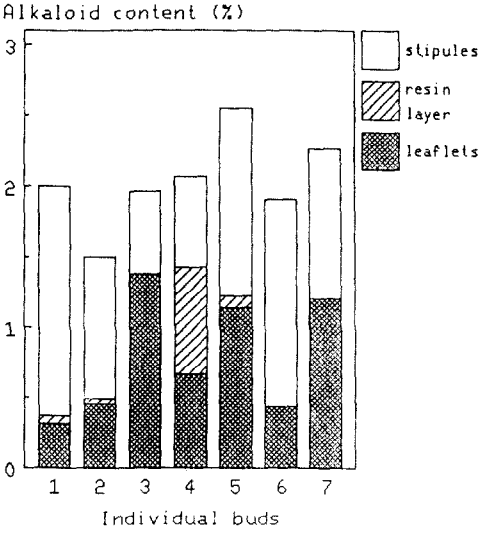

Fig. 4. Alkaloid content (theobromine and caffeine) of seven individual buds. Segments give the relative parts of stipules, resin layer and leaflets.

Velocity of purine alkaloid formation

The method we established to measure caffeine biosynthesis rates in leaves, which is based on the model of single pool tracer kinetics [11], cannot be used in the buds since in this case tracer application is very difficult to carry out *in situ*. In detached leaflets, however, the endogeneous theobromine pool decreases rapidly and is not in a 'steady state' as required in the above method.

In the present paper the rate of caffeine biosynthesis was estimated from the theobromine decrease in leaflets after detachment from the plant. A time course experiment (Fig. 5) demonstrated that the decline of the theobromine pool is exponential within the first 10 hr. Longer periods between detachment and analysis did not evoke a further theobromine decrease, probably because of a shortage of *S*-adenosylmethionine. Since biochemical reactions with theobromine as substrate other than the methylation to caffeine were not found (tested with [2-^{14}C]theobromine), the rate of methylation could be calculated by a multiplication of the regression slope with the mean theobromine content of the leaflets, which were analysed immediately after detachment.

The method described is suitable only for leaflets of stage B3. In older leaflets the endogenous pools of theobromine precursors are too large and in younger leaflets the variation of the theobromine content is too high. At the developmental stage B3 the rates of alkaloid formation are expected to be highest, since, as demonstrated for leaves [11], there exists a positive correlation between theobromine content and the rate of caffeine biosynthesis.

Evaluating the data of Fig. 5 caffeine formation is 17 mg g^{-1} day^{-1} (mean theobromine content 0.69%). We tried to relate the metabolic costs of this particular investment to the costs of general plant metabolism. For this purpose the influx of the carbon atoms into caffeine was compared with that into respiration (Table 1). The calculation showed that the carbon atoms incorporated in caffeine amounted to 15% of the carbon atoms needed for respiration.

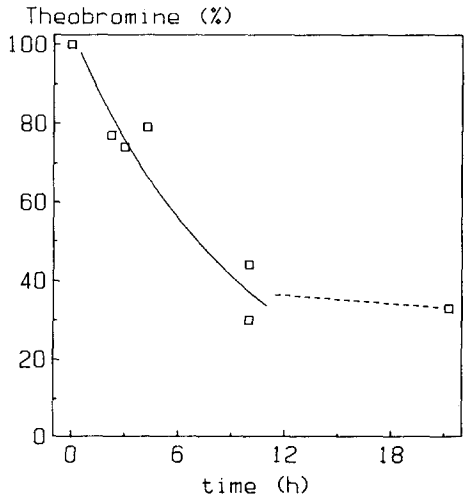

Fig. 5. Time course experiment of theobromine decrease in detached leaflets of developmental stage B3. One leaflets of a pair was analysed at the time of detachment, the other after being preserved for 2.25–22 hr in a humid chamber. The ratio of the two values gives the relative decrease. Regression calculation on the basis of an exponential curve gives a good fit [coefficient of determination (r^2) of 0.89] with a slope of −0.1018. Mean theobromine content of the reference leaflets was 0.69%.

Table 1. Comparison of the metabolic costs (carbon atoms) of general metabolism (respiration) and of purine alkaloid metabolism

	Respiration (g^{-1} day^{-1})	Caffeine formation (g^{-1} day^{-1})
μmol CO_2	4750	
μmol Caffeine		88
μmol Carbon atoms	4750	700
ratio $\frac{\text{Carbon atoms caffeine}}{\text{Carbon atoms respiration}}$	0.15	

DISCUSSION

During the lifespan of a leaf, the pattern of purine alkaloid accumulation may be summarized as follows: in closed buds caffeine and theobromine content of leaflets varies within a wide range (Fig. 3). At the moment of leaflet emergence, alkaloid formation is strongly accelerated; after having totally left the covering stipules the maximum content is reached (Fig. 2). In the following developmental period the rate of caffeine biosynthesis decreases exponentially from 17 mg day^{-1} g^{-1} at stage B3 (Fig. 5) to 0.016 mg day^{-1} g^{-1} when the leaf is fully grown with regard to area and photosynthetic capacity [11]. At that time purine alkaloid content is within the range of 1–1.5%, more than 99% being caffeine. In ageing leaves caffeine breakdown is increased [13], leading to alkaloid-free leaves at the time of shedding [10].

It is most remarkable that the variation in alkaloid content of the leaflets in stage B1 is balanced by the alkaloid content of the stipules (Fig. 4). The coefficient of variation of the alkaloid content in the entire bud is similar to that of leaflets at stages B3 and B4 (Fig. 3). Since in stipules caffeine biosynthesis is low (tested with tracer experiments) alkaloid transport from the leaflets into the stipules is supposed to occur in stage B1. Differences of transport rates would lead to the alkaloid variation observed in the leaflets at this stage.

It is evident that alkaloid formation of uniform high intensity takes place only during a short period after leaf emergence. At that moment the leaflets lack any mechanical protection. They have lost the protection of the covering stipules and lamina rigidity is very low. In addition the dietary value for a predator is high. During further development mechanical durability of the leaves increases and the nutritional value decreases which allows lowering of the rate of alkaloid formation.

An analogous stimulation of caffeine formation was found during germination [14]: coffee seeds are covered by a solid endocarp which decays during the germination process. The mechanically unprotected seedlings double their caffeine content within 8 weeks. A similar change of the defence strategy occurs during fruit development [15]. As long as the pericarp around the seed consists of a uniformly soft tissue, accumulation of caffeine is high

(about 2%). When the differentiation of a hard endocarp starts, caffeine content in the pericarp begins to decrease. At the end of the ripening process it is about 0.24%.

According to these observations, in *Coffea arabica* the following defence strategy is apparent: tissues with a high risk of predation are preferably protected mechanically. Otherwise a chemical defence system based on purine alkaloids may be established for a limited time. This causes considerable metabolic 'extra' costs (Table 1). As a result of growth, however, dilution of the stored purine alkaloids takes place which has to be compensated by a decrease in nutritional value or by mechanical protection. In ageing leaves the purine alkaloids lose their ecological function gradually. Alkaloid conservation by transport into young plant organs is negligible [16]. Re-utilization of purine alkaloid nitrogen [10] however is achieved by increasing catabolic activity.

EXPERIMENTAL

Plant material and cultivation conditions. These studies were carried out with 30- to 36-month-old plants of *Coffea arabica* L. cv Caturra grown in a greenhouse. Six months before the beginning of the experiments plants were transferred to a controlled environment chamber and cultivated under the conditions described in ref. [11].

Alkaloid determination. The leaf material without petioles was dried at 80°, crushed and thereafter extracted at 90° with 0.006 M H_2SO_4 for 20 min. After cooling, a clean up was achieved on a diatomaceous earth column (Extrelut, Merck, Darmstadt, West Germany [17]). Separation and quantitation of caffeine and theobromine were performed with a HPLC-system as described in ref. [9].

Respiration measurements. Respiration was measured with a micro-respirometer constructed according to ref. [18]. The measurements were carried out either *in situ* in the controlled environment chamber or on detached leaflets in a water-bath which improves temperature stability. The two methods gave results which did not differ significantly.

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