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STRESS INDUCED FORMATION OF PURINE ALKALOIDS IN PLANT TISSUE CULTURE OF *COFFEA ARABICA*

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Abstract—Reports that environmental stress may enhance the accumulation of secondary substances in plants led to the idea of introducing stress into tissue cultures with the aim of improving the *in vitro* production of pharmaceutically active compounds. The test was made with low- and high-producing cell suspension cultures of *Coffea arabica*. The production of the purine alkaloid caffeine was shown to be stimulated by stressors such as high light intensity and—depending on the culture type—high NaCl concentration.

INTRODUCTION

Results from diverse research fields show that many secondary plant compounds have ecological significance, e.g. by influencing the interrelation between organisms [1, 2] or by improving adaptation to unfavourable environmental conditions [2]. It is therefore plausible that external factors have a highly modulating effect on secondary metabolism. According to theoretical consideration on optimal defence strategies against herbivores [3], environmental stress conditions are expected to enhance accumulation of qualitative defence substances such as alkaloids, cardenolides, cyanogenic glycosides, and others. This hypothesis is confirmed by recent reports on increased alkaloid formation under water [4–6] and high temperature [7] stress. We tried to take advantage of these ecological connexions as regards a biotechnological application.

Plant tissue culture systems rarely accumulate substantial amounts of secondary metabolites spontaneously, a fact which is usually explained by the morphological dedifferentiation of cells *in vitro*. We hypothesized that low stress culture conditions optimized for rapid cell growth are responsible for this lack of biochemical performance, and that creation of *in vitro* stress situations comparable to those in nature might influence positively the production of medicinally important substances by tissue culture. This view is supported by the observation that secondary product formation often starts only when the cultured cells are in a nutrient stress, i.e. during the stationary phase of growth or if exposed to a deficient medium [8]. There is only one report concerning secondary compound formation during defined stress conditions: in suspension cultures of *Papaver bracteatum* [9] the application of high followed by low temperatures caused the release of thebaine into the medium.

We tested the outlined hypothesis with suspension cultures of *Coffea arabica* L. This represents an advantageous screening system since, as we demonstrated earlier [10], high- and low-producing cell lines can easily be established. Furthermore theobromine and caffeine are always dispersed according to the ratio of volume tissue to volume nutrient medium indicating a free exchange across the membranes.

RESULTS

High and alternating temperatures had no effect, while low temperature and polyethylene glycol had a negative effect on alkaloid formation. High light intensity leads in all cases tested to an alkaloid increase which is highest, relative and absolute, in the low-producing/small-aggregate culture type (Table 1). The effect of salt stress, whether positive or negative, depends on the aggregate size. In cultures with small cell aggregates purine alkaloid production is inhibited whereas in cultures with large cell aggregates it is increased. The combination of light and salt stress results in accumulation of the two single stressor effects. Large cell aggregates exhibit an additional increase of the alkaloid formation, while small cell aggregates have a production intermediate to that with each stressor alone. Under stimulating stress conditions the relative alkaloid amounts generally shift from 30–60% theobromine and 40–70% caffeine to 5–20% theobromine and 80–95% caffeine, independent of the culture type. The latter distribution corresponds to that in young leaves [11]. Dry weight does not appear seriously affected by the stress situation. However, owing to NaCl uptake, dry weight measurements of NaCl-stressed cells are not very reliable. Since in the coffee plant highest rates of purine alkaloid formation were found in young fast growing organs [11], the creation of a stress situation strongly inhibiting growth was not considered as being favourable for alkaloid production in tissue culture but requires also to be tested. However, in the stationary phase of growth the cultures often turned brown indicating that the stress intensity was considerable.

DISCUSSION

The results presented here show that stress may act qualitatively and quantitatively as a regulator of secondary product biosynthesis. The stress response is not

Table 1. Effect of stress on growth and purine alkaloid production in suspension cultures of *Coffea arabica*

Culture type	Condition	Dry weight (mg)	Theobromine	Caffeine (mg/l medium)	Total alkaloids
Large aggregates*	Control	310±11	48±03	132±16	180±17
	NaCl	350±19	9±00	280±28	289±28
Large aggregates†	Control	280±33	16±02	29±07	45±05
	NaCl	250±29	22±02	72±14	94±16
	Light	330±18	27±02	127±29	154±28
	Light+NaCl	240±72	30±04	192±34	222±38
Small aggregates*	Control	290±03	2±01	3±01	5±02
	NaCl	340±20	2±00	4±01	6±01
	Light	280±10	22±10	453±56	475±51
Small aggregates†	Control	220±21	56±10	37±03	93±13
	NaCl	180±11	24±07	20±09	44±15
	Light	230±50	20±10	309±01	329±09
	Light+NaCl	120±03	26±11	196±44	222±54

Stress conditions were 400 μmol m^{-2} sec^{-1} photosynthetically active radiation and/or 7.5 g NaCl l^{-1} leading together with the medium components to a water potential of about −10 bar. Values shown are mean.

*Mean of three cultures.

†Mean of six cultures, analysed three at a time ± standard error of the mean.

uniform and depends on the morphological organisation of the cultured cells. The metabolic background is not known, but it has frequently been reported that under stress conditions low molecular weight compounds are accumulated, for example certain amino acids and polyamines under osmotic stress.

Stress may induce not only differentiation on the secondary metabolism level but also on the morphological level. Recently it was demonstrated [12] that *in vitro* cultures of tobacco pith cones develop cambium-like structures after the application of continuous compressive stress, whereas unstressed regions exhibit an unorganized callus growth. Stress seems to have a general significance for differentiation processes.

Apart from a few exceptions, a profitable exploitation of plant cell cultures in the production of natural compounds has not been achieved within the past two decades. Hitherto in suspension cultures most of the pharmaceutically important substances still gained from plant sources could either not be detected, e.g. cardenolides, or were found only in traces, e.g. morphinane and tropane alkaloids. To overcome these barriers emphasis was laid on selection or biochemical research of regulation of secondary metabolism. Our approach is quite different; we postulate that in cell culture a high complexity of secondary metabolism is reached with a high complexity of the environment. At the present state of knowledge we propose a broad screening of stress factors or of combination of them for biotechnological application. This will allow us to find the optimal stress situation for the production of a desired compound by tissue culture. Future progress in the field of ecological biochemistry will lead to a more sophisticated approach.

EXPERIMENTAL

Cell cultures. Callus cultures were derived from young stem internodes of *Coffea arabica* L. cf 'Caturra' [13] and were grown in darkness at 27° in a Murashige and Skoog [14] medium supplemented with (mg/l) cysteine 10, thiamine HCl 1.0, 2,4-D 1.0 and kinetin 0.2. The cell mass of primary suspension cultures, always heterogeneous with regard to the size of the cell aggregates, was separated by decantation into large (5–10 mm diameter) and small (0.1–1.0 mm diameter) aggregates. This simple selection step yields two cell lines with a relatively stable type of tissue organization. Their growth characteristics are similar, but large aggregates usually have a better productivity [10].

For the experiments randomized cell material of the corresponding aggregate type in the stationary phase was used. Each culture was started with 14 ml medium and 2.5 g cell material corresponding to a dry weight of 60–80 mg (small aggregates) and of 100–140 mg (large aggregates) respectively. At the end of the cultivation period (14–17 days) cells were harvested by filtration and dry weight was measured.

Stress factors. The following stress factors were applied: high light intensity (400 μmol/m^{-2} sec^{-1}), NaCl (7.5 g/l medium) and polyethylene glycol 6000 (148 g/l medium) each creating together with the nutrient solution a water potential of about −10 bar, constant temperatures (37° and 17°) and alternating temperatures (37°/17°) every 12 hr.

Alkaloid analysis. For quantitative alkaloid determination samples of the medium (50 μl) were chromatographed without cleanup on a data processing HPLC-system (Waters associates; Pre-column: Nucleosil C-18, 4 mm × 3 cm, 10 μm packing; Column: Nucleosil C-18, 4 mm × 10 cm, 5 μm packing; Mobile phase: MeOH (25%)/H_2O (75%); Column temperature: room temperature; Flow rate: 1 ml/min; Pressure: 1800–2200 psi; Detection: 271 nm UV).

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