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REDIRECTING CELLULAR METABOLISM BY IMMOBILIZATION OF CULTURED PLANT CELLS: A MODEL STUDY WITH *COFFEA ARABICA*

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Key Word Index—*Coffea arabica*; Rubiaceae; cell metabolism; suspension cultures; cell organization; secondary metabolism; immobilization; theobromine; caffeine; purine alkaloids.

Abstract—Suspension-cultured cells of *Coffea arabica* have been immobilized by entrapment in calcium alginate gels to mimic natural aggregation. The production of methylxanthine alkaloid was increased up to 13-fold by the immobilization. This increased production has been ascribed to organization of the entrapped cells through physicochemical interactions between the polymer (alginate) and the plant cell wall. It has been shown that the metabolic changes induced by the immobilization are reversible.

INTRODUCTION

The differentiation of plant cells in culture is correlated with the accumulation of secondary products in several instances [1]. Compact or organized and slow-growing cultures synthesize higher amounts of secondary products than do friable and rapidly growing cultures. Furthermore, it has been suggested that organization of cells is essential for normal cell metabolism to proceed [1]. Such organization appears to be a major difference between high- and low-producing cultures.

The immobilization of cultured plant cells by entrapment in a gel matrix may provide conditions that are conducive to cell differentiation resulting in higher yields of secondary products. Such immobilized cell preparations may be considered as artificial aggregates of cells and may therefore be employed to investigate the effects of cell aggregation on cell metabolism under defined conditions. A controlled aggregate size and cell concentration can be readily achieved within immobilized cell preparations. In fact, increased product formation has been observed for immobilized plant cell preparations [2–4].

Cell suspension cultures of *Coffea arabica* with large aggregates show a higher production of methylxanthine alkaloids than cultures with finely suspended cells [5]. Thus, this cell culture offers an appropriate model system to study the effects of aggregation on secondary metabolism.

In this study the effects of aggregation on methylxanthine alkaloid production of *C. arabica* cells have been studied. The cells have been aggregated by entrapment in calcium alginate gels. Alginate gels are readily dissolved by addition of a calcium chelating agent to the medium and, therefore, cells can be returned to suspension after being in the immobilized state for some time.

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RESULTS AND DISCUSSION

Cells of *Coffea arabica* were immobilized by entrapment in calcium alginate for studies on the effects of aggregation on methylxanthine alkaloid production. A relatively high cell concentration (50% wet wt of cells) was used in these experiments since the aim was to mimic natural aggregation. The diameter of the beads was 4.5–5.0 mm. Based on a recent study on respiration and oxygen transfer in immobilized *Daucus carota* cells, it can be assumed that the high cell concentration in combination with a large bead size will lead to oxygen depletion in the interior of the particles due to cell respiration and diffusion limitations [6]. Such oxygen gradients should be even more pronounced in natural cell aggregates since the effective cell concentration is even higher than in the immobilized preparations.

Beads or an equivalent amount of freely suspended cells were inoculated into fresh medium and sucrose consumption; dry wt increase and alkaloid production were measured as a function of incubation time. Initial experiments showed that entrapment in alginate was superior to entrapment in kappa-carrageenan and therefore the former polymer was used.

Table 1 compares dry weight and alkaloid production for freely suspended and immobilized cells at two alginate concentrations after 17 days incubation. While growth is limited to essentially the same extent for the two immobilized preparations, a significant difference in alkaloid synthesis is observed. Production based on cell mass and alkaloid level on day 17 is 10–17-fold higher for immobilized cells than for free cells. At the lower polymer concentration some leakage of cells occurs after 2–3 weeks incubation. Attempts to eliminate leakage of cells were made by coating beads with a layer of alginate (around 0.3 mm thick).

A dramatic increase in alkaloid production is seen for immobilized cells upon extended incubation as illustrated in Fig. 1. The freely suspended cells only produce low amounts of methylxanthine alkaloids (around 30 mg/l).

Table 1. Dry weight and alkaloid production by various *Coffea arabica* cell preparations after 17 days incubation

Cell preparation	% alginate (w/w)	Dry weight (g/l)	Alkaloids (mg/l)	Productivity (μg/g day)	Cell leakage
Freely suspended	—	16	9	33	
Entrapped	3	8	78	544	+
Entrapped	5	7	40	336	−

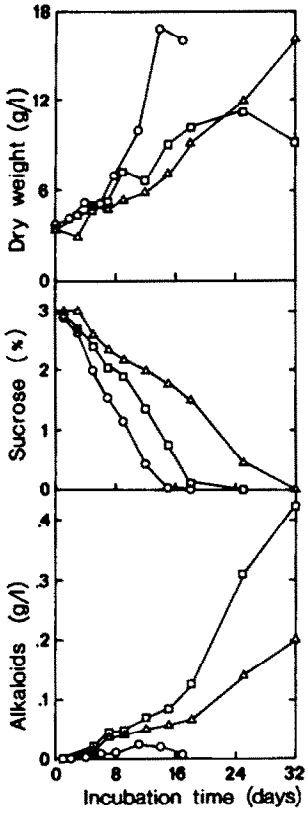

Fig. 1. Dry weight increase, sucrose consumption and alkaloid production as function of incubation time for freely suspended and alginate-entrapped cells of *C. arabica*. (○) Freely suspended cells; (△) cells entrapped in 3% (w/w) alginate; (□) cells entrapped in 3% (w/w) alginate coated with a layer of 5% (w/w) alginate.

After 32 days incubation, the coated and non-coated preparations had produced around 200 and 400 mg/l of alkaloids, respectively. An increased alkaloid production is seen when the sucrose has been completely consumed and the stationary phase reached. For the coated preparation, this stage was not reached until the experiment was terminated and, therefore, less alkaloids were produced. However, one can expect an increased alkaloid production upon extended cultivation of this preparation since the cells appear to be fully viable, as measured by cellular respiration.

Figure 2 shows the amount of theobromine and caffeine (= 1-methyl-theobromine) produced during the experiments depicted in Fig. 1. The ratios of theobromine to caffeine are lower in this experiment than in experiments with young cultures. However, a higher percentage of theobromine is observed for the two immobilized preparations (40 and 33% on day 15 for coated and non-coated preparations, respectively) than for free cells (25% theobromine on day 11). Presumably, the N^1-methyltransferase activity is limited by the level of enzyme during the growth phase and by the amount of substrate (theobromine) during the stationary phase of the cells, as previously suggested [2].

Further experiments were carried out in order to investigate the effects of alginate-entrapment on alkaloid biosynthesis in *C. arabica*. The cell line used in these latter experiments showed different morphological properties due to repeated subcultivations. It consisted primarily of small, dense aggregates and, thus, was already somewhat aggregated even before immobilization. Freely suspended (Fig. 3) and immobilized (Fig. 4) cells were incubated under identical conditions in multiple samples. When sucrose in the medium was depleted (day 11) the cells from half of the suspension cultures were immobilized in alginate and subsequently returned to the depleted medium. Likewise when no sucrose remained in the immobilized preparations (day 14), half of these preparations were treated with citrate buffer to dissolve the alginate and release the cells. The released cells were transferred back to the original medium. The four different set of preparations so obtained were cultivated for an additional 3 weeks.

The cells that were immobilized throughout the experiment produced more alkaloids (180 mg/l) than the other three preparations. When cells of *C. arabica* were immobilized after the sucrose had been consumed and subsequently returned to the original medium the entrapment had a negative effect on alkaloid synthesis. Apparently, some energy is required for the cells to adapt to the immobilized state. When the matrix was dissolved and the immobilized cells were set free, alkaloid synthesis continued at a constant rate. However, after 1 week the synthesis stopped when the concentration of alkaloids had reached the same level as that of cells grown in suspension throughout the experiment (120 mg/l). Thus, immobilization of cells in calcium alginate does not change cell metabolism irreversibly. The high level of alkaloids in the free suspension reflect the greater extent of 'natural' aggregation in the culture [7]. However, it is still evident that further aggregation can increase the alkaloid content.

The enhanced product formation by alginate-

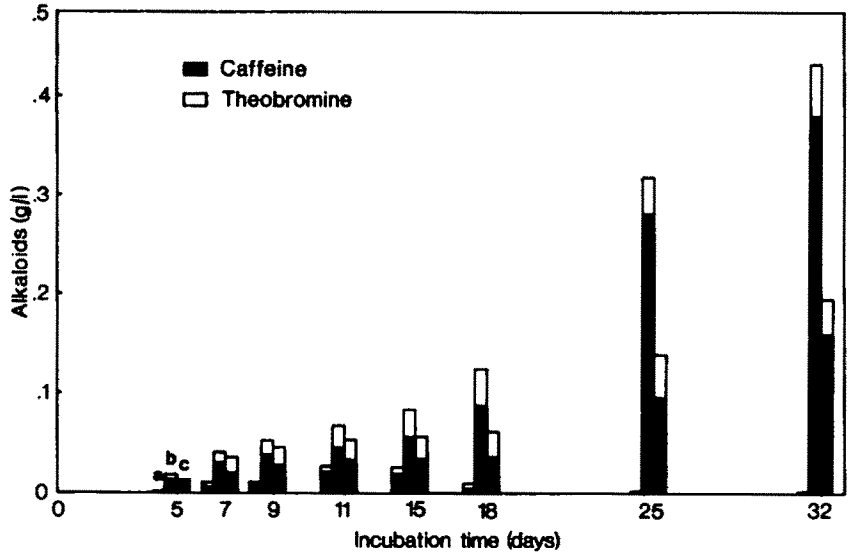

Fig. 2. Methylxanthine pattern of freely suspended and immobilized cells of *C. arabica*. (a) Freely suspended cells; (b) cells entrapped in 3% (w/w) alginate; (c) cells entrapped in 3% (w/w) alginate coated with a layer of 5% (w/w) alginate.

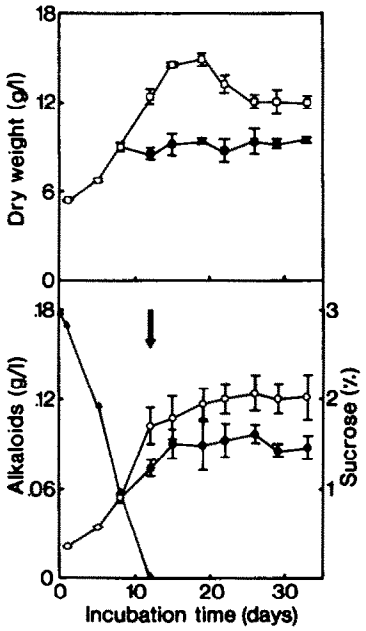

Fig. 3. Growth, sucrose consumption and alkaloid synthesis in freely suspended cells of *C. arabica*. (□) Dry Weight; (▲) sucrose concentration; (○) suspended cells; (●) cells immobilized at time indicated by arrow. The bars represent the s.d. of three parallel samples.

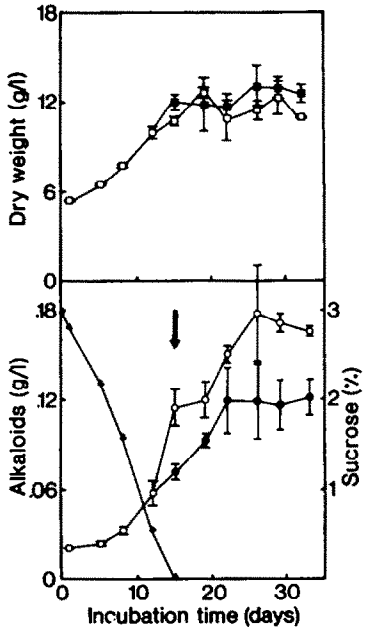

Fig. 4. Growth, sucrose consumption and alkaloid synthesis in immobilized cells of *C. arabica*. (□) Dry weight; (▲) sucrose concentration; (○) entrapped cells; (●) entrapped cells set free at time indicated by arrow. The bars represent the s.d. of three parallel samples.

entrapped cells in this and other studies [2, 3] may be due to the organization of cells. It has been suggested that the morphological organization of cells is required for high alkaloid formation in suspension cultures of *C. arabica* [7]. For immobilized cells this organization may be influenced by physico-chemical interactions between the matrix and cells. In this respect it is interesting to note that cells entrapped in other polymers (e.g. agarose) do not show an enhanced product formation [8]. Agarose is an inert polymer and cells entrapped in this polymer are separated in small aggregates (resembling those in free suspension) within the large aggregate (bead).

However, a high cell–cell contact within an organized cell population is of importance for secondary product formation [1]. Alginate may mediate such cell–cell contact due to its ionic character. Polycarboxylic acids of the plant cell wall (e.g. pectic acid) and alginate both with a high affinity for calcium ions may interact in the presence of such ions. Thus, alginate may function as a 'cross-linker' between cells resulting in an organization of the cells. This type of interaction may stimulate the synthesis of secondary metabolites and would be reversible after removal of the alginate. In fact, the *Coffea* cells used in our experiments behave as freely suspended cells after they have been set free. Therefore, the results support the hypothesis that alginate mediates a cell-to-cell interaction resulting in organization of the cells and an increased alkaloid production.

EXPERIMENTAL

Chemicals. Sodium alginate (Protanal High Viscosity Fraction HF) was obtained from Protan A/S (Drammen, Norway). Murashige and Skoog basal medium was from Flow Laboratories (Irvine, U.K.). Theobromine and caffeine were supplied by Fluka. All other chemicals were obtained from commercial suppliers.

Cultivation of cells. Suspension cultures of *Coffea arabica* L. cv 'Caturra' were kindly supplied by Dr. Th. Baumann, University of Zurich. They were cultivated in darkness at 26° on a gyratory shaker at 120 rpm. The medium was Murashige and Skoog medium [9], supplemented with L-cysteine 10, thiamine–HCl 1.0, 2.4-D 1.0 and kinetin 0.2 (mg/ml). Cells were transferred into fresh medium every 2 weeks with a 15% (w/w) inoculum.

Immobilization procedures. The following procedures were carried out under sterile conditions. 14-Day-old cells of *C. arabica* were sieved through a nylon net of 1 mm mesh size (ZBF AG Zurich, Switzerland). The sieved cells were washed with calcium-free medium on a glass filter. Cells (10 g) were mixed with alginate solution (10 g) in culture medium. Alginate beads of 4.5–5.0 mm in diameter were made by dripping the alginate/cell suspension into medium fortified with 50 mM $CaCl_2$ as previously described [10].

In order to coat the beads with additional layers of alginate, a two-phase system similar to that described in [11] was developed. Alginate beads (15 g) were washed extensively with calcium-free medium and gently mixed with alginate solution (Protanal HF 5% w/w, 7.5 g). A flask containing soy oil (120 ml) was shaken on a gyratory shaker at 300 rpm and the alginate/beads mixture was rapidly added. After 15 sec the additional layer of about 0.3 mm was stabilized by adding calcium (50 mM) containing medium (40 ml) to the suspension. The coated beads were washed extensively on a steel net with 3 mm mesh size and subsequently used for cultivation.

Bead dissolving procedure. Alginate beads (1.0 g) were suspended in medium (10 ml) containing 0.1 M sodium citrate (pH 6.0). The beads were fully dissolved after shaking the suspension for 30 min on a rock'n'roll shaker. The cells were collected by centrifugation (670 *g*) for 1 min and subsequently washed with citrate free medium (2 × 10 ml). The cells were allowed to recover from the citrate treatment by incubating them in fresh medium for 1 hr before the respiration of the cells was monitored. If the cells were to be returned to the original medium (Fig. 4) the whole procedure was carried out with depleted medium from the same culture.

Analytical procedures. Sampling. Weight losses caused by evaporation during incubation were corrected by adding sterile water before sampling. In order to analyse growth and alkaloid production, samples of 5 ml were pipetted aseptically from suspension cultures. For immobilized cultures, a sieve-spoon was used to collect a predetermined number of beads. The appropriate amount of medium was removed with a pipette. *Dry Weight.* Samples were collected on preweighed dry filter papers and dried at 60° until constant weight. *Determination of sucrose and glucose.* Invertase (90 IU; purified by gel filtration over a G-25 column) was added to cotton wool filtered medium (0.1 ml). The mixture was incubated at 37° for 30 min, diluted with 0.9 ml of water and injected into a glucose analyser (Yellow Spring Instruments). Sucrose concentrations were calculated from the estimated glucose concentrations. *Extraction procedure.* Both freely suspended and immobilized cells were extracted with refluxing MeOH. The extracts were evaporated to dryness under red. pres. and the residue was dissolved in (2 ml MeOH and analysed for alkaloid content. *Alkaloid analysis.* Quantitative alkaloid analysis was carried out by reversed phase HPLC of filtered samples (0.2 μm) of extract or medium under the following conditions: Column: Nucleosil C-18 (5 μm) 4.6 × 130 mm. Flow rate: 1 ml/min. Mobile phase: isocratic system for analysis of medium MeOH:H_2O (1 mM MES, pH 5.8) (34:66) and gradient system for analysis of extracts MeOH–H_2O (1 mM MES, pH 5.8) [20–40:80–60] at room temp. Detection: 271 nm (UV).

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REFERENCES

1. Lindsey, K. and Yeoman, M. M. (1983) in *Plant Biotechnology* (Mantell, S. H. and Smith, H., eds) pp. 39–66. Cambridge University Press, Cambridge.
2. Brodelius, P., Deus, B., Mosbach, K. and Zenk, M. H. (1980) in *Enzyme Engineering* (Weetall, H. H. and Royer, G. P., eds) Vol. 5, pp. 373–381. Plenum Press, New York.
3. Brodelius, P., Deus, B., Mosbach, K. and Zenk, M. H. (1981) European Patent Application 80850105.0.
4. Lindsey, K., Yeoman, M. M., Black, G. M. and Mavituna, F. (1983) *FEBS Letters* **155**, 143.
5. Frischknecht, P. M. and Baumann, T. W. (1980) *Planta Med.* **40**, 245.
6. Hulst, A. C., Tramper, J., Brodelius, P., Eijkenboom, L. J. C. and Luyben, K. Ch. A. M. (1985) *J. Chem. Tech. Biotechnol.* **35B**, 198.
7. Frischknecht, P. M. and Baumann, T. W. (1985) *Phytochemistry* **24**, 2255.
8. Brodelius, P. and Nilsson, K. (1980) *FEBS Letters* **122**, 312.
9. Murashige, T. and Skoog, F. (1962) *Physiol. Plant.* **15**, 473.
10. Brodelius, P., Deus, B., Mosbach, K. and Zenk, M. H. (1979) *FEBS Letters* **103**, 93.
11. Nilsson, K., Birnbaum, S., Flygare, S., Linse, L., Schröder, U., Jeppsson, U., Larsson, P.-O., Mosbach, K. and Brodelius, P. (1983) *Eur. J. Appl. Microbiol. Biotechnol.* **17**, 319.