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Activities of NADPH-producing Enzymes and Alkaloid Production in *Catharanthus roseus* Cells

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Abstract. A 2,4-D dependent cell line, accumulating only traces of indole alkaloids under standard conditions of subculture, was grown in two different production media. Removal 2,4-D from maintenance culture medium enhanced the production of ajmalicine and serpentine and increased the activities of the NADPH-producing enzymes: glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, malic enzyme and cytosolic isocitrate dehydrogenase.

Adding zeatin to a 2,4-D depleted medium further increased the alkaloid content but the activities of the NADPH-producing enzymes remained unchanged.

Due to the presence of a number of therapeutically valuable indole alkaloids in *Catharanthus roseus,* cell and tissue cultures of this plant have been extensively investigated for secondary metabolism (Van der Hfijden *et al.* 1989, review). The alkaloids are derived from precursors in the acetate-mevalonate pathway and the tryptophan pathway (Gröger 1985). There is increasing evidence that their production may be regulated at the level of terpenoid biosynthesis, particularly in the post-mevalonate pathway (Mérillon *et al.* 1986). In fact, the activities of the postulated key enzyme, geraniol-10 hydroxylase are increased in cells grown under alkaloid-producing conditions (Schiel *et al.* 1987). This enzyme is NADPH dependent (Madyastha *et al.* 1976), and thus the reduced cofactor must be available for the biosynthesis of 10-hydroxy-geraniol. In *in vitro* cells, the total amount of cytosol is generally very low; Van den Berg *et al.* (1988) suggested that the levels of cofactors (NADPH, ATP) used for biosynthesis might also be low, thus limiting the production of secondary metabolites.

Phytohormones may control the indole alkaloid accumulation in *C. roseus* cells. The inhibitory role of 2,4-D is now well-established (*e. g.* Knoblock and Berlin 1980). On the other hand, adding cytokinins to 2,4-D-depleted media further increases the alkaloid contents (Kodja *et al.* 1989). In order to test the hypothesis that NADPH might be a limiting factor for regulating the indole alkaloid biosyn-

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thesis, we measured the activities of four NADPH-producing enzymes in alkaloid-producing cells, and compared these to the enzyme activities obtained in non-alkaloid producing cells.

MATERIALS AND METHODS

Cell Cultures and Media

Suspension cultures of *Catharanthus roseus* (L.) G. Don (cell line C20) were maintained in the dark at 25 °C, in 250 ml Erlenmeyer flasks filled with 50 ml of the B5 medium of Gamborg *et al.* [1968], supplemented with 58 mM sucrose and 4.5 μM 2,4-D [D-medium]. The pH was set to 5.5 before sterilization. The cells (referred to as C20-D cells) were subcultured every 7th day at a dilution rate of 1 : 10 (*ca*.1g d.m.l^{-1}). Three other media were used for experimental purposes:

- "O-medium": as D-medium but without 2,4-D
- "Z-medium": a 5 μM zeatin - supplemented O-medium
- "DZ-medium": a 5 μM zeatin - supplemented D-medium

Cells cultured in these media will be referred to as C20-0, C20-Z and C20-DZ cells, respectively. Transfers were made by filtering C20-D cells through 30 μm nylon cloth, washing them with O-medium and then resuspending them in the appropriate medium at an inoculum density of 1g (d.m.)l^{-1}.

Enzyme Extracts and Assays

Cells were frozen in liquid nitrogen and stored at – 20 °C. An aliquot of frozen material was used for determining the activities of NADP-linked enzymes. The cells (4 g) were ground under liquid nitrogen with sand in a mortar. Glucose-6-phosphate dehydrogenase (EC 1.1.1. 49, G6PDH), 6-phosphogluconate dehydrogenase (EC 1.1.1. 44, 6PGDH) and NADP-isocitrate dehydrogenase (EC 1.1.1. 42, ICDH) were extracted with 0.1 M sodium phosphate buffer (pH 7.5) containing 5.10^{-4} M EDTA and 5.10^{-3} M mercaptoethanol. Pyrophosphate-dependent phosphofructokinase (EC 2.7.1.90, PPi-PFK), ATP-dependent phosphofructokinase (EC 2.7.1.11, ATP-PFK) and NADP-malic enzyme (EC 1.1.1.40, ME) were extracted with 0,05 M imidazole-HCl buffer (pH 7.6) containing 2.10^{-4} M $MgCl_2$, 10^{-3} M EDTA and 14.10^{-3} M mercaptoethanol.

The homogenates were centrifuged at 100 000 *g* for 30 min, and the supernatants were used for enzymatic activities estimations. All measurements were assayed at 30 °C by following the oxidation or reduction of $NAD(P)^+/H$ at 340 nm with minor modifications to the previously given protocols: G6PDH and 6PGDH (Wagner *et al.* 1987), ICDH (Biochemica Information II, Boehringer Mannheim), PPi-PFK and ATP-PFK (Ashihara *et al.* 1988) and ME (Danner and Ting 1967).

Protein and Alkaloid Measurements

The amount of soluble proteins in the crude extracts was determined as described by Bradford (1976). Ajmalicine and serpentine were dosed by spectrofluorometry, according to Mérillon *et al.* (1983, 1989).

RESULTS AND DISCUSSION

The Effect of Suppressing 2,4-D on the Alkaloid Accumulation and on the Activities of NADPH-Producing Enzymes

The C20-D cells cultured in their maintenance medium (D-medium) accumulated only traces of indole alkaloids (Fig. 1, A-B). These cells, although they are

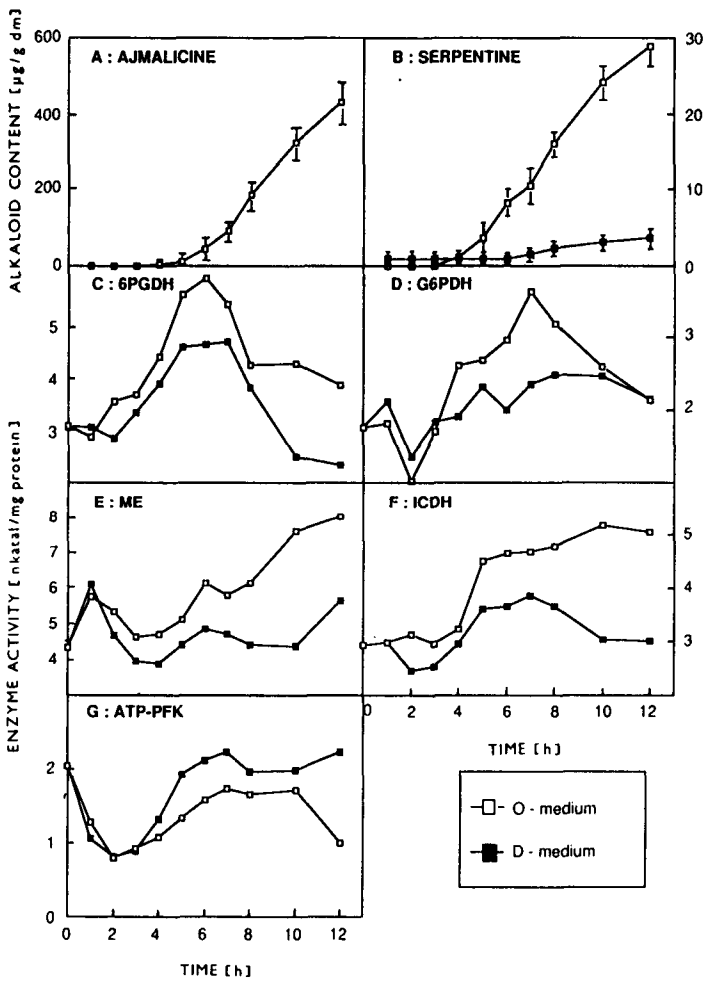

Fig. 1. Alkaloid content and enzyme activities in *C. roseus* cells grown either in O-medium or in D-medium. The data represent one experiment; the experiment was repeated three times and trends were consistent (Ajmalicine is not detectable in D-cells).

2,4-D-dependent, may grow over one cycle of culture in a 2,4-D free medium (O-medium) without their growth being significantly affected (Mérillon *et al.* 1989, Fig. 2). In O-medium, the cells accumulated ajmalicine and serpentine in significant amounts from the fourth day on.

The activities of four NADPH-producing enzymes have been looked at over the whole growth of cells in D- or O-medium. The two enzymes controlling the pentose phosphate pathway, G6PDH and 6PGDH, are of interest, since they are the principal source of NADPH in cells (Turner and Turner 1980). Two other NADPH-producing enzymes have also been studied: malic enzyme (Plumb-Dhindsa *et al.* 1979) and cytosolic CDH (Felix *et al.* 1981). For comparison purposes, we also studied the activities of the two phosphofructokinases, ATP-PFK and PPi-PFK (controlling the Embden-Meyerhof pathway).

Time-courses of activities were somewhat different from one to another NADPH-producing enzymes (Fig. 1, C-F). However, for cells grown in O-medium, as opposed to cells grown in D-medium, higher activities for all four of these enzymes were observed as early as on the 3rd day. On the contrary, higher activities of phosphofructokinases were observed in C20-D cells from day 4 on (Fig. 1G shows results obtained for ATP-PFK but similar results were observed for PPi-PFK).

The Effect of Adding Zeatin on the Alkaloid Accumulation and on the Activities of NADPH-Producing Enzymes

We previously found that under certain conditions and for some cell lines of *Catharanthus roseus,* adding cytokinins to culture medium was another way of stimulating the accumulation of indole alkaloids (Kodja *et al.* 1989). Fig. 2 shows that for cell line C20, the action of zeatin was particularly highlighted when 2,4-D was removed from the culture medium: the alkaloid amount was about three times higher in C20 - Z cells than it was in C20 - O cells. On the other hand, the activities of the four NADP-linked enzymes were similar in C20 - O and C20 - Z cells (Table 1).

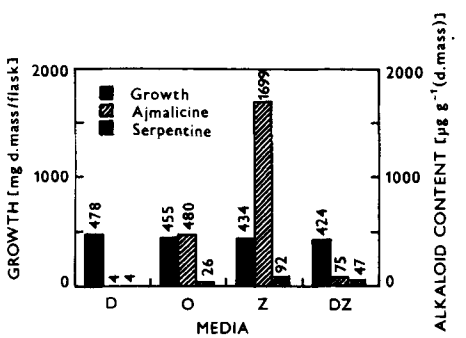

Fig. 2. Growth and alkaloid levels in *Catharanthus roseus* cells grown in D-, O-, Z- and D-Z media. Results at day 12.

TABLE 1

Enzyme activities and ajmalicine levels in *C. roseus* cells grown either in O-medium or in Z-medium. Results at days 5 and 7 for enzymes and at day 12 for ajmalicine

	Enzyme activities [nkatal mg^{-1} protein]								Ajmalicine [$\mu g\ g^{-1}$d.m.]
	G6PDH		6PGDH		ME		ICDH		
Days	+ 5	+ 7	+ 5	+ 7	+ 5	+ 7	+ 5	+ 7	+ 12
O-medium	2.9	2.5	4.6	4.5	4.0	4.8	3.9	3.9	513
Z-medium	2.4	2.9	4.0	4.4	4.7	5.0	4.1	4.1	1610

These results show that depletion in 2,4-D, which enhances the activities of 10-hydroxygeraniol hydroxylase (Schiel *et al.* 1987), also stimulates the production of the reduced cofactor NADPH necessary for this enzyme. Adding zeatin to a 2,4-D depleted medium produces a further increase in the alkaloid contents but the effect of cytokinin seems to differ from the effect of withdrawing auxin, since the activities of the NADP-linked enzymes are not modified. Zeatin might act on more specific steps of the alkaloid pathway after a general enhancement of metabolism following 2,4-D depletion.

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BOOK REVIEW

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Diesel and Gasoline Engine Exhausts and Some Nitroarenes. Volume 46. – International Agency for Research on Cancer, Lyon 1989. Pp. 458. Sw. Fr. 65.–.

In 1980, there were approximately 320 million passenger cars, 75 million trucks and 20 million buses. Since, their numbers had rapidly increased and the adverse effects on our environment are apparent. Engine exhausts are complex mixtures containing chemical compounds in the particulate and gaseous phases. The first part of the IARC Volume 41 covers diesel and gasoline exhausts, their composition, environmental exposure and biological data relevant to the evaluation of their genotoxicity. The second part of the Monographs covers chemical and physical data and biological effects of 15 nitroarenes, found mainly in engine exhausts, *e. g.* 1,3-dinitropyrene, 6-nitrobenzo (a) pyrene, 6-nitrochrysene, 2-nitrofluorene, 1-nitronaphthalene and 1-nitropyrene. Of the tested complexes and compounds, only diesel engine exhaust is considered as probably carcinogenic to humans, while most of the other compounds as possibly carcinogenic. Very useful are the appendixed activity profiles for genetic and related tests. The x-axis of the activity profile represents the bioassays in phylogenetic sequence by endpoints and the values on the y-axis represent the lowest effective doses and the highest ineffective doses tested.

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