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Serpentine Accumulation during Greening in Normal and Tumor Tissues of *Catharanthus roseus*

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Summary

We have developed a model for the study of indole alkaloid accumulation. The model centers on the control of chloroplast biogenesis by light or hormonal regime, and associated serpentine production. *Catharanthus roseus* cells when grown in dark in the presence of 13.3 μM benzyladenine, are achlorophyllous and serpentine is not detected; but if these cultures are grown in the presence of light, they become green with a chlorophyll content of 150 mg Kg^{-1} FM after 24 d. In a parallel manner the serpentine content increases up to 3.1 g Kg^{-1} DM. On the other hand, tumor cells obtained by transformation with *Agrobacterium tumefaciens* strain 4210 are white and do not produce serpentine when grown in the light. When this tumor tissue is transferred to a medium containing 13.3 μM benzyladenine, the chlorophyll content reaches 54 mg Kg^{-1} FM after 204 d, and the serpentine content is 0.3 g Kg^{-1} DM. There seems to be a correlation between the greening of the tissue and their serpentine content that suggests a fundamental function of chloroplasts in serpentine biosynthesis.

Key words: Catharanthus roseus, greening, serpentine.

Abbreviations: BA = benzyladenine; DM = dry mass; FM = fresh mass.

Introduction

Catharanthus roseus (L.) G. Don produces a great variety of monoterpenoid indole alkaloids of economic interest with pharmacological properties, such as ajmalicine and its tetradehydroderivative, serpentine. The production of secondary metabolites by plant tissue cultures has not been completely successful, due partly to the lack of knowledge of the regulatory controls of the enzyme activities involved. Many reports are now focused on obtaining more information about the enzymes that synthesize these alkaloids in plant tissue cultures (Fahn et al., 1985 a; Fahn et al., 1985 b; De Luca and Cutler, 1987; Misawa et al., 1988), for this knowledge could allow us to manipulate the cell cultures in order to obtain higher alkaloid yields.

There is increasing evidence that some level of differentiation is required for the production of secondary metabolites, at both the cellular and the tissue levels (Hirotani and Furuya, 1977; Hagimori et al., 1982; Endo and Yamada, 1985; Hashimoto et al., 1986). Specific reactions of the biosynthetic pathways take place in different cellular compartments, as has been demonstrated for synthesis of vindoline in *C. roseus*, where the first enzymes of the pathway are located in the cytosol, while the last steps take place in the chloroplasts (De Luca and Cutler, 1987; De Luca et al., 1988).

The effect of cytokinin on serpentine and ajmalicine accumulation in *C. roseus* transformed cultures has been studied before by Kodja (1989), who reported that there was a stimulatory effect, but no relationship with chlorophyll content was mentioned. The effect of light on regulation of serpentine biosynthesis has been previously documented (Carew and Krueger, 1976; Roller, 1978; Knobloch et al., 1982; Seibert and Kadkade, 1982; Endo et al., 1987). There is also evidence against the correlation between the chloroplast biogenesis and the alkaloid yield in *Vinca* cell lines (Eilert et al., 1987). Here, we present a model for the study of indole

alkaloid synthesis that focuses on the relationship between chloroplast biogenesis, controlled by light or hormone treatments, and serpentine production.

Materials and Methods

Cell lines

Two *C. roseus* cell lines were used in this study (Loyola-Vargas et al., 1991). The first one was an *Agrobacterium tumefaciens* transformed cell line, obtained through infection with the strain LBA 4210 (cyt$^-$), cultured in Phillips and Collins (1979) medium without hormones and maintained in continuous light; the second was a normal callus line, originally obtained from stems first cultured in Murashige and Skoog (1962) medium with 13.3 µM BA for three years and then transferred to Phillips and Collins medium, also supplemented with 13.3 µM BA and maintained in the dark. Both cultures were unpigmented, and no chlorophyll could be detected under these initial conditions. The ajmalicine content of the callus tissue did not vary along the culture cycle (0.018 ± 0.0018 g Kg^{-1} DM); no ajmalicine could be detected in the tumors. There was a basal content of serpentine in both the callus and tumor tissues, 0.28 ± 0.037 and 0.0047 ± 0.00046, respectively, which did not change along the culture cycle.

Extraction and determination of alkaloids

One gram of freeze-dried *C. roseus* cells was extracted with 50 mL of methanol for 2 min in a Polytron. The homogenate was incubated at 55 °C for 2 h and then filtered. The methanolic extract was evaporated to dryness, and the residue was dissolved in 2.5 % (v/v) H_2SO_4 (15 mL) and washed three times with one volume of ethyl acetate each time. The aqueous phase was adjusted to pH 9.5 with NH_4OH (28.4 %); this alkaline solution was extracted three times with 20 mL of ethyl acetate each time. The organic phase was recovered and evaporated to dryness. The residue was dissolved in 1 mL of methanol and used for the quantitative determinations. This is a modification of the method reported by Kutney et al. (1980).

Serpentine was quantified in TLC plates by fluorescence densitometry scanning. Ajmalicine was determined in TLC plates by absorbance densitometry scanning, after development with Dragendorff reagent and sodium nitrite (Monforte-González et al., 1991).

Chlorophyll determination

Chlorophyll was measured through a modification of Arnon's method (Wintermans and de Mots, 1965).

Results and Discussion

In order to induce the greening process in our cultures, we employed two different strategies. The first one involved the use of light on callus tissue cultured in the presence of BA, and the second, used the addition of the cytokinin, BA, to the medium in which tumors were cultured in light.

In the case of the transformed cell line 4210 (cyt$^-$), subculturing in 13.3 µM BA was chosen because it has been demonstrated that cytokinins promote chloroplast biogenesis in different systems (Parthier, 1979; Caers et al., 1985; Wyndaele et al., 1988; Solís et al., 1989). When this tumor line was subcultured in the media supplemented with BA

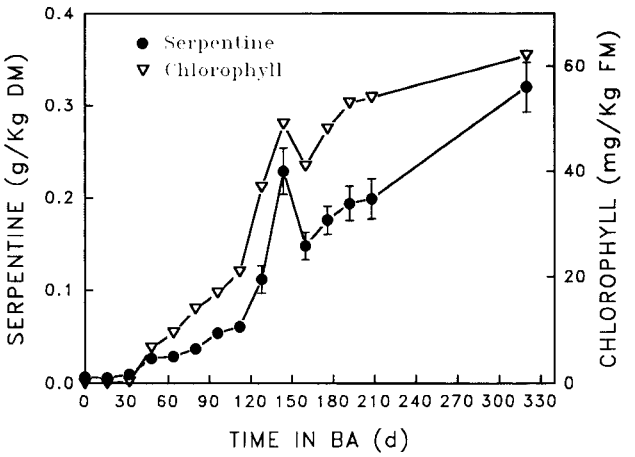

Fig. 1: Effect of BA (13.3 µM) on chlorophyll and serpentine contents in *C. roseus* tumor culture.

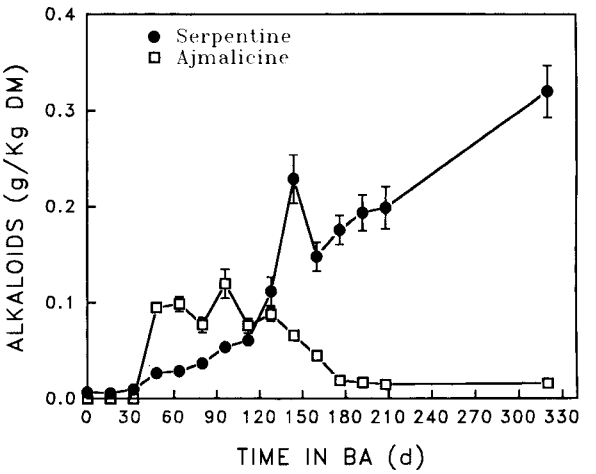

Fig. 2: Effect of BA (13.3 µM) on serpentine and ajmalicine contents in *C. roseus* tumor culture.

every 16 d (exponential growth phase), the chlorophyll content reached 30 mg Kg^{-1} FM after 128 d (Fig. 1). It should be noted that chlorophyll accumulation in the presence of BA was directly related with the accumulation of serpentine. Ajmalicine, the precursor of serpentine, showed a continued increase for 128 d in the tumors subcultured in this medium.

After 128 d, a decrease in the content of ajmalicine was detected, which coincided with a sharp increase in serpentine (Fig. 2). This behavior has also been observed by Knobloch (1982) and Nef et al. (1991): Serpentine accumulation was preceded by an increase and a subsequent decrease of ajmalicine.

In tissue cultures of *C. roseus* it has been shown that light has a stimulating effect on the accumulation of anthocyanins (Carew and Krueger, 1976) and of the indole alkaloid serpentine (Döller et al., 1976; Roller, 1978). Light favors chloroplast development in tissue cultures, so we therefore decided to transfer the *C. roseus* normal callus line from darkness to white light (65.2 W m^{-2}). In approximately 24 h, the calli

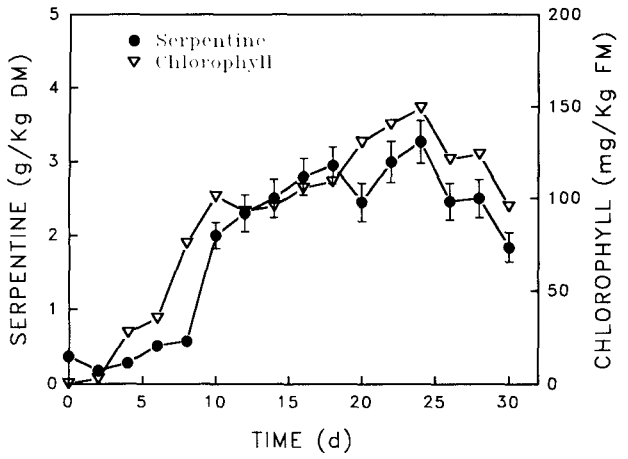

Fig. 3: Effect of white light on chlorophyll and serpentine contents in callus tissue of *C. roseus*.

began to turn green. The chlorophyll content after only 4 d was higher than that reached by the tumor tissues after 128 d.

In the callus tissue cultured in the media supplemented with BA, both chlorophyll and serpentine accumulation showed a similar pattern, suggesting again that there is a correlation between the greening process and the synthesis of serpentine (Fig. 3). These findings contrast markedly with the absence of correlation between the chlorophyll content and the strictosidine lactam yield in *Vinca* cells; furthermore, the time course of greening and alkaloid accumulation was quite different in these cultures (Eilert et al., 1987). It is probable that the accumulation of strictosidine lactam does not require the development of chloroplasts, since the enzymes required for its synthesis are located in the cytosol (De Luca and Cutler, 1987).

In light-regulated events, both the quantity and the quality of light play important roles. Chlorophyll accumulation is favored by high light levels (Yamada et al., 1978). Light quality is a fundamental factor for chloroplast development in cultured plant cells. In the intact plant, the pigment system responsible for chloroplast differentiation responds to both red and blue light. In cell cultures from higher plants, chlorophyll formation and chloroplast development only proceed in blue light (Beauchesne and Poulain, 1966; Bergmann and Berger, 1966; Hüsemann, 1970). We assayed three different light qualities, white, red, and blue, to evaluate the greening of the calli and its relationship with their serpentine and ajmalicine contents. In Fig. 4, it can be observed that the accumulation of both alkaloids through the culture cycle of this line exposed to the different light qualities followed a similar behavior to that observed in the greening process of tumor tissues induced by BA (Fig. 2). In the presence of white light, an accumulation of ajmalicine preceded that of serpentine. However, no such pattern could be observed in both the calli under either red or blue light alone. Their ajmalicine content did not vary significantly throughout the culture cycle, and even when there was a significant accumulation of serpentine during the second phase of the culture cycle, the levels never reached those of the calli in the white light.

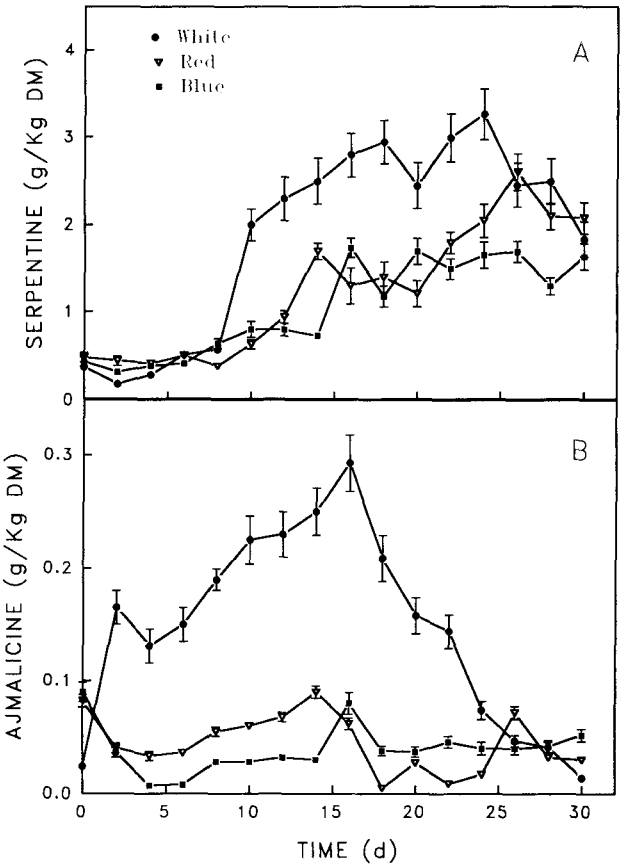

Fig. 4: Effect of white, red and blue light on serpentine (A) and ajmalicine (B) accumulation in callus tissue of *C. roseus*.

This is important since under this culture condition (from darkness to white light), the doubling time of the culture was less than half (0.72 d^{-1}) of that of the white line growing in the dark (2.1 d^{-1}). This same effect has been documented for tobacco cells (Nato et al., 1985). From the biotechnological point of view, it could be said that the alkaloid productivity of the line grown in white light was positively affected.

Summarizing, we have developed a model to study the accumulation of both serpentine and ajmalicine using two different strategies for the greening of the cultures: light for the normal calli, and the use of a medium supplemented with BA for the tumor line. There seems to be a tight correlation between the appearance of chlorophyll and the development of chloroplasts with the presence of serpentine in the cell lines, which suggests a fundamental function of chloroplasts in serpentine biosynthesis. The probable involvement of the chloroplast in the synthesis of this alkaloid could be explained in terms of providing either the required compartmentation for the enzymes involved in this pathway or some metabolites or the reducing power in the form of cofactors ($NADP^+$) that could be limiting the conversion of ajmalicine into serpentine.

One of the proposed models for the synthesis of serpentine requires metabolites synthesized in the chloroplasts and postulates the presence of specific peroxidases in the vacuoles that dehydrogenate ajmalicine (Blom et al., 1991).

A fact that cannot be overviewed is that we are only detecting net alkaloid accumulation, whether this is the result of increased synthesis or a decrease in serpentine turnover will require a detailed study of the biosynthetic and catabolic pathways, which is currently being addressed by our group.

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