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Vindoline synthesis in *in vitro* shoot cultures of *Catharanthus roseus*

Elizabeta Hernández-Domínguez, Freddy Campos-Tamayo & Felipe Vázquez-Flota*
Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Calle 43 No. 130 Chuburná 97200, Mérida Yucatán, México
**Author for correspondence (Fax: +52-999-981-3900; E-mail: felipe@cicy.mx)*

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Abstract

Vindoline, the major alkaloid in cultures of *Catharanthus roseus* shoots, reached 2 mg g^{-1} dry wt after 27 d in culture. Maximal vindoline accumulation coincided with maximum activities of deacetoxyvindoline 4-hydroxylase, deacetylvindoline acetyl-CoA acetyl transferase and tryptophan decarboxylase. Shoot exposure to jasmonate shortened the time required for the maximal vindoline accumulation to 14 d.

Introduction

The leaves of *Catharanthus roseus* are the sole source of the cytotoxic dimeric alkaloids, vinblastine and vincristine, which are formed by the condensation of catharanthine and vindoline subunits. The economic value of these dimers has prompted several efforts to produce them in *in vitro* cultures (Hong *et al.* 1997, Zhao *et al.* 2000); however, even when cell cultures can accumulate catharanthine up to 1.4 mg g^{-1} dry wt, which are similar to amounts found in intact plants, they frequently fail to produce vindoline (Kurz *et al.* 1981). The inability of undifferentiated cultures to produce vindoline is related to the absence of the required cell organization (St-Pierre *et al.* 1999) therefore, *in vitro* shoot cultures have been proposed to overcome this hurdle (Krueger *et al.* 1982). Although vindoline concentration in rootless, *in vitro* shoots is comparable with those in mature leaves (1.5 mg g^{-1} dry wt), the yields can be inconsistent between different batches (Hirata *et al.* 1993). Such variations could be related to a lack of coordination between the enzymes involved in the early and late steps of vindoline biosynthesis. In developing seedlings, the maximal activities of tryptophan decarboxylase and strictosidine synthase, which participate in the early reactions, occurred 24 to 36 h prior to the detection of deacetoxyvindoline 4-hydroxylase and deacetylvindoline acetyl-CoA acetyl transferase, that are involved in the last two steps (Figure 1). We have generated a *C. roseus* vindoline-producing shoot culture and analyzed the enzymes involved both at the early and late biosynthetic reactions, in order to investigate if they are coordinated in the same fashion as in the embryo-derived seedlings. This shoot culture responded to methyl jasmonate by shortening the time required for vindoline accumulation.

Materials and methods

Induction of multiple shoots cultures

Immature *C. roseus* seeds were sterilized by subsequent immersion in 70% (v/v) ethanol, 5% (v/v) sodium hypochlorite and sterile water and were germinated on semi-solid Murashige and Skoog (MS) media without growth regulators. Rootless seedlings were placed on MS agar media supplemented with 1, 3 or 5 mg 6-benzyladenine (BA) l^{-1} and kept at 25 ^{o}C either under continuous light or a 16 h photoperiod. For liquid cultures induction, clusters with 8 to 12 shoots were transferred to MS liquid media supplemented with 1 mg BA l^{-1} and cultured under continuous light on a rotary shaker at 50 rpm. Shoots growth, as fresh weight, was evaluated at different points during a culture cycle. Alkaloid accumulation, as well as

Table 1. Methyl jasmonate (Me-Ja) effects on vindoline accumulation and on the activity of some biosynthetic enzymes in 12 d-old *Catharanthus roseus* shoot cultures. (TDC) tryptophan decarboxylase, (D4H) deacetoxyvindoline 4-hydroxylase and (DAT) deacetylvindoline acetyl CoA acetyltransferase. Figures represent the average of triplicates ± standard deviation.

Time (h)	Vindoline (mg g^{-1} dry wt)		TDC (picokatals mg^{-1} protein)		D4H (picokatals mg^{-1} protein)		DAT (picokatals mg^{-1} protein)	
	Control[a]	Me-Ja[b]	Control	Me-Ja	Control	Me-Ja	Control	Me-Ja
0	0.4 ± 0.1	–	81 ± 13	–	11 ± 5	–	16 ± 4	–
12	0.6 ± 0.1	4.2 ± 0.5	87 ± 15	90 ± 11	6 ± 3	89 ± 12	19 ± 7	94 ± 10
24	0.4 ± 0.1	4.4 ± 0.9	53 ± 12	61 ± 8	12 ± 3	45 ± 15	21 ± 6	82 ± 14

[a]Control shoots were exposed to dimethyl sulfoxide (DMSO).
[b]Methyl jasmonate (Me-Ja) was diluted in DMSO and applied to shoot cultures to a final concentration of 0.1 mM.

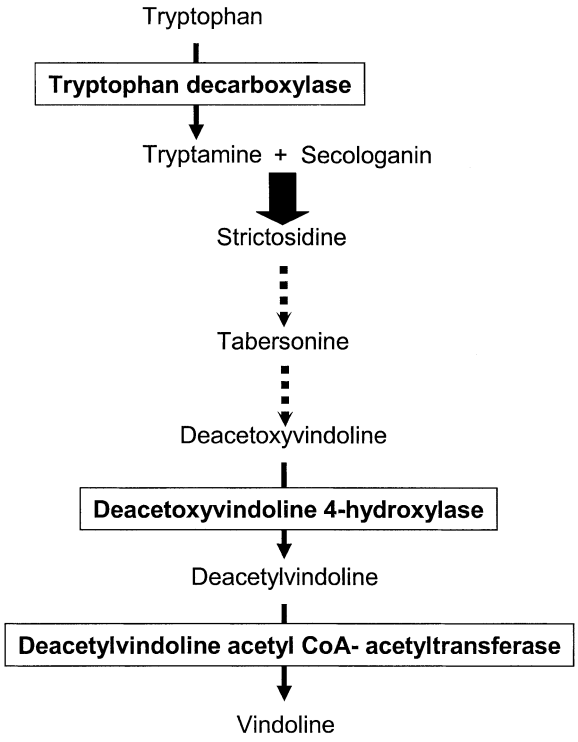

Fig. 1. Diagram of the vindoline biosynthetic pathway. Solid arrows correspond to single enzymatic steps, whereas dashed arrows stand for multiple enzymatic steps. Bold face characters correspond to the enzymes analysed in this work.

tryptophan decarboxylase (TDC), deacetoxyvindoline 4-hydroxylase (D4H) and deacetylvindoline acetyl-CoA acetyl transferase (DAT) enzyme activities, were monitored through out the culture cycle.

Jasmonate induction of alkaloid synthesis

Twelve-day-old shoot cultures were exposed to methyl jasmonate 0.1, 1 or 10 μM (diluted in dimethyl sulfoxide; DMSO). Tissues were collected in triplicate at the times indicated in Table 1 and alkaloid content, as well as TDC, D4H and DAT activities, was analyzed.

Analytical procedures

Alkaloids were extracted from freeze-dried tissues with hot methanol. Ajmalicine, catharanthine and vindoline were quantified as previously described (Vázquez-Flota *et al.* 2002). Enzyme activities were quantified in desalted extracts as reported in Vázquez-Flota *et al.* (2002).

Results and discussion

Establishment of the shoot culture

Shoots were induced on semisolid MS culture medium. After three weeks, an average of six shoots per seedling explant had formed with three assayed concentrations of BA. Shoots cultured with 1 mg BA l^{-1} did not form callus tissue, therefore this concentration was chosen for culture maintenance. No difference in the number of shoot formed was recorded between cultures submitted to either continuous light or a 16 h photoperiod (data not shown). Liquid cultures were induced by transferring shoot clusters into MS media with 1 mg BA l^{-1} under continuous light. Shoots in liquid media showed a quick proliferation and biomass accumulation (Figure 2), with no evidence of vitrification, and neither of root nor callus formation.

Alkaloid production

Shoots in liquid cultures accumulated vindoline as the main alkaloid. Maximal accumulation values (ca. 2 mg g^{-1} dry wt) were reached around day 27 of the culture cycle (Figure 2). This value is in the same order of magnitude than in leaves from plants maintained

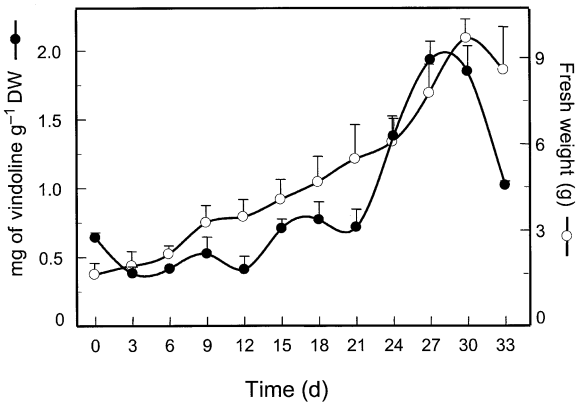

Fig. 2. Biomass (open circles) and vindoline (closed symbols) accumulation in shoots through out a 33-d culture cycle in liquid media. Symbols represent averages of triplicates with their corresponding standard deviation.

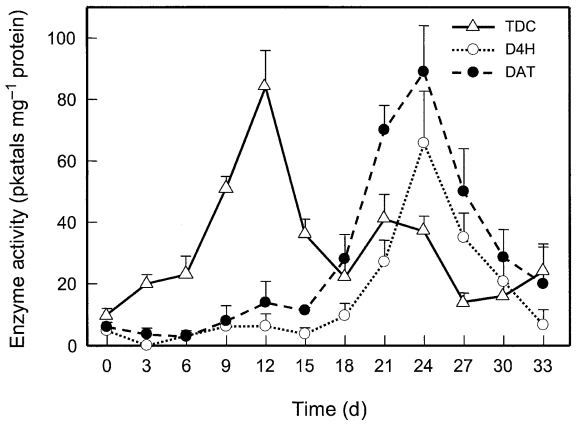

Fig. 3. Tryptophan decarboxylase (TDC; open triangles), deacetoxyvindoline 4-hydroxylase (D4H; open circles) and deacetylvindoline acetyl-CoA acetyltransferase (DAT; closed circles) enzyme activities in shoots through out a 33-d culture cycle. Symbols represent averages of triplicates with their corresponding standard deviation.

in our nursery (4.6 mg g^{-1} dry wt). In comparison, ajmalicine and catharanthine contents in shoots only reached 0.2 and 0.55 mg g^{-1} dry wt respectively (data not shown). These results suggest that *in vitro* shoots behave in a similar way to leaves from intact plants, which barely accumulate ajmalicine and where the vindoline: catharanthine ratio ranges between 3 and 4 (Balsevich & Bishop 1989). Furthermore, these results suggest that root participation is not required for vindoline synthesis in aerial tissues (Krueger *et al.* 1982).

Coordination between the early and late biosynthetic steps of vindoline

TDC showed two activity peaks through out the culture cycle, at days 12 and 21 (Figure 3). The early activity peak was the most prominent (about 85 picokatals mg^{-1} protein), but it did not coincide with alkaloid accumulation. The second activity peak, which occurred just prior to the maximal vindoline accumulation, was 50% lower than the earliest one (Figures 2 and 3). Both D4H and DAT remained slightly above the detection level for most of the culture period, increasing simultaneously shortly before the accumulation of vindoline (Figures 2 and 3).

In seedlings, the earlier induction of TDC, compared to those of D4H and DAT, seems to be related to increasing of vindoline intermediaries concentrations for their later transformation (Balsevich *et al.* 1986). Hence, the second TDC activity peak observed in the shoots, may establish an important difference in the regulation of alkaloids synthesis compared to seedlings, suggesting that the shoot's early activation of TDC may not satisfy later demand of intermediaries for alkaloid synthesis.

Jasmonate elicitation of vindoline synthesis

Vindoline maximal accumulation occurred after a long culture period (around 27 d; Figure 2). To shorten this period, 12 d-old shoots were treated with methyl jasmonate. Exposure to 0.1 μM resulted in a 10-fold increase within the first 12 h (Table 1). Neither a longer exposure (Table 1) nor higher jasmonate concentrations increased vindoline further (data not shown). Despite this increase in vindoline accumulation, TDC remained at the same activity as in unexposed shoots (Table 1). Nevertheless, it should be emphasized that 12 d-old shoots (which were used for this experiment) were at the stage of maximal TDC activity (Figure 3). In unfolding *Catharanthus* seedlings, jasmonate induction of TDC was only noticed when they were at the developmental stages corresponding to low enzyme activity (Vázquez-Flota & De Luca 1998). In contrast to the observed behaviour of TDC activity, shoot exposure to methyl jasmonate produced up to an 8-fold increase of both D4H and DAT activities (Table 1), reaching similar values to those found in untreated shoots at late culture stages (Figure 3). Since D4H and DAT induction coincided with vindoline accumulation, these data suggest that jasmonate promoted the transformation of the previously accumulated intermediaries.

Concluding remarks

Similar trends in alkaloid metabolism were observed between *in vitro* cultured shoots and seed-germinated seedlings, including the differential timing of the maximal TDC activity, in comparison to those of D4H and DAT. However, important differences were also determined, including the occurrence of the second TDC activity peak (Figure 3). The coincidence of TDC activity with alkaloid accumulation has only been described in root cultures (Islas-Flores *et al.* 2002). Although in undifferentiated cell cultures TDC activity is induced by elicitors, it has been shown that this enzyme only becomes limiting in conditions of an excess of terpenoid intermediaries (Canel *et al.* 1998). Shoot exposure to jasmonate revealed that an increase in vindoline accumulation may occur at early culture stages without TDC induction (Table 1). However, the low TDC activity of older cultures needed to be increased in order to reach similar vindoline amounts (Figure 3 and Table 1).

In undifferentiated *Catharanthus* cell cultures, neither the exposure to jasmonate nor the constitutive expression of ORCA 3, a regulatory gene mediating the jasmonate induction of several alkaloid biosynthetic genes, activated vindoline biosynthesis (van der Fits & Memelink 2000). These results confirm the requirement of tissue organization for vindoline synthesis. Such requirements seem to be fulfilled in *in vitro* shoots, where vindoline concentration (2 mg g^{-1} dry wt) was comparable to that in leaves (4.6 mg g^{-1} dry wt), even though it only happened after a prolonged culture period (Figure 3). Interestingly, this period could be shortened by jasmonate treatment. Hence, *in vitro* shoot cultures represent an interesting system, oriented towards the production of the *Catharanthus* alkaloids that cannot be formed in undifferentiated cell cultures.

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