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VARIATION IN VINBLASTINE PRODUCTION BY *CATHARANTHUS ROSEUS* DURING *IN VIVO* AND *IN VITRO* DIFFERENTIATION

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Key Word Index—*Catharanthus roseus*; Apocynaceae; vinblastine; tissue culture; biosynthesis; regeneration; callus; indole alkaloids.

Abstract—Production of vinblastine, an anticancer agent, by *Catharanthus roseus*, increased as the seedlings matured, attaining a steady concentration after the plants become more than 3-month-old. Vinblastine could be detected in the callus lines established from different explants. As the callus differentiated multiple shoots, the vinblastine production increased rapidly, equalizing to that of *in vivo* seedlings of similar age. The high degree of differentiation and maturity in the tissues of *Catharanthus* was correlative to the increased vinblastine production, both *in vivo* and *in vitro*. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

The dimeric indole alkaloids from *Catharanthus roseus*, vinblastine and vincristine are now widely used in treatment of various human cancers. These drugs because of extremely low yields, are among the most expensive in the pharmaceutical market. Attempts to improve their yields through cell and tissue culture have so far met with limited success [1, 2]. Growth and differential stage-dependent biosynthesis of these alkaloids are still poorly understood. It has been proposed that differentiation of storage compartments, such as laticiferous cells are essential for alkaloid production [3]. Studies on the regulation of the biosynthetic pathway of dimeric indole alkaloids have shown that the final steps are modulated by different developmental and environmental mechanisms, and the key enzymes are synthesized only at particular growth phases [4–6].

In order to study the differential vinblastine yield during the growth and morphogenesis of *Catharanthus*, a callus line with detectable amounts of vinblastine was selected and plantlets were regenerated *in vitro*. The yields were compared with the *in vivo* plants during similar morphogenic and maturation processes.

RESULTS AND DISCUSSION

The callus cultures were initiated from different explants of different age. The calli of seedling origin produced only detectable quantities of vinblastine (Table 1). Calli from immature fruits also showed trace quantities of vinblastine. One callus line when cultured on high cytokinin medium turned green and regenerated multiple shoots, after 6 weeks. These shoots could be maintained on basal medium (MS).

Vinblastine was absent in the mature seeds, but in 4-week-old seedlings, the yield was substantial (60% of the mature plants) (Fig. 1). With further growth and maturity, the vinblastine yield increased, until the plants were 3-month-old. Subsequently, the yield became stable at 12 $\mu g\ g^{-1}$ dry weight. In callus induced from the seedlings, the vinblastine yield decreased sharply and the callus produced only 1.6 $\mu g\ g^{-1}$ dry weight (Fig. 1). However, when multiple shoots regenerated from the same callus, vinblastine yield increased rapidly. Vinblastine from regenerated shoots was comparable with that of the seedlings of the same age. However, vinblastine, as well as the regeneration potentiality, decreased with age.

Alkaloids accumulate mostly as salts with organic acids in vacuoles of only some cells in a cluster, termed alkaloid cells. It has been argued that differentiation of storage compartments, such as laticiferous cells, are essential for alkaloid production [7]. That the dimeric indole alkaloids of *Catharanthus* are biosynthesized only in specifically differentiated tissues [3, 4] have been demonstrated through steady state m-RNA levels of two important enzymes, tryptophan decarboxylase and strictosidine synthase, that regulate the biosynthesis of indole alkaloids. It was established that the alkaloid metabolism is restricted to certain tissues and is modulated by different developmental

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Table 1. Vinblastine yield of 4-week-old callus from different explants of *Catharanthus roseus* (pink variety)

Callus line	Source	Explant	Age of explant (weeks)	Vinblastine yield ($\mu g\ g^{-1}$)
I	*In vitro* grown seedlings*	Hypocotyl sections	4	0.9
II	*In vitro* grown seedlings	Cotyledonary leaves	6	0.1
III	*In vitro* grown seedlings	Hypocotyl sections	6	1.6
IV	Young plant†	Nodal sections	8	n.d.
V	Young plant	Leaves	12	n.d.
VI	Mature plant‡	Nodal sections	24	n.d.
VII	Mature plant	Leaves	24	n.d.
VIII	Mature plant	Immature fruits	28	0.2
IX	Mature plant	Mature fruits	32	n.d.

n.d. = Not detectable.
* Whole seedlings contained 7 $\mu g\ g^{-1}$ vinblastine on average.
† Young plants contained 11.5 $\mu g\ g^{-1}$ vinblastine on average.
‡ Mature plants contained 12 $\mu g\ g^{-1}$ vinblastine on average.

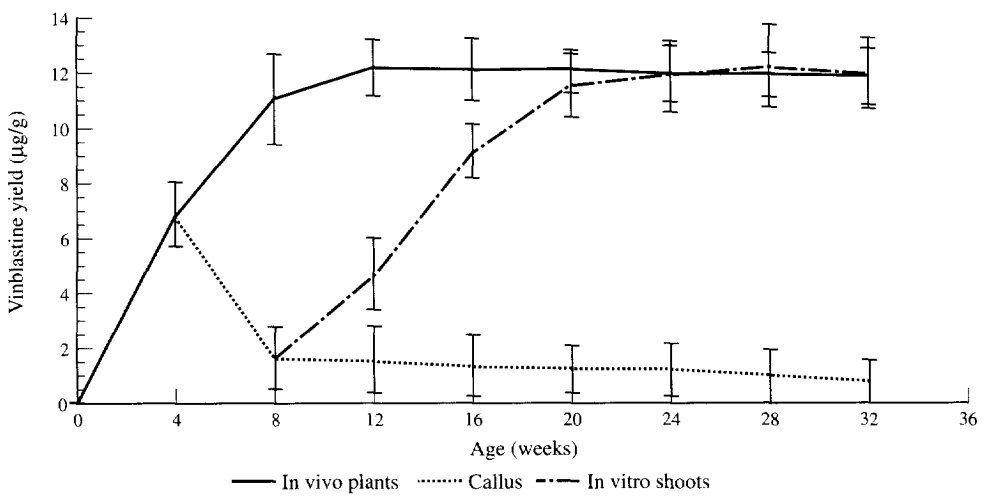

Fig. 1. Variation in vinblastine yield in *Catharanthus roseus* during differentiation *in vivo* and in hypocotyl cultures *in vitro*. Error bars indicate s.e. for $n = 3$.

and environmental mechanisms [8]. The expression of the enzymes involved in the last steps of biosynthesis of vindoline, an important intermediate, was found to be under strong developmental control and modulated by tissue specific and light dependent factors [9]. Dimeric indole alkaloids have earlier been detected in the light induced green callus [10], leaf organ cultures [11] and multiple shoot cultures [4, 12]. Transport of the vacuolar strictosidine to the cytoplasm is also reported to be essential for further alkaloid biosynthesis. In addition, differentiation of chloroplasts is essential for vindoline biosynthesis [6]. Therefore, logically differentiation and maturation of the tissues are clearly the preconditions for the biosynthesis of more complex dimeric alkaloids.

In our experiments, greening of callus and emergence of shoot buds were the critical stages when vinblastine production showed sharp elevations. Callus cultures showing emergence of multiple shoots, and young seedlings, when 4-week-old, exhibited similar sharp increases in the biosynthesis of vinblastine. Conceivably therefore, during these early developmental stages, key biosynthetic enzymes for final stages of vinblastine production are synthesized and the required storage compartments become fully differentiated. Detectability of vinblastine even in callus cultures indicates the possible presence of minute patches of differentiated tissues and that the vinblastine productivity is quantitatively proportional to the extent of differentiation in the tissues.

EXPERIMENTAL

In vitro *techniques*. Mature explants were derived from *Catharanthus roseus* plants grown from seeds in the herbal garden at Hamdard University. Seeds were surface sterilized with solns of 1% cetrimide for 5 min, 10% sodium hypochlorite for 20 min, 0.1% $HgCl_2$ for

5 min and subsequently with 70% alcohol for 3 min. After thorough washing with sterile distilled H_2O, the seeds were inoculated aseptically on MS [13] solidified with 0.6% agar. Four-week-old seedlings (5–7 cm) were used for juvenile explants. The mature (surface sterilized as described earlier) and juvenile explants were inoculated on solidified MS supplemented with naphthaleneacetic acid (NAA, 2 mg l^{-1}), 6-benzylaminopurine (BAP, 5 mg l^{-1}), casein hydrolysate (CH, 1000 mg l^{-1}), and asparagine (100 mg l^{-1}) far callus induction. Four-week-old callus cultures were transferred to MS + NAA (0.1 mg l^{-1}) + BAP (5 mg l^{-1}) + zeatin (1 mg l^{-1}) + asparagine (100 mg l^{-1}) + glutamine (100 mg l^{-1}) for shoot regeneration. Six-week-old regenerants were separated from callus and transferred to MS basal for further growth. All cultures were maintained in an illuminated culture room at $26 \pm 2°$ temp., 55% relative humidity and 10 hr photoperiod of 1045 lux. All the experiments were repeated $3\times$ with replicates.

Analysis of vinblastine. In vivo and *in vitro* samples were dried in a vacuum desicator, finely powdered and 1 g each were extracted with 50 ml MeOH for 24 hr by cold maceration. The filtered extracts were dried and residues were dissolved in 3 ml of 1 N H_2SO_4. pH of each of the acidic extracts were adjusted to 9.0 with 20% NH_4OH soln. and shaken with 5 ml $CHCl_3$. The $CHCl_3$ layer was sepd out and dried. The final residues were dissolved in 1 ml of a 1:4 mixt. of 0.5 N H_2SO_4 and HPLC mobile phase, i.e. MeOH–Me_2CN–2.5 mM KPi buffer (pH 7.0) (48:20:32). The external standard use for quantitative analysis (vinblastine sulphate, Sigma) was also treated in the same way to eliminate any error in extraction. For HPLC analysis, a Merck LiChroCART C_{18} column (125 × 4 mm, 5 μm) and the solvent system were used at a constant flow rate of 1 ml min^{-1} and 2500 Psi pressure. A Diode array detector was employed for detection of peaks, set at a wavelength of 255 nm and band width of 5 [14]. All the analyses were performed with three replicates.

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