Plant Growth Regulation **44:** 53–58, 2004.

Growth, metabolic profiling and enzymes activities of *Catharanthus roseus* seedlings treated with plant growth regulators

Magdi El-Sayed[1,2,*] and Rob Verpoorte[1]
[1]*Department of Pharmacognosy, Section of Metabolomics, Institute of Biology Leiden, Leiden University, Leiden, The Netherlands;* [2]*Department of Botany, Faculty of Science, South Valley University, Aswan, Egypt;* **Author for correspondence (fax: +31 71 5274511, e-mail: m.el-sayed@chem.leidenuniv.nl)*

Received 25 May 2004; accepted 27 August 2004

Key words: abscisic acid, Alkaloids, Catharanthus roseus, gibberellic acid, jasmonate, peroxidase, salicylic acid, seedlings, strictosidine glucosidase.

Abstract

The effect of different growth regulators on growth and the production of terpenoid indole alkaloids as well as some enzymes involved in the biosynthesis were studied in *Catharanthus roseus* seedlings. The seedlings were grown on MS solid medium containing different concentrations of each growth regulator for a period of one month. Extracted alkaloids were analyzed by HPLC for determination of terpenoid indole alkaloid quantities. Continuous availability of growth regulators induced different alkaloids with variable effects among the regulators. Gibberellic acid at concentration of either 5.8 μM or 11.6 μM resulted in elongation of shoots with lowering the number of leaves. Abscisic acid has a retardant effect on growth. Ethylene did not effect the growth pattern at concentration of 100 μM but seedlings were not tolerant to higher concentrations. Methyljasmonate reduced the growth of the root system. Methyljasmonate was a general inducer for all alkaloids and increased the activity of strictosidine glucosidase. Ethylene applications promoted the pathways towards ajmalicine, serpentine, tabersonine and vindoline. Similar effect as for ethylene was observed for abscisic acid. Salicylic acid treatment increased the production of serpentine, tabersonine and higher concentration of salicylic acid induced vindoline accumulation. Peroxidase activity was also induced by salicylic acid. Gibberellic acid has little effect on alkaloid levels.

Introduction

The Madagascar periwinkle (*Catharanthus roseus*) produces several pharmaceutically important alkaloids. Among these valuable compounds are the powerful antineoplastic agents vinblastine and vincristine. The monomeric alkaloids ajmalicine and serpentine are used in the treatment of circulatory diseases (Verpoorte et al. 1991, 1997). The synthesis of *C. roseus* indole alkaloids is regulated under developmental and environmental control. The developmental regulation of tryptophan decarboxylase (TDC), strictosidine synthase (STR), and the enzymes involved in vindoline biosynthesis (T16H, NMT, D4H and DAT) have been studied in detail. These enzymes are developmentally controlled in *C. roseus* developing seedlings (De Luca et al. 1986, 1988). Strictosidine β-glucosidase (SGD) plays an important role in the monoterpenoid indole alkaloid biosynthesis. It is a branching enzymatic step to many different types of indole alkaloids. A few studies have been published concerning SGD in *C. roseus*. All previous studies focused on purification and characterization of this

enzyme in cell cultures (Luijendijk et al. 1998; Geerlings 1999). Basic peroxidases are involved in the conversion of ajmalicine to serpentine and also the coupling of monomeric alkaloids vindoline and catharanthine to bisindole alkaloids.

Phytohormones play a crucial role in the regulation and coordination of plant growth, morphogenesis and metabolism. It is thus postulated that they also will play a role in the biosynthesis of alkaloids. Much research has been devoted to the improvement of alkaloid production in *C. roseus* cell suspension cultures (Kargi 1988; Verpoorte et al. 1997). The results were not as successful as expected as dimeric alkaloids are produced only in the green shoots of the plant and productivity of other alkaloids in cell cultures is still too low to permit commercialization. Only very few studies with seedlings or young plants cultivated *in vitro* were carried out. In the present study, we report on the effect of continuous long-term presence of growth regulators gibberelic acid GA, salicylic acid (SA), abscisic acid (ABA), ethylene (ETH) and methyljasmonate (MJ) on alkaloid contents as well as enzyme activities of *C. roseus* seedlings.

Materials and methods

Growth media and preparation of growth regulators

All growth regulators were dissolved in EtOH (60%), filter sterilized and added to 150 ml Erlenmeyer flask containing 20 ml of MS medium (Murashige and Skoog 1962) just before solidification under sterile conditions. The concentrations of the growth regulators were as follows: salicylic acid (Sigma, St. Louis, MO, USA) with a final concentration in the medium of 14.5 and 29 μM; gibberellic acid (Sigma, St. Louis, MO, USA) 5.8 and 11.6 μM; abscisic acid (Aldrich Chem. Co., Milw. WI) 7.5 and 15 μM; methyljasmonate (Aldrich, Steinheim, Germany) 100 and 500 μM. and Ethylene (Ethophon, Aldrich Chem. Co., Milw. WI) 100 and 500 μM. The control flasks received the same amount of EtOH (60%).

Seed germination

Seeds of *C. roseus* were surface sterilized and allowed to germinate on MS hormone free medium in Petri dishes. After one week 6 seedlings were transferred to each experimental Erlenmeyer flask containing growth regulators and grown under 16 hours of artificial light (81 μmol m^{-2} s^{-1}) at 25 °C. Each treatment was represented by two flasks. Four weeks later, the seedlings were harvested and the growth parameters (shoot length, root length and number of leaves) were recorded before the seedlings were frozen in liquid nitrogen.

Extraction and alkaloids determination

Seedlings of each flask were ground in mortar using a pestle to fine powder. 100 mg of this powder was transferred to 1.5 ml microtube and alkaloids were extracted by addition of 500 μl of 0.1% trifluoroacetic acid solution. The vials were sonicated for 30 minutes, centrifuged at 13000g for 30 minutes. 50μl of the supernatant was analyzed by HPLC using a Waters 991 photo-diode array detector. A C18 RP Vydac column (no. 218 MS54, 4.6 × 250 mm, USA) with a guard column (Vydac, Hesperia, CA, USA) was used with an isocratic elution system of trifluoroacetic acid/ acetonitrile/ water (0.1:21:79, by vol.) at 1 ml min^{-1}.

Enzymes extractions and assays

50 mg Polyvinylpolypyrrolidone (PVPP) were added to the seedlings powder in 1.5 ml microtube and homogenized in 500 μl of 50 mM phosphate buffer pH 7.0 containing 2 mM EDTA, 1 mM DTT and 10 μM leupeptin. The tubes were centrifuged at 5000g for 30 min. at 4 °C. The supernatant (crude enzyme) resulting after centrifugation was used for the determination of strictosidine glucosidase and peroxidase activity. Protein concentrations were determined according to Bradford (1976) using bovine serum albumin as standard. Strictosidine glucosidase was assayed according to Stevens et al. (1992) and basic peroxidase activity was assayed according to Maehly and Chance (1954) using Guaiacol as a substrate and hydrogen peroxide (H_2O_2) in 50 mM phosphate buffer pH 9.0. The reaction was started by adding the enzyme and absorbance was measured at 470 nm after exactly 3 minutes.

Table 1. Growth parameters of *C. roseus* seedlings as influenced by different growth regulators.

	control	GA5.8	GA11.6	ABA7.5	ABA15	SA14.5	SA29	ETH100	MJ100
Root	1.58 ± 0.24	1.04 ± 0.22	1.4 ± 0.29	1.1 ± 0.15	1.2 ± 0.22	1.07 ± 0.11	1.52 ± 0.46	1.28 ± 0.36	0.83 ± 0.31
Shoot	3.03 ± 0.23	4.56 ± 0.57[a]	5.2 ± 0.22[a]	3.07 ± 0.30	2.32 ± 0.63	2.28 ± 0.34	2.75 ± 0.35	2.95 ± 0.11	3.13 ± 0.49
Leaves No	4 ± 1	2 ± 0	2 ± 0	2 ± 0	2 ± 0	3 ± 1	3 ± 1	4 ± 1	3 ± 1

GA5.8: gibberellic acid (5.8 μM); GA11.6: gibberellic acid (11.6 μM); ABA7.5: abscisic acid (7.5 μM); ABA 15 abscisic acid (15 μM); SA14.5: salicylic acid (14.5 μM); SA29: salicylic acid (29 μM); ETH100: ethephon (100 μM) and MJ100: methyljasmonate (100 μM).
[a] Promoted lateral branches.

Results and discussion

Effect of growth regulators on growth of C. roseus seedlings

The variation in shoot length, root length and the number of leaves apart of cotyledonary leaves as growth parameters of *C. roseus* seedlings treated with different concentrations of different growth regulators for a time period of one month was determined (Table 1). GA application at concentration of 5.8 & 11.6 μM resulted in an increase in shoot height of the seedlings to 4.56 ± 0.57 and 5.2 ± 0.22 cm respectively. These treatments did not result in an increase of the number of leaves (2 ± 0) compared to the controls (4 ± 1) but to an elongation of the internode and formation of branches from the node bearing the cotyledonary leaves. GA reduced the growth of the root system of the seedlings. ABA and SA have a similar effect on growth of shoots and roots of the seedlings but with higher number of leaves in SA treatments. Ethylene did not affect the growth of the seedlings except for root length that was decreased. Higher concentration of ethylene (500 μM) led to the death of seedlings in a period of one week.

MJ at concentration of 100 μM severely decreased the root growth but did not affect the shoots. The same effect of ethylene at higher concentrations was observed with higher concentration of MJ.

Effect of growth regulators on alkaloid biosynthesis

Figure 1 shows the effect of different growth regulators on ajmalicine content in *C. roseus* seedlings. All treatments resulted in a variable increase of ajmalicine, the highest production was observed in ethylene treatment for which a 5-fold increase was recorded. ABA at concentration of 15 μM and MJ resulted in 3-fold increase of ajmalicine while GA (11.6 μM), SA (14.5 μM) and ABA (7.5 μM) resulted in 2-fold increase. Exogenous application of ethephon (ethylene releaser) to *C. roseus* cell suspension cultures greatly enhanced ajmalicine accumulation (Yahia et al. 1998), thus ethylene seems to up-regulate the alkaloid production pathways. MJ treatment to *C. roseus* cell suspension cultures fed with loganin and tryptamine resulted in a 2-fold increase in ajmalicine accumulation (El-Sayed and Verpoorte 2002).

Similarly, serpentine was increased by the treatments of growth regulators with a range from 4-fold (GA at 11.6 μM) to a 7-fold increase (ABA at 15 μM). MJ resulted in a 6-fold increase in serpentine content (Figure 2). Although Aerts et al. (1996) reported that ABA and SA had no effect or decreased alkaloid levels in *C. roseus* seedlings and only MJ induced alkaloids, our observation for a longer contact period with these compounds resulted in up-regulation of the alkaloid biosynthesis.

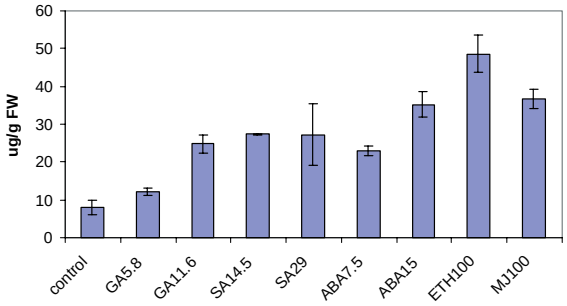

Figure 1. Effect of different growth regulators on ajmalicine biosynthesis in *C. roseus* seedlings. GA5.8: gibberellic acid (5.8 μM); GA11.6: gibberellic acid (11.6 μM); ABA7.5: abscisic acid (7.5 μM); ABA 15 abscisic acid (15 μM); SA14.5: salicylic acid (14.5 μM); SA29: salicylic acid (29 μM); ETH100: ethephon (100μM) and MJ100: methyljasmonate (100μM).

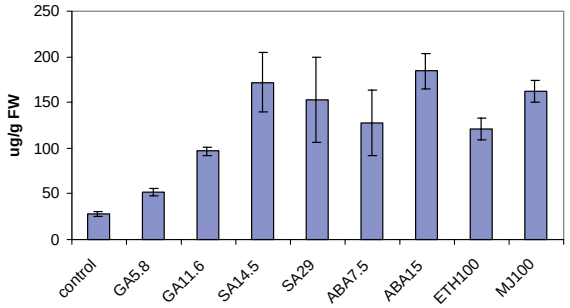

Figure 2. Effect of different growth regulators on serpentine biosynthesis in *C. roseus* seedlings.

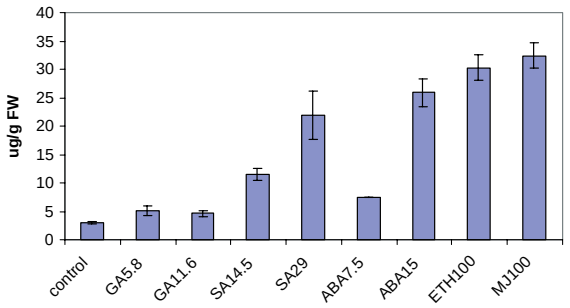

Figure 4. Effect of different growth regulators on tabersonine biosynthesis in *C. roseus* seedlings.

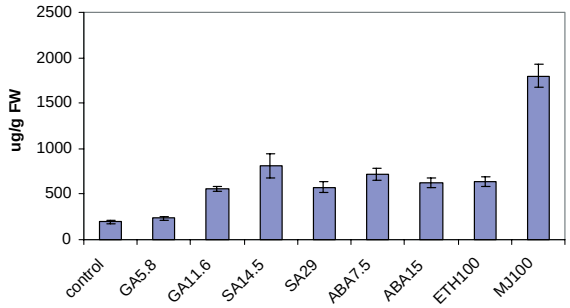

Figure 3. Effect of different growth regulators on catharanthine biosynthesis in *C. roseus* seedlings.

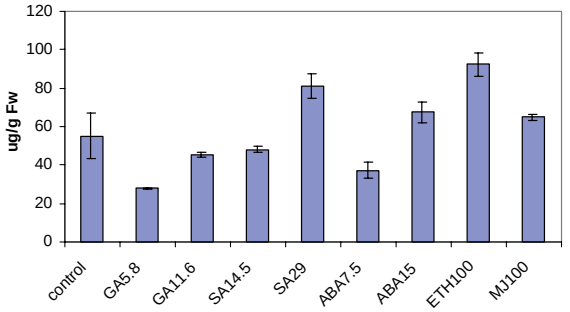

Figure 5. Effect of different growth regulators on vindoline biosynthesis in *C. roseus* seedlings.

ABA stimulates accumulation of the indole alkaloids catharanthine and ajmalicine in both flask and fermenter-scale systems (Smith et al. 1986). Also a combination of ABA and choleratoxin induced alkaloid accumulation 32-fold in *Sanguinaria canadensis* cell suspension cultures compared to choleratoxin application alone where the induction was 25-fold (Mahady et al. 1998). Application of acetylsalycilic acid to *C. roseus* cell suspension cultures increased the total alkaloids in both medium and cells (Godoy-Hernandez and Loyola-Vargas 1997). SA increased the amount of scopolamine in *Scopolia parviflora* without negative effects on growth (Kang et al. 2004).

Catharanthine biosynthesis was increased in a similar range in all treatments (2 to 3-fold) except for GA at 5.8 μM where no effect was observed and MJ which caused a 9-fold increase (Figure 3). The latter observation is similar as found by Aerts et al. (1996) that catharanthine content increased 203% in *C. roseus* seedlings exposed to MJ.

GA did not affect the biosynthesis of tabersonine. SA, ABA, ethylene and MJ treatments resulted in high accumulation of tabersonine ranging from 3.5-fold to 11-fold (Figure 4). MJ highy increased the tabersonine contents in *C. roseus* seedlings to 309% (Aerts et al. 1996). MJ vapor significantly enhanced alkaloid synthesis in the seedlings of *Catharanthus* and *Cinchona* and increased enzyme activities. Combination of MJ and tryptophan did not result in further increase in alkaloids (Aerts et al. 1994).

Vindoline biosynthesis pattern was more or less similar to that of tabersonine. Vindoline content was reduced in GA (5.8 μM) treatment and no effect of GA (11.6 μM) and SA (14.5 μM) was observed. The highest content of vindoline was obtained in ethylene treatment where a 2-fold increase was recorded (Figure 5). Low stimulation of vindoline by SA is in consistent with the results obtained from transformed roots of *Atropa belladonna* where 35% induction was observed (Lee

et al. 2001). Aerts et al. (1996) found low induction of vindoline by MJ compared to other alkaloids.

Effect of growth regulators on enzyme activities

The continuous availability of growth regulators in the growth media did not affect the activity of strictosidine glucosidase except for MJ. Three-fold increase in glucosidase activity was found when seedlings were grown in a medium containing MJ (Figure 6). The increasing content of different types of alkaloids in the seedlings treated with different growth regulators and the lack of effect of most of these growth regulators on strictosidine glucosidase suggest that this enzymatic step is not limiting in the pathway. In *C. roseus*, Vázquez-Flota and De Luca (1998) reported that MJ treatment induced tryptophan decarboxylase and desacetylvindoline 4-hydroxylase but SA was ineffective in activating both of these enzymes.

Basic peroxidase activity was increased in all treatments. The highest activity was observed in SA (14.5 μM) where a 5-fold increase was recorded (Figure 7). These activities resulted in an increase in the serpentine content in most of treatments as basic peroxidase is involved in conversion of ajmalicine to serpentine.

Figure 8 summarizes the induction of different alkaloid pathways and some connected enzymes by application of growth regulators to *C. roseus* seedlings. MJ may be considered a general inducer, as it appears to up-regulate all intermediates and products examined in this study. It is also known that MJ itself induced *Tdc* and *Str* gene expression when added exogenously to *C. roseus* cell suspension culture (Menke et al. 1999).

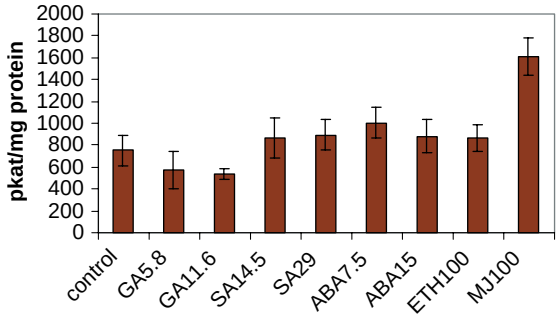

Figure 6. Influence of different growth regulators on strictosidine glucosidase activity of *C. roseus* seedlings.

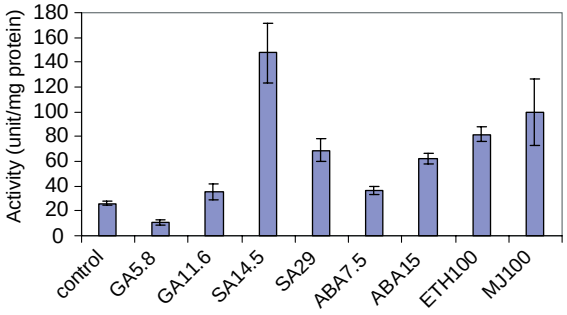

Figure 7. Influence of different growth regulators on basic peroxidase activity of *C. roseus* seedlings.

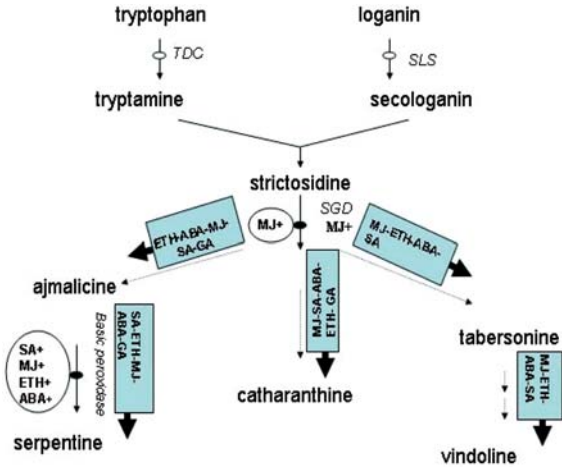

Figure 8. Induced different alkaloid pathways after strictosidine by growth regulators. *TDC: tryptophan decarboxylase*; *SLS:secologanin synthase*; *SGD: strictosidine glucosidase*. *enzymatic step* ○ *product step*.

Conclusions

Application of growth regulators to *C. roseus* seedlings affected the growth and the alkaloid productivity. Our results from continuous availability of growth regulators are different from those obtained by Aerts et al. 1996 for short time treatments. MJ is a fast and long response inducer of terpenoid indole alkaloids. SA, ABA, ethylene that in most studies were found to have no effect, induced different pathways to different classes of alkaloids. Gibberellins have no or weak induction effect on *Catharanthus* alkaloid biosynthesis. Strictosidine glucosidase activity was induced by MJ but not affected by other growth regulators although these regulators highly promoted some

alkaloids. Increased basic peroxidase activity was found in the treatments of SA, ethylene and MJ.

References

Aerts R.J., Gisi D., De Carolis E, De Luca V. and Baumann T.W. 1994. Methyl jasmonate vapor increases the developmentally controlled synthesis of alkaloids in *Catharanthus roseus* and *Cinchona* seedlings. Plant J. 5: 635–643.

Aerts R.J., Schafer A., Hesse M., Baumann T.W. and Slusarenko A. 1996. Signalling molecules and the synthesis of alkaloids in *Catharanthus roseus* seedlings. Phytochemistry 42: 417–422.

Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

De Luca V., Balsevich J., Tyler R.T., Eilert U., Panchuk B.D. and Kurz W.G.W. 1986. Biosynthesis of indole alkaloids: developmental regulation of the biosynthetic pathway from tabersonine to vindoline in *Catharanthus roseus*. J. Plant Physiol. 125: 147–156.

De Luca V., Fernandez J.A., Campbell D. and Kurz W.G.W. 1988. Developmental regulation of enzymes of indole alkaloid biosynthesis in *Catharanthus roseus*. J. Plant Physiol. 86: 447–450.

El-Sayed M. and Verpoorte R. 2002. Effect of phytohormones on growth and alkaloid accumulation by a *Catharanthus roseus* cell suspension cultures fed with alkaloid precursors tryptamine and loganin. Plant Cell Tiss. Org. Cult. 68: 265–270.

Geerlings A., Memelink J., van der Heijden R. and Verpoorte R. 2000. Molecular cloning and analysis of strictosidine β-D-glucosidase, an enzyme in terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. J. Biol. Chem. 275: 3051–3056.

Godoy-Hernandez G. and Loyola-Vargas V.M. 1997. Effect of acetylsalicylic acid on secondary metabolism of *Catharanthus roseus* tumor suspension cultures. Plant Cell Rep. 16: 287–290.

Kang S., Jung H., Kang Y., Yun D., Bahk J., Yang J. and Choi M. 2004. Effects of methyl jasmonate and salicylic acid on the production of tropane alkaloids and the expression of PMT and H6H in adventitious root cultures of *Scopolia parviflora*. Plant Sci. 166: 745–751.

Kargi F 1988. Alkaloids formation by *Catharanthus roseus* cells in a packed column biofilm bioreactor. Biotechnol. Lett. 10: 181–186.

Lee K., Hirano H., Yamakawa T., Kodama T., Igarashi Y. and Shimomura T. 2001. Responses of transformed root cultures of *Atropa belladonna* to salicylic acid stress. J. Biosci. Bioeng. 91: 586–589.

Luijendijk T., Stevens L.H. and Verpoorte R. 1998. Purification and characterization of strictosidine β-D-glucosidase from *Catharanthus roseus* cell suspension cultures. Plant Physiol. Biochem. 36: 419–425.

Mahady G.B., Liu C. and Beecher C.W. 1998. Involvement of protein kinase and G proteins in the signal transduction of benzophenanthridine alkaloid biosynthesis. Phytochemistry 48: 93–102.

Maehly A.C. and Chance B. 1954. The assay of catalases and peroxidases. Meth. Biochem. Anal. 1: 357–424.

Menke F.L.H., Parchmann S., Mueller M.J., Kijne J.W. and Memelink J. 1999. Involvement of the Octadecanoid Pathway and Protein Phosphorylation in Fungal Elicitor-Induced Expression of Terpenoid Indole Alkaloid Biosynthetic Genes in *Catharanthus roseus*. Plant Physiol. 119: 1289–1296.

Murashige T. and Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol. 15: 473–497.

Smith J.I., Smart N.J., Kurz W.G.W. and Misawa M. 1987. Stimulation of indole alkaloid production in cell suspension cultures of *Catharanthus roseus* by abscisic acid. Planta Med. 53: 470–474.

Stevens L.H., Schripsema J., Pennings E.J.M. and Verpoorte R. 1992. Activities of enzymes involved in indole alkaloid biosynthesis in suspension cultures of *Catharanthus*, *Cinchona* and *Tabernaemontana* species. Plant Physiol. Biochem. 30: 675–681.

Vázqyuez-Flota F. and De Luca V. 1998. Jasmonate modulates development- and light-regulated alkaloid biosynthesis in *Catharanthus roseus*.. Phytochemistry 49: 395–402.

Verpoorte R., van der Heijden R., Van Gulik W.M. and ten Hoopen H.J.G. 1991. Plant biotechnology for the production of alkaloids: present and prospects. In: Brossi A. (ed.), The Alkaloids. Vol. 40. Academic Press, San Diego.

Verpoorte R., van der Heijden R. and Moreno P.R.H. 1997. Biosynthesis of terpenoid indole alkaloids in *Catharanthus roseus* cells. In: Cordell G.A. (ed.), The Alkaloids Vol. 49. Academic Press, pp. 221–299.

Yahia A., Kevers C., Gaspar T., Chénieux J., Rideau M. and Créche J. 1998. Cytokinins and ethylene stimulate indole alkaloid accumulation in cell suspension cultures of *Catharanthus roseus* by two distinct mechanisms. Plant Sci. 133: 9–15.