COMMUNICATION TO THE EDITOR

Quantification of Metabolites in the Indole Alkaloid Pathways of *Catharanthus roseus:* Implications for Metabolic Engineering

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Abstract: In this article, we present a review of the current state of metabolic engineering in *Catharanthus roseus.* A significant amount of research has contributed to characterization of several individual steps in the biosynthetic pathway of medicinally valuable alkaloids. However, knowledge of the regulation of these pathways is still sparse. Using hairy root cultures, we studied the responses of alkaloid metabolism to environmental stimulation such as light and elicitation. Through precursor feeding studies, the putative rate-limiting steps of the terpenoid pathway in hairy root cultures also have been examined. Relating this knowledge to specific events at the molecular level, and the cloning of corresponding genes are the next key steps in metabolic engineering of the *C. roseus* alkaloids. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng* **58:** 333–338, 1998.

Keywords: *Catharanthus roseus;* hairy roots; indole alkaloids; tabersonine; elicitation

INTRODUCTION

Metabolic engineering of plant cell and tissue cultures for enhanced production of pharmaceutically valuable alkaloids has promise due to recent advances in plant molecular biology. A growing body of knowledge exists in the cloning of genes from alkaloid biosynthetic pathways, in their expression by sense and anti-sense techniques, and in gene activation and gene expression studies in response to environmental perturbations such as elicitation and light (Kutchan, 1995). However, rational genetic manipulation of an alkaloid-producing pathway requires determination of the points in the pathway at which flux is most severely restricted. For identification of these points, sufficient understanding of the biochemistry and regulation of the pathway under consideration is necessary. Over two decades of work has gone into the biosynthesis and regulation of the indole alkaloid pathways of the periwinkle, *Catharanthus roseus* (van der Heijden et al., 1989). Thus, it may be one of the best systems to test the feasibility of the metabolic engineering of alkaloids. At present, however, only one gene of a key step has been over-expressed in *C. roseus,* but indole alkaloid production did not increase (Goddijn et al., 1995). This result may be due to the complex interactions between biosynthetic pathways in which several steps in the network share flux control (Kacser and Burns, 1973).

At this stage, an iterative approach must be used to try to optimize production. Flux into the alkaloid pathways, diversion of flux at intermediate branches, and lack of final conversion at the end of a specific branch all appear to affect alkaloid production in *C. roseus* cell and tissue culture. Experiments employing light adaptation and genetic modification of a single step are useful in gaining information about the pathways. Perturbation-response type experiments, such as adding enzyme inhibitors, elicitors, and precursors are also useful in trying to pinpoint rate-limiting branches and develop a ''working model'' (Srinivasan et al., 1996) of the pathways. These steps then can be pursued for genetic modification, and further studies can be targeted to obtain additional information. Clearly, iteration of these procedures is necessary. In this article, we will describe the challenges in metabolic engineering of *C. roseus* for indole alkaloid production, and present a review of our work of quantifying several alkaloids simultaneously to identify points of possible flux limitation. Uniquely, we analyzed the response of the levels of several alkaloids in different branches of the pathways in response to light adaptation, precursor feeding, and fungal elicitation.

INDOLE ALKALOID TARGETS AND PATHWAYS

Catharanthus roseus produces a wide range of indole alkaloids as a part of its secondary metabolism. Figure 1 is a

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simplified schematic of the biosynthetic pathways of the terpenoid indole alkaloids. Six alkaloids can be viewed as production targets. Four are obtained commercially from the intact plant: the powerful antineoplastic agents, vinblastine and vincristine, and the anti-hypertensive agents, ajmalicine and serpentine. Two other possible targets are catharanthine and vindoline, which can be coupled enzymatically in vitro to form vinblastine and vincristine (Kutney et al., 1988; Pennanen and Huhtikangas, 1990). The low yield of these valuable indole alkaloids in plants has been the major motivation to produce them by cell and tissue cultures. However, vinblastine and vincristine have not been able to be produced in an economically viable cell or tissue culture bioprocess. The absence of vindoline in cell suspensions and hairy root cultures is the key limitation. Ajmalicine, serpentine, and catharanthine can be produced in cell and tissue cultures, but at levels too low for commercialization. Metabolic engineering may aid in the overproduction of these alkaloid targets.

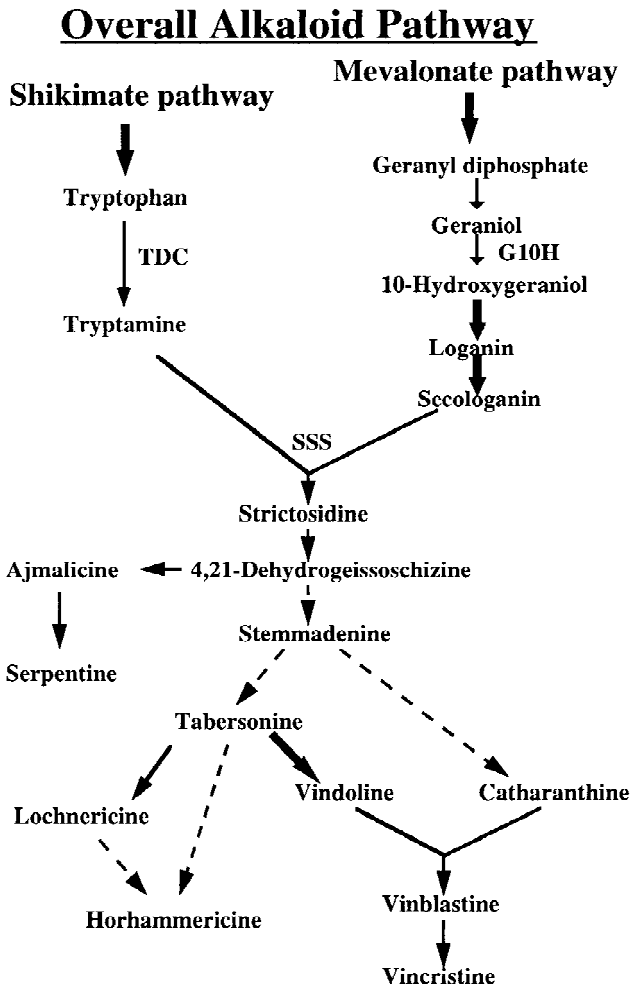

Figure 1. Schematic diagram of the biosynthesis of indole alkaloids in *Catharanthus roseus.* Thin arrows represent single enzymatic steps; thick arrows represent multiple steps; dashed arrows represent uncharacterized steps. Abbreviations: SSS, Strictosidine Synthase; TDC, Tryptophan Decarboxylase; G10H, Geraniol 10-hydroxylase.

The complexity of the genetic, catalytic, and transport processes of the terpenoid indole alkaloid pathway presents a formidable challenge to the metabolic engineering of these compounds. Although over 30 steps are involved in the synthesis of these six alkaloids, only 16 enzymes have been characterized, and the cloning of three genes has been reported (Meijer et al., 1993b). Subcellular compartmentation of enzymes—tissue-specific and developmental control—and environmental factors such as light and fungal elicitors are implicated in the regulation of these pathways.

Identification, characterization, and regulation of the genes and enzymes of the indole alkaloid pathways has been reviewed in detail by Meijer et al (1993b). As shown in Figure 1, the shikimate and mevalonate pathways from primary metabolism feed the terpenoid indole alkaloid pathways. Tryptamine is produced from tryptophan by the enzyme tryptophan decarboxylase (TDC), which has been cloned (De Luca, 1989). Secologanin is formed through the terpenoid pathway, and a key enzyme to flux limitation in this branch is thought to be geraniol 10-hydroxylase (G10H). The provacuolar location of G10H has made the cloning of this cytochrome P-450 enzyme difficult. The co-enzyme to G10H, a NADPH:cytochrome P-450 reductase, has been cloned (Meijer et al., 1993a). The indole and terpenoid pathways converge with the condensation of tryptamine and secologanin to form strictosidine, the central precursor of all terpenoid indole alkaloids. This step is catalyzed by the enzyme strictosidine synthase (SSS), which has been cloned (Kutchan et al., 1988). The current hypothesis is that the terpenoid branch is more limiting than tryptamine formation (Moreno et al., 1993). Further evidence in support of this hypothesis was a study in which TDC was overexpressed by an oncogenic transformation, and no increase in alkaloid production downstream was observed (Goddijn et al., 1995).

The intermediate branchpoints leading to the different classes of alkaloids are not as well-defined. Considerable progress has been made in characterizing the tabersonine to vindoline branch. The metabolic engineering of this branch is perhaps the most critical for commercial success of vinblastine production in plant cell and tissue culture. Catharanthine and vindoline are coupled by a peroxidase to form vinblastine. Many cell and hairy root cultures produce catharanthine and tabersonine, and thus the limitation exists in the conversion of tabersonine to vindoline. Five of the six enzymes in the tabersonine to vindoline branch have been characterized (St.-Pierre and De Luca, 1995), and progress is being made in cloning steps in this pathway (De Carolis and De Luca, 1993).

CATHARANTHUS ROSEUS HAIRY ROOTS

The potential of hairy roots of *C. roseus* as a production system for indole alkaloids has been a subject of investigation since an initial report in a review by Flores in 1987 (Flores et al., 1987). Hairy roots tend to be genetically and biochemically stable, and possess a higher level of differ-

entiation compared to cell suspensions, and are amenable to genetic transformation. In an early study, Parr et al. (1988) reported an intriguing result: the detection by immunoassay of a low level of vinblastine in *C. roseus* hairy root cultures (Parr et al., 1988). The differentiated characteristics of the hairy roots were speculated to perhaps have allowed vindoline to be synthesized. To date, however, several other investigations of *C. roseus* hairy root cultures (Bhadra and Shanks, 1997; Brillanceau et al., 1989; Ciau-uitz et al., 1994; Davioud et al., 1989; Jung et al., 1992; Toivonen et al., 1989) have not demonstrated evidence for vindoline or vinblastine. Nonetheless, hairy root cultures are a stable system for performing a series of metabolic studies.

We established hairy root clones of *C. roseus* by the infection of seedlings of Little Bright Eye with *Agrobacterium rhizogenes* ATCC 15834 (Bhadra et al., 1991; Bhadra et al., 1993). The fastest growing clones are characterized by thin, straight, and regular branches with thin tips in liquid culture. The growth kinetics and transient alkaloid profiles of this clone has been described (Bhadra and Shanks, 1997), and successful scale-up to 1 L has been achieved (Vani, 1996, Ph.D. thesis).

ANALYSIS OF INDOLE ALKALOID COMPOUNDS

In quantification of indole alkaloids, two conflicting constraints exist. Identification of compounds implies isolation of a pure compound, in large enough amounts, for standard chemical analysis. However, the desire for transient measurements of several compounds requires a method for rapid analysis. Consequently, we used a two-step approach. We identified several indole alkaloids using mass spectrometry and HPLC photodiode array (PDA) detection of TLC separated compounds. We used HPLC PDA detection for more rapid quantification of compounds in transient experiments. With the PDA detector, the purity of a peak can be estimated. Because of the large number of alkaloids present in our root cultures (Vani, 1996), determination of the UV-VIS spectrum by PDA makes peak identification more reliable than just relying upon retention time.

Analysis of alkaloid extracts revealed the presence of several expected compounds: ajmalicine, serpentine, catharanthine, tabersonine, hörhammericine, and lochnericine (Vani, 1996). Hörhammericine and lochnericine are two tabersonine-derived compounds, and thus represent a diversion of flux away from the tabersonine to vindoline branch (see below). Akuammine, which had not been reported previously in either cell or hairy root cultures, was also present (Vani, 1996). Notably absent were vindoline and vinblastine.

Akuammine is *N*-methylated at an analogous position to vindoline, and thus may indicate the presence of an enzyme related to the *N*-methyl transferase involved in the tabersonine to vindoline pathway. Earlier, we had obtained a mass spectrum of a compound at the retention time of vindoline that had a fragment consistent with a *N*-methylated fragment (Bhadra et al., 1993). However, we tested the extracts for deacetylvindoline, the *N*-methylated precursor of vindoline, and the result was negative. Furthermore, the first two compounds between tabersonine and vindoline, 16-hydroxytabersonine and 16-methoxytabersonine, were not found in the extracts. The absence of these two compounds in roots could be due to the lack of a specific monooxygenase demonstrated to catalyze the formation of 16-OH tabersonine (St.-Pierre and De Luca, 1995), or to a low activity of this enzyme relative to the enzymes converting tabersonine to lochnericine and hörhammericine (see below).

In the HPLC chromatogram, the peaks for ajmalicine, serpentine, tabersonine, hörhammericine, and lochnericine were essentially pure, and thus were reliably quantified. Unfortunately, several attempts to obtain a resolved catharanthine peak by changing HPLC conditions was not successful due to co-eluting compounds. Presently, work is in progress to develop an independent method for the analysis of catharanthine. The quantities of ajmalicine and serpentine in our roots, typical maximum yields of 5 mg/L and 10 mg/L at 3% sucrose and B5/2 media (Bhadra and Shanks, 1997), are within what was reported previously (Toivonen et al., 1989).

In contrast to ajmalicine and serpentine, quantification of tabersonine, hörhammericine, and lochnericine has not been previously reported for *C. roseus* hairy root cultures. The combined specific yields of hörhammericine and lochnericine range from 5–15 times the level of tabersonine (Fig. 2), and thus reflect a significant diversion of flux away from the tabersonine to vindoline branch. Figure 2 shows the transient profiles of the specific yields of tabersonine, hörhammericine, and lochnericine for cultures in 3% sucrose, B5/2 media. Tabersonine levels decline with the onset of stationary phase of the cultures, while lochnericine and hörhammericine accumulate. Lochnericine accumulates to higher levels than either tabersonine or hörhammericine, and declines later, while hörhammericine continually increases.

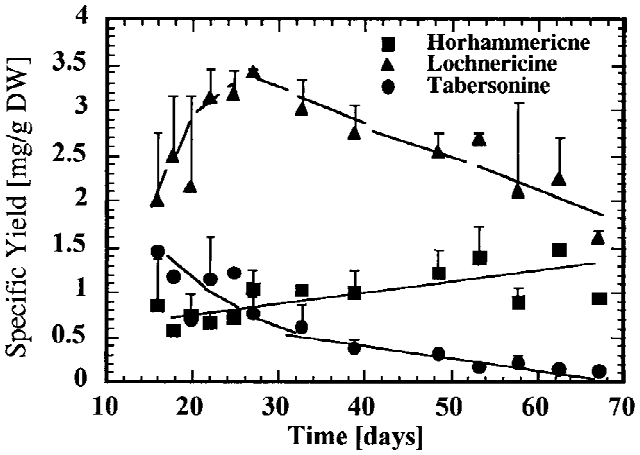

Figure 2. Transient accumulation profiles of specific yields of tabersonine, lochnericine, and hörhammericine in hairy roots. Hairy roots were cultured in the dark at 26°C in media containing 3% sucrose and Gamborg's B5/2 salts. Error bars represent the standard deviation of at least two data points.

The exact pathway and enzymes that convert tabersonine to lochnericine and hörhammericine are unknown. Hörhammericine may be derived from tabersonine (Kutney et al., 1980) or possibly through lochnericine (Vani, 1996).

METABOLIC MANIPULATIONS

Light

Light is thought to affect both enzyme induction and activity. In cell and tissue culture, light reduces ajmalicine accumulation while stimulating serpentine and catharanthine production (Loyola-Vargas et al., 1992; van der Heijden et al., 1989). NMT activity is also associated to thylakoid membranes in the chloroplast (Dethier and De Luca, 1993), and three other enzymes in the tabersonine to vindoline pathway are light inducible (St.-Pierre and De Luca, 1995). Therefore, one possible strategy we tried was developing ''green'' hairy roots, and analyzing if tabersonine could be converted to vindoline, or an intermediate in the tabersonine-to-vindoline branch.

Catharanthus roseus hairy root clone LBE-6-1 was adapted to light through successive generations of increased photoperiod and reduced exogenous sugar (Shanks and Bhadra, 1997). Light-adapted hairy roots were maintained on 2% sucrose. These cultures exhibited green pigmentation, restricted mainly to sections of older roots located in the center of the bunch (Shanks and Bhadra, 1997). Analysis of extracts of the light-adapted cultures did not reveal the presence of vindoline, deacetylvindoline, 16-hydroxytabersonine, or 16-methoxytabersonine (Bhadra et al., submitted for publication). As shown in Figure 3, light-adapted cultures had increased specific yields of ajmalicine and serpentine, while tabersonine levels were suppressed as compared to dark-grown controls. This result could imply that tabersonine either was not synthesized or was diverted to other compounds. We have preliminary evidence that indicates tabersonine, hörhammericine, and lochnericine levels are all depressed in light adapted cultures (Bhadra et al., submitted for publication). Interestingly, while light is necessary for induction of later stages of vindoline biosynthesis, it apparently reduces flux to tabersonine in roots.

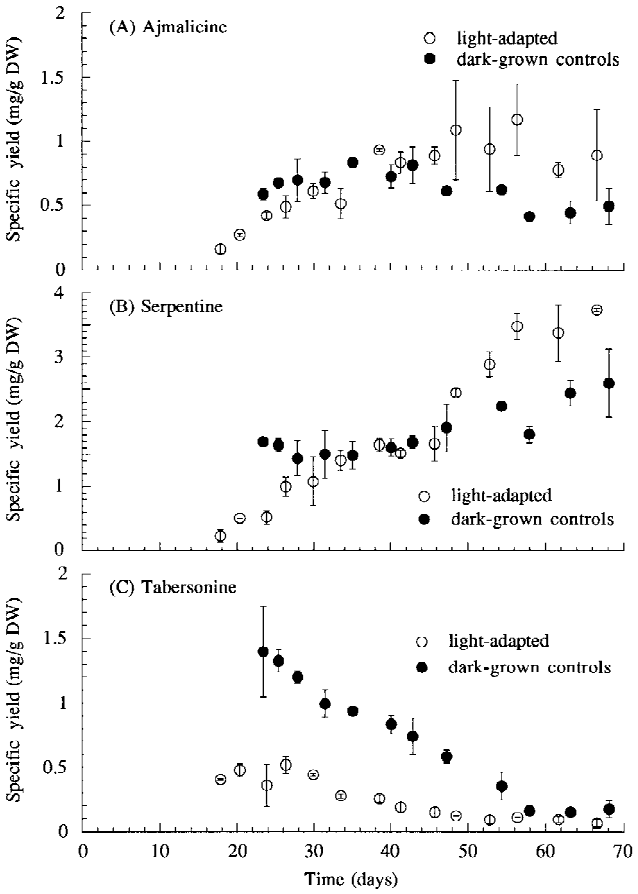

Figure 3. Specific yields (mg/ g DW) of (A) ajmalicine, (B) serpentine, and (C) tabersonine in heterotrophic and light-adapted cultures. Hairy roots were cultured heterotrophically in 2% sucrose and Gamborg's B5/2 salts. Light-adapted cultures were maintained in the same at a photoperiod of 12 h d^{-1}. Data are the mean of at least two data points, and error bars indicate standard deviation.

Precursor Feeding

Precursor feeding is a method that is used to probe rate-limiting steps by increasing the substrate supply. By identifying precursors, which after feeding significantly enhance the production of alkaloids, the specific part of the pathway that may have flux limitations can be identified. Enhanced enzyme levels would not be expected to occur in these experiments. In separate experiments, each with a control, four precursors, mevalonic acid, geraniol, 10-hydroxygeraniol, and loganin were fed to stationary phase cultures (refer to Figure 1). The relative changes in alkaloid accumulation in response to precursor feeding are shown in Table I. The addition of loganin, a precursor from the terpenoid portion of the pathway was found to enhance the level of tabersonine, while feeding mevalonic acid had no effect. These results corroborated the findings in similar studies using cell suspension cultures (Moreno et al., 1993).

Table I. Percent change in alkaloid accumulation, relative to controls, upon feeding precursors to early stationary-phase hairy root cultures. Cultures were harvested 72 h after addition of precursor.

Precursor-fed Alkaloid	Mevalonic Acid	Geraniol	10-hydroxygeraniol	Loganin
Tabersonine	5	66[a]	50[a]	55[a]
Hörhammericine	12	−24[a]	−8	−24[a]
Lochnericine	6	28	8	6
Ajmalicine	12	11	−10	10
Serpentine	3	−14	−7[a]	3

[a]Indicates statistically significant difference ($p < 0.05$) between treatment and control ($n = 3$).

The unique portion of this study, however, was feeding geraniol and 10-hydroxygeraniol, the substrate and product of a proposed rate limiting enzyme. Interestingly, while the addition of equal amounts of these precursors increased tabersonine accumulation ($p < 0.05$), there was no significant difference between the individual treatments. This result suggests that in hairy roots, flux limitations in the terpenoid pathway may occur upstream of geraniol (Morgan and Shanks, submitted for publication).

Fungal Elicitation

In contrast to precursor feeding studies, addition of fungal elicitors is expected to induce enzyme levels in the indole alkaloid pathway. The mechanism of fungal elicitation for induction of enzymes in response to elicitors is not completely understood (Whitehead and Threlfall, 1992). Therefore, we chose to elicit with well-defined elicitors as compared to crude fungal homogenates. The three elicitors chosen were pectinase, an endopolygalacturonase; chitin, a component of fungal cell walls; and jasmonic acid, a signal transducer in the defense response.

These elicitors were added to late exponential-phase hairy root cultures, which were harvested 48 h later. Selective effects on indole alkaloid yields were observed upon elicitation. Pectinase increased levels of tabersonine approximately 2.5 times the control, but had no significant effect on the other alkaloids. Chitin selectively enhanced ajmalicine levels (by 50%), but no significant effect was observed on the other alkaloids. In contrast, addition of jasmonic acid affected all of the alkaloid levels (Rijhwani and Shanks, submitted for publication). Jasmonic acid increased the specific yields of ajmalicine by 80% and serpentine by 60% (data not shown). The effect of jasmonic acid on the tabersonine branchpoint alkaloids is shown in Figure 4. Lochnericine and hörhammericine levels increased with increasing jasmonic acid concentration. Tabersonine declined at lower levels of jasmonic acid then increased with increasing jasmonic acid concentration (Rijhwani and Shanks, submitted for publication).

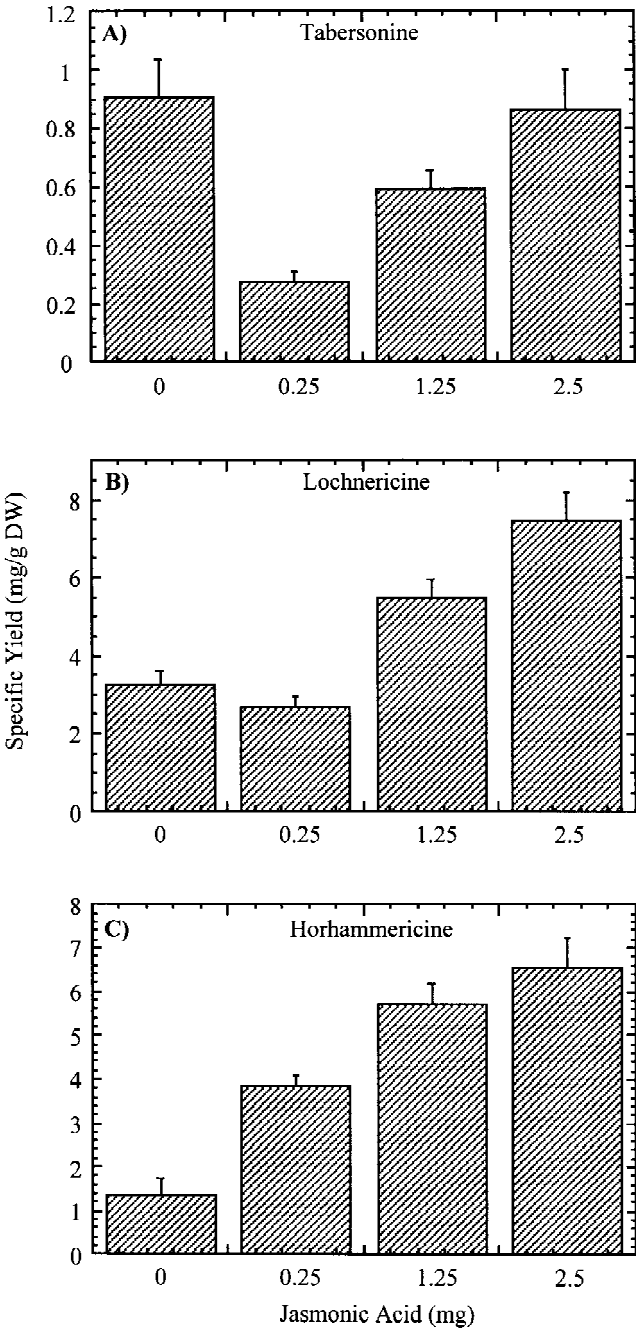

Figure 4. Specific yield of (A) tabersonine, (B) lochnericine, and (C) hörhammericine vs. jasmonic acid concentration. Late exponential stage cultures were elicited with varying concentrations of jasmonic acid. Cultures were harvested in triplicate, 48 h after the addition of elicitor.

CONCLUSIONS AND FUTURE DIRECTIONS

The key drawback of hairy roots and cell suspension cultures at present is the lack of vindoline accumulation. Perhaps, genes in the tabersonine to vindoline pathway will eventually be cloned and then expressed. Using multicomponent measurements, we discovered that considerable flux is diverted at tabersonine into two ''undesired'' compounds, lochnericine and hörhammericine. A similar scenario likely occurs in plant cell suspension cultures (Furuya et al., 1992). Thus, for an economical bioprocess, this diversion of flux should be blocked. Insertion of the tabersonine 16-hydroxylase gene may allow this to occur, depending on the relative activities of the other enzymes surrounding tabersonine. Therefore, the enzymology and molecular biology surrounding the tabersonine branchpoint is an area worthy of investigation. Also critical to the pinpointing of targets for metabolic engineering will be understanding the molecular controls involved in light and elicitor perception, cell signaling, and the consequent expression of specific enzymes involved in alkaloid biosynthesis.

Standards of catharanthine and vindoline were gifts from Eli Lilly & Co.; tabersonine was a gift from Prof. H. Hamada, Okayama University, Japan; deacetylvindoline was a gift from Prof. V. De Luca, Montreal.

References

Bhadra, R., Ho, C., Sharks, J. V. 1991. Growth characteristics of hairy root lines of *Catharanthus roseus.* In vitro Cell Dev Biol **27**: 290A.

Bhadra, R., Shanks, J. V. 1997. Transient studies of nutrient uptake, growth and indole alkaloid accumulation in heterotrophic cultures of hairy roots of *Catharanthus roseus.* Biotechnol. Bioeng. **55**: 527–534.

Bhadra, R., Vani, S. N., Shanks, J. V. 1993. Production of indole alkaloids by selected hairy root lines of *Catharanthus roseus.* Biotechnol. Bioeng. **41**: 581–592.

Brillanceau, M., David, C. Tempe, J. 1989. Genetic transformation of *Catharanthus roseus* G. Don by *Agrobacterium rhizogenes.* Plant Cell Rep **8**: 63–66.

Ciau-uitz, R., Mirandaham, M. L., Coellocoello, J., Chi, B., Pacheco, L. M., Loyolavargas, V. M. 1994. Indole alkaloid production by transformed and nontransformed root cultures of *Catharanthus roseus.* In Vitro Cell Dev Biol-Plant **30P**: 84–88.

Davioud, E., Kan, C., Hamon, J., Tempe, J., Henri-philippe, H. 1989. Production of indol alkaloids by in vitro root cultures from *Catharanthus Trichophyllus.* Phytochemistry **28**: 2675–2680.

De Carolis, E., De Luca, V. 1993. Purification, characterization, and kinetic-analysis of a 2-oxoglutarate-dependent dioxygenase involved in vindoline biosynthesis from *Catharanthus roseus.* J Biol Chem **268**: 5504–5511.

De Luca, V., Marineau, C., Brisson, N. 1989. Molecular cloning and analysis of cDNA encoding a plant tryptophan decarboxylase: Comparison with animal dopa decarboxylases. Proc. Natl. Acad. Sci. **86**: 2582–2586.

Dethier, M., De Luca, V. 1993. Partial-Purification of an N-Methyltransferase involved in vindoline biosynthesis in *Catharanthus roseus.* Phytochemistry **32**: 673–678.

Flores, H. E., Hoy, M. W., Pickard, J. J. 1987. Secondary metabolites from root cultures. *Tibtech* **5**: 64–69.

Furuya, T., Sakamoto, K., Iida, K., Asada, Y., Yoshikawa, T., Sakai, S.-I., Aimi, N. 1992. Biotransformation of tabersonine in cell suspension cultures of *Catharanthus roseus.* Phytochemistry **31**: 3065–3068.

Goddijn, O., Pennings, E., van der Helm, P., Shilperoort, R., Verpoorte, R., Hoge, J. 1995. Overexpression of a tryptophan decarboxylase cDNA in *Catharanthus roseus* crown gall calluses results in increased tryptamine levels but not increased terpeniod alkaloid production. Transgenic Res **4**: 315–323.

Jung, K. H., Kwak, S. S., Kim, S. W., Lee, H., Choi, C. Y., Liu, J. R. 1992. Improvement of the catharanthine productivity in hairy root cultures of *Catharanthus roseus* by using monosaccharides as a carbon source. Biotechnol Lett **14**: 695–700.

Kacser, H., Burns, J. A. 1973. The control of flux. Symp. Soc. Exp. Biol. **27**: 65–105.

Kutchan, T. M. 1995. Alkaloid biosynthesis—The basis for metabolic engineering of medicinal plants. Plant Cell **7**: 1059–1070.

Kutchan, T. M., Hampp, N., Lottspeich, F., Beyreuther, K., Zenk, M. H. 1988. The cDNA clone for strictosidine synthase from *Rauyolfia serpentina:* DNA sequence determination and expression in *Escherichia coli.* FEBS Lett. **237**: 40–44.

Kutney, J. P., Boulet, C. A., Choi, L., Gustowski, W., McHugh, M., Nakano, I., Nikaido, T., Tsukamoto, H., Hewitt, G. M., Suen, R. 1988. Alkaloid production in (L.) G. Don cell cultures. XV. Synthesis of bisindole alkaloids by use of immobilized enzymes. Heterocycles **27**: 621–628.

Kutney, J. P., Choi, L. S. L., Kolodziejczyk, P., Sleigh, S. K., Stuart, K. L., Worth, B. R., Kurz, W. G. W., Chatson, K. B., Constabel, F. 1980. Alkaloid production in *Catharanthus roseus* cell cultures: Isolation and characterization of alkaloids from one cell line. Phytochemistry **19**: 2589–2595.

Loyola-Vargas, V. M., Mendez-Zeel, M., Monforte-Gonzalez, M., Miranda-Ham, M. L. 1992. Serpentine accumulation during greening in normal and tumor tissues of *Catharanthus roseus.* J. Plant Physiol **140**: 213–217.

Meijer, A. H., Cardoss, M., Voskuilen, J., de Waal, A., Verpoote, R., Hoge, J. 1993a. Isolation and characterization of a cDNA clone from *Catharanthus roseus* encoding NADPH: cytochrome P-450 reductase, an enzyme essential or reactions catalyzed by cytochrome P-450 monooxygenases in plants. Plant J **4**: 47–60.

Meijer, A. H., Verpoorte, R., Hoge, J. H. C. 1993b. Regulation of enzymes and genes involved in terpenoid indole alkaloid biosynthesis in *Catharanthus roseus.* J Plant Res. Special Issue, 145–164.

Moreno, R. P. H., van der Heijden, R., Verpoorte, R. 1993. Effect of terpenoid precursor feeding and elicitation of formation of indole alkaloids in cell suspension cultures of *Catharanthus roseus.* Plant Cell Rep **12**: 702–705.

Parr, A. J., Peerless, A. C. J., Hamill, J. D., Walton, N. J., Robins, R. J., Rhodes, M. J. C. 1988. Alkaloid production by transformed root cultures of *Catharanthus roseus.* Plant Cell Rep **7**: 309–312.

Pennanen, S., Huhtikangas, A. 1990. Photochemical one-pot synthesis of vinblastine and vincristine. Photochem and P **51**: 515–518.

Shanks, J. V., Bhadra, R. 1997. Characteristics of selected hairy root lines of *Catharanthus roseus,* pp. 51–63. In: P. Doran (ed.), Hairy roots. Harwood Academic, Amsterdam.

Srinivasan, V., Ciddi, V., Bringi, V., Shuler, M. L. 1996. Metabolic inhibitors, elicitors and precursors as tools for probing yield limitation in taxane production by *Taxus chinensis* cell cultures. Biotechnol. Prog. **12**: 457–465.

St.-Pierre, B., De Luca, V. 1995. A cytochrome P-450 monooxygenase catalyzes the first step in the conversion of tabersonine to vindoline in *Catharanthus roseus.* Plant Physiol **109**: 131–139.

Toivonen, L., Balsevich, J., Kurz, W. G. W. Indole alkaloid production by hairy root cultures of *Catharanthus roseus.* Plant Cell Tiss Org Cult **18**: 79–93.

van der Heijden, R., Verpoorte, R., Ten Hoopen, J. G. 1989. Cell and tissue cultures of *Catharanthus roseus* (L.) G. Don. a literature survey. Plant Cell Tiss. Organ Cult. **18**: 231–280.

Whitehead, I. M., Threlfall, D. R. 1992. Production of phytoalexins by plant tissue cultures. J. Biotechnol. **26**: 63–81.