Continuous Selective Extraction of Secondary Metabolites from *Catharanthus roseus* Hairy Roots with Silicon Oil in a Two-Liquid-Phase Bioreactor

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A two-liquid-phase bioreactor was designed to extract indole alkaloids from *Catharanthus roseus* hairy roots with silicon oil. Partition studies between silicon oil and culture medium showed that the silicon oil did not alter the availability of nutrients. The affinity of tabersonine and löchnericine for silicon oil is nine times higher than for the aqueous phase. Cultures were elicited with 25 mg/L of jasmonic acid. The growth of the hairy roots was not significantly modified by the presence of silicon oil. The overall specific yields of tabersonine and löchnericine were increased by 100−400% and 14−200%, respectively, with the use of silicon oil in nonelicited control cultures. In elicited cultures, these values were 10−55% for tabersonine and 20−65% for löchnericine. Serpentine was never found in the silicon oil. All measured alkaloids' specific yields were higher using silicon oil and elicitation, suggesting that the silicon oil, while acting as a metabolic sink for tabersonine and löchnericine, was efficient in increasing metabolic fluxes of the secondary metabolism pathways.

Introduction

Plant biotechnology has become an active field of study because of its potential as a source of new pharmaceutical compounds. Plants have been studied for their production of secondary metabolites as well as for the production of foreign molecules using recombinant DNA technology. For example, the genes encoding for the bacterial *Alcaligenes eutrophus* polyhydroxybutyrate acid (PHB) were successfully inserted into the canola plant (Monsanto, USA), and genes of diverse molecules of mammalian origin were also successfully inserted in plants (Daniell et al., 2001). Field culture seems economically attractive, but in vitro cultures represent important advantages because they are not exposed to diseases and pests, appear not to be subject to seasonal variations, and in the case of differentiated cell cultures, are genetically stable. Moreover, in vitro culture conditions highly simplify the validation approval for the production and the purification processes.

Many plant species have been evaluated for their potential to produce molecules of high pharmaceutical interest, and *Catharanthus roseus* (Madagascar periwinkle) is considered to be an interesting model system. *Catharanthus roseus* has been widely studied for its production of the anticancer drugs vinblastine and vincristine, as well as the antihypertensive compounds ajmalicine and serpentine. Many studies have been conducted to produce these compounds in vitro using *C. roseus* cell suspension cultures, but the absence of production of vindoline, a precursor of vinblastine, has always been reported (Moreno et al., 1995). The use of *Agrobacterium rhizogenes* transformed hairy root cultures was similarly unsuccessful (Van der Heijden et al., 1989; Toivonnen et al., 1989) except in rare lines of hairy roots of *C. roseus* (Parr et al., 1988). Serpentine and ajmalicine can be produced by hairy root cultures of *C. roseus* but only in small amounts, with a yield of approximately 1 mg per g dry weight of hairy roots (Rijwhani and Shanks, 1998). Other indole alkaloids were found in these cultures, such as löchnericine and hörhammericine. These compounds are believed to be tabersonine derivatives but unfortunately seem devoid of pharmaceutical value. St-Pierre et al. (1999) have shown that at least two essential enzymes involved in the pathway to vindoline are only expressed in the leaves of the plant. Moreover, vinblastine and vincristine biosynthesis requires at least two cell types, thus explaining that to date these alkaloids are still only produced commercially by extraction from whole plants (DiCosmo and Misawa, 1995). Successful production of these compounds from in vitro cultures might require genetic manipulations or metabolic engineering to bypass intercellular transport of precursors.

In contrast to other alkaloids such as sanguinarine produced by cell suspensions of *Papaver somniferum* (Archambault et al., 1996), indole alkaloids of *C. roseus* have never been detected in the culture medium (Bhadra et al., 1993) or in amounts as small as 2−5% of the total production (Toivonen et al., 1989). Because secondary metabolites are usually extracted from lyophilized plant tissues, their harvest is destructive to the culture and therefore limits the potential productivity of an industrial scale process. Nondestructive secondary metabolite extraction from culture medium has been studied using polymeric resins in situ. Williams et al. (1992) showed that the production level of total sanguinarine was even

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improved using Amberlite XAD-7 polymeric resins. The addition of polymeric resins to *C. roseus* suspension cell cultures has also been shown to increase the production of catharanthine and ajmalicine (Payne et al., 1998; Sim., 1994). Moreover, this extractive phase allowed the harvest of indole alkaloids known to remain intracellular. Another approach consists of the use of a nonaqueous liquid phase for the continuous extraction of secondary metabolites. A silicon oil antifoam was shown to accumulate benzophenanthridine in *Eschscholtzia californica* cell suspension culture (Byun and Pedersen, 1994). Tricaprilyn (1,2,3-trioctanoylglycerol) was shown to be efficient in accumulating and even enhancing the production of taxol in a *Taxus brevifolia* cell suspension culture (Collins-Pavao et al., 1996). The circulation of the culture medium in an external loop containing a nontoxic organic phase was also shown to be efficient for the extraction of secondary metabolites of *Hyoscyasmus muticus* hairy root cultures (Corry et al., 1993).

The literature presents extractive phases (solid and liquid) that are highly efficient with hydrophobic molecules such as secondary metabolites. However, the present study has investigated the use of a low affinity organic phase to selectively extract precursors and end secondary metabolites using an air-lift type bioreactor. The aim of this study was to evaluate the capacity of silicon oil (polymethyl siloxane) to continuously extract intracellular indole alkaloids from *C. roseus* hairy roots. The use of silicon oil was motivated by the fact that this low density organic phase may flow freely throughout a hairy root bed as compared to rigid adsorbent beads. Also, it was proposed that an organic phase would extract the intracellular indole alkaloids produced by *C. roseus* hairy roots. The experiments were conducted in lab-scale 1-L bioreactors. The differences in the primary and secondary metabolisms were also investigated.

Materials and Methods

Hairy Root Transformation and Cultures. Hairy roots of *C. roseus* (L.) G. Don were established as described by Bhadra et al. (1993) with the A4 strain of *A. rhizogenes*. Six fast-growing root lines were obtained (Tikhomiroff, 2001); root line LAO was used in this study. Hairy roots were transferred every month into Petri dishes with 20 mL of minimum medium (Bécard and Fortin, 1988) supplemented with 3% (w/v) sucrose, a 10-fold KH_2PO_4 (0.352 mM final), and a 3-fold $Ca(NO_3)_2$ (8.11 mM final) concentration.

Two-Liquid-Phase Bioreactor. On the basis of previous work by Jolicoeur et al. (1999), an airlift bioreactor was designed with a closed loop configuration allowing the use of a small quantity of silicon oil (Figure 1). The bioreactors were made with 1.2-L total volume (11 cm i.d. × 13 cm height) autoclavable polycarbonate jars (Nalgene, Sybron International, Rochester, NY) with a modified cover. The hairy roots were inoculated and immobilized in a 45 mm × 45 mm × 20 mm (height) 316 stainless steel screen mesh (mesh size 20) box placed 1 cm from the bottom of the reactor in the aqueous phase. The second, lighter phase was DC 200 silicon oil (Sigma-Aldrich, Oakville, ONT, Canada) with a density (ρ = 0.937) lower than that of water. Silicon oil was circulated with a peristaltic pump (Masterflex, Labcor, Anjou, QC, Canada) at a flow rate of 7 mL/min from the upper layer to the bottom of the bioreactor, under the root bed. The silicon oil loop, whose length was minimized, was made of viton, norprene, and stainless steel tubes (Masterflex). The silicon oil trickled up through the hairy root bed and returned to the upper layer. This configuration allowed both the silicon oil and the medium to be in continuous contact with the hairy roots. Air was fed through a porous (2 μm) stainless steel sparger, which generated fine bubbles at the bottom of the aqueous phase far from the root area at a rate of 50 mL/min (0.1 VVM). Air filters (bacterial air vent, Pall-Gelman, VWR Canlab, Ville Mont-Royal, QC, Canada) ensured sterility, and a water condenser minimized water losses by evaporation throughout the cultures' duration. The bioreactor was designed to handle 500 mL of culture medium and 150 mL of silicon oil to obtain a ratio of 23% (v/v), which is within the range (20−25% (v/v) of silicon oil) previously found optimal for a tricaprilyn two-liquid-phase bioreactor (Byun et al. 1990). Single-liquid-phase bioreactors with the same design but without silicon oil were used as controls. All stainless steel parts were cleaned as follows. Grid boxes and tubes were treated for 1 h in 1% (w/v) citric acid, thoroughly rinsed with water, treated for 1 h in 0.5% (w/v) NaOH and again thoroughly rinsed with water. Culture medium and bioreactors were steam sterilized separately for 35 min (121 °C, 1 bar). Cultures were grown at 23 ± 1 °C in the dark.

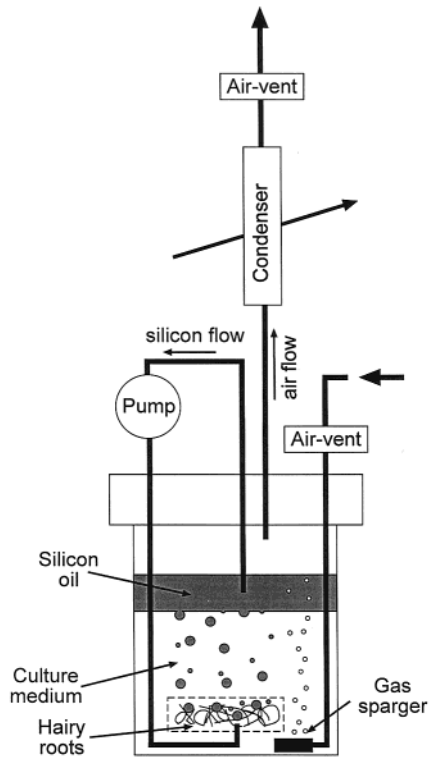

Figure 1. Two-liquid-phase bioreactor with 500 mL supplemented M medium (Bécard and Fortin, 1988) in a 1.2-L working volume. Hairy roots are inoculated in a stainless steel mesh box. A lighter phase of 150 mL of silicon oil is circulated at a flow rate of 7 mL/min from the upper layer to the bottom of the bioreactor, under the root bed. Air is provided at 50 mL/min by a 2-μm sparger at the bottom of the reactor. See Materials and Methods for details.

Ion Analysis. Major ions (Cl^-, NO_3^-, $H_2PO_4^-$, SO_4^{2-}, NH_4^+, K^+, Na^+, Ca^{2+}) were analyzed using a Dionex HPLC system (Dionex Canada Ltd, Oakville, ONT, Canada) equipped with an isocratic pump, an automated AS-3500 sampler, and a pulsed electrochemical detector in the conductivity mode, controlled by the Dionex AI-450 software for cations and the Dionex Peaknet software for anions. Anions were separated using a 4 mm × 250 mm Dionex IONPAC AS14A-SC analytical column, a Dionex IONPAC AG14A-SC guard column, and a Dionex ASRS-II anion self-regeneration suppressor to improve

the signal-to-noise ratio. The mobile phase consisted of an aqueous buffer of 2 mM Na_2CO_3/1 mM $NaHCO_3$ solution flowing at a rate of 1.0 mL/min. Cations were separated using a 4 mm × 250 mm Dionex IONPAC CS-12A analytical column, a Dionex IONPAC CG-12A guard column, and a Dionex CSRS-I cation self-regenerating suppressor. The mobile phase was an aqueous 20 mM methanesulfonic acid (Sigma-Aldrich) solution flowing at a rate of 0.9 mL/min.

Indole Alkaloid Extraction. To measure the concentration of indole alkaloids in hairy roots, fresh hairy roots were frozen at −80 °C and then lyophilized for 12 h in a commercial freeze-dryer (Lyph.Lock4.5, Labconco, Kansas City, MO) at pressures <1 Pa with a condenser refrigerated to −63 °C. Approximately 100 mg DW was blended with 1 mL of methanol (Fisher Scientific, Nepean, ONT, Canada) in a tissue grinder (VWR Canlab). The extract was centrifuged for 5 min and the supernatant was filtered through a PTFE 0.45-μm filter (Pall-Gelman) before HPLC analysis.

Alkaloids were extracted from 50 mL samples of silicon oil by addition of 50 mL of methanol and overnight agitation on an orbital shaker (150 rpm). Each silicon oil sample was extracted twice, and the pooled methanolic extract was isolated after centrifugation and evaporated under vacuum at 45 °C. As methanol is partially miscible in silicon oil, some silicon oil drops remained after evaporation of the methanol. The mixture of alkaloids and silicon oil was resuspended in 1 mL of methanol and centrifuged, and both phases were separated. The above procedure was repeated three times in order to obtain a silicon-oil-free methanolic extract, which was analyzed by HPLC. Alkaloids were extracted from the culture medium using the method described by Morris et al. (1985). Mass balances have shown that methanol completely recovers the alkaloids.

Indole Alkaloid HPLC Analysis. The HPLC analysis was performed using a Beckman Coulter (Mississauga, ONT, Canada) pump model 126, a Beckman Coulter auto-sampler model 508, a C18 guard column (Upchurch Scientific, Oak Harbor, WA), a Zorbax Eclipse XDB-C18 4.6 mm × 250 mm column, 3.5 μm (Hewlett-Packard, Missisauga, ONT, Canada), and a Beckman Coulter PDA detector 168. The alkaloids were separated on the column starting with an 80:20 mixture of 5 mM KH_2PO_4, pH = 6/ACN, decreasing linearly in 20 min to a final 20:80 mixture at a flow rate of 2.0 mL/min. Peaks were identified by comparison of UV spectra and retention times of standards of serpentine, tabersonine, catharanthine, vinblastine, and vincristine. Only small amounts of pure löchnericine were available, and thus the quantification of löchnericine was not possible. Löchnericine quantities are given in arbitrary units, proportional to the area of the chromatrographic peak. The method development has been previously described (Tikhomiroff and Jolicoeur, 2002).

Partition Studies of Silicon Oil. For ion partition studies, four replicates each of 20-mL samples of fresh culture medium at pH 5, 6, and 7 were mixed with 6 mL of silicon oil in 50-mL tubes (ratio of 23% (v/v)). The mixtures, along with blanks without silicon oil, were agitated overnight on a shaker (150 rpm) and centrifuged, and the aqueous phase was analyzed by HPLC. Jasmonic acid partition studies were performed as described above for the culture medium using 250 and 500 mg/L jasmonic acid concentrations in the aqueous phase. Jasmonic acid concentration was measured with a spectrophotometer at 285 nm (DU 640, Beckman Coulter, Missisauga, ONT, Canada). For alkaloid partition studies, a methanolic extract of alkaloids was first obtained as previously described. Then the extract was evaporated under N_2 atmosphere and resuspended in a mixture of 15:85 tetrahydrofurane/methanol. Next, 3 mL of the extract, along with 200 μL tabersonine standard (4.59 g/L) were incorporated into 120 mL of silicon oil. The tabersonine standard was added to increase the intrinsically low concentration in the extract. The silicon oil mixture was sonicated for 5 min until all of the extract was dissolved. In 50-mL tubes, 6 mL of silicon oil mixture was added to four replicates each of fresh culture medium at pH 5, 6, and 7. Addition of silicon oil did not significantly modify the pH of the aqueous phase. The mixtures, along with blanks without medium, were agitated overnight on a shaker (150 rpm). Each phase was isolated and treated for alkaloid extraction.

Oxygen Mass Transfer Measurement. The $k_L a$ of the bioreactors were measured in triplicate by the gassing (air) method using a polarographic, temperature-compensated, dissolved oxygen probe (Ingold, Urdorf, Switzerland). Degassing was performed using N_2 gas fed at the same flow rate as air. No hairy roots were inoculated for the measurement.

Inoculation of the Bioreactors. All bioreactors were inoculated with 5 g fresh weight (FW) of late exponential phase *C. roseus* hairy roots in 500 mL of M medium supplemented with 3% sucrose (w/v) and a 3-fold KH_2PO_4 (0.105 mM final) salt concentration. The LAO root line was selected for its high maximum specific growth rate (∼0.12 d^{-1}) in this medium (Tikhomiroff, 2001). Seven single-liquid-phase and seven two-liquid-phase bioreactors were inoculated.

Elicitation of the Hairy Roots. The bioreactors were elicited with 5 mL of a 2.5 g/L jasmonic acid solution in ethanol to obtain a final concentration of 25 mg/L in the medium. This concentration has been determined to be efficient in enhancing the production of indole alkaloids in *C. roseus* hairy root cultures (Rijhwani and Shanks, 1998). Both single-liquid-phase and two-liquid-phase bioreactor cultures were elicited on days 14, 21, and 28 and harvested 3 days later.

Harvest of the Bioreactors. The hairy roots were harvested as follows (Jolicoeur et al., 1999): clinging liquid was removed by placing the roots between absorbent towels (Kimwipes, Kimberly-Clark, USA) and applying gentle pressure. There was no damage to the roots. Biomass wet weight and residual liquid medium volume were measured and retained for further analysis. The roots were then frozen at −80 °C and lyophilized for 12 h for measurement of the dry weight and alkaloid extraction. The fresh and dry weights of the roots cultivated in two-liquid-phase bioreactors were biased because some silicon oil remained after wiping the fresh root and after lyophilization. The bias was evaluated by treatment with silicon oil of a known fresh weight of hairy roots. Fresh and dry weights of treated roots were then measured as described above. Nonelicited cultures were harvested on days 14, 21, 28, and 51, and elicited cultures were harvested on days 17, 24, and 31. No culture was elicited on day 51.

Results and Discussion

Partition of Ionic Nutrients between Aqueous and Silicon Oil Phases. Major ion (Cl^-, NO_3^-, $H_2PO_4^-$, Mg^{2+}, K^+, Ca^{2+}) concentrations were measured in the culture medium with and without the addition of silicon

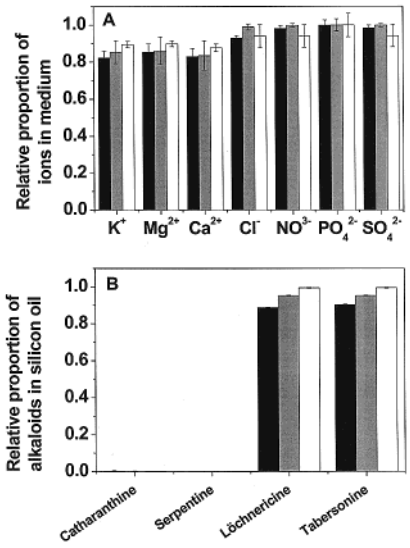

Figure 2. Partition ratios of major ions (A) in culture medium and indole alkaloids (B) in silicon oil at different pH values. See the text for the calculation of the partition ratio. Solid bars are for medium at pH 5, dark gray for medium at pH 6, and light gray for medium at pH 7. Error bars represent standard deviations ($n = 4$).

oil (23% v/v final) in order to estimate a possible effect of silicon oil on hairy roots' nutrition. The ionic concentrations in the silicon oil were calculated from mass balances. Figure 2A presents the ratio of each major ion in the aqueous phase normalized to the sum of the ion concentration in the two phases. A value of 0.5 indicates that the concentration is the same in both phases, whereas a value of 1 indicates that the ionic concentration in the aqueous phase is not modified by the addition of silicon oil. Results suggest that nutrient affinity for the aqueous phase is higher than for silicon oil, with all ratios above 0.8. All analyzed major ions seem to behave similarly, suggesting that silicon oil has no specific affinity for a particular ion. No significant pH effect was observed. Therefore, the presence of silicon oil should not have altered the availability of nutrients in the aqueous phase or the composition of culture medium and thus the growth of the hairy roots.

Partition of Indole Alkaloids between Aqueous and Silicon Oil Phases. The affinity of silicon oil for indole alkaloids was investigated. An extract from elicited *C. roseus* hairy roots was used to perform this study. Particular attention was paid to catharanthine, serpentine, tabersonine, and löchnericine (Figure 2B). Catharanthine and serpentine showed a high affinity for the aqueous phase (ratios of 1 and 0.99, respectively). After overnight mixing, these compounds were not detected in the silicon oil phase. In contrast, tabersonine and löchnericine seem to have a higher affinity for the organic phase. Indeed, more than 90% of these alkaloids remained in the silicon oil at pH 6 and 7, which are pH values typically encountered during hairy root culture. The pH seems to have an influence on the partition of these alkaloids as the affinity of tabersonine and löchnericine for silicon oil rises with increasing pH. These results are in accordance with the polarity of the molecules. Indeed, tabersonine and löchnericine are less polar than serpentine and catharanthine and therefore are less prone to be hydrated. These partition studies demonstrated that silicon oil is a selective extractive phase for tabersonine and löchnericine.

Partition of Jasmonic Acid between Water and Silicon Oil. Jasmonic acid was used to elicit the hairy roots. Since the aim of this study was to compare the indole alkaloid production upon elicitation in bioreactors with and without silicon oil, it was important to evaluate the affinity of silicon oil for jasmonic acid. Indeed, jasmonic acid concentration has a differential effect on the response of hairy roots (Rijhwani and Shanks, 1998). Jasmonic acid was added to a culture medium sample in order to obtain final concentrations of 250 and 500 mg/L. The detection limit of the spectrophotometer did not allow the use of smaller concentrations. It was observed that the jasmonic acid concentration in the aqueous phase was not affected after addition of silicon oil (23% v/v final) to culture medium (data not shown).

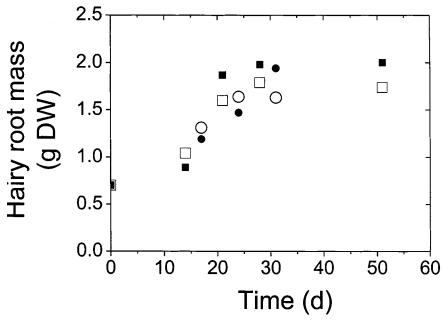

Figure 3. *Catharanthus roseus* hairy roots growth in single-liquid-phase cultures (solid symbols) and two-liquid-phase cultures (empty symbols). Control cultures are shown with squares and elicited cultures with circles. Each data point represents a bioreactor culture.

Oxygen Transfer in the Bioreactors. The $k_L a$ of the single-liquid-phase and the two-liquid-phase bioreactors were measured. The presence of the silicon oil did not significantly affect the $k_L a$ of the bioreactors with values of 5.79 ± 0.94 (standard deviation) h^{-1} for the single-liquid-phase bioreactor and of 7.29 ± 0.39 h^{-1} for the two-liquid-phase bioreactor. Oxygen is much more soluble in silicon oil, and it has been studied as an oxygen transporter (Doran, 1998). The presence of silicon oil did not affect the oxygen transfer rate in the aqueous phase.

Hairy Root Growth and Nutrient Consumption. Presence of silicon oil in the two-liquid-phase cultures significantly biased the measurements of fresh and dry weight of hairy roots. The error was estimated on the basis of the hypothesis that some silicon oil remained on the harvested roots after removal of the clinging liquid and lyophilization. It was found that silicon oil accounted for 5% of the fresh weight. The correction was applied systematically to fresh and dry weights. The growth rates of hairy roots cultivated in the single- and two-liquid-phase cultures were not significantly different (Figure 3). The silicon oil did not seem to interfere with the growth behavior of hairy roots. The maximum specific growth rate of 0.11 d^{-1} was similar to the maximum specific growth rate measured in Petri dish cultures ($\sim$0.12 d^{-1}) using the same *C. roseus* hairy root line and culture medium (Tikhomiroff, 2001). Continued growth after exhaustion of phosphate and nitrate in the culture medium suggested accumulation of these nutrients (Figure 4). Similarly, the consumption of nutrients did not seem to be affected by the presence of silicon oil. Nitrate was entirely consumed by the hairy roots by day 21, whereas phosphate was depleted by day 14 and then interestingly released back into the culture medium.

Alkaloid Production in Single-Liquid-Phase Bioreactors. Studied alkaloids were never detected in the culture media. The specific yields of tabersonine, löchnericine, and catharanthine in nonelicited cultures (Figure 5) suggest that the production of these compounds

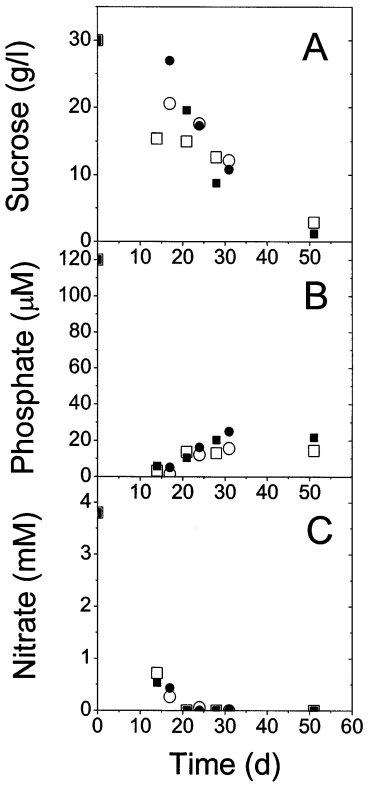

Figure 4. Sucrose concentration (A), phosphate concentration (B), and nitrate concentration (C) in single-liquid-phase cultures (solid symbols) and two-liquid-phase cultures (empty symbols). Control cultures are shown with squares and elicited cultures with circles.

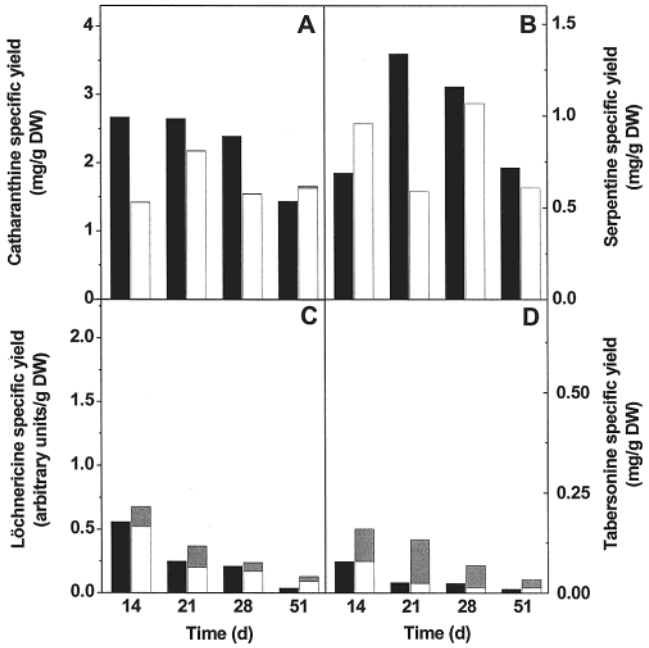

Figure 5. Catharanthine (A), serpentine (B), löchnericine (C), and tabersonine (D) specific yields in hairy roots of nonelicited single-liquid-phase cultures (solid bars) and two-liquid-phase cultures (empty bars). Grey bars indicate the quantity of alkaloids in silicon oil per g dry weight of biomass for two-liquid-phase cultures. Time at harvest is given for cultures. The alkaloid specific yields in silicon oil are the quantity of each alkaloid in silicon oil divided by the dry weight of the hairy roots at harvest. Löchnericine is shown in arbitrary units proportional to the area of the chromatographic peak.

may be growth related as observed by Bhadra and Shanks (1997). Tabersonine and löchnericine specific

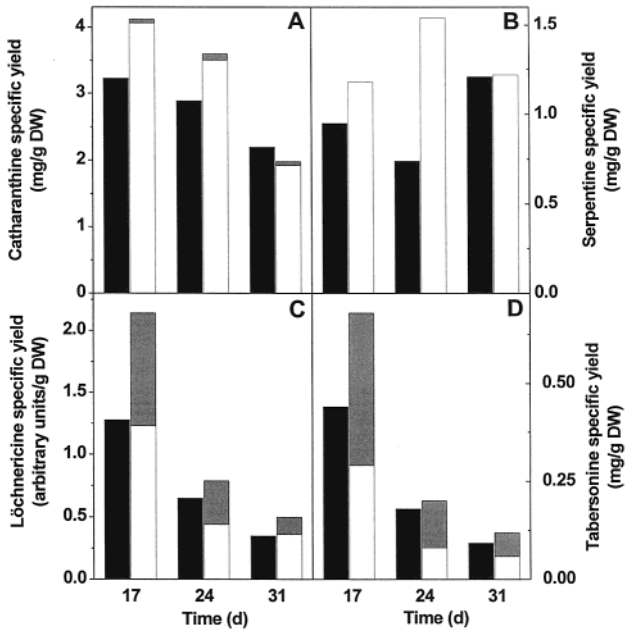

Figure 6. Catharanthine (A), serpentine (B), löchnericine (C), and tabersonine (D) specific yields in hairy roots of elicited single-liquid-phase cultures (solid bars) and two-liquid-phase cultures (empty bars). Grey bars indicate the quantity of alkaloids in silicon oil per g DW of biomass in two-liquid-phase cultures. Time at harvest is given for cultures. The alkaloid specific yields in silicon oil are the quantity of each alkaloid in silicon oil divided by the dry weight of the hairy roots.

yields were maximal at day 14 during the exponential growth phase (0.08 mg/g DW for tabersonine). The specific yield of catharanthine was constant throughout the culture (2.65 mg/g DW), except during the stationary growth phase when a decrease was observed. In the case of serpentine, the production yield profile does not seem to be growth related as observed by Bhadra and Shanks (1997). Indeed, its specific yield reached a maximum at day 21, which corresponds to the end of the exponential growth phase. The yield profile of the two end metabolites, serpentine and catharanthine, was thus significantly different. Catharanthine did not seem to be accumulated throughout the culture while serpentine appeared to be accumulated until growth cessation. Different half-lives or regulatory kinetics may be involved. These results were even more pronounced in elicited cultures (Figure 6). In general terms, addition of jasmonic acid to the culture medium increased the specific yields of tabersonine (maximum value of 0.44 mg/g DW) and löchnericine, which are the central intermediates of the secondary metabolism of *C. roseus* (Figures 5 and 6). However, no elicitor effect was observed on the yield levels of catharanthine and serpentine. In the case of serpentine, the specific yield evolved differently than for nonelicited cultures, showing a minimum at day 24 and a maximum at day 31.

Other studies on elicited cultures have reported that the maximum tabersonine specific yield of 1.2 mg/g DW occurred at the end of the exponential growth phase (Bhadra and Shanks, 1997). Similar values were also reported for catharanthine (Bhadra and Shanks, 1997), but without a decrease during the stationary growth phase. Such dissimilar results may have been induced by the use of minimal culture medium in this study, as compared to the more commonly used Gamborg's B5 medium. Difference in hairy root line may also be involved, as significant disparities in the primary and secondary metabolisms have been observed between root

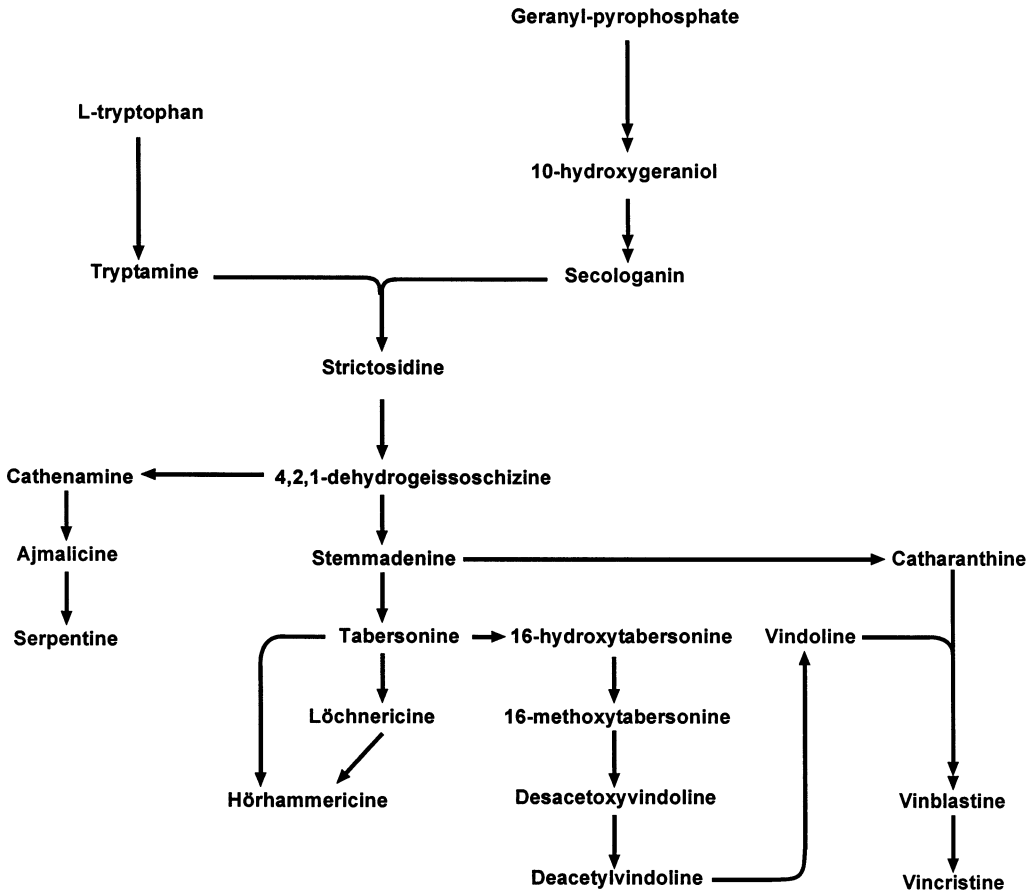

Figure 7. Indole alkaloid biosynthesis pathway in *C. roseus* (adapted from Morgan and Shanks, 2000). Double arrows indicate several steps in the biosynthesis.

lines emerging from a single *A. rhizogenes* transformation experiment (Tikhomiroff, 2001).

Effects of Silicon Oil on Indole Alkaloid Production. The use of silicon oil enhanced the overall production levels of tabersonine and löchnericine by 100−400% and 14−200% in nonelicited cultures (Figure 5). In elicited cultures, the yields were increased by 10−55% and 20−65% (Figure 6). Again, studied alkaloids were never detected in the culture media. Tabersonine and löchnericine both accumulated in the silicon oil phase.

As observed for the control cultures without silicon oil, tabersonine and löchnericine total specific yields declined during the culture. Intracellular yields of these alkaloids were similar or lower than in control cultures without extractive phase. Indeed, it appears that the additional production accumulated mostly in the silicon phase. Thus, results suggest that the silicon oil was efficient in extracting intracellular tabersonine and löchnericine. Moreover, this extracting capability of silicon oil may have induced a positive effect on specific pathways of the secondary metabolism.

As predicted by partition studies, only traces of catharantine were detected in the silicon oil and serpentine was undetectable. Similarly as in control cultures without silicon oil, the specific yields of catharanthine and serpentine progressed differently throughout the two-phase culture. However, similar levels of yields as for the control cultures were observed. Therefore, it seems that silicon oil did not influence the production of these two secondary metabolites.

Since tabersonine and catharanthine have a common intermediate in their biosynthesis pathways (dehydrogeissoschizine) (Figure 7) and catharanthine is an end

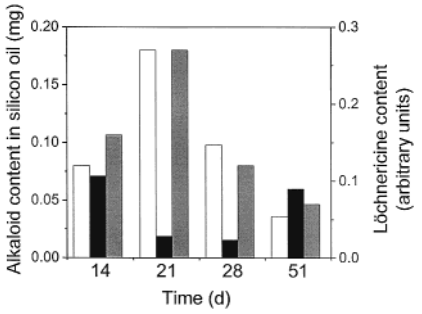

Figure 8. Total content of tabersonine (empty bars), catharanthine (solid bars), and löchnericine (grey bars) in the silicon oil of control cultures in two-liquid-phase cultures. Löchnericine is given in arbitrary units (see text).

metabolite here, it is suggested that the tabersonine pathway has thus been favored. For some specific secondary metabolites, silicon oil may have acted as a sink, pulling on the secondary metabolism. It can thus be suggested that the rise in tabersonine and löchnericine production as a result of elicitation did not reach its full potential. The silicon oil sink effect may have been close to saturating some of the metabolic fluxes. However, the alkaloids extracted in the silicon oil did not seem to be protected from degradation as their amount clearly declined with time (Figure 8). This decrease may correspond to their breakdown or transformation into other end-compounds.

Conclusion

The potential of a continuous extraction of specific secondary metabolites of *C. roseus* hairy roots by silicon

oil has been demonstrated. Indeed, the use of silicon oil improved the production of tabersonine and löchnericine but did not affect serpentine and catharanthine yields. Thus, selective extraction of tabersonine and löchnericine alters *C. roseus* hairy roots' secondary metabolism. In fact, silicon oil may alleviate negative feedback and thus up-regulate the pathways of these compounds. The presence of an organic phase did not seem to alter the primary metabolism in terms of root specific growth rate and behavior; therefore it allows simultaneous growth, production, and extraction in a culture. Other extractive phases are under investigation as well as the use of silicon oil with other plant species. Comprehension of the extraction's mechanism of action could allow optimization of the process.

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