Chapitre 6

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

Dans le chapitre précédent, les lignées établies de racines transformées de *C. roseus* ont été étudiées et il apparaît qu'il existe une grande diversité d'une part entre les lignées elle-même et d'autre part en comparant ces lignées à celles décrites dans la littérature. Cette grande variabilité pose des difficultés pour l'optimisation d'un procédé industriel de production d'alcaloïdes à noyau indole par des racines transformées de *C. roseus*. Afin de réduire cette variabilité, l'orientation volontaire du métabolisme secondaire des racines transformées vers certaines voies a été étudiée en utilisant une phase extractive sélective non-miscible à l'eau.

Ce chapitre présente l'étude des effets de l'huile de silicone sur la croissance et le métabolisme secondaire de racines transformées de *C. roseus* cultivées dans un bioréacteur de 0,5 l de volume utile. Il apparaît que l'huile de silicone a une forte affinité avec la tabersonine et la löchnericine, et une affinité faible ou nulle pour les autres alcaloïdes. Elle n'altère pas

la disponibilité des nutriments ioniques et le métabolisme primaire, mesuré par la croissance des racines, n'est pas modifié par la présence d'huile de silicone. En revanche, l'huile de silicone a eu un effet sur le métabolisme secondaire. La tabersonine et la löchnericine se sont accumulées dans l'huile de silicone. L'utilisation d'huile de silicone a permis d'augmenter la production totale de tabersonine et de löchnericine tandis que la production totale de catharanthine a diminué. Ceci suggère que l'utilisation d'huile de silicone a permis de dévier les flux métaboliques vers la production de la tabersonine et de ses dérivés époxyde au dépens du flux métaboliques menant à la production de catharanthine.

Ces travaux montrent qu'il est possible d'orienter sélectivement le métabolisme secondaire des racines transformées de *C. roseus* en utilisant de l'huile de silicone. A long terme, il est réaliste de penser qu'il sera possible d'orienter le métabolisme secondaire des racines transformées de *C. roseus* en optimisant la nature de la phase extractive. Ceci ajoute à l'élicitation un nouvel outil de contrôle du métabolisme secondaire de ces racines.

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MANUSCRIT # 2

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

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6.1 Abstract

A two-liquid phase bioreactor was designed to extract indole alkaloids of *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 and that tabersonine and löchnericine have the same affinity for silicon oil as for the aqueous phase. Cultures were elicited with 0.25 mg/l of jasmonic acid. The growth of the hairy roots was not significantly modified by the presence of silicon oil. The overall specific yield of tabersonine and löchnericine were respectively increased by 100-400% and 40% with the use of silicon oil in non-elicited control cultures. In elicited cultures, these values were 50% for tabersonine and 65% for löchnericine. Serpentine was never found in the silicon oil. Catharanthine specific yield was lower in control cultures, suggesting that the silicon oil acted as a metabolic sink for tabersonine and löchnericine and diverted the metabolic fluxes to these alkaloids at the expense of the catharanthine and serpentine fluxes.

6.2 Key Words

Selective extraction; silicon oil; *Catharanthus roseus* ; indole alkaloids; bioreactor; Hairy roots

6.3 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 in canola plant (Monsanto, USA) and diverse molecules of mammalian origin were also successfully inserted in plants (Daniell et al., 2001). Field culture seems economically attractive but in vitro culture represents important advantages since it is not exposed to diseases and pests, and seems not to be subject to seasonal and somatic variations. Moreover, in vitro culture conditions highly simplify the validation process for the production and the purification steps.

Many plant species have been evaluated for their potential to produce some molecules of high pharmaceutical interest and *Catharanthus roseus* (Madagascar periwinkle) is considered to be an interesting model system. C. 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 and De Luca (1999) have recently shown that at least two essential enzymes involved in the pathway to vindoline are only expressed in the leaves of the plant, thus explaining that the vinblastine and vincristine bisindole alkaloids are still 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 which could de-silence some genes involved in the secondary metabolism.

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 (Toivonnen et al., 1989). At an industrial scale, the harvest of the secondary metabolites is destructive as they are usually extracted from lyophilized plant tissues. Non-destructive secondary metabolite extraction from culture medium has been studied using polymeric resins in situ. Williams et al. (1996) showed that the production level of total sanguinarine was even 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., 1988, Sim et al., 1994). Moreover, this extractive phase allowed the harvest of indole alkaloids known to remain intracellular. Another approach consists in the use of a non-aqueous 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 non-toxic 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 experiments were conducted in lab-scale 1 l bioreactors. The differences in the primary and secondary metabolisms were also investigated.

6.4 Materials and Methods

6.4.1 Hairy Roots Transformation and Cultures

Hairy roots of *Catharanthus roseus* (L.) G. Don were established as described by Bhadra et al. (1993) with the A_4 strain of Agrobacterium rhizogenes. Six fast-growing root lines were obtained (Tikhomiroff, 2001) and the root line LAO was used in this study. Hairy roots were transferred every month into Petri dishes into 20 ml of minimum medium (Bécard and Fortin, 1988) supplemented with 3% (w/v) sucrose, a tenfold KH_2PO_4 (0.352 mM final) and a threefold $Ca(NO_3)_2$ (8.11 mM final) concentration.

6.4.2 The Two-liquid-phase Bioreactor

A closed loop configuration allowing the use of a small quantity of silicon oil was designed (Figure 6.1). The bioreactors were made with 1.2-L total volume (11 cm ID x 13 cm height) autoclavable polycarbonate jars (Nalgene, Sybron International, Rochester, NY, USA) with a modified cover. The hairy roots were inoculated and immobilized in a 45 x 45 x 20 (height) mm 316 stainless steel screen mesh (mesh size of 20) box placed 1 cm from the bottom of the aqueous phase. DC 200 Silicon oil (Sigma-Aldrich, Oakville, ONT, Canada) was the light phase with a lower density ($\rho = 0.937$) than water. Silicon oil was pumped with a peristaltic pump (Masterflex, USA) 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 length was minimized. Tubes of viton, norprene and stainless steel were used. The silicon oil trickles up through the hairy

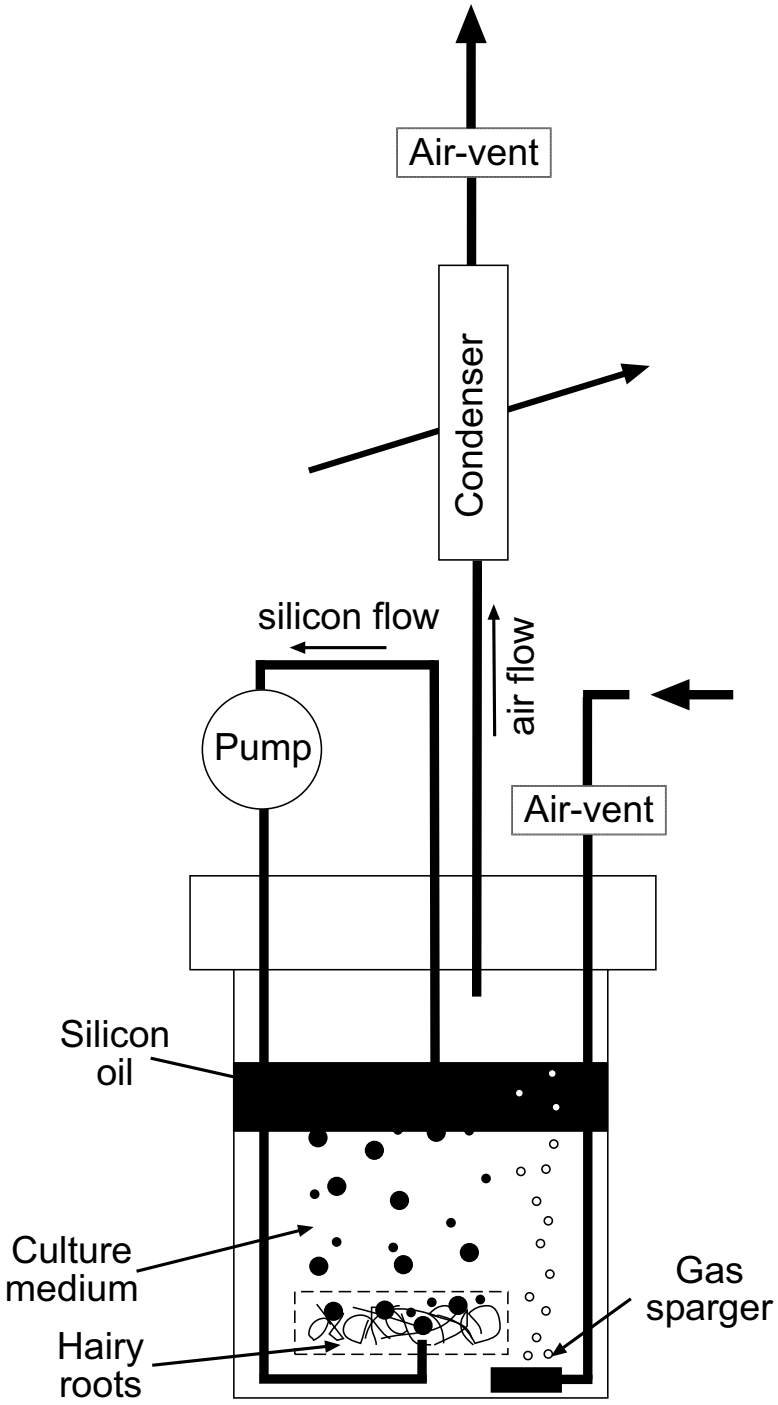

Figure 6.1 – Two-liquid-phase bioreactor.

root bed and returns 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

mm) 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). Sterile air filters (bacterial air vent : Gelman Sciences, Ann Arbor, MI, USA) and a liquid condenser ensured sterility and minimal 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. 1994). Single-liquid-phase bioreactors with the same design but without silicon oil were used as control. All stainless steel parts were cleaned as follows. Grid boxes and tubes were treated for 1 hour in 1% (w/v) citric acid, thoroughly rinsed with water, treated for 1 hour 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.

6.4.3 Ions 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, Canada) equipped with an isocratic pump, an automated sampler AS-3500, and a pulsed electrochemical detector in the conductivity mode, controlled by the Dionex Al-450 software for cations and the Dionex Peaknet software for anions. Anions were separated using a 4 x 250 mm IONPAC AS14A-SC analytical column, an IONPAC AG14A-SC guard column and a ASRS-1 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 x 250 mm IONPAC CS-12 analytical column, a IONPAC CG-12 guard column, and a CSRS-1 cation self-regenerating suppressor. The mobile phase was an aqueous 20 mM methanesulphonic acid solution flowing at a rate of 0.9 ml/min.

6.4.4 Indole Alkaloids Extraction

To measure the concentration of indole alkaloids in hairy roots, fresh hairy roots were frozen at -80oC and then lyophilized for 12 hours. Approximately 100 mg DW were blended with 1 ml of methanol in a tissue grinder (VWR Canlab, Ville Mont-Royal, Canada). The extract was centrifuged for 5 min and the supernatant was filtered through a PTFE 0.45 mm filter before HPLC analysis. Alkaloids were extracted from the silicon oil with the same volume of methanol on an orbital shaker (150 min-1) overnight. The methanolic sample 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, centrifuged and both phases were separated. The above procedure was repeated 3 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).

6.4.5 Indole Alkaloids HPLC Analysis

The HPLC analysis was performed using a Beckman Coulter pump model 126, a Beckman Coulter auto-sampler model 508, a C18 guard column (Upchurch Scientific, Oak Harbor, WA, USA), a Zorbax Eclipse XDB-C18 4.6mm x 250 mm column 3.5mm (Hewlett Packard, Missisauga, ONT, Canada), and a Beckman Coulter PDA detector 168. The alkaloids were separated on the column starting with a 20:80 mixture of ACN:5 mM KH_2PO_4 pH=6 increasing linearly in 20 min to a final 80:20 mixture at a flow rate of 2.0 ml/min. Peak identification was done 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. Lochericine quantities are given in arbitrary units, proportional to the area of the chromatrographic peak. Ajmalicine was detected but was not quantified since it co-eluted with an unknown compound.

6.4.6 Partition Studies of Silicon Oil

For ion partition studies, a sample of fresh culture medium was first analyzed by HPLC. Then a mixture of 23% (v/v) silicon oil in culture medium was agitated on a shaker (150 min^{-1}) overnight, centrifuged, and the aqueous phase was analysed by HPLC. Jasmonic acid partition studies were performed as described above for the culture medium using 250 mg/l 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 N2 atmosphere and resuspended in a mixture of 15:85 tetrahydrofurane:methanol. 100 ml of the extract were incorporated in either 5 ml of culture medium or 5 ml of silicon oil. The medium and the silicon oil were sonicated separately for 5 minutes and both appeared as a single yellow liquid phase. The two phases were then mixed, sonicated for 5 minutes and centrifuged for 10 minutes. Each phase was isolated and treated for alkaloid extraction.

6.4.7 Oxygen Masse Transfer Measurement

The k_La of the bioreactors were measured by the gassing (air) and degassing (N_2) methods using a dissolved oxygen probe (polarographic : Ingold, Urdorf, Switzerland). No hairy roots were inoculated for the measurement.

6.4.8 Inoculation of the Bioreactors

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

6.4.9 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, which 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 29, and harvested 3 days later.

6.4.10 Harvest of bioreactors

The hairy roots were harvested as follows: 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 hours 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. Non-elicited cultures were harvested on days 14, 21, 28 and 51 and elicited cultures were harvested on day 17, 24 and 31. No culture was elicited on day 51.

6.5 Results and Discussion

6.5.1 Bioreactor Characterization

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

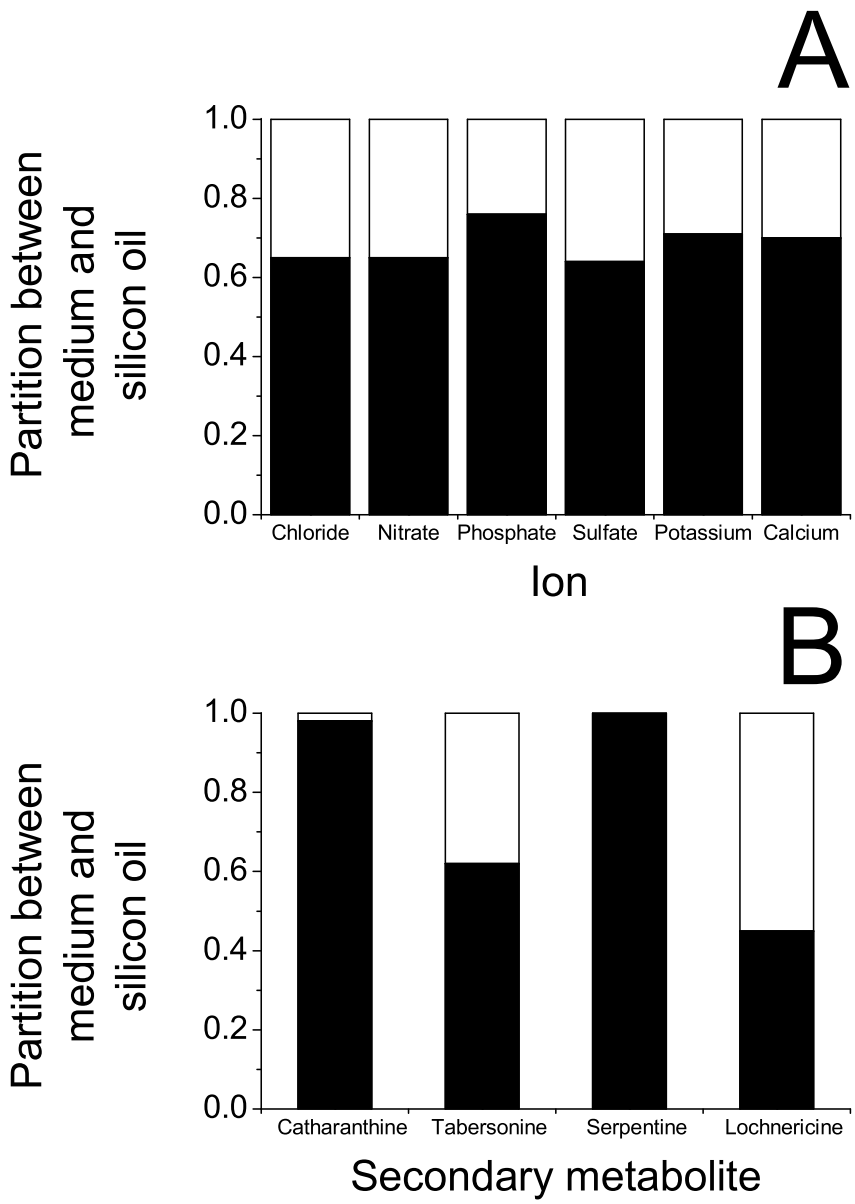

Figure 6.2 – Partition ratio of major ions (A) and indole alkaloids (B) between silicon oil and medium culture. See the text for the calculation of the partition ratio. Solid bars are for the aqueous phase and empty bars are for the silicon oil.

from mass balances. The Figure 6.2A presents the ratio between the concentration of each major ion in aqueous and silicon oil phases 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 water is higher than for silicon oil, with an average normalized ratio of 0.75. All analyzed major ions seemed to behave similarly, suggesting that silicon oil has no specific affinity for a particular ion. 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 Water and Silicon Oil. The affinity of silicon oil for indole alkaloids was investigated. An extract from elicited C. roseus hairy roots was used to perform this study. Catharanthine, serpentine, tabersonine and löchnericine were studied (Figure 6.2B). The alkaloids showed a high affinity for water, except tabersonine and löchnericine which seemed to have the same affinity for both phases (normalized ratio near 0.5). Unlike the other alkaloids, no vinblastine (data not shown) and only traces of serpentine were detected in the silicon oil phase.

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. Jasmonic acid was added to a culture medium sample in order to obtain final concentrations of 250 mg/l 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.

Oxygen Transfer in the Bioreactors. The k_La 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_La of the bioreactors with values of 5.31 ± 2.5 h^{-1} for the single-liquid-phase

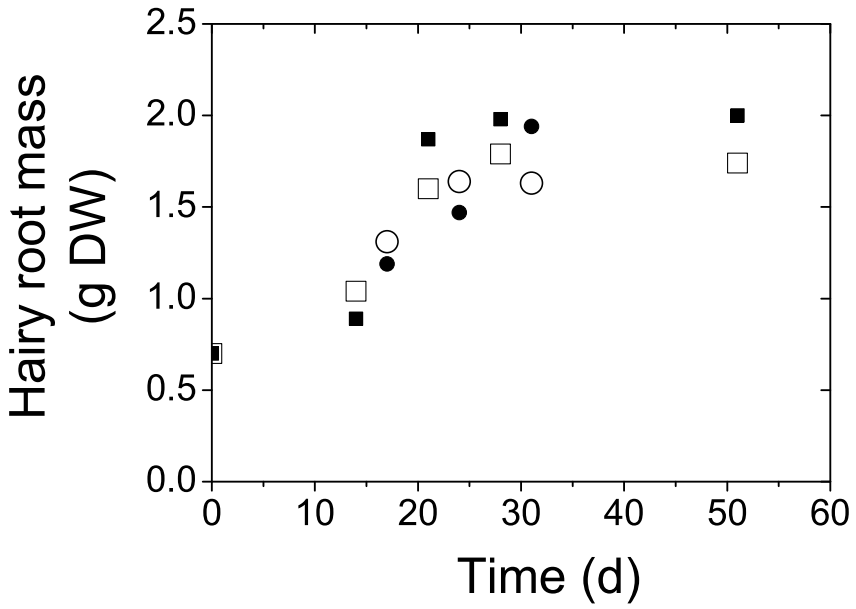

Figure 6.3 – *C. roseus* growth in single-liquid-phase cultures (solid symbols) and two-liquid-phase cultures (empty symbols). Control cultures are shown with squares and elicited cultures with circles. Each data point represents a bioreactor culture.

bioreactor and of 3.96 ± 1.1 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 $k_L a$ values obtained here were thus lower than expected.

6.5.2 Hairy Roots 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 based upon 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 of hairy roots cultivated in the single and two-liquid-phase cultures were not significantly different (Figure 6.3). The silicon oil did not seem to interfere with the growth behaviour of hairy roots. The

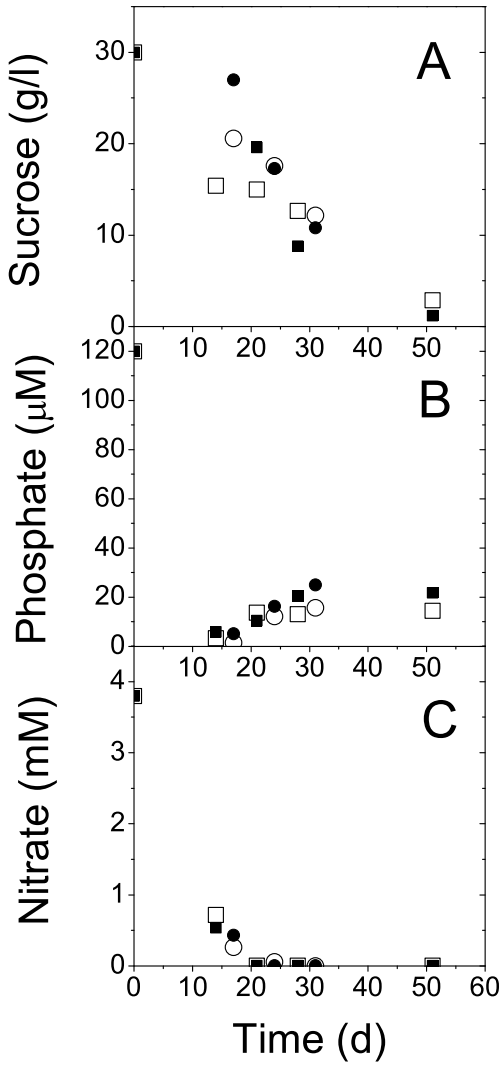

Figure 6.4 – Sucrose consumption (A), phosphate consumption (B) and nitrate consumption (C) in single-liquid-phase cultures (solid symbols) and two-liquid-phase cultures (empty symbols). Control cultures are shown with squares and elicited cultures with circles.

maximum specific growth rate of 0.11 d^{-1} was similar to the maximum specific growth rate measured in Petri dish cultures ($\approx$ 0.12 d^{-1}) using the same C. roseus hairy root line and culture medium (unpublished data). Continued growth after exhaustion of phosphate and nitrate in the culture medium suggested accumulation of these nutrients (Figure 6.4). Similarly to the specific growth rate, the consumption of nutrients did not seem to be affected by the

presence of silicon oil. Nitrate was entirely consumed by the hairy roots at day 21, whereas phosphate was depleted at day 14 and then interestingly released back into the culture medium.

6.5.3 Secondary Metabolism of Hairy Roots

The use of silicon oil was motivated by the fact that indole alkaloids produced by C. roseus hairy roots and suspension cells are kept intracellular. Known alkaloids (from our standards) were never detected in the culture media. However, alkaloids were present in hairy roots and the silicon oil.

Alkaloids Production in Single-liquid-Phase Bioreactors. The specific yields of tabersonine and löchnericine (Figure 6.5) in non-elicited control cultures suggested that the production of these compounds may be growth related. The tabersonine and löchnericine specific yields were maximal at day 14 (0.078 mg/g DW for tabersonine), which was during the exponential growth phase. Other studies have reported that the maximum tabersonine specific yield occured at the end of the exponential growth phase with a value of 1.2 mg/g DW (Bhadra and Shanks, 1997). Serpentine specific yields (Figure 6.6B) were maximal (1.34 mg/g DW) at day 21, which was at the end of the exponential growth phase. The specific concentration in serpentine then decreased to 0.72 mg/g DW by day 51. The catharanthine specific yield (Figure 6.6A) remained constant during the culture, except during the stationary growth phase when a significant decrease was observed. Similar values were reported in the literature (Bhadra and Shanks, 1997), with, however, no decrease during the stationary growth phase. Such differences may have been induced by the use of minimal culture medium in this study, as compared to the most common Gamborg's B5 medium. Difference in hairy root line may also be involved as the primary and secondary metabolism behaviours seem to be highly related to root lines (Tikhomiroff, 2001). Addition of jasmonic acid to the culture medium significantly increased the specific yields of tabersonine, löchnericine and serpentine from 40% to 300% (Figures 6.5 and 6.6) by day 28. At the end of the culture, 28 days, the specific yield of tabersonine was still increased (280%) by elicitation, and löchnericine's to a

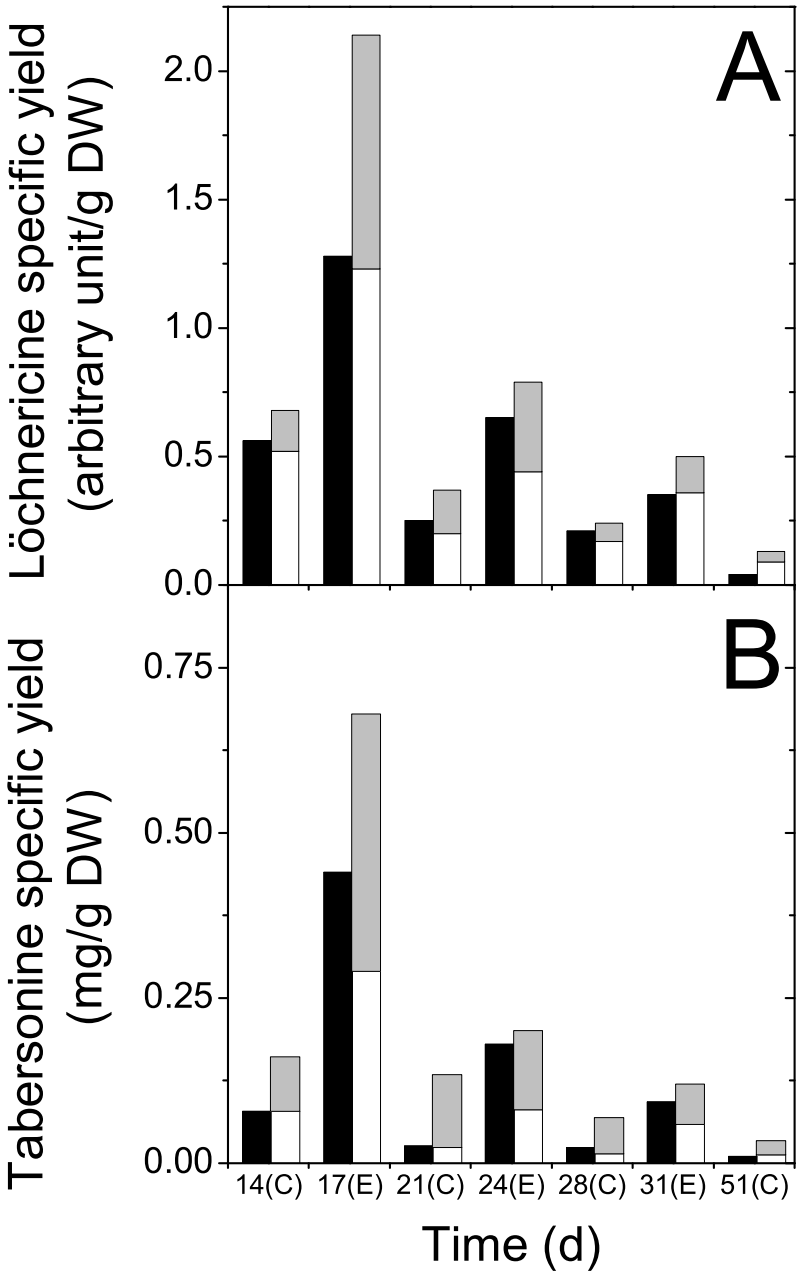

Figure 6.5 – Löchnericine (A) and tabersonine (B) specific yields in hairy root for the single-liquid-phase cultures (solid bars) and two-liquid-phase cultures (empty bars). Grey bars indicate the quantity of alkaloids in silicon oil per g dry weight of biomass for two-liquid-phase cultures. Time at harvest is given for control cultures (C), and elicited cultures (E). 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 .

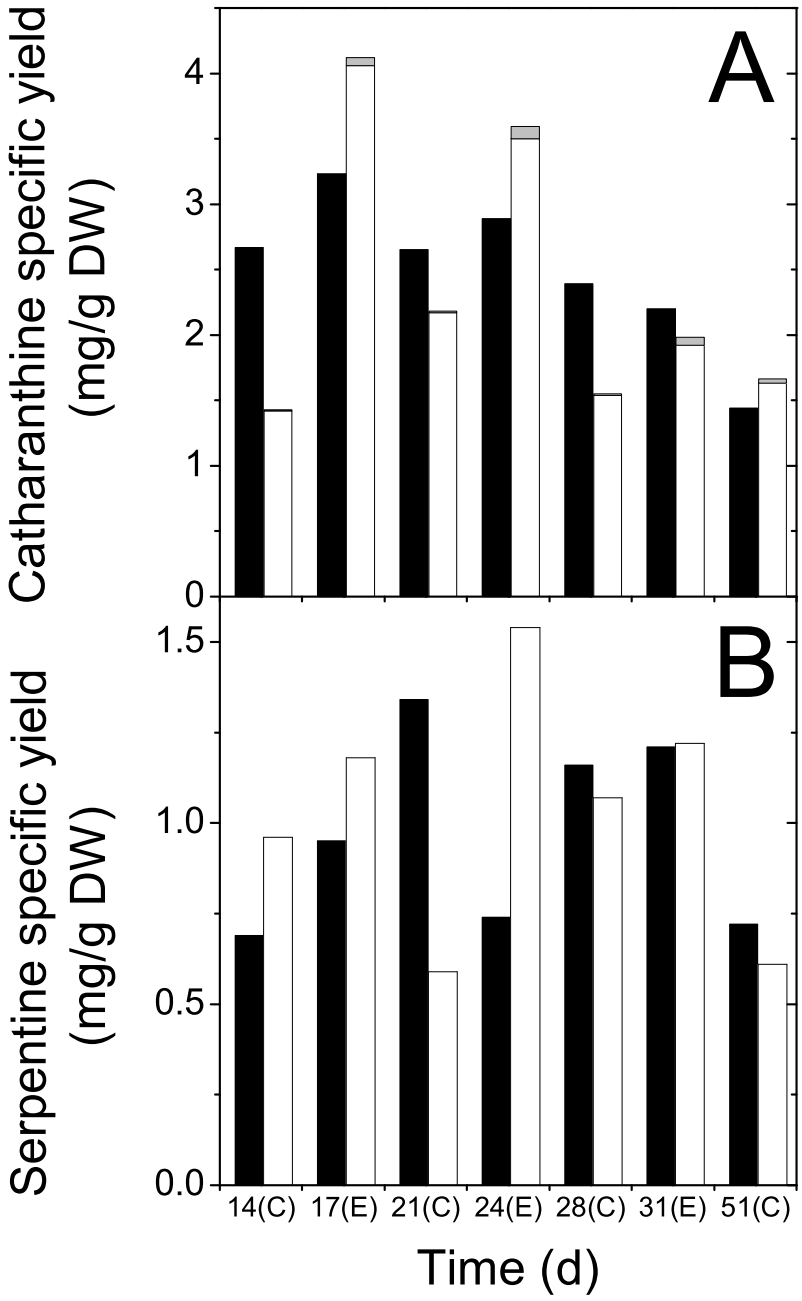

Figure 6.6 – Catharanthine (A) and serpentine (B) specific yields in hairy root of single-liquid-phase cultures (solid bars) and two-liquid-phase cultures (empty bars). Grey bars indicate the quantity alkaloid in silicon oil per g dry weight of biomass in two-liquid-phase cultures. Time at harvest is given for control cultures (C) and elitited cultures (E). 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.

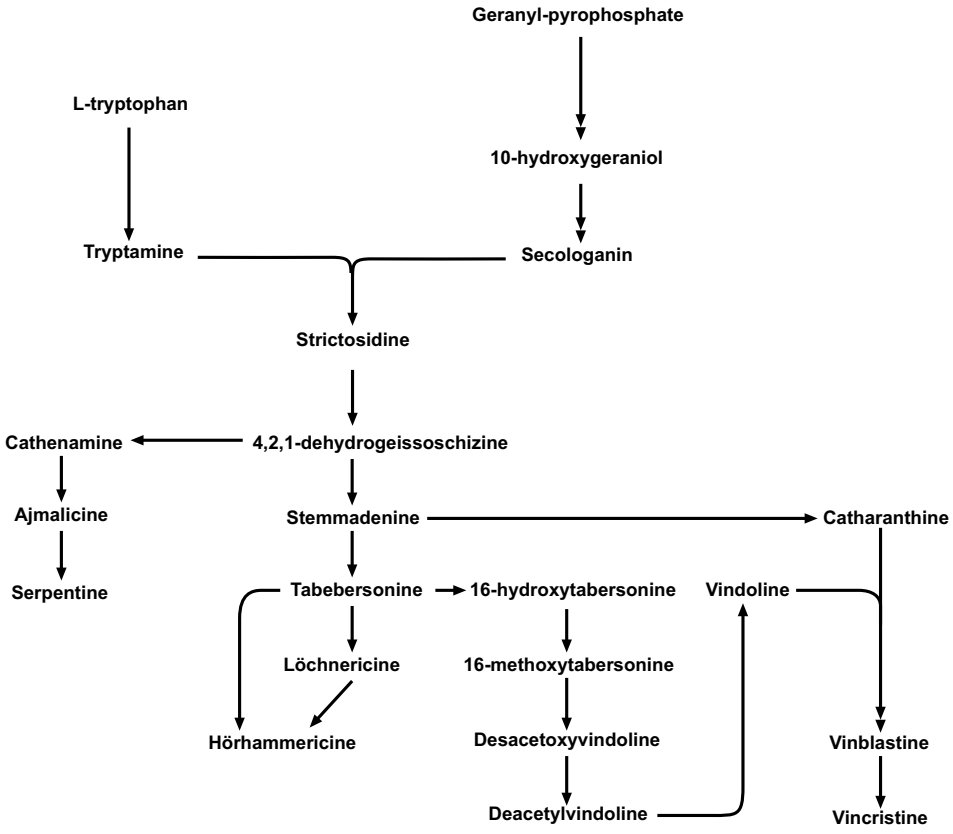

Figure 6.7 – Indole alkaloids biosynthesis pathway in *Catharanthus roseus* (adapted from Morgan and Shanks, 2000). Double arrows indicate several steps in the biosynthesis.

lesser extend (66%). For serpentine, the specific yield was similar to that of the non-elicited cultures. Catharanthine specific yield did not appear to be increased in elicited cultures.

Effects of Silicon Oil on Indole Alkaloids Production. The use of silicon oil significantly enhanced the production of tabersonine and löchnericine (Figure 6.5). In non-elicited control cultures the total (intracellular plus silicon) specific yields were increased by a factor of 100% to 400% for tabersonine and 15% to 40% for löchnericine. Tabersonine and löchnericine were accumulated in the silicon oil, reaching a maximum at day 14. Results thus suggest that for non-elicited hairy roots, transfer and accumulation of tabersonine and löchnericine in silicon oil had a significant positive effect on specific pathways of the secondary metabolism. For the elicited cultures, the use of silicon oil increased the total specific yield up to 50%

for tabersonine and 65% for löchnericine. However, specific yield of intracellular tabersonine was lower using silicon oil. For intracellular löchnericine, the specific yield was similar with the exception of day 21 when it was lower. Catharanthine was not significantly accumulated in the silicon oil (Figure 6.6A). However, with the exception of days 14 and 21 of elicited cultures, the total catharanthine specific yield was even lower in the two-liquid-phase cultures. As tabersonine and catharanthine have a common intermediate in their biosynthesis pathways (dehydrogeissoschizine) (Figure 6.7) and catharanthine is an end metabolite here, it is suggested that tabersonine pathway has thus been favoured. Silicon oil showed traces of serpentine, an end metabolite. However, for some specific secondary metabolites, silicon oil may act as a sink pulling on the secondary metabolism. It can thus be suggested that the effect of elicitation on the secondary metabolism did not reach its full potential as the silicon oil sink effect may have been close to saturate some of the metabolic fluxes. It was clear that tabersonine and löchnericine did not accumulate during the whole culture (Figure 6.8) since the total production of these alkaloids decreased from day 21 to day 51. This decrease may correspond to their degradation at the end of the culture. The same fate seemed to occur to catharanthine. However, the sudden increase in accumulation of catharanthine in silicon oil on day 51 is unclear.

6.6 Conclusion

The potential of a continuous extraction of specific secondary metabolites of C. roseus hairy roots by silicon oil has been demonstrated. The presence of an orgarnic phase did not seem to alter the primary metabolism in terms of root specific growth rate and behaviour. The use of silicon oil did not improve the production of serpentine or ajmalicine. Catharanthine production was devreased and only tabersonine and löchnericine productions were improved. The main interest of this study is the alteration of C. roseus hairy roots secondary metabolism by silicon oil. Other extractive phases are currently under investigation as well as the use of silicon oil on other plant species.

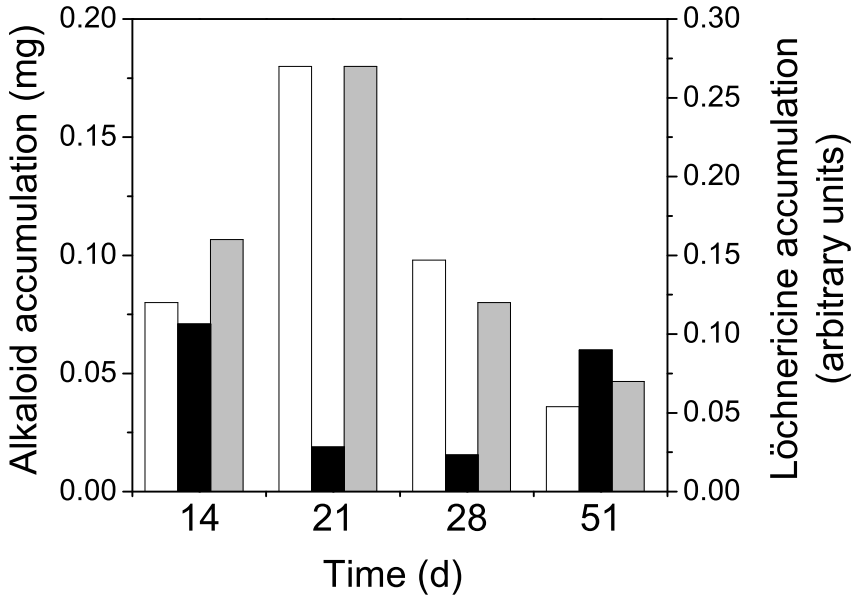

Figure 6.8 – Accumulation of tabersonine (empty bars), catharanthine (solid bars) and löchnericine (grey bars) in the silicon oil of control cultures in two-liquid-phase cultures. Löchnericine is given in arbitrary units (see text).

6.7 Acknowledgments

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