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STRESS AS A MEANS TO ENHANCE SECONDARY METABOLITE PRODUCTIVITY AND TO PROBE METABOLIC PATHWAYS

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

Doctor of Philosophy

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July, 1997
To

My Parents
ABSTRACT

STRESS AS A MEANS TO ENHANCE SECONDARY METABOLITE PRODUCTIVITY AND TO PROBE METABOLIC PATHWAYS

Sushil K. Rijhwani

The objectives of this research were to study metabolic pathways in \textit{C. roseus} hairy root cultures, and to enhance secondary metabolite productivity using fungal elicitation as the technique. The effects of age of inoculum were studied by adapting the cultures to three subculture cycle routines. The 2 week subculture cycle yielded the fastest, while the 4 week cycle yielded the lowest, specific growth rates. Specific yields of tabersonine decreased from day 21 to 35 while the total yields of hörhammericine increased in all three subculture cycles. Lochnericine yields were highest in the 2 week cycle while serpentine yields were lowest.

The effects of dosages and exposure times of specific elicitors on several compounds in the indole alkaloid pathway were studied. A 150\% increase in tabersonine specific yield was observed upon addition of 72 units of pectinase. Levels of serpentine, tabersonine and lochnericine decreased transiently after addition of pectinase in time course studies. Jasmonic acid was found to be a unique elicitor leading to an enhancement in flux to several branches in the alkaloid pathway. Time
course studies with jasmonic acid showed a transient increase in lochnericine and tabersonine levels and a continuous increase in levels of ajmalicine, serpentine and hörhammericine.

NMR spectroscopy was utilized as the tool to study primary metabolism of hairy roots non-invasively. $^{31}$P NMR spectroscopy studies indicated that vacuolar and cytoplasmic pH were maintained at 7.4 ± 0.06 and 5.25 ± 0.08 respectively. $^{13}$C NMR spectroscopy studies indicated activities of pentose phosphate pathways, non-photosynthetic CO$_2$ fixation and glucan synthesis pathways. Recycling of triose phosphate was evident from scrambling of label in glucans.

In vivo $^{31}$P and $^{13}$C NMR spectroscopy was subsequently utilized to study the effects of elicitors on primary metabolism. A transient short-term decline in the cytoplasmic pH was observed upon addition of pectinase while a prolonged decrease in vacuolar pH was observed upon addition of jasmonic acid. Enhanced accumulation of glucans was detected upon addition of pectinase. A reduction in the levels of pyruvate and glutamine was observed, upon addition of jasmonic acid, indicating a decrease in flux to glycolysis or an increase in the drain on these pools.
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CHAPTER I: INTRODUCTION

Plant derived natural products continue to stimulate the interest of several researchers interested in determination of the structure and biosynthetic pathways of these compounds (Kutney, 1993). The natural systems which produce compounds finding applications in pharmaceutical, agrochemical and the fragrance industries are of particular interest. The structural complexity of these natural products demands multistep processes for chemical synthesis, making large-scale industrial applications economically unfeasible (Kutney, 1993). Plant cell biotechnology has evolved as a promising area in the past two decades, focusing on the production of these secondary metabolites (Verpoorte et al., 1994). Progress in this area has widespread implications on development of cell cultures as efficient sources of production of secondary metabolites.

The plant Catharanthus roseus (Periwinkle) is an important research system since it is a source of a wide array of pharmacologically active secondary metabolites called indole alkaloids (Verpoorte et al., 1993). Two of these indole alkaloids, vinblastine and vincristine, have been approved by the FDA as antineoplastic drugs for use in chemotherapy (Flores et al., 1987; Verpoorte et al., 1991). Another alkaloid produced by the plant, ajmalicine, is used to treat hypertension (Hamill et al., 1987). At present these chemicals are produced by extraction of the whole plant (DiCosmo, 1990). Other strategies, such as production from cell suspension cultures have not
succeeded as yet (Verpoorte et al., 1993). Hairy roots, due to their morphological differentiation and genetic stability overcome the limitations imposed by plant cell suspensions cultures as a production system. Furthermore, hairy root cultures are amenable to genetic transformations and can be metabolically engineered to synthesize the required compounds and therefore provide an excellent system for metabolic studies. However a detailed knowledge of metabolic pathways at the molecular level is essential for systematic genetic engineering of the cultures.

The indole alkaloid pathway of C. roseus is presented in Figure 1-1 (For detailed overview of pathway see Meijer et al. (1993). Strictosidine is the first indole alkaloid which is formed by condensation of secologanin (derived via the mevalonate pathway) and tryptamine (derived via the shikimic acid pathway). Strictosidine is further converted to 4,21 dehydrogeissoschizine which either enters the cathechamine pathway to form ajmalicine and serpentine or is converted to stemmadenine. Stemmadenine is further converted to catharanthine (a precursor of vinblastine and vincristine) and tabersonine via some unknown reactions. Tabersonine goes through six steps to form vindoline, the second precursor of the dimeric anti-cancer alkaloids, and is also converted to lochnericine and hörhammericine (Vani, 1996). Hence the secondary metabolic pathways of these cultures are very complex, not fully known and their regulation is poorly understood.
Figure 1-1 Biosynthetic pathway and enzymes involved in alkaloid biosynthesis. Dotted arrows represent unknown steps and multiple arrows represent more than one step.
In our laboratory hairy root cultures of *C. roseus* have been generated previously (Bhadra *et al.*, 1993). These cultures were characterized for growth rates, nutrient consumption rates and secondary metabolite productivities. The effect of carbon source and illumination on growth and indole alkaloid productivity was studied (Bhadra, 1995). However, the anti-cancer alkaloids and their key precursor (vindoline) could not be detected in both the dark grown and the light grown root cultures (Bhadra, 1995). Furthermore, lochnericine and hörhammericine were also detected and quantified in our root cultures (Vani, 1996). Ajmalicine and serpentine were synthesized by the root cultures but they were produced at levels too low to be commercialized.

The broad goal of this research was to enhance the productivity of secondary metabolites synthesized by *C. roseus* hairy root cultures and to study both the primary and the secondary metabolism of the root cultures. The research encompasses subculture cycle studies for growth improvements, elicitation studies for specific yield enhancements, non-invasive primary metabolism studies of the root cultures using labeled isotopes and the study on the effect of elicitors on primary metabolism. It was conceived that this research would help devise strategies for enhancement of secondary metabolite productivity by *C. roseus* hairy root cultures and also help study the primary and secondary metabolic pathways in elicited and unelicited cultures.

The organization of this thesis proceeds with background in Chapter 2. The strategies employed for enhancement in secondary metabolite productivity are discussed. The theory behind fungal elicitation induced enhancement in secondary
metabolite productivity and the previously studied affects of fungal elicitors on *C. roseus* cultures is presented. The basic concepts of NMR spectroscopy and applications of NMR spectroscopy for studying the metabolism of a perturbed and an unperturbed system are also presented.

Productivity is a combination of growth rates and specific yields. Higher growth rates can lead to greater accumulation of biomass in a smaller period of time and hence improve the economics of a process. The inoculum used in a subculture can have a significant effect on the specific growth rates of a root line. Chapter 3 presents a study in which the root cultures were adapted to three different subculture cycles and their growth rates were determined. The effects of subculture cycle on accumulation of indole alkaloid is also presented in this chapter.

In combination with high growth rates, a strategy was required to enhance the specific yields of secondary metabolites, to induce the synthesis of the compounds that the roots could not synthesize and to probe the complex secondary metabolite pathways of *C. roseus*. Fungal elicitation was utilized as the technique to achieve these objectives. Fungal elicitation has been an important technique for enhancement of secondary metabolite synthesis by tissue cultures and a means to study secondary metabolism (Whitehead and Threlfall, 1992; Singh, 1996). Elicitation can also lead to induction or activation of enzymes, not induced or active in the plant systems, and can also lead to biosynthesis of new compounds. The binding of an elicitor to the receptor on plant plasma membrane leads to production of secondary messengers. Due to the
fast speed of the response it is believed that a short signal transduction cascade leads to activation of genes involved in secondary metabolite pathway and a response (Cramer et al., 1993). Hypersensitive response, accumulation of lignins, hydroxyproline linked glycoproteins (HPRG), cell wall components and secondary metabolites are other responses induced by elicitors (Dixon, 1986).

The current engineering strategies for studying and utilizing elicitation response for secondary metabolite synthesis involve the use of fungal extracts/filtrate solutions. However, these preparations contain several components and the active components sometimes are very difficult to determine. Hence specific elicitors, namely cell wall components (chitin and polygalacturonic acid), pectinase (a fungal endopolygalacturonase) and jasmonic acid (a signal transducer), were utilized in Chapter 4 to induce secondary metabolite biosynthesis. Studies of elicitor dosage and elicitor exposure times are important from a production standpoint. Low dosages of elicitor may not produce a response while high dosages can lead to cell death therefore dosage studies are essential to determine the right concentration of elicitor required. Transient induction of genes is also observed in response to elicitation hence, transient metabolite profiles also needs to be quantified to determine the right harvest times for maximum production. The affects of aforementioned elicitor dosages and exposure times on biomass and indole alkaloid accumulation is presented in Chapter 4.

Biomass is an integral part of overall productivity of a metabolite. Since the precursors for biomass are provided by primary metabolism, a study of flux
distribution in the basic primary metabolism pathways is important. The feasibility of non-invasively studying the primary metabolism of hairy root cultures using NMR spectroscopy was demonstrated earlier in our laboratory (Ho, 1994). A microperfusion reactor system was developed for non-invasive metabolic studies of hairy root cultures. The reactor system was characterized and utilized to determine the short term transient intracellular concentrations of several intermediates in the primary metabolism pathways of *C. roseus* hairy root cultures using $^{13}\text{C}$ and $^{31}\text{P}$ NMR spectroscopy, with [1- $^{13}\text{C}$] labeled glucose as the sugar source (Ho, 1994).

As an extension of that work, the NMR reactor system was utilized in long term experiments to determine the concentrations of several intermediates in primary metabolism. Glucose labeled in [1-$^{13}\text{C}$], [2-$^{13}\text{C}$] and [6-$^{13}\text{C}$] positions was utilized as the carbon source in separate experiments. The flow of $^{13}\text{C}$ label in the metabolic pathways was followed in all experiments to study the distribution of carbon fluxes in the glucan synthesis, glycolytic and the pentose phosphate pathway. $^{31}\text{P}$ NMR spectroscopy was utilized simultaneously in all experiments to obtain information on pH regulation and compartmentation. The results obtained from those experiments are presented in Chapter 5.

The NMR reactor system was further utilized to study the metabolism of hairy root cultures in response to addition of specific elicitors. The changes in compartmental pH, phosphate accumulation, distribution of carbon fluxes in the carbohydrate synthesis, glycolytic and the pentose phosphate pathway were
monitored upon addition of pectinase, polygalacturonic acid and jasmonic acid. The results obtained from these studies are presented in Chapter 6.

A few suggestions for future work are presented in Chapter 7.
CHAPTER 2: BACKGROUND

*C. roseus* has been an important system for research in the area of secondary metabolite production. This plant produces a large variety of secondary metabolites (at least 75 indole alkaloids), several of which are pharmacologically active. The indole alkaloid pathway for *C. roseus*, depicted in Figure 1-1, shows the two FDA approved anti-neoplastic agents: vinblastine and vincristine, and the anti-hypertensive drugs: ajmalicine and serpentine. Plant cell and tissue cultures have been explored as an alternative source for the production of these alkaloids (Kutney *et al.*, 1980; Eilert *et al.*, 1986; Morris, 1986; Parr *et al.*, 1988; Asada and Shuler, 1989; Toivonen *et al.*, 1990; Bhadra *et al.*, 1991; Jung *et al.*, 1992; Loyola-Vargas *et al.*, 1992; Vazquez-Flota *et al.*, 1994). These cultures are important as they are free from environmental concerns and seasonal variations, and also offer a source for uniform and controlled production of desired compounds. However, although ajmalicine, serpentine, catharanthine and tabersonine can be synthesized by cell and tissue cultures of *C. roseus*, vindoline (one of the precursors of the anti-neoplastic agents) is absent.

Optimization of conditions for maximization of production of secondary metabolites involves knowledge of several factors, including biochemical pathways, relationship between cell differentiation and product formation, nutrient formulation of the medium, hormone levels required for optimal growth and secondary metabolite
biosynthesis, and environmental factors such as medium pH, light, temperature and stress. In most cases, optimization of conditions for maximization of secondary metabolite production involves separate studies for growth and production. Two stage processes are often designed based upon such determinations, with the cell mass being produced in the first stage and secondary metabolites being produced in the second.

2.1 Economic Aspects

Volumetric productivity is the key factor in commercialization of plant cell based products. The revenue derived from a variety of fermentations processes fall in the range of 7 $\$/L/day to 23 $\$/L/day (Payne et al., 1992). Payne et al. (1992) have also calculated the required price to achieve a revenue of 15 $\$/L/day and in some cases a wholesale price for several compounds of interest. Several cost-price estimations for industrial plant cell biotechnology products have been published, the objective of these studies being an economic assessment of the feasibility of an industrial process (Sahai and Knúth, 1985; Drapeau et al., 1986; van Gulik et al., 1988). Sahai & Knúth (1985) compared the price of production of a metabolite in culture, based upon the hypothetical yield of various compounds, from reactor systems, with the market price of the compound. A more realistic approach was adapted by Drapeau et al. (1986), who compared the cost of producing ajmalicine from C. roseus in plant culture with the cost of producing it from the whole plant. It was shown that the specific rate of biosynthesis
in cell culture (0.2 mg/gDW/day) will have to be increased 40 fold for the cell culture process to compete with the naturally grown plant as the source of production. Hence multifold increases in productivity are desirable for substantial improvements in process economics.

2.2 Nutrient and environmental factors influencing accumulation of indole alkaloids

Environmental factors as well as plant material genotype exert control on the production of alkaloids by plant tissue cultures. Over the last decade several approaches, involving nutrient and environmental condition optimizations, have been tried to enhance the production of the secondary metabolites in plant cell and tissue cultures. The effect of components of the medium including carbohydrate source, nitrogen, phosphate and addition of precursors to the medium have been tried to enhance secondary metabolite production. Furthermore, the effect of light, temperature and pH of the culture medium have also been studied as a means to enhance secondary metabolite production (for a review see van der Heijden et al. (1989)).

These strategies lead to an enhancement in the alkaloid production; however, the increase in production is at most 3-4 times the basal levels. Such an enhancement is not enough to be able to produce these alkaloids commercially. Fungal elicitation is an important technique used to enhance the production of secondary metabolites
(Whitehead and Threlfall, 1992; Singh, 1996). It has been shown that fungal elicitation could enhance the alkaloid yields by as much as 40 times the basal levels (Eilert et al., 1987).

2.3 Fungal Elicitation

Fungal elicitation technique provides a single stage strategy for enhancement in secondary metabolite production, which is important from commercial viewpoint. Fungal elicitation leads to an inducible response in the form of deposition of callose, lignin and glucans or accumulation of phytoalexins (Dixon, 1986). The induction of these defense reactions in response to a pathogen attack is believed to be mediated by an initial natural recognition process between plant and the pathogen. This involves detection of certain unique structural features of incompatible pathogens by receptor like molecules in plants, with a subsequent resultant cascade of biochemical events that lead ultimately to expression of resistance (A pictorial representation of induction of this response is presented in Figure 2-1). Elicitors are also believed to modulate the activity of preexisting transcription factors causing rapid induction or repression of enzymes (Pasquali et al., 1992).
Figure 2-1 A simplified picture of the interaction of elicitors with plant cells. The steps involved in manifestation of the defense response include recognition by receptors present on the plasma membranes, signal transduction via one or more signal transducers, gene activation, gene expression and response (Modified from (Cramer et al., 1993))
Different kinds of classifications have been proposed for elicitors

a) **Classification based on specificity**: Elicitors are said to be specific if they have different elicitor activity in various plant cultures. If they do not have any difference in activity they are called non specific (Yoshikawa et al., 1993).

b) **Classification based on origin**: In this classification the elicitors are said to be

1) Biotic elicitors :- If they are obtained from biological sources. These include fungal cell wall components, fungal enzymes and lipids.

2) Abiotic elicitors :- If they are of physical or chemical origin. e.g. UV light, heavy metal ions etc.

3) Constitutive/endogenous elicitors :- If they are produced in elicitor treated or microbially infected plant cells (Whitehead and Threlfall, 1992).

To ensure optimal product accumulation, several criteria have to be determined experimentally. These include elicitor specificity, elicitor concentration and duration of exposure to the culture (Kurz et al., 1989). The primary process of elicitor cell interactions can occur at various levels such as binding to membrane-linked receptors, membrane depolarization and changes in membrane permeability (Kurz et al., 1989). Most strategies involving fungal elicitation use elicitors prepared by autoclaving or homogenizing fungi (Table 2-1). In some cases the fungus chosen is a natural pathogen while in other cases several elicitors, not necessarily the natural pathogen, are selected and the most active elicitor is chosen.
Table 2-1: Elicitor mediated accumulation of secondary metabolites in plant cell cultures.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Culture type</th>
<th>Elicitor</th>
<th>Phytoalexins formed</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tabernaemontana</em> 4-species</td>
<td>Cells</td>
<td><em>Aspergillus niger</em>&lt;sup&gt;(E)&lt;/sup&gt; <em>Candida albicans</em>&lt;sup&gt;(E)&lt;/sup&gt; Cellulases</td>
<td>10 compounds including Ursolic acid, Oleanolic acid</td>
</tr>
<tr>
<td><em>Ipomoea batatas</em> Sweet potato</td>
<td>Callus</td>
<td>Yeast extract in culture media</td>
<td>Ipomeamarone Dehydroipomeamarone 4-Hydroxyxymyoporone</td>
</tr>
<tr>
<td><em>Gossypium</em> Cotton</td>
<td>Cells</td>
<td><em>Saccharomyces cervisiae</em> <em>Verticillium dahliae</em> (live, E)</td>
<td>Hemigossypol, Gossypol, 6-O-Methyl gossipol 6-O-Methylhemigossypol</td>
</tr>
<tr>
<td><em>Dianthus caryophyllus</em> Carnation</td>
<td>Callus</td>
<td><em>Phytophthora parasitica</em> (&lt;E, CF&gt;)</td>
<td>Dianthalexin Dianthramide A,B</td>
</tr>
<tr>
<td><em>Morinda citrifolia</em></td>
<td>Cells</td>
<td>Chitin</td>
<td>Anthraquinones</td>
</tr>
<tr>
<td><em>Sanguinaria canadensis</em> <em>Papaver bracteatum</em></td>
<td>Cells</td>
<td><em>Verticillium dahliae</em> (cells) <em>Dendryphion</em> (E)</td>
<td>Sanguinarine</td>
</tr>
<tr>
<td><em>Cicer arietinum</em> Chick pea</td>
<td>Cells</td>
<td>Subculturing yeast extract</td>
<td>Medicarpin Maackian</td>
</tr>
<tr>
<td><em>Glycine max</em> Soybean</td>
<td>Callus, cells</td>
<td><em>Endopolygalacturonase</em> <em>Phytophthora megasperma</em> <em>Pseudomonas syringae</em></td>
<td>Glyceollin I, II, III Daidzein Coumestrol</td>
</tr>
<tr>
<td><em>Phaseolus vulgaris</em> French bean</td>
<td>Callus, cells</td>
<td><em>Aspergillus niger</em>&lt;sup&gt;(E)&lt;/sup&gt; <em>Botrytis cinerea</em></td>
<td>Diadzein 2-Hydroxydiadzein</td>
</tr>
<tr>
<td>Dwarf bean</td>
<td></td>
<td><em>Colletotrichium lindemuthianum</em> (E) <em>Macrophonina phaseolina</em></td>
<td>2-Hydroxygenistein Keivitone Phaseollin</td>
</tr>
<tr>
<td>Plant Species</td>
<td>Cell Type</td>
<td>Pathogens</td>
<td>Chemicals</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-----------</td>
<td>------------------------------------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td><em>Capsicum annuum</em> (Sweet potato)</td>
<td>Callus</td>
<td>Pectinase Cellulase</td>
<td>Capsidol</td>
</tr>
</tbody>
</table>
| *Nicotiana tabacum* (Tobacco)  | Callus, cells | **Phytophthora parasitica**
|                               |           | *Pseudomonas solanacearum*                      | Capsidol Debneyol                              |
|                               |           | *P. syringae pv. tabaci*                        | Rishitin, Phytuberin, Phytuberol               |
| *Solanum tuberosum* (Potato)   | Callus    | *Phytophthora infestans*                        | Lubimin, Phytuberin, Rishitin, Solavetivone    |
| *Petroselinum* (Parsley)       | Cells     | *Alternaria carthamal*                          | Bergapten, Graveolone, Psoralen, Xanthotoxin  |
|                               |           | *Phytophthora megasperma* f. sp. glycines      |                                               |
| *Daucus carota* (Carrot)       | Cells     | *Botryis cinerea* (CF)                            | Dihydroisocoumarin                             |
|                               |           | Endopolygalacturonase                            | 6-Hydroxymellein                               |
|                               |           | *Fusarium monoliforme*                          | 6-Methoxymellein                              |
|                               |           | *Helminthosporium oryzae*                       |                                               |
|                               |           | Pectinase                                       |                                               |
| *Hyoscyamus muticus*           | Hairy roots | *Rhizoctonia solani* (E)                       | Sesquiterpene                                  |
| *Tagetes patula*               | Hairy roots | *Fusarium conglutinans* Aspergillus niger | Thiophene                                      |

Abbreviations:- E, Elicitor preparation; CF, Culture Filtrate.

Reference:- (Whitehead and Threlfall, 1992; Singh, 1996)
Some of the active components, including branched β-glucans present in the fungal cell walls, in elicitor extracts/homogenates have been characterized. Albersheim et al. (1992) have determined the structure and size of glucan elicitors from mycelial walls of *Phytospora meagasperma*. The primary structure of the smallest elicitor fragment, a hepta-β-glucopyranoside, was confirmed by chemical synthesis (Albershiem et al., 1992). Chitinous substances are another class of compounds in the cell walls of many fungi which may be important for induction of defense response. Chitin has been found to be an active elicitor of pisatin in pea (Hadwinger and Beckman, 1980) and was also found to be capable of inducing lignification of cell walls in wheat (Pearce and Ride, 1982).

Endogenous components of plant cell walls have also been shown to elicit phytoalexin production. Davis et al. (1986) have demonstrated that exogenous application of enzymes can release endogenous elicitors from plant cell walls. Hence, it is possible that polygalacturonases secreted by plant cell walls, upon activation due to the cell damage caused by fungal infection or elicitor treatment, release endogenous elicitors which trigger phytoalexin production (Yoshikawa et al., 1993). Structural analysis using specific glucan degrading enzymes and $^{13}$C NMR has shown that the released elicitors were composed of β-1,4 linked main chain which were bound to the cell walls by β-1,3 linked side chains (Figure 2-2). The attack of endoglucanases on the side chains leads to release of β-1,4 linked chains with one to three β-1,3 linked moieties on
the side chain (Yoshikawa et al., 1993). These elicitor fragments bind to receptors present on the plasma membrane and lead to a signaling cascade which leads to transcription of DNA and translation for phytoalexin production. However, the final levels of phytoalexins accumulated in the plant cell are not regulated by induced biosynthetic activity alone but by degradation rates also. Hence there exists a balance between the synthesis and degradation rates of the phytoalexins in plant cells (Yoshikawa et al., 1993).
Figure 2-2 Proposed structures of elicitors bound to cell walls of *Phytophthora megasperma* f. sp. *glycinea* and the released form that result from attack by β-1,3 endoglucanase. Upon infection, β-1,3 side chains are attacked by the endo-type soybean β-1,3 glucanase, with release of elicitor active β-1,6-chains of various chain lengths with occasional side branches of one or two β-1,3 linked glucose moities and dimers or trimers of β-1,3-glucans derived from the attack by the endoglucanase β-1,3 side chains of cell wall-bound elicitors (adapted from Yoshikawa *et al.*, 1993).
2.4 Elicitation of *C. roseus* cultures

Effects of several fungal elicitors on *C. roseus* suspension and organ cultures have been studied. Elicitation of several cell lines has lead to induction of alkaloid biosynthetic pathway (van der Heijden *et al.*, 1989). Response to elicitation is highly dependent on the cell line. The diversity and variation of elicitor activity emphasizes the necessity to screen a wide variety of compounds for elicitor activity (Eilert *et al.*, 1986). Table 2-2 presents a summary of elicitors used to induce defense response in *C. roseus* cultures.
Table 2-2 Fungal elicitors used to enhance alkaloid production by *Catharanthus roseus* cultures.

<table>
<thead>
<tr>
<th>Elicitor used</th>
<th>Type of Cultures</th>
<th>Alkaloids monitored</th>
<th>Control levels (µg/gDW)</th>
<th>Maximum Elicited levels (µg/gDW)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alternaria zinnac</em></td>
<td>Cell suspension</td>
<td>tryptamine</td>
<td>245</td>
<td>1304</td>
<td>(Eilert <em>et al.</em>, 1986)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strictosidine</td>
<td>3</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ajmalicine</td>
<td>12</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Catharanthine</td>
<td>0</td>
<td>112</td>
<td></td>
</tr>
<tr>
<td><em>Colletotrichium gloeosporoides</em></td>
<td></td>
<td>tryptamine</td>
<td>245</td>
<td>902</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strictosidine</td>
<td>3</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ajmalicine</td>
<td>12</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Catharanthine</td>
<td>0</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td><em>Pythium aphanidermatum</em></td>
<td></td>
<td>tryptamine</td>
<td>245</td>
<td>1438</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strictosidine</td>
<td>3</td>
<td>165</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ajmalicine</td>
<td>12</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Catharanthine</td>
<td>0</td>
<td>136</td>
<td></td>
</tr>
<tr>
<td><em>Sclerotina sclerotiorum</em></td>
<td></td>
<td>tryptámine</td>
<td>245</td>
<td>1498</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strictosidine</td>
<td>3</td>
<td>114</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ajmalicine</td>
<td>12</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Catharanthine</td>
<td>0</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td><em>Verticillium dahliae</em></td>
<td></td>
<td>tryptamine</td>
<td>245</td>
<td>2068</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strictosidine</td>
<td>3</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ajmalicine</td>
<td>12</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Catharanthine</td>
<td>0</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Organism</td>
<td>Culture Type</td>
<td>Alkaloids</td>
<td>Concentration</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>--------------</td>
<td>------------------------------------------</td>
<td>-------------------</td>
<td>------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td><em>Pythium vexans</em></td>
<td>Cell suspension</td>
<td>Ajmalicine, Serpentine, Catharanthine</td>
<td>1.96 (µg/100ml)</td>
<td>(Nef et al., 1991; Nef-Campa et al., 1993)</td>
<td></td>
</tr>
<tr>
<td><em>Pythium aphanidermatum</em></td>
<td>Cell suspension</td>
<td>Tryptamine, strictosidine, ajmalicine</td>
<td>60 µmoles/L, 5 µmoles/L</td>
<td>(Moreno et al., 1993)</td>
<td></td>
</tr>
<tr>
<td><em>Geotrichium candidum</em> + Vandyl sulphate + Potassium chloride</td>
<td>Cell suspension</td>
<td>Total alkaloid</td>
<td>0.069 mg/gDW</td>
<td>(Kargi and Potts, 1991)</td>
<td></td>
</tr>
<tr>
<td><em>Phytophthora megasperma f. sp. glycinea</em></td>
<td>Cell suspension</td>
<td>DHBA, Ajmalicine</td>
<td>0 µg/ml, 5 µg/gFW</td>
<td>(Frankmann and Kauss, 1994; Moreno et al., 1994)</td>
<td></td>
</tr>
<tr>
<td><em>Pythium aphanidermatum</em></td>
<td>Cell suspension</td>
<td>Total alkaloids</td>
<td>20 µg/gDW</td>
<td>(Seitz et al., 1989)</td>
<td></td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td>Cell suspensions</td>
<td>Total alkaloids</td>
<td>50 µg/gDW</td>
<td>(Godoy-Hernández and Loyola-Vargas, 1997)</td>
<td></td>
</tr>
<tr>
<td><em>Phytophthora cactorum</em></td>
<td>Cell suspension</td>
<td>Ajmalicine, Serpentine</td>
<td>40 mg/L, 6 mg/L</td>
<td>(Asada and Shuler, 1989)</td>
<td></td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>Hairy roots</td>
<td>Ajmalicine, Catharanthine</td>
<td>2.7 mg/L, 6 mg/L</td>
<td>(Sim et al., 1994)</td>
<td></td>
</tr>
<tr>
<td><em>Penicillium spp.</em></td>
<td></td>
<td>Ajmalicine, Catharanthine</td>
<td>2.7 mg/L, 6 mg/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>Hairy roots</td>
<td>Ajmalicine</td>
<td>Catharanthine</td>
<td>Ajmalicine</td>
<td>Catharanthine</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------</td>
<td>------------</td>
<td>---------------</td>
<td>------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Macerzyme</td>
<td></td>
<td>3.75 mg/L</td>
<td>1 mg/L</td>
<td>7.5 mg/L</td>
<td>1 mg/L</td>
</tr>
<tr>
<td>Cellulase</td>
<td></td>
<td>4 mg/L</td>
<td>1 mg/L</td>
<td>6 mg/L</td>
<td>1 mg/L</td>
</tr>
<tr>
<td><em>Aspergillus</em> spp.</td>
<td></td>
<td>8 mg/L</td>
<td>1.5 mg/L</td>
<td>13 mg/L</td>
<td>1.7 mg/L</td>
</tr>
<tr>
<td><em>Trichoderma reesei</em></td>
<td></td>
<td>8 mg/L</td>
<td>1.5 mg/L</td>
<td>7 mg/L</td>
<td>0.6 mg/L</td>
</tr>
<tr>
<td>Methyl jasmonate</td>
<td></td>
<td>20 mg/L</td>
<td>1.5 mg/L</td>
<td>30 mg/L</td>
<td>3 mg/L</td>
</tr>
</tbody>
</table>
2.4.1 Cell suspension cultures

The effect of several elicitors on indole alkaloid production by \textit{C. roseus} cell suspension cultures has been studied. Eilert \textit{et al}. (1986) studied the effect of homogenates of \textit{Alternaria zinniae}, \textit{Pythium aphanidermatum}, \textit{Verticillium dahliae}, and \textit{Rhodotorula rubra} on 5 cell lines of \textit{C. roseus}. These studies revealed an increase in the level of several alkaloids (including tryptamine, strictosidine lactam, ajmalicine, tabersonine and catharanthine) in cells and medium after 12-24 hours of treatment. Treatment with elicitors also caused a browning effect in the cell and medium. After the addition of homogenized \textit{Pythium aphanidermatum} preparation to a \textit{C. roseus} cell line, Eilert \textit{et al}. (1987) saw a rapid accumulation of indole alkaloids preceded by rapid transient increases in tryptophan decarboxylase (TDC) and strictosidine synthetase (SS) activity. It has been shown that this increase in activity occurs as a result of specific increases in TDC and SS mRNAs (Roewer \textit{et al}., 1992). Modulation of activity of pre-existing transcription factors has been suggested to lead to the transcriptional response. These genes are known to be rapidly downregulated by auxin in suspension cell cultures (Pasquali \textit{et al}., 1992). After addition of \textit{Pythium aphanidermatum} filtrate, Seitz \textit{et al}. (1989) found an increase in accumulation of phenolic compounds correlated with the induction of phenylalanine ammonia lyase (PAL) activity.

Nef \textit{et al}. (1991) studied the effect of \textit{Pythium vexans} extracts on alkaloid accumulation and compartmentation. At low elicitor concentration, increase in
ajmalicine production was observed within the first 24 hours but no effect on serpentine accumulation was observed. Catharanthine was synthesized de novo. At higher elicitor concentrations, ajmalicine production was decreased 8-fold compared to control and catharanthine biosynthesis was inhibited. Elicitation also caused excretion of alkaloids into the medium. Nef-Campa et al. (1993) studied the affect of long term treatment of Pythium vexans extracts on growth and alkaloid production of C. roseus cell suspensions. Repeated treatments were found to have a negative long term effect on both growth and alkaloid accumulation and did not appear to be feasible for production purposes. It was postulated that long term treatment could act on cell wall structure, ionic equilibrium or membrane permeability leading to undesirable effects.

Moreno et al. (1993) observed an increase in tryptamine accumulation on addition of Pythium aphanidermatum filtrate to C. roseus cell suspension. The combination of elicitation with feeding terpenoid precursors did not improve alkaloid accumulation. However, the decline in strictosidine levels was postulated to be due to strictosidine catabolism. C. roseus cultures were also found to produce large amounts of 2,3 dihydrobenzoic acid (DHBA) after elicitation. The accumulation of this compound was strongly correlated with isochorismate mutase synthase induction (Frankmann and Kauss, 1994; Moreno et al., 1996).

Synergistic effects have also been explored in the cell suspension culture studies. Kargi and Potts (1991) determined the affect of three factors (fungal elicitor Geotrichium candidum, vandyke sulphate and potassium chloride) on indole alkaloid
production. Low concentration of these stress factors increased alkaloid accumulation while higher quantities resulted in toxic effects leading to loss of cell viability. Asada and Shuler (1989) studied the effect of in situ adsorption, elicitation with Phytophthora cactorum and immobilization of cells in calcium alginate. The amount of ajmalicine in the medium for the cells subjected to all three treatments was 65 times more than that in the control cultures.

2.4.2 Hairy root cultures

Effects of hydrolytic enzymes (Chitinase, macerozyme and cellulase), fungal homogenates (Aspergillus spp., Trichoderma virise, Trichoderma reseii), a yeast (Rhoditurula marina) and methyl jasmonate on catharanthine and ajmalicine synthesis has been studied in Catharanthus roseus hairy root cultures (Vazquez-Flota et al., 1994). Catharanthine levels did not change when hydrolytic enzymes were employed. Ajmalicine liberation into the media was observed on addition of 0.1% macerozyme or 1% cellulase. Ajmalicine content in roots treated with 10 μl of methyl jasmonate was four fold with respect to untreated roots. Use of Aspergillus homogenates also resulted in increase in both the accumulation and yield of ajmalicine, whereas, no response was found with other fungi or yeast.

Jung et al. (1994) studied the effects of in situ adsorption, fungal elicitation by Aspergillus niger and Penicillium spp. homogenates and permeabilization on production and secretion of alkaloids by C. roseus hairy root cultures. Catharanthine
production increased 2.5 times when 0.01g/l of *A. niger* homogenate was added and both catharanthine and ajmalicine showed a significant increase on addition of 0.01 g/l *Penicillium* sp. homogenate. Synergistic action of *in situ* adsorption, permeabilization and fungal elicitation resulted in enhancement in total production of ajmalicine and catharanthine.

2.5 Nuclear Magnetic Resonance (NMR) Spectroscopy

2.5.1 Basic NMR Concepts

NMR spectroscopy utilizes the principle of detection of the spin of a nuclei placed in a magnetic field. Certain atomic nuclei (having odd mass numbers e.g. \(^1\text{H}, \quad ^{31}\text{P}, \quad ^{13}\text{C}\) etc.) possess a nuclear magnetic moment and an angular momentum. *In vivo* NMR experiments involve application of two magnetic fields to a cellular sample. The first field, applied in the z direction, is a strong field which causes the nuclear dipoles to align in the z direction. Since more nuclei are aligned in the direction of the magnetic field (low energy state) than against it (high energy state), a net magnetization in the direction of the field is obtained. These nuclei precess about the static magnetic field at a characteristic frequency called larmor frequency.

The second field (a superimposed magnetic field) is applied in form of short powerful radio frequency (RF) pulses to induce transitions between energy levels. The band width of these pulses is adjusted sufficiently to allow resonance of all nuclei. The population difference between the energy levels (the sole cause of nuclear transitions)
is dependent upon the frequency of pulses. Rapid pulsing can lead to equalization of
nuclei at the two energy levels (saturation), decreasing the absorption signal intensity
to zero. Hence saturation correction factors are required for quantitative analysis of a
rapid pulsing experiment.

The differences in the chemical and physical environment of a nuclei in various
compounds is the basis for an NMR signal. These differences cause nuclei to resonate
at frequencies, slightly altered from their larmor frequency (expressed as chemical
shifts in dimensionless form). The receiver detects the decay of transverse
magnetization after the application of the RF pulse. This signal (called free induction
decay or FID) decays due to field inhomogeneities or relaxation processes. A series of
RF pulses are applied and the FIDs collected are summed up to obtain a better signal
to noise ratio (S/N) in the frequency domain. Exponential multiplication is also applied
to further improve S/N.

2.5.2 Intracellular pH/Compartmentation

In one of the first in vivo applications of NMR, Moon and Richards (1973)
found that the chemical shift of the P_i resonance was dependent upon the pH of the
environment and used this property to measure intracellular pH. Since then several
phosphorylated compounds like ATP, 1,3 diphosphoglycerate (Moon and Richards,
1973), inorganic phosphate, glucose-6-phosphate (Shanks and Bailey, 1988) and
orthophosphate (Gout et al., 1992) have been used as pH probes. The principal
advantage of NMR methods for pH measurements are that measurements of several compartments can be made simultaneously, nondestructively and noninvasively as compared to other techniques. The pH value obtained using NMR is dependent upon the intercellular ion content of the cellular compartments. Hence, calibration curves need to be made under conditions that mimic the intercellular environment as close as possible.

pH plays an important role in regulating the transport of several metabolites within the various compartments of the plant cell and the activities of enzymes therein. Gout et al. (1992) have studied the regulation of pH in cytoplasm and vacuole of sycamore (Acer pseudoplantus) cells. They found that the rate of oxygen consumption increases and the cytoplasmic ATP concentration decreases as the external pH is decreased. The cytoplasmic ATPase was found to play an important role in pH maintenance as at low ATP concentrations the pH of the cytoplasm was not maintained at a constant value. As the external pH was increased, production of organic acids (malate and citrate) was observed due to activation of PEP carboxylase owing to an increase in cytoplasmic bicarbonate concentration. The metabolic pathways in Catharanthus roseus are under very complex regulatory control and pH gradients are known to play an important role. The knowledge of pH of the various compartments and the effect of elicitation on pH of the various compartments is essential for understanding the complex regulatory control mechanism in Catharanthus roseus.
2.5.3 Applications of NMR to metabolic studies

Intracellular kinetics of a cell population growing in a bioreactor is very difficult to study. Most of the techniques utilized involve an analysis of nutrients and products present extracellularly by conventional analytical methods. These methods do not provide us with enough information about the complex transport and kinetic parameters governing growth. NMR spectroscopy has emerged as a powerful technique for metabolic studies of several cell types (Gadian, 1982; Shulman, 1983). The ability to monitor several labeled intermediates in the metabolic pathways, in one scan, overrides the tedious analysis required for each compound. Furthermore NMR spectroscopy, owing to its non-invasiveness, offers an excellent technique for simultaneous monitoring of intracellular concentrations of metabolites, annulling the need for separate extraction and quantification of each compound.

$^{31}$P NMR is very popular for in vivo studies since $^{31}$P nucleus is 100% naturally abundant, and most of the phosphorylated metabolites (e.g. ATP, sugar phosphates, inorganic phosphorous) are present in relatively high concentrations. Several systems have been studied using $^{31}$P NMR including citrus (Ben-Hayyim and Navon, 1985), C. roseus (Martin et al., 1982; Vogel and Brodelius, 1984; Guern et al., 1989), Glycine max (Martin et al., 1982), Acer pseudoplatanus (Martin et al., 1982), maize root tips (Tu et al., 1990), and Nicotiana tabacum (Wray et al., 1983). $^{31}$P NMR has also been used to determine pH in both cytoplasmic and vacuolar compartments of plant cells and to study uptake and intracellular distribution of
phosphorous (Roberts et al., 1981; Vogel, 1987; Chang and Roberts, 1988; Guern et al., 1988; Fox and Ratcliffe, 1990).

$^{13}$C NMR has been used to obtain information about a variety of non-phosphorylated metabolites. Because of its low receptivity (receptivity=$1.8 \times 10^{-4}$ for $^{13}$C, 0.066 for $^{31}$P and 1 for $^1$H), and low natural abundance (1.1%), $^{13}$C NMR experiments require isotopic enrichment. $^{13}$C resonances are narrow and occupy a wide range (200 ppm) providing good spectral resolution to get dynamic metabolic information and to follow fluxes of NMR isotopes. $^{13}$C NMR studies have been used to study metabolic pathways and calculate metabolic fluxes in hybridoma cells, *Corynebacterium melassecola* (Rollin et al., 1995), astrocytes (Sonnewald et al., 1996), renal cells (Jans and Willem, 1991), yeast (Den Hollander et al., 1979; Den Hollander and Shulman, 1983), and in solanaceous cell suspensions (Marty et al., 1997).

$^{15}$N NMR spectroscopy has been used to study secondary metabolism in transformed root cultures of *Datura stramonium* and *Nicotiana tabacum* (Ford et al., 1994). Since nitrogen metabolism pathways are limited as compared to carbon, the principle nitrogen pathways were utilized to study synthesis and accumulation of nitrogen containing secondary metabolites. Using $^{15}$N NMR subcellular distribution of nicotine in *Nicotiana tabacum* was also studied (Ford et al., 1994).
2.5.4 Metabolic analysis

$^{13}$C Nuclear magnetic resonance provides a new approach to analysis of metabolic pathways. Several compounds can be identified based upon their chemical shifts and their concentrations can be monitored noninvasively. By using selectively labeled precursors, the path of the labeled atom can be followed during growth and accumulation of several intermediates and endproducts can be studied. $^{13}$C has a natural abundance of 1.1% and hence expensive isotopic enrichment is required to study endogenous metabolites. However, the low natural abundance can also be an advantage as it eliminates the possibility of background interference from endogenous metabolites. This technique can be used with several tissues and is an excellent tool in vivo studies. $^{13}$C NMR has been used to study metabolic pathways in rat heart (Chance et al., 1983; Malloy et al., 1988; Sherry et al., 1988; Malloy et al., 1990a; Malloy et al., 1990b), E. coli (Urgbil et al., 1978), yeast (Den Hollander et al., 1979; Den Hollander and Shulman, 1983), and mammalian cells (Scharfstein et al., 1993).

In plant cells the metabolic network includes multiple branch points, few secreted products and several transport steps between the various compartments. $^{13}$C NMR can be used to determine the intracellular fluxes and to study their response to environmental changes (e. g. elicitation), thereby providing unique information about biosynthetic pathway and their regulation. In this study [1-$^{13}$C], [2-$^{13}$C] and [6-$^{13}$C] labeled glucose will be used to elucidate the complex primary metabolic pathways in
*Catharanthus roseus* hairy root cultures. Figures 2-3, 2-4, and 2-5 shows the fate of C-1, C-2 and C-6 atoms respectively of glucose as they go through the metabolic pathway. The effect of competing pathways (Pentose Phosphate Pathway and glycolysis), anaplerotic pathway (Pyruvate to Malate) and accumulation (Glucans, alanine, glutamine, glutamate) can be studied using NMR. The relative fluxes through the glycolytic, pentose phosphate pathway and carbon storage pathway can be determined by measuring the peak areas from the spectra of tissues supplied with $^{13}$C-enriched substrates.
Figure 2-3 Incorporation of label from [1-\(^{13}\)C] glucose into PPP pathway, glycolysis and TCA cycle. Intermediates: G, Glucose; F, Fructose; GA, Glyceraldehyde; PE, Phospho-enolpyruvate; A, Acetyl CoA; C, Citrate; K \(\alpha\)-Ketoglutarate; N, Glutamine; S, Succinate; M, Malate; O, Oxaloacetate. Bold letters represent \(^{13}\)C label. Labeling in the first turn of TCA cycle has been shown.
Figure 2-4 Incorporation of label from [2-^{13}C] glucose into PPP pathway, glycolysis and TCA cycle. Intermediates: G, Glucose; F, Fructose; GA, Glyceraldehyde; PE, Phospho-enolpyruvate; A, Acetyl CoA; C, Citrate; K \( \alpha \)-Ketoglutarate; N, Glutamine; S, Succinate; M, Malate; O, Oxaloacetate. Bold letters represent \(^{13}C\) label. Underlined letters in TCA cycle represent labeling from [1-\(^{13}C\)] acetyl CoA. Labeling in the first turn of TCA cycle has been shown.
Figure 2-5 Incorporation of label from $[6^{-13}C]$ glucose into PPP pathway, glycolysis and TCA cycle. Intermediates: G, Glucose; F, Fructose; GA, Glyceraldehyde; PE, Phospho-enolpyruvate; A, Acetyl CoA; C, Citrate; K, α-Ketoglutarate; N, Glutamine; S, Succinate; M, Malate; O, Oxaloacetate. Bold letters represent $^{13}C$ label. Labeling in the first turn of TCA cycle has been shown.
CHAPTER 3: EFFECT OF SUBCULTURE CYCLE ON GROWTH AND INDOLE ALKALOID PRODUCTION BY C. ROSEUS HAIRY ROOT CULTURES.

(this chapter in its current form has been submitted to The Journal of Biotechnology)

3.1 Abstract

The effect of age of the inoculum on C. roseus hairy root culture growth and indole alkaloid production was investigated by adapting the cultures to 2 week, 3 week and 4 week subculture cycle routines. Conductivity of the culture medium was measured every 2-3 days and the root fresh weight determined using a conductivity-fresh weight correlation. Cultures were harvested, in triplicate, at 21, 28 and 35 days and analyzed for final biomass and indole alkaloid content. These experiments demonstrated that age of inoculum could lead to up to 25% increase in the specific growth rates of hairy root cultures when the subculture cycle routine is changed from 4 weeks to 2 weeks. The 2 week subculture cycle yielded the fastest, while the 4 week cycle yielded the lowest, specific growth rates. Specific yields of tabersonine decrease from day 21 to 35 while the total yields of hörhammericine increase in all three subculture cycles. Lochnericine yields are highest in the faster growing cultures (2 week cycle) indicating a strong correlation to growth rate. Serpentine yields are lowest in the 2 week cycle and highest in the 4 week cycle indicating inverse proportionality of serpentine accumulation to growth rate.

Keywords: - Hairy roots; C. roseus; Indole alkaloids; growth rate.
3.2 Introduction

Hairy root cultures represent a promising alternative to plant cell suspensions for production of secondary metabolites (Hamill et al., 1987; Flores and Curtis, 1992). The advantages of hairy roots as compared to plant cells include genotypic and biochemical stability, morphological differentiation, and growth in hormone-free media. Hairy root cultures of several plant species have been developed and explored for synthesis of medicinally important chemicals but the processes have not been commercialized as yet due to low production levels as compared to the whole plant (Verpoorte et al., 1994). The success of a hairy root culture system is dependent upon fast growth rates and high accumulation of secondary metabolites (Verpoorte et al., 1991). Strategies such as selection of high producing cell lines and optimization of growth and production media (DiCosmo, 1990; Verpoorte et al., 1993; Moreno et al., 1995) have been applied to improve the growth rate of hairy root culture. However, a uniform basis for inoculum age selection does not exist.

Inoculation of hairy root cultures is performed by transferring a defined amount of biomass to fresh medium. A statistical design has been reported for optimization of inoculum conditions, emphasizing that the length of individual root tips had the most significant effect on the specific growth rate (Bhadra and Shanks, 1995). The effect of inoculum morphology on hairy root cultures of Atropa belladonna was reported recently. This study indicated that the presence or absence of laterals in the inoculum
did not influence the growth rate or the hyoscyamine content (Falk and Doran, 1996). However, inoculum age was not a variant in either of these studies. In cell suspension cultures, an inoculum of rapidly dividing young cells demonstrated the greatest growth rates and hence faster biomass accumulation (Stafford et al., 1985). No studies, to our knowledge, have been performed on the effect of the age of the culture used for inoculation on the growth rates of hairy root cultures.

Hairy root cultures of *C. roseus* have been established in our laboratory for biosynthesis of valuable indole alkaloids, and the kinetics of growth and the transient accumulation of the indole alkaloids have been investigated (Bhadra and Shanks, 1997). The nature of association of total yields of ajmalicine, serpentine (antihypertensory alkaloids), and tabersonine (key precursor of vindoline) have been determined (Bhadra and Shanks, 1997). The transients of accumulation of lochnericine and hörhammericine, derived from tabersonine, have also been studied in our laboratory (Morgan, J. A.; Bhadra, R. and Shanks, J. V., manuscript under preparation). These studies have demonstrated that accumulation of tabersonine and lochnericine is strongly growth associated, accumulation of ajmalicine and hörhammericine is largely growth associated while serpentine accumulation is non-growth associated. The objective of this study was to determine the effect of age of the culture, at inoculation, on growth rate and on the production of indole alkaloids tabersonine, lochnericine, hörhammericine, ajmalicine and serpentine.
3.3 Materials and Methods

3.3.1 Hairy Root Cultures

The experiments were conducted with the *C. roseus* hairy root clone LBE 6-1 (Bhadra *et al.*, 1993). Approximately 0.05 grams fresh weight of roots (5 primary root tips with no lateral branches present, 35-40 mm long) were inoculated into a 250 ml Erlenmeyer flask containing 50 ml of Gamborgs B5/2 media with 30 g/L sucrose (Bhadra and Shanks, 1995). The pH of the medium was adjusted to 5.7 prior to filter sterilization. The cultures were grown on an orbital shaker at 100 rpm, at 26°C in the dark. Evaporation control was also done simultaneously and autoclaved water was added every week to account for the water lost due to evaporation. The cultures were adapted to their respective cycles for at least two subcultures.

3.3.2 Conductivity and biomass measurements

The conductance of the medium was measured using a conductivity electrode (Phoenix electrode company; Cell constant k=1.0) and a YSI conductance meter (Model 35). The electrode was calibrated periodically using a 100 μmho standard and milli-Q water, 0.8-1.0 μmho. Conductivity of the culture media was correlated to the fresh weight of the root cultures by harvesting the root cultures at different stages of growth and measuring their fresh weight and medium conductance. A calibration curve was set up to determine the relationship between the fresh weight of the roots and the
conductivity of the medium. The conductance correlation obtained from the calibration curve is shown in Equation 3-1

\[ W = 5.1109 - 2.3648 \times C \quad (R^2 = 0.988) \quad (3-1) \]

where \( W \) = Fresh Weight, \( C \) = Conductance.

For the three subculture cycles, about 30 ml of medium was pipetted out using a sterile pipette and was transferred into aseptic tubes every 2-3 days. The conductivity electrode, sterilized by keeping in UV light for at least 48 hours, was used to measure the conductance and then the medium was transferred back to the respective flasks aseptically. The fresh weight of the roots in each flask was determined using the conductivity correlation (Equation 3-1). At harvest, the hairy roots were dried by blotting all the external moisture and were weighed to determine the final fresh weight. The conductance of the medium did not correlate to hairy root fresh weight during the first 10 days of growth.

The fresh weights and dry weights of the root cultures were measured previously for the root line LBE 6-1 by harvesting cultures at several time points during growth (Bhadra and Shanks, 1997). A plot between the fresh weight to dry weight ratio (FW/DW) vs the fresh weight (FW) of the root cultures was used to calculate the dry weights of the root cultures between 10 and 21 days.
3.3.3 Alkaloid Analysis

The alkaloid extraction and fractionation procedure described by Bhadra et al. (1993) was used for sample preparation. The eluents were analyzed using HPLC. The HPLC system consisted of two Waters 510 pumps, a Waters Wisp 712 injector, a Phenomenex C18 bondclone reverse phase column (300 mm length, 3.9 mm diameter), a Phenomenex C18 bondclone reverse phase guard column (30 mm length, 3.9 mm diameter), and a Waters 996 PDA detector. Millennium 2010 software (Waters Corporation) was used for acquisition and analysis on a NEC Image 466es workstation. Isocratic condition using 32:32:36 mixture of methanol : acetonitrile : 5 mM (NH₄)₂PO₄ at flow rate of 1ml/min were used to separate the alkaloids. Calibrations were done on authentic standards and quantification was performed on chromatograms extracted at 254 nm and 329 nm. Peak identification was based on comparison of retention time and UV spectra with authentic standards of ajmalicine (Fluka), serpentine (Research Plus, Bayonne, NJ) and tabersonine (gift of Dr. Hamada, Okayama University, Okayama, Japan).

Hörhammericine and lochnericine were identified earlier (Vani, 1996). The amount of these compounds was based upon tabersonine response factor due to lack of standards. Since the UV spectra of these compounds was very similar to that of tabersonine (as there is no substitution on the conjugated double bond system), this quantification is believed to give realistic estimates of hörhammericine and lochnericine.
concentrations (as the substitution away from the chromophore, in both lochnericine and hörhammericine, would not affect the absorbance at 329 nm).

3.3.4 Mathematical Analysis

Since graphical interpolation and differentiation of transient batch data can lead to subjective errors, the data was represented mathematically by an equation of the form

\[ X(t) = \frac{X_m}{1 + \exp(b - ct)} \]  \hspace{1cm} (3-2)

where \( X(t) \) = biomass at time \( t \), \( X_m \) = maximum biomass, \( b \) & \( c \) are the model parameters.

The parameters \( b \) and \( c \) were independently determined for all three subculture cycle. These parameters for each subculture cycle were subsequently used to determine the instantaneous specific growth rates using the exponential growth model presented in Equation 3-2

\[ \frac{dX(t)}{dt} = \mu X(t) \]  \hspace{1cm} (3-3)

The instantaneous specific growth rate can hence be represented as

\[ \mu(t) = \frac{d(\ln X(t))}{dt} \]  \hspace{1cm} (3-4)

Substituting for \( X(t) \) from Equation 3-2

\[ \mu(t) = c \cdot \exp(b - ct)/(1 + \exp(b - ct)) \]  \hspace{1cm} (3-5)
Microsoft Excel 5.0 (Microsoft Corporation) software was used to process the results and to calculate the model parameters by regression analysis using least squares method. Student t-test was used to compare the means.

3.4 Results and Discussion

The effect of age of the inoculum on *C. roseus* hairy root growth and indole alkaloid production was tested by using inoculum from 2 week, 3 week and 4 week subculture cycles. For each of the three subculture cycles, nine flasks were inoculated with tips from the respective subculture inoculum and their conductance was measured every 2-3 days. Flasks were harvested, in triplicates, after 21, 28 and 35 days of inoculation and their biomass and indole alkaloid content was determined. *Figure* 3-1 shows the fresh weight and the dry weight data obtained from the three subculture cycles. Also shown in *Figure* 3-1 are the ‘fits’ of the data to Equation 3-2 (see Materials and Methods). As apparent from *Figure* 3-1, biomass data from the initial growth phase were not available, thus applicability of Equation 3-2 was verified by applying it to measured biomass data reported previously by Bhadra *et al.* (1997). This analysis demonstrated that Equation 3-2 can be used to describe the whole range of data and hence can be used for further analysis (*Figure* 3-2).
Figure 3-1 Fresh weight and dry weight profiles of 2 week, 3 week and 4 week subculture cycles. Open markers represent weights determined using the conductivity correlation while the solid markers represent weights measured by harvesting root culture. The lines represent least square fits of Equation 3-2 to experimental data. Error bars represent standard deviations.
Figure 3-2: Fresh weight (○) and dry weights (□) of the *C. roseus* hairy root line LBE 6-1 from Bhadra and Shanks (1997). The solid line represents a least square fit of Equation 3-2 to the experimental data.
The results of the effect of subculture cycle on growth are summarized in Table 3-1. The parameters \( b \) and \( c \) obtained for each subculture cycle are shown. These parameters were then used to generate time course data for biomass and the specific growth rate based on exponential growth model was determined from the linear portion of the curves for the three subculture cycles. The maximum specific growth rates (\( \mu_r \)) and the doubling times (\( t_{Df} \)) obtained by this method are shown in Table 3-1. These values are in excellent agreement with the maximum specific growth rates (\( \mu_d \)) and doubling times (\( t_{Dd} \)) obtained by applying the exponential model to the measured biomass data, obtained in the first 20 days of growth (Table 3-1).

The maximum specific growth rate of the root cultures decreases as the age of the inoculum increases from 2 to 4 weeks (Table 3-1). The doubling time of the hairy root cultures decreased significantly (\( p<0.05 \)) from 4.01 to 3.18 as the subculture cycle is changed from 4 weeks to 2 weeks. These trends in increased growth rate are consistent with the experimental data on growth index. The growth index (ratio of final FW to initial FW, based upon the average inoculum size of 0.05 grams) of the root cultures is a measure of the rate of accumulation of biomass. The growth indices of the three subculture cycles are shown in Table 3-2. At the end of 21 days the two week subculture cycle has a growth index of 89.78 and the fresh weight of the roots is about 4.5 grams. As compared to this, the three week subculture cycle has a growth index of 66.4 and the fresh weight of roots is about 3.3 grams, while the four week subculture
cycle has a growth index of 44.58 and the fresh weight of the roots is 2.2 grams. This trend is expected for the exponentially modeled phase of growth.

Table 3-I Parameters obtained for 2 week, 3 week and 4 week subculture cycles. The parameters c and b are obtained by fitting the dry weight data to Equation 3-2. The values $\mu_f$ and $t_{DF}$ represent the specific growth rates and the doubling times respectively, obtained from the data generated by Equation 3-2, using the determined values of b and c. The values $\mu_d$ and $t_{DD}$ represent the maximum specific growth rates and the doubling times respectively, obtained from the actual dry weights. The values of $\mu_f$, $t_{DF}$, $\mu_d$ and $t_{DD}$ are significantly different in the 2 week, 3 week and 4 week subculture cycles ($p<0.05$).

<table>
<thead>
<tr>
<th>Cycle</th>
<th>c (days $^{-1}$)</th>
<th>b</th>
<th>$\mu_f$ (days $^{-1}$)</th>
<th>$t_{DF}$ (days)</th>
<th>$\mu_d$ (days $^{-1}$)</th>
<th>$t_{DD}$ (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 week</td>
<td>0.273</td>
<td>4.711</td>
<td>0.219</td>
<td>3.16</td>
<td>0.218±0.007</td>
<td>3.18±0.09</td>
</tr>
<tr>
<td>3 week</td>
<td>0.244</td>
<td>4.994</td>
<td>0.194</td>
<td>3.57</td>
<td>0.191±0.012</td>
<td>3.62±0.2</td>
</tr>
<tr>
<td>4 week</td>
<td>0.210</td>
<td>5.155</td>
<td>0.173</td>
<td>4.01</td>
<td>0.168±0.006</td>
<td>4.01±0.25</td>
</tr>
</tbody>
</table>
**Table 3-2** Growth indices for the three subculture cycle determined at the end of 21, 28 and 35 days respectively. The growth indices are based upon the initial biomass of 0.05 grams.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>GI 21 days</th>
<th>GI 28 days</th>
<th>GI 35 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 week</td>
<td>89.78±4.1</td>
<td>103.45±4.2</td>
<td>115.36±2.3</td>
</tr>
<tr>
<td>3 week</td>
<td>66.4±10.2</td>
<td>99.85±1.1</td>
<td>112.31±5.7</td>
</tr>
<tr>
<td>4 week</td>
<td>44.58±3.2</td>
<td>87.39±4.6</td>
<td>100.93±3.0</td>
</tr>
</tbody>
</table>

Hence, a difference in growth rates explains the different growth indices observed at day 21 in the three subculture cycles. As the cultures approach stationary phase, it is expected that the growth indices would converge to a similar value for all three subculture cycles, since the final biomass is dependent upon initial nutrient concentration (same for all three subculture cycles). Indeed, the growth indices of the 2 week and the 3 week subculture cycle are not significantly different at days 28 and 35 however, they are slightly higher than the growth index of the 4 week cycle at day 28 and 35 respectively (Table 3-2). As the subculture progresses, the final fresh weight and dry weights (and hence the growth indices) measured at day 35 approach the same value.
Figure 3-3 shows the instantaneous specific growth rates for the three subculture cycles obtained using Equation 3-2, and the instantaneous specific growth rate of the inoculum for each subculture cycle. The inoculum used in the 2 week subculture cycle was derived from mid-exponential phase and had a high instantaneous specific growth rate (Figure 3-3). As compared to this, the inoculum in the 3 week subculture cycle was obtained from late exponential stage, while that in the 4 week cycle was obtained from stationary phase and hence had the lowest instantaneous specific growth rate. An argument can be made that the cells in the root tips derived from different subculture cycles should be the same as only the primary tip without any lateral branches is used for inoculum. However, the nutrient condition experienced by the 2 week culture cycle inoculum, when transferred from the depleted to fresh medium, is more similar when compared to the change experienced by the 3 and 4 week cycle inocula. This difference in change in nutrient concentration experienced by the three subculture cycles causes minimum nutrient shock in the 2 week subculture cycle and the maximum nutrient shock in the 4 week subculture cycle.
Figure 3-3 Instantaneous specific growth rates of the 2 week, 3 week and 4 week subculture cycles at different time points during growth. The values have been obtained by using parameters b and c, in Table 1, into the relationship for instantaneous growth rate as a function of time (Equation 3-5). Black circles indicate the instantaneous specific growth rates of the root cultures at the time of inoculation, for each subculture cycle.
Secondly, the FW/DW profile for a complete growth cycle of the root line, presented previously (Bhadra and Shanks, 1997), shows that the FW/DW ratio of *C. roseus* root cultures goes through a maxima just prior to stationary phase. This indicates that prior to reaching the maxima, the increase in FW/DW is caused by cell elongation while loss of water or deposition could be responsible for the decline thereafter. Hence, the inoculum derived in the three subculture cycles differs in the FW/DW ratio. These differences may also contribute to the greater growth rates of the 2 week subculture cycle as compared to the 4 week cycle.

In addition to growth, the effect of age of inoculum on production of *C. roseus* alkaloids was also studied. The indole alkaloid pathway of *C. roseus* has been reviewed by Meijer *et al.* (1993). The cathenamine pathway leads to synthesis of the antihypertensive indole alkaloids ajmalicine and serpentine. Tabersonine is a key precursor of vindoline, that condenses with catharanthine to form the anti-cancer alkaloids vinblastine and vincristine. Large amounts of two tabersonine-derived compounds, lochnericine and hörhammericine, have been found in our root cultures (Vani, 1996). Since they may divert flux away from the vindoline branch, understanding the kinetics of their accumulation is important to design strategies aimed at reduction of flux to these compounds. The levels of tabersonine, lochnericine, hörhammericine, ajmalicine and serpentine were monitored in this study.
Figure 3-4 shows the specific yields of tabersonine, as determined at 21, 28 and 35 days after inoculation, in the three subculture cycles. The specific yields of tabersonine decrease linearly with biomass in all 3 subculture cycles. The overlapping tabersonine profiles in the three subculture cycle indicate that tabersonine accumulation is dependent upon the total amount of biomass and not on the growth rates. The decline in tabersonine levels is likely caused by a combination of dilution (due to increase in biomass) and degradation or transformation to other alkaloids (eg. lochnericine and hörhammericine).
Figure 3-4 Specific yields of tabersonine obtained after 21, 28 and 35 days in the three subculture cycles studied. Error bars represent standard deviations.
Stoichiometrically, the decrease in levels of tabersonine cannot account for the increase in lochnericine and hörhammericine yields, hence it is possible that tabersonine is continually being synthesized but the rate of transformation/degradation is greater than the rate of biosynthesis. No similar correlation was observed between biomass (dry weight) and specific yields of the remaining four alkaloids.

Figures 3-5A and 3-5B show the total yields of lochnericine and hörhammericine respectively obtained in the three subculture cycles. Highest yields of lochnericine are obtained in the 2 week subculture cycle (Figure 3-5A) as compared to 3 and 4 week cycles. This indicates that lochnericine yields are growth rate dependent as higher lochnericine yields are obtained in faster growing cultures. The total yield of hörhammericine increases with biomass in all three subculture cycles (Figure 3-5B). No effect of growth rate on hörhammericine levels is observed indicating that accumulation of hörhammericine is independent of growth rates, unlike lochnericine.

The total yields of serpentine and ajmalicine obtained at days 21, 28 and 35 are shown in Figures 3-6A and 3-6B respectively. As observed previously (Bhadra and Shanks, 1997), total yield of serpentine increases from day 21 to 35 in all three subculture cycles. From the ordering of the profiles for the three subculture cycle, it is clear that the total yield of serpentine is higher in the 4 week subculture cycle as compared to the 3 and 2 week cycles. This increase in total yield of serpentine with the decrease in growth rate is consistent with the results of Parr et al. (1988), who
observed highest serpentine concentration in slow growing cultures. The total yield of ajmalicine also increases with the increase in biomass in all three subculture cycles, as seen from Figure 3-6B. However nothing can be said about the nature of association of ajmalicine yields with growth rates as no definite order is observed in the three profiles with variation in growth rates.
Figure 3-5 Total yields of (A) lochnericine and (B) hörhammericine obtained on day 21, 28, and 35 in the three subculture cycles. Error bars represent standard deviations.
Figure 3-6 Total Yields of serpentine (A) and ajmalicine (B) obtained on day 21, 28 and 35 in the three subculture cycles. Error bars represent standard deviations.
3.5 Conclusions

The results of these experiments indicate that the age of inoculum has a significant effect on the growth rates of *C. roseus* hairy root cultures. The nature of association of the key alkaloids varied with growth and is consistent with previously obtained results (Bhadra and Shanks, 1997), (Morgan, J. A.; Bhadra, R. and Shanks, J. V., manuscript under preparation). These results can be used to in the design of a successful commercial process (Payne et al., 1992). A typical growth cycle of *C. roseus* hairy root cultures, grown in a single/two stage commercial process, would be continued until the culture reaches stationary phase. This would mean that the culture would go through about 7 doublings during its growth period. A simple optimization of the subculture cycle could lead to a reduction in doubling times and hence reduce the reactor operation times in two stage processes. Secondary metabolite accumulation studies are very important for development of a successful commercial process. For harvest of alkaloids whose yields are growth-associated, e.g. tabersonine, a fast growing culture will go through a maxima earlier as compared to the slow growing cultures and hence cut short the operation time of reactor. Similar reduction in operation cost can be observed if the rate of accumulation of alkaloid is dependent upon rate of biomass synthesized (e.g. lochnericine). For biosynthesis of alkaloids like serpentine, which accumulate to higher levels in slower growing culture, the growth rate and the operation time can be optimized for minimal operation cost. Hence, an application of this study
to hairy root and other culture types could have a significant impact on process economics.
CHAPTER 4: EFFECT OF ELICITOR DOSAGE AND EXPOSURE TIME ON BIOSYNTHESIS OF INDOLE ALKALOIDS BY CATHARANTHUS ROSEUS HAIRY ROOT CULTURES.

(This chapter in its current form has been submitted to Biotechnology Progress)

4.1 Abstract

Late exponential phase hairy root cultures of Catharanthus roseus were elicited with pectinase, chitin and jasmonic acid. The effects of elicitor concentration and exposure time on growth and levels of several compounds in the indole alkaloid biosynthetic pathway were monitored. Pectinase decreased the fresh weight to dry weight ratio of the roots while addition of chitin and jasmonic acid had no significant effect. Selective effects on indole alkaloid yields were observed upon addition of elicitors. An increase of 150% in tabersonine specific yield was observed upon addition of 72 units of pectinase. Transient studies at the same level demonstrated possible catabolism as serpentine, tabersonine and lochnericine levels decreased immediately after elicitation. The levels of these compounds recovered back to control levels or were higher than the control levels after some time. Chitin did not have a significant and consistent effect on alkaloid production. Jasmonic acid was found to be a unique elicitor leading to an enhancement in flux to several branches in the indole alkaloid. Jasmonic acid addition caused an increase in the specific yields of ajmalicine (80%), serpentine (60%), lochnericine (150%) and hörhammericine (500%) in dosage studies. Tabersonine, the likely precursor of lochnericine and hörhammericine, decreased at lower levels of
jasmonic acid and then increased with increasing jasmonic acid concentration. Transient studies showed that lochnericine and tabersonine levels go through a maxima, then decrease back to control levels and reduce below control levels respectively. The yields of ajmalicine, serpentine and hörhammericine increased continuously after the addition of jasmonic acid. The methods described in this article could generally be used in devising strategies for enhancement in productivity of secondary metabolites and for probing and studying the complex secondary metabolite pathways in plant tissue cultures.

Keywords: elicitation, jasmonic acid, pectinase, tabersonine, ajmalicine

4.2 Introduction

*Catharanthus roseus* is an important source of the anti-cancer alkaloids, vinblastine and vincristine, and the anti-hypertensive compounds ajmalicine and serpentine (Moreno et al., 1995). Currently these drugs are produced by extraction of the whole plant (DiCosmo and Misawa, 1995). Plant cell and tissue cultures have been investigated as an alternate source of production; however low yields of these compounds and the absence of vindoline (a precursor of vinblastine and vincristine) are the key bottlenecks to this technology (Moreno et al., 1995). Hairy roots cultures of *C. roseus*, with their apparent genetic stability, high levels of differentiation and amenability to genetic transformations, have been investigated in the last decade for production of indole alkaloids (Parr et al., 1988; Toivonen et al., 1989; Bhadra et al.,
1991; Jung et al., 1992; Vazquez-Flota et al., 1994). The complexity of the indole alkaloid biosynthetic pathways has presented a major challenge for enhancement of secondary metabolite productivity and a combination of approaches will be essential for enhanced production.

The secondary metabolite pathways of *C. roseus* have been reviewed in detail by (Meijer et al., 1993). A simplified portion of the pathway leading to biosynthesis of indole alkaloids is shown in Figure 4-1. Ajmalicine, serpentine, catharanthine and tabersonine are readily synthesized in hairy root cultures of *C. roseus*; however, vindoline is absent (van der Heijden et al., 1989; Moreno et al., 1995; Vani, 1996). Lochnericine and hörhammericine, recently quantified in our root cultures, are believed to be derivatives of tabersonine, a key precursor of vindoline (Vani, 1996). The magnitude of concentration of these compounds indicates that conversion of tabersonine into lochnericine and hörhammericine possibly diverts flux away from the tabersonine to vindoline branch (Vani, 1996). Despite an initial report of low levels of vinblastine in hairy roots (Parr et al., 1988), vinblastine and its key precursor vindoline have not been able to be produced in subsequent studies. Also ajmalicine and serpentine are produced in too low of quantities for commercialization. Advances in cloning of genes in the indole alkaloid pathway indicate that metabolic engineering may eventually be used to eliminate some of the bottlenecks in the pathways (Kutchan, 1995); however, metabolic studies with standard tools such as fungal elicitation to manipulate flux will be necessary and complimentary to genetic approaches in the advancement of production goals.
**Figure 4-1** Part of the pathway showing some of the quantified indole alkaloids of *Catharanthus roseus* (for complete pathway see Meijer et al., 1993). 4,21 dehydrogeissoschizine is the precursor that enters the cathenamine pathway and is converted to antihypertensory alkaloids ajmalicine and serpentine. Stemmadenine is also synthesized from 4,21 dehydro-geissoschizine through an unknown step. Tabersonine and catharanthine emerge from stemmadenine via some unknown reactions. Tabersonine further goes through six reactions to produce vindoline. Tabersonine is also the putative precursor of lochnericine and hörhammericine (Vani, 1996). Both vindoline and catharanthine condense together to form anti-cancer bisindole alkaloids vinblastine and vincristine (Dotted arrows represent unknown steps, solid arrows represent known steps and multiple arrows represent several steps).
Fungal elicitation has been an effective technique for enhancement of levels of secondary metabolites (Eilert et al., 1986) and as a tool to study metabolism (Srinivasan et al., 1996). Most of the strategies employing fungal elicitation utilize fairly undefined mixtures such as autoclaved fungal homogenates (Yoshikawa et al., 1993b), or the fungal culture filtrates (Pasquali et al., 1992; Ciddi et al., 1995). Effects of fungal homogenates and other elicitors on C. roseus cultures have been studied but only the accumulation of ajmalicine and catharanthine has been monitored (Asada and Shuler, 1989; Sim et al., 1994; Vazquez-Flota et al., 1994). The use of specific elicitor components to determine their effects on several indole alkaloids in the pathway may lead to more precise strategies to enhance synthesis of desired alkaloids. In addition to elicitor specificity, elicitor dosage and timing of harvest after elicitation are important factors in studying the enhancement of alkaloid levels. High dosages of elicitors has been known to induce hypersensitive response (Collinge and Susarenko, 1987; Mukundan and Hjorsoto, 1990; Roewer et al., 1992) leading to cell death while a minimal level is required for induction of a response. Transient changes in concentrations of several secondary metabolites have been observed in response to elicitation, due to transient induction of genes (Seitz et al., 1989; Pasquali et al., 1992; Roewer et al., 1992). Thus determination of the secondary metabolite profiles in response to elicitation is also essential for maximization of yield by optimization of the harvest time.

In this study three different types of elicitors, each with a different role in the plant defense response to pathogens, were tested to stimulate indole alkaloid
production. *A. niger* pectinase is an endopolygalacturonase which attacks plant cell walls (Alghisi and Favaron, 1995), chitin is a component of cell walls of many fungi (Ryan, 1987) and jasmonic acid is a signal transducer (Gundlach et al., 1992; Müller et al., 1993; Farmer, 1994). Indole alkaloids in the cathenamine branch (ajmalicine and serpentine) and those around the tabersonine branch point (tabersonine, lochnericine and hörhammericine) were quantified in response to elicitation. In this paper, we report both on the effects of elicitor dosage and elicitor exposure time on indole alkaloid accumulation by *C. roseus* hairy root cultures.

4.3 Materials and Methods

4.3.1 Hairy Root Cultures

The experiments were conducted with the *C. roseus* hairy root clone LBE 6-1 (Bhadra et al., 1993). Approximately 0.05 grams fresh weight (5 root tips, 35-40 mm long (Bhadra and Shanks, 1995)) of roots, derived from a 3 week subculture cycle (Rijhwani and Shanks, 1997), were inoculated into a 250 ml erlenmeyer flask containing 50 ml of Gamborgs B5/2 media with 30 g/L sucrose. The pH of the medium was adjusted to 5.7 prior to filter sterilization. The cultures were grown on an orbital shaker at 100 rpm, at 26°C in dark, for 3 weeks. Evaporation controls were also done simultaneously and autoclaved water was added every week to account for the water lost due to evaporation. After three weeks, late exponential phase root cultures were used to study the effect of elicitor dosage and elicitor exposure times.
4.3.2 Dosage Studies

Pectinase, chitin and jasmonic acid were added separately to late exponential phase root cultures. No elicitor was added to the control cultures. *A. niger* pectinase (solution in 40% glycerol, Sigma) was sterilized using a 0.22 μm filter and was added at five different levels of 24, 48, 72, 96 and 120 units per flask. Chitin suspensions were prepared by grinding 50 mg of chitin flakes (from crab shells, Sigma) to a fine powder and then resuspending in 50 ml Milli-Q water. This suspension was sterilized by placing in UV light for 48 hours and was subsequently used for elicitation at five different levels of 1, 2, 3, 4 and 5 mg chitin per flask. The jasmonic acid solution was prepared by mixing 250 mg of jasmonic acid in 10 ml of Milli-Q water. The jasmonic acid solution was filter sterilized using a 0.22 μm filter and was added to late exponential stage cultures at three different levels of 10, 50, 100 μL/flask corresponding to 0.25, 1.25 and 2.5 mg/flask respectively. The solution was vortexed thoroughly before addition to shake flasks. Triplicate flasks were run for each dosage and control. Independent controls were performed with each dosage experiment to compare treatments with controls at the same stage in growth cycle. All cultures were harvested 48 hours after the addition of elicitor and were then analyzed for biomass and indole alkaloid content.

4.3.3 Transient Studies

For the transient studies, chitin and jasmonic acid solutions were prepared as in dosage studies. The pectinase solution was a new formulation (pectinase solution in
KCl and sorbitol) purchased from Sigma. Pectinase (72 units), chitin (1 mg) and jasmonic acid (0.25 mg) were added to late exponential phase cultures (21 days after inoculation). The transient was conducted for approximately 4-5 days after the addition of elicitor. In all three transient studies, shake flasks were harvested in triplicate at each time point and several time points were analyzed for biomass and alkaloid content.

Two different types of control experiments were conducted. Previously indole alkaloid profiles for C. roseus hairy root cultures were reported (Bhadra and Shanks, 1997) and some of the alkaloids (tabersonine and ajmalicine) were observed to go through a maxima in yields. For each transient experiment, controls were performed from the same source culture as the elicited culture but were harvested only at the beginning, middle and at the end of each experiment to obtain transient control profiles. In a separate experiment, possible fluctuations in the alkaloid profiles of the control cultures were tested by harvesting shake flasks (in triplicates) at different times during the 5 day period (21-26 days after inoculation). This strategy was employed to reduce tedious redundant analysis and yet obtain a control profile in each experiment for the purpose of comparison.

4.3.4 Biomass Measurements

Hairy roots were dried by blotting all the external moisture and were weighed to determine the final fresh weight (FW). These roots were then transferred to preweighed lyophilizer bottles and frozen to -40° C. The frozen roots were dried by
lyophilization until no weight loss occurred. The final weight of the roots and bottle was measured and the dry weight (DW) of the roots was calculated.

4.3.5 Extraction, fractionation and HPLC analysis

Hairy roots were extracted and fractionated as in Bhadra et al. (1993). Alkaloids were extracted from 180-210 mg of freeze dried root cultures with 85-90 ml of methanol for 3 hours in a soxhlet extractor. The extract was concentrated to 1.6 ml in a rotary evaporator and was diluted by adding 2.4 ml of 5 mM (NH₄)₂PO₄ solution. Fractionation was performed using a C-18 Maxi-Clean cartridge (Alltech, Deerfield, IL). The cartridge was conditioned by pretreating with 10 ml methanol, 10 ml Milli-Q water and 4 ml of 40:60 methanol:5 mM (NH₄)₂PO₄ mixture. The sample was loaded onto the cartridge and the eluent collected at a constant rate using a syringe pump. The cartridge was subsequently washed with 60:40 methanol:5 mM (NH₄)₂PO₄ solution followed by 95:5 methanol:5 mM of (NH₄)₂PO₄ solution. Culture medium was loaded on a C-18 Maxi-Clean cartridge (Alltech, Deerfield, IL) preconditioned by treating with 10 ml methanol and 10 ml Milli-Q water. The cartridge was subsequently washed with 4 ml methanol. HPLC analysis was performed for all eluents to determine the alkaloid content.

The HPLC system consisted of two Waters 510 pumps, a Waters Wisp 712 injector, a Phenomenex C18 bondclone reverse phase column (300 mm length, 3.9 mm diameter), a Phenomenex C18 bondclone reverse phase guard column (100 mm length, 3.9 mm diameter), and a Waters 996 PDA detector. Millennium 2010 software
(Waters) was used for acquisition and analysis on a NEC Image 466es workstation. Isocratic condition using 41:59 methanol:5 mM (NH₄)₂PO₄ solution at a flow rate of 1 ml/min were used to separate the alkaloids. Calibrations were done on authentic standards and quantification was performed on chromatograms extracted at 254 nm and 329 nm. Peak identification was based on comparison of retention time and UV spectra with authentic standards of ajmalicine (Fluka), serpentine (Research Plus, Bayonne, NJ), catharanthine (gift of Dr. Hamada, Okayama University, Okayama, Japan) and tabersonine (gift of Dr. Hamada). Hörmhammericine and lochnericine were quantified based upon HPLC response factor of tabersonine, as in Vani (1996).

4.3.6 Statistical Analysis

Students t-test (Microsoft Excel 5.0) was used to compare the means.

4.4 Results

Late exponential phase hairy root cultures of *C. roseus* were elicited with three specific elicitors and the effects on indole alkaloid accumulation was studied. Dosage studies were performed to determine the effects of elicitor concentration on growth and indole alkaloid production. Cultures were elicited with different concentrations of the elicitor, were harvested 48 hours after the addition of the elicitor and then analyzed for indole alkaloids. One concentration of each elicitor from the dosage studies was chosen for transient experiments and cultures were harvested and analyzed for intracellular indole alkaloid accumulation at different time points after elicitation. Excretion of
indole alkaloids into the medium was not detected in our root cultures in response to elicitation. Consequently all results reported are for intracellular concentrations of indole alkaloids.

4.4.1 Transient Control Experiment

Since previous work in our group showed that tabersonine is growth associated and hence could experience a maximum in yield during exponential phase (Bhadra and Shanks, 1997), a separate transient control experiment was performed on a finer time scale for all of the alkaloids monitored. This experiment tested for fluctuations in the alkaloid profiles of the control cultures during the 5 day period between 21-26 days after inoculation. Figure 4-2 shows the time resolution of the profiles of ajmalicine and serpentine (Figure 4-2A), and those of tabersonine, lochnericine and hörhammericine (Figure 4-2B). The alkaloid profiles determined in this experiment show that no sudden changes in the indole alkaloid yields occur during the 5 day experimental period. Hence, for the transient elicitation studies, control cultures were harvested at three points only in each experiment to determine the control alkaloid profiles for comparison with those of elicited cultures.
Figure 4-2 Indole alkaloid profiles of control cultures for the 5 day period from day 21 to day 26. Cultures were harvested in triplicate at each time point. Error bars indicate standard deviation.
4.4.2 Effects of Pectinase

The fresh weight to dry weight (FW/DW) ratio was monitored for the elicited and control cultures in both dosage and transient studies. In pectinase dosage studies, the fresh weight to dry weight ratio of the elicited cultures was lower than that of controls, as shown in Figure 4-3A. As the amount of pectinase was increased from 0 to 120 units/flask, the FW/DW decreased from 15.25 to 14.5 (p≤0.05), indicating the dependence of FW/DW ratio on the concentration of pectinase added. The fresh weight of the root cultures remained constant in the range of 3.9 ± 0.3 grams/flask for the entire range of pectinase dosage used (Figure 4-3A). In the transient study, a similar decline in the FW/DW ratio of both the control and the elicited root cultures was observed. A decline in the FW/DW ratio of unelicited late exponential phase C. roseus hairy root cultures was reported previously (Bhadra and Shanks, 1997). However, the FW/DW ratio of the elicited root cultures was lower than that of controls, as shown in Figure 4-3B. The FW/DW ratio of the elicited cultures decreased from 15 to 10.25 in 120 hours while in case of control cultures, the FW/DW ratio decreased from 15.5 to 12.5 in the same time. No significant difference in the fresh weights of the elicited and the control cultures was observed (data not shown). Hence, the difference in the FW/DW ratio is due to high dry weight of the elicited cultures as compared to that of controls. This increase in dry weight is likely caused by new material deposition on the cell walls as a barrier to the invading agent which is an important component of the defense response (Yoshikawa et al., 1993a; Robertson et al., 1995).
Figure 4-3 A) Fresh weight (O) and FW/DW (■) of root cultures versus pectinase concentration. Late exponential stage root cultures were elicited with 0, 24, 48, 72, 96 and 120 pectinase units per flask and harvested, in triplicate, 48 hours after elicitation. B) Fresh weight to dry weight ratio of the pectinase elicited and control cultures in transient studies. Cultures were harvested in triplicate at each time point. Error bars indicate standard deviation.
In dosage studies, pectinase addition resulted in a significant effect on tabersonine levels only. No significant effect of pectinase dosage was seen on the levels of ajmalicine and serpentine. The specific and total yields of ajmalicine remained constant around 0.75 mg/gDW and 4 mg/L respectively (data not shown). This result is consistent with the observations of (Garnier et al., 1996) who did not observe any effect of pectinase addition on ajmalicine synthesis by *C. roseus* cell suspension cultures. Serpentine levels were slightly lower than that of ajmalicine (data not shown). The specific yield of tabersonine increased significantly (p<0.05) in dosage experiments, as the amount of pectinase added was increased beyond 48 units (Figure 4-4). The maximum specific yield of tabersonine (2.5 times that of control) was observed when 72 units of pectinase were added (Figure 4-4). Beyond the maximum, the levels of tabersonine decreased to 1.9 times the control values at 120 units of pectinase. Despite the alteration in flux observed in tabersonine, no significant effect of pectinase dosage was seen on the two tabersonine derived compounds, hörhammericine and lochnericine, as compared to controls. The specific and total yields of hörhammericine remained constant at 1.1 mg/gDW and 6 mg/L respectively while lochnericine specific and total yields were 3.9 mg/gDW and 20 mg/L respectively (data not shown).
Figure 4-4 Specific yield of tabersonine versus pectinase concentration. Late exponential stage cultures were elicited with varying concentrations of pectinase. Cultures were harvested, in triplicate, 48 hours after the addition of elicitor. Error bars indicate standard deviation.
Since dosage experiments only give one timepoint, transient experiments were performed. A pectinase concentration of 72 units per flask was used in transient study, since a 2.5 times increase in tabersonine specific yield was obtained at this level. As in the dosage study, no significant effect of pectinase exposure time on the specific yield of ajmalicine was observed (data not shown). However, an analysis of the indole alkaloid content of the elicited and the control cultures in transient studies showed that serpentine apparently was catabolised or transformed to other compounds immediately after elicitation. The specific yield of serpentine decreased from 0.6 mg/gDW, immediately after the addition of the elicitor, and reached a minimum value of 0.35 mg/gDW, 6 hours after elicitation (Figure 4-5). The specific yield of serpentine then recovered back to 0.55 mg/gDW, 24 hours after elicitation. At 120 hours, there was no significant difference in serpentine specific yields between elicited and control cultures (Figure 4-5).
Figure 4-5 Specific yield of serpentine versus elicitor exposure time. Late exponential stage cultures were elicited with 72 units of pectinase per flask. Cultures were harvested, in triplicate, at different time periods (for 120 hours) after the addition of elicitor. Error bars indicate standard deviation.
The initial decline observed with serpentine was also seen with tabersonine and lochnericine. Tabersonine specific yield decreased from 1.1 mg/gDW, reaching a minimum value of 0.5 mg/gDW at 10 hours. The specific yield of tabersonine increased continuously after 10 hours and reached control levels, 50 hours after the addition of the elicitor. At 120 hours the specific yield of tabersonine in elicited cultures was 1.15 mg/gDW, as compared to the control level of 0.6 mg/gDW (Figure 4-6A). Lochnericine levels decreased rapidly from 3.25 mg/gDW to 1.5 mg/gDW, 3 hours after the addition of pectinase. After 3 hours, the specific yields of lochnericine increased beyond the control levels, crossing the control levels approximately 48 hours after elicitation (Figure 4-6B). Hörhammericine specific yields, on the other hand, appear to increase immediately after addition of pectinase but this increase was not statistically significant (Figure 4-6C). Furthermore, the initial decrease in tabersonine and lochnericine does not result in an equivalent increase in hörhammericine levels. Thus catabolism or conversion to other unknown compounds could be responsible for this observation.
Figure 4-6 Specific yields of A) tabersonine, B) lochnerine and C) hörhammericine versus pectinase exposure time. Cultures were harvested, in triplicates, at different time periods (for 120 hours) after the addition of pectinase. Error bars indicate standard deviation.
The preparation of pectinase did affect tabersonine levels. The tabersonine levels at 72 units of pectinase in the dosage studies (at 48 hours) did not correspond to the tabersonine specific yields obtained 48 hours after the addition of 72 units of pectinase in transient studies. This difference exists due to a compositional change (by vendor) in elicitor solutions used in these studies. To test this a short term (48 hours) transient elicitation experiment was performed using 120 units of the pectinase solution preparation used in dosage studies. The results obtained from that experiment corroborate the yields obtained in dosage studies (data not shown), indicating that the tabersonine levels are sensitive to the enzyme formulation.

4.4.3 Effects of Chitin

In both dosage and transient studies, no significant effect on FW/DW ratio was observed upon addition of chitin. Alkaloid analysis in dosage studies showed that specific yield of ajmalicine increased 45% ($p \leq 0.05$), as compared to controls, upon addition of 1 mg chitin. No significant effect of chitin dosage was seen on the remaining alkaloids (data not shown). However, in transient studies, no significant effect of chitin exposure times was observed on any of the five alkaloids monitored (data not shown), and thus the result of ajmalicine production was not consistent.

4.4.4 Effects of Jasmonic acid

Jasmonic acid did not have a significant effect on the FW/DW ratio in either dosage or transient experiments (data not shown). Jasmonic acid stimulated changes in alkaloid
levels for all five compounds monitored in dosage studies. The specific yield of ajmalicine increased significantly (p≤0.05) as the dosage of jasmonic acid was increased. Ajmalicine levels increased from 0.5 mg/gDW in controls to 0.9 mg/gDW upon addition of 2.5 mg/flask of jasmonic acid (Figure 4-7A). Specific yield of serpentine was not significantly altered when 0.25 mg or 1.25 mg of jasmonic acid were added to each flask; however, on addition of 2.5 mg of jasmonic acid per flask, serpentine specific yield increased significantly (p≤0.05) to 1.1 mg/gDW as compared to the control levels of 0.7 mg/gDW (Figure 4-7A).

Specific yields of tabersonine decreased significantly (p≤0.05), from 1.08 mg/gDW in controls, to 0.2 mg/gDW upon addition of 0.25 mg of jasmonic acid per flask. As the amount of jasmonic acid was increased to 1.25 and 2.5 mg per flask, tabersonine specific yields increased but were lesser than the control levels (Figure 4-7B). Specific yields of hörhammericine and lochnericine were strongly dependent upon jasmonic acid dosage. Lochnericine levels decreased from 3.2 mg/gDW (controls) to 2.6 mg/gDW upon addition of 0.25 mg of jasmonic acid per flask. However, upon addition of higher concentrations of jasmonic acid, the specific yield of lochnericine increased significantly and reached a concentration of 7.5 mg/gDW (2.3 times control levels). The specific yield of hörhammericine increased as the concentration of jasmonic acid was increased from 0.25 to 2.5 mg/flask. The specific yield of hörhammericine was 1.37 mg/gDW in controls and it increased 5 fold to 6.5 mg/gDW upon addition of 2.5 mg of jasmonic acid per flask (Figure 4-7B).
Figure 4-7 Specific yield of A) ajmalicine and serpentine, B) tabersonine, lochnerine and hörhammericine, versus jasmonic acid concentration. Late exponential stage cultures were elicited with varying concentrations of jasmonic acid. Cultures were harvested, in triplicates, 48 hours after the addition of elicitor. Error bars indicate standard deviation.
Since we were interested in studying fluxes around the tabersonine branch point, the concentrations of 0.25 mg jasmonic acid per flask was chosen for transient experiments to probe the decrease in tabersonine levels. As observed at this jasmonic acid concentration in dosage studies, no significant effect of jasmonic acid exposure time on ajmalicine specific yield was seen in transient experiments (data not shown). Specific yields of serpentine increased continuously upon addition of jasmonic acid. At time 0 the specific yield of serpentine was similar to controls at 0.5 mg/gDW and it increased to 0.87 mg/gDW 100 hours after elicitation while the control levels were about 0.5 mg/gDW at that time (Figure 4-8).

The specific yields of tabersonine and lochnericine increased rapidly upon the addition of jasmonic acid. Tabersonine specific yields increased from 0.55 mg/gDW to 1.2 mg/gDW, 14 hours after the addition of the elicitor. After the maximum at 14 hours, the levels of tabersonine decreased and were below the control levels by 48 hours after the addition of the elicitor, corroborating the decrease in tabersonine levels observed in the dosage studies (Figure 4-9A). Lochnericine levels increased from 5 mg/gDW at time 0 to 7.2 mg/gDW, 24 hours after the addition of jasmonic acid. Subsequently the specific yield of lochnericine decreased continuously and was same as controls after 70 hours of elicitation (Figure 4-9B). Hörrhammericine levels, on the other hand, increased continuously after the addition of the jasmonic acid. The specific yield of hörrhammericine increased from 1.15 mg/gDW at time 0 to 6.8 mg/gDW (a 6 fold increase), 100 hours after the addition of jasmonic acid (Figure 4-9C).
Figure 4-8 Specific yield of serpentine versus jasmonic acid exposure time. Late exponential stage cultures were elicited with varying concentrations of jasmonic acid. Cultures were harvested, in triplicates, 48 hours after the addition of elicitor. Error bars indicate standard deviation.
Figure 4-9 Specific yields of A) tabersonine, B) lochnericine and C) hörhammericine versus jasmonic acid exposure time. Cultures were harvested, in triplicates, at different time periods (for 120 hours) after the addition of jasmonic acid. Error bars indicate standard deviation.
4.5 Discussion

Dosage and transient studies, with hairy root cultures of *Catharanthus roseus*, were conducted to demonstrate the importance of both elicitor concentration and exposure times on indole alkaloid production. Since the secondary metabolites produced by our hairy roots are intracellular, elicitor addition was performed in late exponential stage of the cultures (i.e. when sufficient biomass had accumulated). Three different elicitors, a fungal polygalacturonase, a fungal cell wall component and a signal transducer, were used and the growth and yield parameters were determined.

Fast accumulation of biomass, and an increase in indole alkaloid specific yield are essential for enhancement in total yields. The fresh weights and the dry weights of the elicited and control root cultures were monitored to determine if any elicitor had detrimental effects on growth. Only pectinase had a significant effect on growth. The action of pectinase (an endopolygalacturonase) leads to breakdown of plant cell wall pectins into small oligogalacturonides and causes wall loosening (Cervone *et al.*, 1989). The damage caused by the enzyme could lead to enhanced deposition of wall bound phenolics on the cell wall to patch up the damage and also form a barrier to prevent further spread of the pathogen. As the concentration or exposure time of pectinase increases, more damage could be caused to the cells. In order to combat this damage plant cells deposit more wall bound phenolics to prevent further degradation of cell walls (Negeral and Javelle, 1995). This deposition perhaps lead to an enhancement in the dry weight. This explains lower FW/DW ratio in elicited cultures, compared to
controls, as observed in Figure 4-2. Unlike pectinase, chitin and jasmonic acid have no effect on the FW/DW ratio of the root cultures.

Identification of the points of metabolic control is essential for desired manipulation of plant cell cultures (Srinivasan et al., 1996). Signal transducers like jasmonic acid are believed to play an important role in defense gene regulation and induction (Gundlach et al., 1992; Müller et al., 1993; Farmer, 1994). Low concentrations of jasmonic acid have been shown to be present constitutively in plant cells while higher amounts are responsible for cell death or growth inhibition (Cree:man and Mullet, 1995), hence dosage of jasmonic acid is an important parameter. In our studies, an increase in the concentration of jasmonic acid caused an increase in the levels of all the alkaloids quantified with no effect on growth, indicating an overall enhancement in flux to the alkaloid pathway. A rigorous optimization of jasmonic acid dosage could perhaps further enhance the levels of indole alkaloids.

Implementation of precise strategies for enhancement in production of valuable secondary metabolites is also hampered by the rapid and, in some cases, complete turnover of secondary metabolites. The phytotoxic nature of these compounds has been proposed as the probable reason for active catabolism (Whitehead and Threlfall, 1992). In plants, the necrotic tissue at the site of infection provides a sink for these compounds. However, high accumulation of secondary metabolites is not observed in cultured cells, even though the activities of enzymes involved in their biosynthesis are enhanced upon fungal elicitation (Moreno et al., 1996). Involvement of chemical degradation and biotransformation in catabolism of indole alkaloids, synthesized by C.
*roseus* cell suspension cultures, has been shown using isotope labeling studies (Schripsema *et al.*, 1994). The transient decline in specific yields of tabersonine, lochnericine and serpentine observed upon addition of pectinase indicates the possibility of short-term activation of catabolic processes in response to pectinase elicitation. Thus pectinase elicitation may be a method to study catabolic processes in the indole alkaloid pathway if transformation to other unknown compounds, or a temporary reduction in biosynthesis, can be quantified or ruled out.

Elicitor exposure time also plays a significant role in transient induction of genes, activation of enzymes and accumulation of several products (Seitz *et al.*, 1989; Pasquali *et al.*, 1992; Roewer *et al.*, 1992). Therefore, transient studies can be used to study the regulation of the secondary metabolite pathways. The contrasting transient profiles of tabersonine and lochnericine, obtained after the addition of jasmonic acid versus pectinase, suggest that jasmonic acid is not a part of the signal transduction cascade that leads to manifestation of the response produced by pectinase. The effect of jasmonic acid on the various points of the pathway is evident from the striking difference in the profiles of serpentine and hörhammericine (end point alkaloids) which, unlike tabersonine and lochnericine (intermediates), increase continuously after elicitation. Determination of transient metabolite profiles and hence the optimal exposure time for enhanced productivity can also have a significant impact on the elicitor requirement in bioreactor scale-up. Based upon the transient profiles of a specific metabolite, binding curves at the optimal exposure times should be determined
and utilized in the elicitor binding model (Singh et al., 1994) to predict dosage requirements for secondary metabolite production from root cultures.

Jasmonic acid and its ester, methyl jasmonate, appear to have a positive effect on secondary metabolite production in over 36 plant species (Gundlach et al., 1992). Therefore these compounds may be effective universal elicitors for enhancement of secondary metabolite levels in a plant cell or tissue culture process. Multi-fold enhancements in secondary metabolite production in response to jasmonic acid treatments have been reported in Catharanthus and Cinchona seedlings (Aerts et al., 1994), in suspension cultured rice cells (Nojiri et al., 1996), and in suspension cultures of Taxus species (Yukimune et al., 1996). We saw an enhancement in the levels of several alkaloids upon addition of jasmonic acid to our root cultures. Thus jasmonic acid seems to be an excellent choice as an elicitor for enhancement in secondary metabolite yields.

4.6 Conclusions

In summary, we have demonstrated the effect of three different elicitor components on synthesis of indole alkaloids by C. roseus hairy roots. Jasmonic acid appears to be an excellent elicitor leading to an overall enhancement of flux in several branches of the indole alkaloid pathway of C. roseus, without any deleterious effects on growth. Transient induction of genes or increase in enzyme activities in response to elicitation likely leads to rapid enhancement in biosynthesis (as seen upon jasmonic acid addition) or catabolism (as seen upon pectinase addition) of secondary
metabolites. Metabolic studies of the responses induced by the known fungal cell wall components, fungal enzymes and signal transducers independently can give information on the specific effects of each component. Using these specific components in correct dosage, or their mixtures in different proportions, more precise manipulations could be used to probe the complex secondary metabolite pathways and their regulation and to induce a targeted branch of the secondary metabolite pathway for enhancement in production of desired compounds.
CHAPTER 5: NMR SPECTROSCOPY AS A TOOL TO STUDY PLANT TISSUE CULTURE METABOLISM.

5.1 Abstract

Metabolic engineering of plant tissue cultures is hampered by poor understanding of the intracellular metabolism. NMR spectroscopy is being pursued as an important technique for studying the metabolism of growing cells non invasively. However, limited applications to studying the metabolism of plant tissue cultures are present in literature. An NMR perfusion reactor system was designed to allow C. roseus hairy root cultures to grow for 3-6 weeks, during which time NMR spectroscopy was performed to study metabolism. $^{31}$P NMR spectroscopy was performed to monitor phosphorous uptake and to monitor pH in the cytoplasmic and vacuolar compartments. Cytoplasmic pH was used as the marker for oxygenation and the constant value of cytoplasmic pH ($5.25 \pm 0.08$), observed during the entire experiment indicated adequate oxygenation. $^{13}$C NMR spectroscopy was performed to determine the activity of pathways involved in glucose catabolism. Hairy root cultures were grown in solutions containing [1-$^{13}$C], [2-$^{13}$C], and [3-$^{13}$C] labeled glucose in separate experiments and the flow of label was monitored. Activities of pentose phosphate pathways, non-photosynthetic CO$_2$ fixation and glucan synthesis pathways were evident from the experiment results. Scrambling of label in glucans also indicated recycling of triose phosphate and their subsequent conversion to hexose phosphates.
5.2 Introduction

Understanding of metabolic processes is essential for targeted manipulation of pathways of an organism via metabolic engineering (Stephanopoulos and Sinskey, 1993; Varma and Palsson, 1994). Detailed knowledge of the biosynthetic pathway kinetics and control is essential for purposeful metabolic engineering of plants and plant tissue cultures; however, this information is very difficult to obtain. This lack of quantitative information on control and integration of metabolic pathways also makes it difficult to predict the response of metabolism to genetic and environmental perturbations.

*In vivo* NMR spectroscopy has emerged as an important technique to study intracellular metabolism of a growing culture non invasively (Shulman *et al*., 1979; Gadian, 1982). A combination of high-field NMR instruments and highly enriched $^{13}$C precursors has led to broader applications of NMR to understanding several aspects of plant metabolism. Specifically, in plants cell suspensions, NMR spectroscopy has been utilized as a tool for biosynthetic studies (Hano *et al*., 1994), to monitor substrate utilization (Dijkema *et al*., 1988), and to study the metabolic changes in response to a perturbation (Ojalvo *et al*., 1987). NMR spectroscopy has been also applied to plant tissues to study glycolysis (Hatzfeld and Stitt, 1990; Lutterbach and Stokigt, 1994) and starch synthesis pathways (Viola *et al*., 1991) and to understand intracellular compartmentation and transport of metabolites (Chang and Roberts, 1988). Difficulties, however, exist in maintaining adequate oxygen and nutrient supplies, to
slow growing plant tissue cultures, in the constricted space of an NMR tube (Hartbrich et al., 1996). Several reactor systems have been designed previously (Fernandez et al., 1988; Fox et al., 1989; Minichiello et al., 1989; de Graff et al., 1992; Chen and Bailey, 1993), but most of those schemes are not suitable for plant organ cultures.

Plant tissue culture represents a promising model system for understanding the intracellular metabolism of differentiated plant cells (DiCosmo and Misawa, 1995). In this study, Catharanthus roseus hairy root culture, an alternative source for production of valuable anti-cancer and anti-hypertensive vinca pharmaceuticals (van der Heijden et al., 1989; Moreno et al., 1995), has been used as a model system. The characteristics of a NMR perfusion reactor system, designed specifically for in situ metabolic studies of hairy root cultures, have been presented. These hairy root cultures have been grown for long time periods in the NMR perfusion reactor containing $^{13}$C labeled glucose and the concentration changes of several metabolic intermediates have been monitored using $^{13}$C NMR spectroscopy. The contributions of pentose phosphate pathway, anaplerotic pathways and pathways for glucan synthesis have also been studied using $^{13}$C glucose labeled in C-1, C-2 and C-6 positions. Intracellular inorganic phosphate measurements are important as intracellular Pi is believed to have important regulatory effects on plant cell metabolism (Vogel and Brodelius, 1984; Li and Asihara, 1989). Intracellular pH measurements are also important as the activities of several enzymes are dependent upon environmental pH (Voet and Voet, 1990).
Chemical shifts of several intermediate phosphorous metabolites are dependent upon pH and can be used as markers for pH measurement (Moon and Richards, 1973; Shanks and Bailey, 1988). Therefore, $^{31}$P NMR has been used simultaneously to monitor $P_i$ uptake, compartmental pH, and the oxygenation state of the root cultures during growth.

5.3 Materials and Method

5.3.1 Hairy Root Cultures

The experiments were conducted with the *Catharanthus roseus* hairy root clone LBE 6-1 (Bhadra *et al.*, 1993). The cultures were grown on an orbital shaker at 100 rpm, at 26°C in dark. Half strength Gamborgs B5 (B5/2) medium containing 30 g/l of sucrose was used for maintenance of subculture. Roots were maintained on a 3 week subculture cycle (Rijhwani and Shanks, 1997) by transferring 0.05 grams of 4-5 cm root tips into a flask containing fresh medium. The pH of the medium was adjusted to 5.7 prior to filter sterilization.

5.3.2 Perfusion Experiment

The microperfusion system contains an NMR reactor, a reservoir, a peristaltic pump and a flow control mechanism (Figure 5-1A). The medium is oxygenated in the reservoir by passing pre-humidified oxygen. This oxygenated medium is pumped to the NMR reactor and back through the flow control mechanism. The pH of the
medium was monitored on line with a 200 μl micro pH probe (Phoenix Electrode, Houston, TX) connected to a pH meter (pH 7615, phoenix Electrode). Dissolved oxygen was monitored with an in line DO probe (Phoenix Electrode) connected to a DO meter (DO 7615, Phoenix Electrode). The NMR reactor, shown in Figure 5-1B, comprised of a 15 mm screw cap NMR tube fitted with a custom made teflon insert (modified from the design of Foxall and Cohen, 1983). The top of the insert was fitted with inlet and outlet tubing. The inlet reaches the bottom of the NMR tube through the center of the insert, and a filter sequesters the outlet perfusate and keeps the sample from flowing out of the detection region. This NMR reactor could be lifted in and out of the magnet with ease. In order to minimize the circulation volume of the perfusate, fine diameter, non permeable tubing was used for medium inlet and outlet.
Figure 5-1 A) NMR microperfusion system. B) Detailed outline of the NMR reactor in the perfusion system (Modified from Ho, 1994).
Prior to experiment, the system was steam sterilized for 20 minutes. The DO and the pH electrodes were submerged in 70% ethanol and sterilized by UV light overnight. 0.27 grams of [1-13C], [2-13C] and [6-13C] labeled glucose (CDN Isotopes, Quebec, Canada), along with 0.63 grams of unlabelled glucose (Sigma Chemicals) was dissolved in 90 ml of the Gamborgs salt solution free of paramagnetics. 0.1 ml of Gamborgs vitamin (Sigma Chemicals) solution was added and the media was transferred to the reservoir after filtration through a 0.22 μm membrane. 10 ml of deterated water (D₂O) was added to the reservoir to provide lock for the NMR signal. Approximately 1.5 grams of 4-5 cm long hairy root tips were harvested from a late exponential stage cultures (3 weeks old) and were aseptically transferred to the NMR reactor. Care was taken to avoid physical damage to the roots. The NMR reactor was sealed using a silicon sealant and medium perfusion was started. The outlet was pumped at a flow rate slightly higher than the inlet to prevent leakage inside the magnet. Oxygenation was started in the reservoir and the media was stirred to allow mixing and gas liquid mass transfer.

5.3.3 NMR Spectroscopy

In situ NMR spectroscopy was performed in Fourier Transform mode on Bruker AMX 500 spectrometer. 31P and 13C{1H} spectra were acquired alternatively in a custom built 15 mm dual 31P and 13C{1H} broad band probe. 31P spectra were acquired at 202.46 MHz at 300K, using 45° pulses and a delay time of 0.97 seconds. A
pulse width of 24.7 μ seconds was used based on the compounds of interest. The spectra were acquired with 12,000 scans corresponding to an acquisition time of 3 hr 15 min. (16K files). The acquisition time was set to 0.69 seconds and the spectral width was 12195 Hz. At the end of each experiment, GPC (glycerylphosphocoline) was added and the final spectrum was acquired for chemical shift calibration. GPC resonates at 0.49 ppm downfield from 85% phosphoric acid, and is used as a chemical shift reference.

$^{13}$C NMR spectra were obtained at 125.75 MHz at 300k, using 60° pulses and a delay time of 2 seconds. A pulse width of 12 μ seconds was used and spectra were acquired with 6000 scans corresponding to an acquisition time of 3 hour 47 min. Gated-proton decoupling was achieved by using composite pulses and WALTZ-16 sequence. C-1 β carbon of glucose, which resonates at 96.7 ppm downfield from trimethylsilane, was used as an internal chemical shift reference.

Determination of saturation factors are critical for data quantification. Saturation factors of $in$ $vivo$ $^{31}$P NMR signals of $C$. $roseus$ hairy roots were measured in perfusion experiments. $^{31}$P NMR spectra of 3,000 scans were obtained at 202.46 MHz at 26°C using 45° pulses and a spectral width of 12,100 Hz. Repetition times for saturated and fully relaxed acquisitions were 0.97 sec and 20.6 sec respectively. Two saturated spectra were acquired immediately prior to and after the fully relaxed acquisition respectively.
Saturation factors of $^{13}$C-$^1$H signals were measured in perfusion experiments in which the *C. roseus* hairy root samples were perfused with oxygenated medium containing 50 mM $^1$-^{13}$C-glucose. After $^{13}$C-label enhanced signals were sufficiently developed, saturated spectra and relaxed spectra were acquired alternately at 125.76 MHz at 26°C, using 60° pulses and a spectral width of 27,770 Hz. Gated-proton decoupling was applied using composite pulses and a WALTZ-16 sequence. The saturated factors were then determined by a fully relaxed spectrum and two saturated spectra which were acquired immediately before and after the fully relaxed spectrum, according to

$$\text{Saturation factor} = \frac{\text{average of saturated signal intensities}}{\text{relaxed signal intensity}}$$

Average values of saturation factors were used for the quantification of NMR data (Ho, 1994).

### 5.3.4 Data Processing

The NMR data files obtained were transferred to an Indy computer (Silicon Graphics Inc.) for analysis. The spectra were zero filled and baseline corrected. Line broadening (25Hz for $^{31}$P and 4Hz for $^{13}$C) were applied to the FIDs and they were subsequently fourier transformed. These spectra were then phase corrected and baseline flattened using a 5th degree polynomial. Deconvolution of overlapping peaks was performed by fitting the individual peaks to a lorentzian line shape. All analysis
was performed using FELIX95 software (BIOSYM). The concentrations were based on peak areas which were corrected with saturation factors. Biomass estimates for intracellular metabolite concentrations at various time points were obtained by simulating a growth curve from the initial and endpoint measurements of several independent experiments. All concentrations were based upon per unit medium volume (extracellular components) or per unit cell volume (intracellular components).

5.3.5 OUR and $k_La$ Measurement

The measurements were based on the mass balance of dissolved oxygen in the reservoir

$$V_r \frac{dC_i}{dt} = k_La \ (C^* - C_i) \ V_r + F \ C_i - F \ C^* \quad (5-1)$$

and the reactor

$$FC^* - F \ C_i - rW = V_b \ \frac{dC_i}{dt} \quad (5-2)$$

From 5-1 and 5-2 we get

$$V_t \ \frac{dC_i}{dt} = k_La \ (C^* - C_i) \ V_r - r \ W \quad (5-3)$$

Measurements of $k_La$ of the microperfusion system was made without hairy root samples in the NMR reactor. Dissolved oxygen concentration in the medium was first lowered to 30% air saturation, then oxygen supply was resumed and the resulting
DO profile was recorded. $C_I$ was plotted against $\frac{d C_I}{dt}$ to give a slope of $\frac{-1}{k_L a} * \frac{V_t}{V_r}$, as indicated in the rearranged form of Eq.(5-3)

$$C_I = C^* - \frac{1}{k_L a} * \frac{V_t}{V_r} * \frac{d C_I}{dt}$$  \hspace{1cm} (5-4)

Oxygen uptake rate of hairy roots grown in B5/2 medium was measured in shake flasks. Prior to measurements, shake flasks containing hairy root cultures at specified age were filled with B5/2 medium containing 30 g/L sucrose and were oxygenated to 120% air saturation. When oxygen supply was shut off, the resulting decline in dissolved oxygen concentration was recorded. The specific oxygen uptake rate ($r$) was calculated with the decline slope in DO, $\frac{d C_I}{dt}$, and the hairy root fresh weight, $W$, according to

$$r = - \frac{d C_I}{dt} * \frac{V_s}{W}$$  \hspace{1cm} (5-5)

### 5.3.6 Intracellular pH Determination

Intracellular pH was determined from chemical shifts of inorganic phosphate ($P_i$) peaks according to in vitro correlations of pH and $P_i$ chemical shift (Moon and Richards, 1973; Shanks and Bailey, 1988). Two correlation curves, as shown in Figure 2, were prepared with two different medium compositions: 0.1 M KCl, 0.1 M $P_i$, 5 mM glucose-6-phosphate, 5 mM fructose-6-phosphate and 5 mM MgCl$_2$; and 0.2 M
KCl, 0.1 M P_i, 5 mM glucose-6-phosphate, 5 mM fructose-6-phosphate and 5 mM MgCl_2. The pH value of the *in vitro* sample was measured with a Beckman F50 pH electrode (Beckman, Fullerton, CA); and ^31^P NMR spectra were acquired with 100 scans at 202.46 MHz at 26°C using 55° pulses, repetition time of 1.33 sec and a spectral width of 8,000 Hz. From each *in vivo* P_i chemical shift, two intracellular pH values were estimated from the two calibration curves respectively, and final estimate was the average of those values. This protocol was employed because ionic strength and divalent cation concentrations, in particular, Mg^{2+}, also affect P_i chemical shift and the intracellular milieu of many biological systems can be mimicked by a Mg^{2+} concentration of 5 mM and the KCl concentration between 0.1 to 0.2 M (Roberts *et al.*, 1981).
Figure 5-2 Chemical shift variation of inorganic phosphate with pH. Two curves were obtained by varying the pH of two *in vitro* samples 5 mM Mg$^{2+}$, 0.2 mM KCl and 5 mM Mg$^{2+}$, 0.1 mM KCl (adapted from Ho, 1994).
5.4 Results and Discussion

In vivo NMR has proved to be a powerful technique for metabolic studies because of its noninvasive nature and its abilities to detect subcellular compartmentation (Gadian, 1982; Vogel, 1987). However, maintenance of oxygen and nutrient supply is difficult because high cell densities and long acquisition times are often required to obtain adequate signal-to-noise ratio. In order to overcome this problem, an NMR microperfusion system was developed for in situ metabolic studies of plant organ cultures. The NMR perfusion system was designed for long term studies, i.e. the length of culture growth cycle (3-6 weeks). The aims of our metabolic investigations and the constraints of the NMR spectrometer required the following design considerations: (1) minimization of interference of the magnetic field; (2) maintenance of sterile conditions for extended periods; (3) efficient transfer of nutrients; (4) rapid online introduction of a stimulus and withdrawal of a sample; (5) reproducibility for dissolved oxygen, pH and NMR measurements and (6) ease of lifting the reactor in and out of the magnet between measurements without compromising sterility.

5.4.1 System Characterization

In order to ensure sufficient oxygen provision, oxygen uptake rates (OUR) by C. roseus hairy roots and the volumetric mass transfer coefficient of the microperfusion system, $k_{La}$, were measured. The maximal specific oxygen uptake rate by C. roseus
hairy roots was approximately 5.5 µmol/gFW/hr and the $k_{La}$ of the microperfusion system was approximately 0.095 min$^{-1}$ at an oxygen flowrate of 20 mL/min (Ho, 1994). Based on this data, it was shown that the microperfusion system could support 46.0 g (450% of reactor volume) of most actively growing hairy roots when operated at the medium flowrate of 12ml/min (Ho, 1994). With adequate oxygen supply achieved, the possibility of severe channeling inside the NMR reactor which could result in local hypoxia still remained. In order to clear this concern, the mixing characteristics in the NMR reactor were determined. A 1.5 g-hairy root sample was placed in the NMR reactor and perfused with ADP-depleted medium at 12 mL/min. The outlet medium from the NMR reactor was collected as waste and was not recycled back to the reservoir. After a steady state was established, a step change was introduced at time 0 by adding 10 mM ADP in the reservoir, and the transient ADP profile in the NMR reactor was monitored by acquiring a series of 1-min $^{31}$P NMR spectra, using 45° pulses and repetition time of 0.97 sec. A new steady-state was reached in 2 min after ADP front arrived in the NMR reactor (Ho, 1994). A long tailing, which would have taken place to reflect the slow diffusion processes, was not observed before the establishment of the new steady state, indicating that no significant channeling occurred inside the NMR reactor.

5.4.2 Characteristics of $^{31}$P NMR spectra
$^{31}$P NMR spectroscopy was performed every 2-3 days after inoculation of the reactor. Figure 5-3 shows the $^{31}$P NMR spectra of C. roseus hairy root cultures perfused with oxygenated medium, obtained 925 hours after inoculation. Resonance assignments were performed according to the literature data on plant cultures (Vogel and Brodelius, 1984; Brodelius and Vogel, 1985; Vogel, 1987; Fan et al., 1988; Bligny et al., 1989; Minichiello et al., 1989). Two distinct intracellular organic phosphate peaks, a vacuolar Pi peak at 0.8 ppm and a cytoplasmic Pi peak at approximately 2.8 ppm, were observed. The extracellular Pi peak was observed at 1.4 ppm however this peak disappeared as inorganic phosphate was taken up by the root cultures. The peaks at 4.9 and 3.9 ppm respectively were assigned to glucose-6-phosphate and fructose-6-phosphate respectively. The resonances of ATPγ and ATPβ at -4.9 ppm, ATPα and ADPα at -9.9 ppm, NADP and NDPG at -10.5 ppm, NDPG at -12.2 ppm and ATPβ at -18.7 ppm were also observed. A possible new peak at -0.28 ppm was observed in the old hairy root cultures (Figure 5-3). A similar observation in C. roseus suspension cultures was also made by Vogel and Brodelius (1984) who suggested that this peak belongs to a free phospholipid however, the identity was not confirmed.
Figure 5-3  $^{31}$p NMR spectra of the hairy root cultures obtained after 920 hours of perfusion.
The time course of phosphorous NMR spectra indicated rapid uptake of inorganic phosphate from the extracellular medium and its accumulation in the vacuole, as evident from the increase in concentration of Pi vacuole (Figure 5-4). This increase in vacuolar Pi concentration is consistent with the hypothesis that the vacuole functions as the storage site for Pi (Vogel, 1987). The cytoplasmic Pi concentration increased initially from 0.2 mM to 1.4 mM, at 100 hours, and declined thereafter (Figure 5-4A). Phosphorous plays an important role in regulating the activity of several enzymes in the cytoplasm of the plant cells (Salisbury and Ross, 1986). Phosphorous supply of the cytoplasm is maintained by continuous influx of Pi from the vacuole to account for the metabolic fixation of cytoplasmic Pi (Wray et al., 1983).

The concentrations of glucose-6-phosphate (G6P) was determined from the area under the peak at 4.9 ppm. The levels of glucose-6-phosphate followed the cytoplasmic Pi profiles i.e. G6P concentration increased initially from 0.6 to 2.2 mM, at 100 hours, and decreased thereafter (Figure 5-4B). Poor resolution of fructose-6-phosphate (F6P) peak was observed and the concentration of F6P could not be determined.
Figure 5-4 Time course profiles of A) cytoplasmic and vacuolar inorganic phosphate, and B) Glucose6-phosphate during growth of *C. roseus* hairy root cultures in the perfusion reactor.
5.4.3 Intracellular pH

Chemical shifts of several compounds, like inorganic phosphate, sugar phosphates, ATP, have been used as markers for measurement of intracellular pH (Shanks and Bailey, 1988). A calibration curve correlating the chemical shift of the inorganic phosphate (Pi) peak with pH was used to determine the intracellular pH of the cytoplasmic and the vacuolar compartments. Two solutions mimicking the intracellular ion content were utilized to obtain the calibration curves (Figure 5-2). From these curves the intracellular pH of the cytoplasmic and the vacuolar compartments was determined by averaging the values obtained from the two curves. The extracellular pH was measured using an online pH micro probe as the extracellular Pi signal could not be detected after the uptake of inorganic Pi by the root cultures. A decline in the extracellular pH followed by a continuous increase was observed in all three experiments. The chemical shifts of the cytoplasmic and the vacuolar inorganic phosphate peaks indicated that the pH of the cytoplasmic and the vacuolar compartments was maintained at 7.4 ± 0.06 and 5.25 ± 0.08 respectively (Figure 5-5). These values are consistent with the previously observed values for cytoplasmic and vacuolar pH (Vogel, 1987; Tu et al., 1990; Roberts et al., 1991).
Figure 5-5 Extracellular, cytoplasmic and vacuolar pH profiles of *C. roseus* hairy root cultures during growth in the perfusion reactor.
A significant enhancement in oxygen consumption and a reduction in cytoplasmic ATP concentration was reported in sycamore cell suspensions upon decreasing the extracellular pH (Gout et al., 1992). However, the cytoplasmic and the vacuolar pH was maintained at a constant value by the action of membrane bound H⁺-ATPase. When the pH of the medium was reduced below 4, the rate of ATP synthesis could not keep up with the ATP requirement for maintenance of constant pH and acidification of the cells was observed (Gout et al., 1992). Hence, plant cells have an ability to maintain the intracellular pH constant within the physiological pH range. Cytoplasmic pH has been used as a marker for oxygenation (Melvin and Shanks, 1996). Onset of anaerobic conditions, induced by replacing oxygen by nitrogen, lead to a decline in the level of the cytoplasmic pH in immobilized C. roseus cells (Vogel and Brodelius, 1984) and in our root cultures (Ho, 1994). Hence, cytoplasmic pH was used as the marker for oxygenation and the constant value of cytoplasmic pH observed throughout the experiments indicated that adequate oxygen was being supplied to the root cultures.

5.4.4 $^{13}$C NMR Spectra

$^{13}$C NMR spectroscopy was used to measure the concentrations of intermediates in the glycolysis and the TCA cycle. Hairy roots, fed with unlabeled sucrose as the carbohydrate source, were harvested from shake flasks and were transferred to the NMR reactor. These roots were perfused with glucose labeled in
different positions (C-1, C-2 and C-6) and the distribution of label in the hairy root cultures was monitored using $^{13}$C NMR. Figure 5-6 shows the NMR spectra of the hairy root cultures of *C. roseus* obtained at 870 hours in the experiment using [1-$^{13}$C] labeled glucose. Two distinct resonances from $\beta$ [1-$^{13}$C] and $\alpha$ [1-$^{13}$C] glucose are observed at 96.7 and 92.9 ppm respectively (Figure 5-6). Figure 5-7 shows the NMR spectra from the experiment using [2-$^{13}$C] labeled glucose, obtained at 900 hours in the experiment. The $\beta$ [2-$^{13}$C] and the $\alpha$ [2-$^{13}$C] resonances were not be resolved and were observed at 75 ppm (Figure 5-7). Figure 5-8 shows the NMR spectra of the hairy root cultures in the experiment using [6-$^{13}$C] labeled glucose, obtained at 890 hours. The resonances from $\beta$ [6-$^{13}$C] and $\alpha$ [6-$^{13}$C] are observed at 61.5 and 61.35 ppm respectively (Figure 5-8). The assignments were made in two steps: first, chemical shifts of the resonances were compared with literature data (Gorin, 1981; Dais and Perlin, 1982; Canioni et al., 1983; Stidham et al., 1983; Bock et al., 1984; Barany et al., 1985; Halliday et al., 1988; Klein et al., 1990) and/or in vitro spectra; and then tentative assignments were checked with consistency with metabolic pathways. At the beginning of each experiment only natural abundance resonances belonging to other unlabeled glucose carbon are observed besides the labeled carbon. Since this first spectra is obtained immediately after inoculation of the roots into a known concentration of glucose media, these spectra are used as calibrations to obtain concentrations. The NMR spectra of the initial and the final medium was also obtained
to determine the localization of some resonances i.e. if the compound is present in the culture medium or inside the cells.
Figure 5-6 $^{13}$C NMR spectra obtained from *C. roseus* hairy root cultures perfused with [1-13C] glucose.
Figure 5.7 13C NMR spectra obtained from hairy roots perfused with [2-13C] glucose.
Figure 5-8 $^{13}$C NMR spectra obtained from hairy roots perfused with [6-$^{13}$C] glucose.
5.4.5 Glucose

Extracellular concentration of glucose in the solution could be calculated using NMR. A decrease in the intensity of glucose resonances in the culture medium was observed with time, indicating uptake of glucose by the root cultures. The concentrations of both β [1-13C] and α [1-13C] glucose resonances was observed to decrease with time (Figure 5-9A). The time course NMR spectra for the three experiments also show appearance of several new resonances with time. The accumulation of these resonances, assigned to intermediates in glycolysis and TCA cycle pathways, indicates assimilation and flow of glucose through primary metabolic pathways.

The ratio of β [1-13C] and α [1-13C] glucose, in solution, at the beginning of the experiment was observed to be 66:34, however this ratio decreased with time (Figure 5-9B). Different rates of disappearance of β and α glucose have been observed in Escherichia coli (Urgbil et al., 1978). An increase in the ratio of [1-13C] glucose β:α ratio was observed in suspensions of yeast cells by Den Hollander et al. (1979) (Den Hollander et al., 1979). Anomeric specificity of glucose uptake with both β and α anomers competing for the same site was proposed as the reason for this effect. An anomeric specificity, opposite of that observed by Den Hollander et al. (1979), could be one reason for the increase in ratio of [1-13C] glucose β/α ratio. The α [1-13C] resonance of glucose overlaps with the α [1-13C] glucose resonance from sucrose
(Dijkema et al., 1988). Thus synthesis of sucrose by the root cultures could lead to an increase in this resonance and hence cause a decrease in the $\beta$:$\alpha$ ratio.
Figure 5-9 Time course profiles of A) $\alpha \ [1^{-13}\text{C}]$ and $\beta \ [1^{-13}\text{C}]$ glucose, and B) ratio of $\beta \ [1^{-13}\text{C}]$ to $\alpha \ [1^{-13}\text{C}]$ glucose obtained from hairy root cultures perfused with $[1^{-13}\text{C}]$ glucose.
5.4.6 [3-\textsuperscript{13}C] pyruvate and [4-\textsuperscript{13}C] Glutamine

Several intermediates in the glycolytic and the TCA cycle could be detected in these experiments. The concentrations of [3-\textsuperscript{13}C] pyruvate (Figure 5-10A) and [4-\textsuperscript{13}C] glutamine (Figure 5-10B) were found to increase with time due to flow and accumulation of the \textsuperscript{13}C label in these pools. These concentrations were higher when [6-\textsuperscript{13}C] labeled glucose was used as the carbohydrate. This indicated activity of pentose phosphate pathway because, if glycolysis alone was the pathway for the flow of carbon flux, the C-1 and the C-6 labels should be indistinguishable after the triose phosphate point (as both C-1 and C-6 labels of glucose would appear in C-3 position of triose phosphates). Therefore the concentration of label determined in subsequent metabolites should be the same when either [6-\textsuperscript{13}C] or [1-\textsuperscript{13}C] labeled glucose is used as the sugar source. However, the lower concentration of [3-\textsuperscript{13}C] pyruvate and hence [4-\textsuperscript{13}C] glutamate (derived from C-3 label of pyruvate) in the [1-\textsuperscript{13}C] labeled experiment, as compared to the [6-\textsuperscript{13}C] labeled experiment, indicates that there is some loss of [1-\textsuperscript{13}C] label.

Pentose phosphate pathway activity in plant cells is essential for synthesis of reducing equivalents of NADPH and for the synthesis of precursors for amino acid and secondary metabolite pathways (Salisbury and Ross, 1986). When glucose enters the pentose phosphate pathway, the [1-\textsuperscript{13}C] label is lost as CO\textsubscript{2} in the first step while the [6-\textsuperscript{13}C] label is recycled back into the glycolysis pathway (Voet and Voet, 1990). Therefore, flux to the pentose phosphate pathway causes the final concentration of [3-
labeled pyruvate and [4-\(^{13}\text{C}\)] labeled glutamate to be less in the roots using \([1-^{13}\text{C}]\) labeled glucose, as compared to the roots using \([6-^{13}\text{C}]\) labeled glucose.

The contribution fractions of glycolysis, at different times, were calculated directly from the ratio of concentration of \([3-^{13}\text{C}]\) pyruvate in \([1-^{13}\text{C}]\) and \([6-^{13}\text{C}]\) labeled experiments. This analysis indicated that the contribution of glycolysis to pyruvate synthesis decreased as the root culture grew. The contribution of glycolysis to pyruvate synthesis was 85% at 200 hours while at 800 hours this contribution reduced to 35%. These results indicate that during growth phase, there is a higher flux of carbon through the glycolysis pathway while there is a higher flux through the pentose phosphate pathway after the growth phase.
Figure 5-10 Time course profiles of A) [3-^{13}C] pyruvate and B) [4-^{13}C] glutamine in C. roseus hairy root cultures perfused with [1-^{13}C] glucose.
5.4.7 Non photosynthetic CO₂ fixation

A peak from [4-¹³C] label of malate was detected at 179.3 ppm when glucose labeled at position C-1 was used as the sugar source (Figure 5-6). pH dependence of the chemical shifts of [1-¹³C] and [4-¹³C] malate has been used to determine the localization of malate (Chang and Roberts, 1988). In order to determine the origin of the [4-¹³C] malate peak in our experiments, calibration curves correlating the chemical shift of [1-¹³C] and [4-¹³C] malate to solution pH were obtained (Ho, 1994). From these curves it was determined that chemical shift of [4-¹³C] malate corresponds to a pH of 5.3 indicating that malate was localized in the vacuole. To determine the source of [4-¹³C] malate peak, glucose labeled in position [6-¹³C] was used as the sugar source. The [4-¹³C] malate peak was not detected when [6-¹³C] labeled glucose was used as the sugar source. If this [4-¹³C] malate peak was being derived from malate in TCA cycle, not only should a higher concentration of this peak be observed due to a greater flow of label in the pathways when [6-¹³C] labeled glucose is used (explained previously), but also α [1-¹³C] malate peak of equal concentration should be observed due to scrambling of label at fumarase. However, the C-1 peak was not detected when either [1-¹³C] or [6-¹³C] labeled glucose was used as the carbohydrate source. Secondly, the absence of [4-¹³C] malate peak in the [6-¹³C] glucose experiment confirmed that [4-¹³C] resonance does not originate from the malate pool participating in TCA cycle.
Figure 5-11 Time course profiles of [4- $^{13}$C ] malate in *C. roseus* hairy root cultures perfused with [1- $^{13}$C] glucose.
Non-photosynthetic fixation of carbon dioxide has been known to occur in plants (Basra and Malik, 1985). In these reactions, CO₂ combines with phosphoenolpyruvate (PEP) to form oxaloacetate (OAA) which is further reduced to malate. Conventionally, PEP formed in glycolysis is regarded as being converted to pyruvate. However, it has been found that PEP carboxylase directs a significant proportion of the glycolytic carbon in form of PEP towards OAA synthesis. This OAA formed by the PEP Carboxylase in the cytoplasm is very rapidly converted to malate in the cytoplasm because of the widespread activity of NAD-malate dehydrogenase (Basra and Malik, 1985). A concerted action of the enzymes PEP Carboxylase, NAD-Malate dehydrogenase and NADP-malic enzyme in the cytoplasm will affect the conversion of PEP to pyruvate and Pi, though less directly than the reaction catalyzed by pyruvate kinase. This set of reactions could provide OAA and NADPH for biosynthesis and lead to accumulation of malate in the vacuoles for osmoregulation and maintenance of pH.

Since this malate molecule is not being produced via fumarase in the TCA cycle, scrambling of label is not observed and hence no label appears in C-1 position of malate. This hypothesis is consistant with the results obtained in our experiments as ¹³C labeled CO₂ is produced when [1-¹³C] labeled glucose is used (when glucose enters Pentose phosphate pathway) which can be fixed via CO₂ fixation pathway to form [4-¹³C] labeled malate. The lack of synthesis of [1-¹³C] labeled malate by TCA cycle was further confirmed by using [2-¹³C] labeled glucose. C-2 label of glucose should show
up in the C-4 and C-1 position of malate after the first round in TCA cycle. However malate labeled in C-1 position only, resonating at 181.4 ppm and corresponding to a pH of 5.35, could be detected in these experiments indicating that malate accumulation in vacuole occurs via the non photosynthetic CO₂ fixation pathway.

An increase in the concentration of [4-\(^{13}\)C] malate was observed with time, when [1-\(^{13}\)C] labeled glucose was used as the carbohydrate source (Figure 5-11). This increase consistent with the contribution ratios of glycolysis and pentose phosphate pathway calculated previously. An increased contribution of pentose phosphate pathway in the latter period of growth would cause to greater release of \(^{13}\)CO₂, which can be fixed to lead to an increase in the concentration of [4-\(^{13}\)C] malate.

5.4.8 Glucans

Glucans are polymers of glucose and fall under the category of storage molecules or plant cell wall components. Several resonances obtained from glucans were observed in all three experiments. Four resonances from C-1 carbon of glucans were resolved at 102.6, 103.1, 103.4 and 104.4 ppm when [1-\(^{13}\)C] labeled glucose was used (Figure 5-6). The time course profiles of glucan resonances are presented in Figures 5-12 and 5-13. The concentrations of the different glucan peaks vary significantly with time. As seen in Figure 5-12, the 104.4 ppm peak was the first to appear. Peaks at 103.4 and 102.6 were not detected until 150 hours after perfusion with C-1 labeled glucose but increased continuously thereafter.
[6-^{13}C] labeled glucan resonances are also observed to build up in the [1-^{13}C] labeled experiment. Similarly, a build up of the [1-^{13}C] labeled glucans is observed (Figure 5-13) along with the [6-^{13}C] labeled glucans when [6-^{13}C] labeled glucose is used as the sugar source. These resonances are observed probably because of scrambling of label between fructose 1,6 di-phosphate, glyceraldehyde-3-phosphate and dihydroxy acetone phosphate. Similar scrambling of label has also been observed in yeast (Den Hollander and Shulman, 1983) and in heterotrophic Chenopodium cells, potato and maize (Hatzfeld and Stitt, 1990). This scrambling of label, due to backflux of carbon from the triose phosphates to hexose phosphates, could lead to accumulation of label in the symmetrically opposite position of the parent labeled molecule.
Figure 12 Time course profiles of C-1 glucans in C. roseus hairy root cultures perfused with [1-^{13}C] glucose.
Figure 13 Time course profiles of C-1 glucans in *C. roseus* hairy root cultures perfused with [6-^{13}C] glucose.
5.5 Conclusions

An NMR perfusion reactor was designed to study the metabolism of hairy root cultures for prolonged periods of time. The reactor system provided adequate oxygen and nutrients to the root culture and maintained a sterile environment for growth of the root cultures. Results from $^{31}$P NMR spectroscopy study, indicating rapid increase in vacuolar Pi content (Figure 5-4), are consistent with previous results stating that vacuole is the storage site for phosphorous taken up from the medium (Vogel, 1987). It was observed that the cytoplasmic pH decreases when the cultures were exposed to anaerobic conditions (Ho, 1994). Hence cytoplasmic pH was used as the marker for state of oxygenation of the root cultures growing inside the NMR perfusion reactor. A constant value of the cytoplasmic pH was obtained indicating adequate oxygenation during the three reactor runs presented in this study.

$^{13}$C NMR spectroscopy studies indicated activity of several pathways in central metabolism. Flow of flux through glycolysis and the TCA cycle pathways was evident from the detection of labeling in intermediates in the pathway. Synthesis of glucans was observed in all three experiments indicating a significant diversion of the glucose flux into cell wall and storage products synthesis. The scrambling of label in the glucans synthesized indicated recycling of the triose phosphates to hexose phosphates. Scrambling of label was also observed in the studies of Hatzfeld & Stitt (1990) (Hatzfeld and Stitt, 1990), who found a considerable recycling of triose phosphates in heterotrophic Chenopodium cells, potato and maize. Activity of the non
photosynthetic CO₂ fixation pathway, as evident from the synthesis of C-4 labeled malate in C-1 labeled glucose experiment was also confirmed in these experiments. These studies have demonstrated the applicability of NMR spectroscopy to non-invasive long-term studies of hairy root cultures. The response of metabolism of these root cultures to environmental perturbations is currently being studied by NMR spectroscopy.

5.6 Notation

- \( C_l \) dissolved oxygen concentration, mmol/L
- \( C^* \) equilibrium dissolved oxygen concentration, mmol/L
- \( k_La \) volumetric mass transfer coefficient, min\(^{-1}\)
- \( r \) specific oxygen uptake rate by hairy roots, mmol/g.FW-min
- \( W \) hairy root fresh weight, g
- \( V_b \) reactor volume
- \( V_r \) reservoir volume
- \( V_t \) total volume of the reactor system (\( V_r + V_b \))
- \( V_s \) medium volume in the shake flask
CHAPTER 6: ELICITATION INDUCED METABOLIC CHANGES IN
CATHARANTHUS ROSEUS HAIRY ROOT CULTURES: AN NMR
SPECTROSCOPICALLY BASED STUDY.

6.1 Abstract

Fungal elicitation has been pursued as an important strategy for enhancement in secondary metabolite production from plant tissue cultures, and as a means for studying the secondary metabolite pathways; however, the effects of fungal elicitors on primary metabolism are not known. Hairy root cultures of Catharanthus roseus were grown in a perfusion NMR reactor containing [1-\(^{13}\)C] glucose as the carbon source. \textit{In vivo} \(^{31}\)P and \(^{13}\)C NMR spectroscopy was utilized to monitor intracellular pH and to determine the concentration profiles of phosphorous and carbon metabolites in response to addition of three elicitors. Poor growth was observed upon elicitation with pectinase while jasmonic acid and PGA did not affect growth. A transient short-term decline in the cytoplasmic pH was observed upon addition of pectinase while a prolonged decrease in the vacuolar pH was observed upon addition of jasmonic acid. Enhanced accumulation of glucans was observed upon addition of pectinase. The profiles of intermediate metabolites in glycolysis and TCA cycle indicated a possible redistribution of carbon fluxes in glycolysis and the pentose phosphate pathway upon addition of jasmonic acid. These studies help explain some of the secondary metabolite changes observed in \textit{C. roseus} hairy root cultures upon addition of elicitor.
6.2 Introduction

Fungal elicitation is utilized as a technique for enhancement of secondary metabolites biosynthesis from plant cell and tissue cultures (Whitehead and Threlfall, 1992; Singh, 1996). The addition of a fungal elicitor to a plant cell or tissue culture simulates the phenomenon of actual infection of a plant host by a pathogen. Fungal elicitation involves addition of fungal extracts, filtrates or specific compounds to the tissue for induction of plant defense response, a part of which is secondary metabolite synthesis (Dixon, 1986). Several studies have been performed previously to study the effect of fungal elicitors on biosynthesis of secondary metabolites by plant tissue cultures (Singh, 1996). However, studies of elicitation induced changes in primary metabolism have been restricted to measurements of intracellular pH and intracellular phosphate concentrations (Ojalvo et al., 1987; Kneusel et al., 1989; Hagendoorn et al., 1991; Horn et al., 1992).

Hairy root cultures of C. roseus, an important source of valuable secondary metabolites, represent a promising model system for several biological studies. We have previously studied the effects of specific components of elicitors, their dosage and exposure times on indole alkaloid biosynthesis by C. roseus hairy root cultures at shake flask level (Rijhwani and Shanks, 1997a). Since the non-invasive nature of NMR spectroscopy allows for observations of biochemical phenomenon in cells, without extrapolations from cell extracts (Gadian and Radda, 1981), $^{13}$C and $^{31}$P NMR spectroscopy were utilized to study the primary metabolism of hairy root cultures.
(Rijhwani et al., 1997). In this paper the effects of addition of elicitors on *C. roseus* hairy root culture metabolism are presented. NMR spectroscopy has been utilized previously to study the effect of elicitation on plant cell suspension cultures, however non specific elicitors were utilized and only the effect on intracellular pH was measured (Ojalvo et al., 1987; Kneusel et al., 1989; Hagendoorn et al., 1991; Horn et al., 1992). In this manuscript, we report on the effect of addition of three specific elicitors: pectinase (an endopolygalacturonase), polygalacturonic acid (a fungal cell wall component) and jasmonic acid (a signal transducer) on intracellular pH of *C. roseus* hairy root cultures, determined using $^{31}$P NMR spectroscopy. Furthermore, $^{13}$C NMR spectroscopy was performed simultaneously during growth and the effect of elicitation on the concentrations of glucans and several intermediate primary metabolites is also presented.

6.3 Materials and Method

6.3.1 Hairy Root Cultures

The experiments were conducted with the *Catharanthus roseus* hairy root clone LBE 6-1 (Bhadra et al., 1993). The cultures were grown on an orbital shaker at 100 rpm, at 26°C in dark. Half strength gamborgs B5 (B5/2) medium containing 30 g/l of sucrose was used for maintenance of subculture. Roots were maintained on a 3 week subculture cycle (Rijhwani and Shanks, 1997b) by transferring 0.05 grams of 4-5 cm
root tips into a flask containing fresh medium. The pH of the medium was adjusted to 5.7 prior to filter sterilization.

6.3.2 Perfusion Reactor Startup

The microperfusion system described in section 5.3.2 was used in these experiments. Prior to experiment, the system was steam sterilized for 20 minutes. The DO and the pH electrodes were submerged in 70% ethanol and sterilized by UV light overnight. 0.27 grams of [1-$^{13}$C] (CDN Isotopes, Quebec, Canada), along with 0.63 grams of unlabelled glucose (Sigma Chemicals) was dissolved in 90 ml of Gamborgs salt solution free of paramagnetics. 0.1 ml of Gamborgs vitamin (Sigma Chemicals) solution was added and the media was transferred to the reservoir after filtration through a 0.22 μm membrane. 10 ml of deterated water (D$_2$O) was added to the reservoir to provide lock for the NMR signal. Approximately 1.5 grams of 4-5 cm long hairy root tips were harvested from a late exponential phase culture (3 weeks old) and were aseptically transferred to the NMR reactor. Care was taken to avoid physical damage to the roots. The NMR reactor was sealed using a silicon sealant and medium perfusion was started. The outlet was pumped at a flow rate slightly higher than the inlet to prevent leakage inside the magnet. Oxygenation was started in the reservoir and the media was stirred to allow mixing and gas liquid mass transfer.
6.3.3 Elicitation

To make sure that the perfusion reactor has not been contaminated, it was operated for a period of 2-3 days before the addition of elicitor. Elicitation was performed by adding 1 ml PGA solution (50 mg PGA in 50 ml water), 72 units of pectinase and 10 μl of jasmonic acid solution (250 mg jasmonic acid in 10 ml water) through a 0.22 μm filter via the stimulus injector port (Figure 5-1) in separate experiments. A $^{31}\text{P}$ and a $^{13}\text{C}\{^1\text{H}\}$ spectra was acquired immediately before the addition of elicitor. Immediately after the addition of the elicitor, a series of $^{31}\text{P}$ NMR spectra were acquired for a period of 18-24 hours to monitor the changes in intracellular pH. Subsequently $^{31}\text{P}$ and $^{13}\text{C}\{^1\text{H}\}$ spectra were acquired every 2-3 days in a custom built 15 mm dual $^{31}\text{P}$ and $^{13}\text{C}\{^1\text{H}\}$ broad band probe.

6.3.4 NMR Spectroscopy

*In situ* NMR spectroscopy was performed in Fourier Transform mode on Bruker AMX 500 spectrometer. $^{31}\text{P}$ spectra were acquired at 202.46 MHz at 300K, using 45° pulses and a delay time of 0.97 seconds. A pulse width of 24.7 μ seconds was used based on the compounds of interest. The spectra were acquired with 12,000 scans corresponding to an acquisition time of 3 hour 15 minutes (16K files). To enhance time resolution in the jasmonic acid experiment, spectra were acquired with 6,000 scans corresponding to an acquisition time of 1 hour and 37 minutes. A spectral width of 12195 Hz was used. At the end of each experiment, glycercylphosphocholine
(GPC) was added and the final spectrum was acquired for chemical shift calibration. GPC resonates at 0.49 ppm downfield from 85% \text{H}_3\text{PO}_4 and is used as a chemical shift reference.

$^{13}\text{C}$ NMR spectra were obtained at 125.75 MHz at 300k, using 60° pulses and a delay time of 2 seconds. A pulse width of 12 μs seconds was used and spectra were acquired with 6000 scans corresponding to an acquisition time of 3 hour 47 min. Gated-proton decoupling was achieved by using composite pulses and WALTZ-16 sequence. C-1β carbon of glucose, which resonates at 96.7 ppm downfield from trimethylsilane, was used as an internal chemical shift reference.

### 6.3.5 Data Processing

The NMR data files obtained were transferred to an Indy computer (Silicon Graphics Inc.) for analysis. The spectra were zero filled and baseline corrected. Line broadening (25 Hz for $^{31}\text{P}$ and 4 Hz for $^{13}\text{C}$) were applied to the FIDs and they were subsequently fourier transformed. These spectra were then phase corrected and baseline flattened using a 7th degree polynomial. Deconvolution of overlapping peaks was performed by fitting the individual peaks to a lorentzian line shape. All analysis was performed using FELIX95 software (BIOSYM). The concentrations were based on peak areas which were corrected with saturation factors. Biomass estimates for intracellular metabolite concentrations at various time points were obtained by simulating a growth curve from the initial and endpoint measurements of several
independent experiments. All concentrations were based upon per unit medium volume (extracellular components) or per unit cell volume (intracellular components).

6.3.6 Intracellular pH Determination

Intracellular pH was determined from chemical shifts of inorganic phosphate (P_i) peaks according to *in vitro* correlation of pH and P_i chemical shift (Moon and Richards, 1973; Shanks and Bailey, 1988). pH versus chemical correlation curves, obtained previously (Ho, 1994), were used to determine the pH based on the inorganic phosphate chemical shifts.

6.4 Results

\(^{31}\text{P}\) and \(^{13}\text{C}\) NMR spectroscopy were utilized to study the affect of elicitation on pH and primary metabolism of *C. roseus* hairy root cultures. Approximately 1.5 grams of hairy roots were inoculated into the NMR perfusion reactor and were monitored for a period of 2-3 days. The elicitors: *A. niger* pectinase, jasmonic acid and polygalacturonic acid were added, 2-3 days after inoculation, in separate experiments to probe the changes in intracellular pH and primary metabolism in response to elicitation. \(^{31}\text{P}\) and \(^{13}\text{C}\) NMR spectra were acquired for a period of 30-40 days during which intracellular pH and the concentrations of several metabolites were determined.
6.4.1 Biomass yields

Host pathogen interactions during pathogenic invasion of a plant involve a multitude of signals. The plant responds by causing localized cell death and lignin, callose deposition to prevent the spread of the pathogen. It also secretes several enzymes and phytoalexins to kill the pathogen (Dixon, 1986; Yoshikawa et al., 1993). If the pathogen, however, can survive these attacks and thrive on the plant material, it can lead to plant death. During elicitation of a plant cell or tissue culture, elicitor dosage plays an important role as high dosage could lead to cell death and cause a reduction in secondary metabolite productivity. The effect of jasmonic acid, pectinase and PGA on biomass yield was determined by measuring the final biomass and the glucose concentration in the medium at the end of each experiment. The net increase in biomass and the net consumption of glucose was calculated and utilized to determine the biomass yields (Table 6-1).
Table 6-1: The initial and final weight of the hairy root cultures as obtained after growth in the NMR reactor. The results obtained in the control experiment and the elicitation experiments are shown. The amount of glucose consumed was calculated from the final glucose concentration in the medium and was utilized to determine the biomass yield in each experiment.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Inoculum Weight (gm)</th>
<th>Final Biomass (gm)</th>
<th>Experiment duration (days)</th>
<th>Glucose consumed (gm)</th>
<th>Biomass Yield (g FW/g glucose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.56</td>
<td>4.05</td>
<td>45</td>
<td>0.645</td>
<td>3.86</td>
</tr>
<tr>
<td>Pectinase</td>
<td>1.56</td>
<td>2.40</td>
<td>31</td>
<td>0.453</td>
<td>1.86</td>
</tr>
<tr>
<td>PGA</td>
<td>1.45</td>
<td>2.63</td>
<td>31</td>
<td>0.347</td>
<td>3.40</td>
</tr>
<tr>
<td>Jasmonic acid</td>
<td>1.46</td>
<td>3.85</td>
<td>36</td>
<td>0.601</td>
<td>3.98</td>
</tr>
</tbody>
</table>
The final biomass yield of the hairy root cultures elicited with pectinase was lower than that of the control cultures. The biomass yields of the hairy root cultures elicited with PGA and jasmonic acid were similar to the control cultures. Previously we had observed a reduction in the fresh weight to dry weight (FW/DW) ratio of pectinase elicited root cultures while the FW/DW ratio of the jasmonic acid elicited root cultures was same as the controls (Ridge and Shanks, 1997a). A decreased biomass yield in case of pectinase elicitation experiment indicates that pectinase addition has a significant affect on growth of the root cultures.

6.4.2 \[^{31}P\] NMR spectroscopy

Identification of the peaks observed in \[^{31}P\] NMR spectra of the hairy root cultures of *C. roseus* was presented in section 5.4.2. Accumulation of Pi in the vacuole, evident from the increasing intensity of the vacuolar phosphate peak, was observed in all the experiments. Figure 6-1 shows the vacuolar concentration profiles in the controls and the elicited root cultures. The vacuolar phosphate concentration reached the highest value in case of pectinase elicited root cultures while the vacuolar phosphate levels were similar to the control values in the jasmonic acid elicited root cultures.

Figure 6-2 shows the concentration profiles of glucose-6-phosphate (G6P) observed in the control and the elicitation experiments. The maximum concentrations of G6P obtained in the control, jasmonic acid elicitation and PGA elicitation
experiments were in the range of 1.6-2 mM however, in pectinase elicitation experiment the maximum concentration of G6P was 3 mM. The concentration of G6P decreased after reaching a maximum in all the experiments.
Figure 6-1 Vacuolar phosphate concentration profiles in the control and the elicitation experiments. Pectinase was added at 56 hours, PGA was added at 45 hours and jasmonic acid was added at 100 hours in the respective experiments.
Figure 6-2 Glucose-6-phosphate concentration profiles in the control and the elicitation experiments. Pectinase was added at 56 hours, PGA was added at 45 hours and jasmonic acid was added at 100 hours in the respective experiments.
6.4.3 Intracellular pH

Intracellular pH measurements were based upon the chemical shifts of the inorganic phosphate (section 5.4.3). The cytoplasmic and the vacuolar pH of the control cultures were 7.4 ± 0.06 and 5.25 ± 0.08 respectively (Figure 5-5). No significant changes in these values were observed upon addition of PGA (data not shown); however, a rapid decline in the cytoplasmic pH of the hairy root cultures was observed upon addition of pectinase (Figure 6-3A). The cytoplasmic pH decreased from 7.4 to 7.15 within 3 hours and recovered back to the control levels within 18 hours. No significant effect on vacuolar pH was observed in this experiment. The extracellular pH decreased immediately after the addition of pectinase and attained values lower than the vacuolar pH after the addition of pectinase.

Addition of jasmonic acid on the other hand had a significant impact on the vacuolar pH only (Figure 6-3B). The cytoplasmic pH did not demonstrate any change upon the addition of jasmonic acid however the vacuolar pH decreased by 0.2 units, 48 hours after the addition of jasmonic acid. The vacuolar pH decreased from 5.34 ± 0.06 to 5.147 ± 0.004 after the addition of jasmonic acid. The vacuolar pH stayed at this level for about 200 hours and then recovered back to the preelicitation value of 5.38 ± 0.04. The extracellular pH profile observed in the jasmonic acid elicitation experiment was similar to that of the control cultures.
Figure 6-3 Cytoplasmic, vacuolar and extracellular pH profiles obtained from hairy root cultures elicited with A) *A. niger* Pectinase and B) jasmonic acid. The dotted lines represent the time of addition of elicitors.
6.4.4 Glucose

The resonances of $\alpha$ [1-\textsuperscript{13}C] and $\beta$ [1-\textsuperscript{13}C] were resolved in all experiments. Glucose uptake was determined from the decrease in extracellular concentration of $\beta$ glucose resonance only, assuming equilibrium between the $\alpha$ and the $\beta$ glucose in solution. The glucose uptake rate did not change upon the addition of the elicitors (data not shown). On the other hand when the ratio of $\beta/\alpha$ glucose was compared for the 4 reactor runs, a higher value of the $\beta/\alpha$ glucose ratio was observed upon addition of pectinase (Figure 6-4). The NMR spectra of the pectinase elicitation experiment shows that the $\alpha$ glucose peak is lower than the $\beta$ glucose peak, upon addition of pectinase (Figure 6-5). An increase in $\alpha$ glucose peak was observed in all other experiments which leads to the difference in the $\beta/\alpha$ ratios of these runs.
Figure 6-4 The ratio of $\beta/\alpha [1^{-13}C]$ glucose concentrations in the elicitation and the control experiments. Pectinase was added at 56 hours, PGA was added at 45 hours and jasmonic acid was added at 100 hours in the respective experiments.
Figure 6-5 $^{13}$C NMR spectra of hairy roots of *C. roseus* perfused with $[1-{^{13}}\text{C}]$ glucose and elicited with pectinase. Addition of pectinase did not lead to an increase in the $\alpha [1-{^{13}}\text{C}]$ glucose resonance. The inset shows an enlarged spectra of the $[1-{^{13}}\text{C}]$ glucan region.
6.4.5 Glucans

The accumulation of glucans was not affected upon addition of jasmonic acid and PGA (data not shown) on the other hand, addition of pectinase lead to formation of several new glucans (Figure 6-6). Furthermore the rates of accumulation of the glucans also found in controls were higher upon the addition of pectinase. A continuous increase in the concentration of glucans was observed in controls however, upon addition of pectinase the concentration of glucans increased rapidly for the first 450 hours but then decreased. This decline in glucan concentrations could be due to reduction in G6P levels or cell death due to prolonged exposure to pectinase.
Figure 6-6 Concentration profiles of [1-\textsuperscript{13}C] glucans in \textit{A. niger} pectinase elicitation experiment. Pectinase was added at 56 hours.
6.4.6 Carbon Fluxes

The profiles of [4-\textsubscript{13}C] glutamine are shown in Figure 6-7A. A comparison of the [4-\textsubscript{13}C] glutamine profiles shows that the concentration of glutamine is lowest upon elicitation with jasmonic acid. Since the C-4 label of glutamine is derived from the C-3 label of pyruvate, the concentration profiles of [3-\textsubscript{13}C] pyruvate were also quantified and were observed to follow similar trends (Fig 6-7B). This decrease in concentrations of [4-\textsubscript{13}C] glutamine and [3-\textsubscript{13}C] pyruvate could be explained based upon a reduced flux in the Glycolysis pathway or an increased drain on the pyruvate and glutamine pools.

[4-\textsubscript{13}C] Malate label was derived from non-photosynthetic fixation of CO\textsubscript{2} via phosphoenol pyruvate carboxylase (section 5.4.7). When C-4 malate profiles in the elicitation experiments were compared, no significant difference in malate profiles was observed upon addition of the elicitors.
**Figure 6-7** Concentration profiles of A) [4-\(^{13}\)C] glutamine and B) [3-\(^{13}\)C] pyruvate in the control and elicitation experiments. Pectinase was added at 56 hours, PGA was added at 45 hours and jasmonic acid was added at 100 hours in the respective experiments.
Figure 6-8 [4-\textsuperscript{13}C] Malate concentration profiles as obtained in the control and the elicitation experiments. Pectinase was added at 56 hours, PGA was added at 45 hours and jasmonic acid was added at 100 hours in the respective experiments.
6.5 Discussion

Long term perfusion experiments were performed to study the effect of elicitors on primary metabolism and intracellular pH profiles. $^{31}$P and $^{13}$C NMR spectroscopy were utilized and the several resonances were quantified. Inorganic phosphate chemical shift was used to determine the intracellular pH. The concentration of metabolites was determined from the areas under the individual peaks.

The perfusion medium utilized in all these experiments (100 ml) contained 1 mM inorganic phosphate. It has been shown previously that plant cells take up inorganic phosphate from the culture medium and store it in the vacuole (Vogel and Brodelius, 1984; Vogel, 1987). Since the concentrations reported in Figure 6-1 are based upon cell volume, the final biomass level will have an impact on the measured phosphate concentration. In experiments involving pectinase and PGA elicitation, the final biomass observed is lower than that observed in the controls and the jasmonic acid elicitation experiment (Table 6-1). Thus lower biomass implies that the concentration of inorganic phosphate will be higher in the PGA and pectinase elicited root cultures as all extracellular phosphate is taken up by a smaller volume of cells.

Glucose-6-phosphate is formed by phosphorylation of glucose in the glycolytic pathway. Pectinase being an endopolygalacturonase attacks the pectins present in the plant cell walls (Alghisi and Favaron, 1995). This attack leads to alterations in metabolism of plant cells, a big part of which is depositions at the cell
wall to prevent damage. This requires synthesis of cell wall components or glucans, and several new glucan resonances are observed upon addition of pectinase (Figure 6-6). The enhanced synthesis of glucans implies a high supply of glucose-1-phosphate, the precursor of glucan synthesis. This could lead to enhanced accumulation of G6P as G6P can be converted to G1P by phosphoglucomutase (Salisbury and Ross, 1986). Furthermore, a reduction in the ratio of β/α glucose observed upon the addition of pectinase. This reduction probably arises due to a decrease in biosynthesis of sucrose, the C-1 glucopyranose resonance of which overlaps with α [1- 13C] glucose resonance. Since sucrose synthesis, like glucan synthesis, requires G1P as the precursor, a decrease in synthesis of sucrose implies redirection of resources away from storage compounds to cell wall components.

Intracellular pH can act as a signal transducer and can also have a significant impact on the activities of several intracellular enzymes (Kneusel et al., 1989). Hence, a study of intracellular pH in response to elicitation might help explain some phenomenon in the elicitation response. In order to differentiate between elicitation and a reduction in external medium pH, the effect of medium pH alone on the intracellular pH was also studied (Ho, 1994). It was observed that the external pH did not have a significant effect upon the intracellular pH. Intracellular pH changes in response to elicitation of plant cell cultures have also been studied previously and a species dependent and exposure time dependent response has been observed (Ojalvo et al., 1987; Horn et al., 1992). In the present studies, however, totally different effects
on intracellular pH were observed upon addition of pectinase and jasmonic acid. In contrast to a transient short-term reduction in cytoplasmic pH upon addition of pectinase, a prolonged reduction of vacuolar pH was observed upon addition of jasmonic acid. pH is known to be the driving force for transport of ajmalicine to the vacuole and its storage in form of serpentine (Neumann et al., 1983). Furthermore, pH also has an affect on the activities of enzymes involved in secondary metabolite pathways (Kneusel et al., 1989).

Elicitation induced pH changes thus could play a significant role in regulation of indole alkaloid biosynthesis by C. roseus hairy root cultures. Experiments at shake flask levels have indicated that jasmonic acid addition leads to an overall enhancement in flux to the indole alkaloid pathway. Transient enhancements in the levels of tabersonine and lochnericine and continuous increase in the levels of serpentine and horhammericine were observed in the transient studies (Rijhwani and Shanks, 1997a). NMR studies of elicitation with jasmonic acid indicate a prolonged decrease in vacuolar pH induced by jasmonic acid. This decline could be responsible for activation of certain enzymes or could have an impact on the intracellular transport processes.

A study of the concentration profiles of intermediate metabolites in glycolysis and the TCA cycle indicated a decrease in the levels of [3-^{13}C] pyruvate and [4-^{13}C] glutamine upon elicitation with jasmonic acid. This could imply that jasmonic acid leads to an alteration in distribution of fluxes in glycolysis and Pentose phosphate pathway, reducing the fluxes to glycolysis as compared to pentose phosphate
pathway. Another reason for the reduction in the levels of [3-\(^{13}\)C] pyruvate and [4-\(^{13}\)C] glutamine could be an increase in the drain upon these pools, in response to addition of jasmonic acid.

6.6 Conclusions

These studies have helped understand the effect of addition of three different kinds of elicitors on intracellular pH and the primary metabolism of the root cultures. A transient decrease in the cytoplasmic pH was observed after the addition of pectinase and a prolonged decrease in the vacuolar pH was observed after addition of jasmonic acid. The concentration profiles of glucans and the intermediates in glycolysis and TCA cycle helped understand the redistribution of the carbon flux in response to the addition of elicitors. Pentose phosphate pathway provides the reducing equivalents (NADPH) and the precursors for the shikimic acid pathway. As evident from \(^{13}\)C NMR spectroscopy of the root cultures, an increase in flux to the pentose phosphate pathway could lead to enhanced synthesis of reducing equivalents and the precursors for the shikimic acid pathway and could be responsible for an increase in flux to the indole alkaloid pathway. An analysis of these experiments indicates that NMR spectroscopy could play an important role in understanding plant defense response.
CHAPTER 7: FUTURE DIRECTIONS

This research has helped understand several aspects of primary and secondary metabolism of C. roseus hairy root cultures. Dosage and transient studies in response to fungal elicitors at the shake flask level, coupled with NMR spectroscopy studies have provided several insights in indole alkaloid pathway and flux distribution in primary metabolism. Based on the observations here, the following are recommended for future research:

(1) Four specific elicitors were utilized in this research and their effects on indole alkaloid biosynthesis were determined. However, since elicitation remains a trial and error strategy, studies with other specific elicitors could further help understand the indole alkaloid metabolism. Secondly, synergistic approaches could be utilized to induce enhancements in the levels of a desired metabolite. For example jasmonic acid caused an overall enhancement in flux to the indole alkaloid pathway while pectinase led to an enhancement in tabersonine only. A combination of the two elicitors might be able to bring a greater enhancement in tabersonine levels as compared to individual elicitors. Thirdly, utilization of specific inhibitors of transcription, translation, pathway enzymes and intracellular transport, in combination with elicitors, could bring about critical advancement in understanding of cellular metabolism.

(2) A detailed understanding of the indole alkaloid pathway is crucial for utilization of hairy root cultures in an economic production process. NMR
spectroscopy could help understand several unknown steps in the complex indole alkaloid pathway of *C. roseus*. Hairy root cultures can be grown in cultures containing specifically labeled intermediates in the indole alkaloid pathway of *C. roseus*. Extraction of the labeled intermediates and end products followed by an *in vitro* NMR analysis of peak couplings and isotopomer distribution patterns could help resolve several unknowns in the indole alkaloid pathway.
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