CHARPTER V

DISCUSSION

1. Establishment of *in vitro* Cultures of *A. salviifolium*

In this study, we have tried to establish callus, suspension and root cultures of *A. salviifolium* in order to study their ability to produce emetine alkaloids. Although it seemes that all these three types of cultures could be established, their stable cultures could not be maintained to obtain substantial biomass. Furthermore, our preliminary results on the alkaloid detection in these *A. salviifolium* cultures showed that absolutely no alkaloids produced in both the callus and cell suspension cultures (Figures 10 and 11). Therefore, it was unlikely to use both cultures of *A. salviifolium* for studying the biosynthesis of emetine alkaloids. However, our unsuccessful results on the establishment of *A. salviifolium* cultures producing emetine alkaloids do not mean complete cell cultures of this plant can not be obtained. Culture initiation and plant regeneration are still accomplished empirically by varying conditions until the desired response is obtained (Henshaw, O'Hara, and Webb,1982). It is not known obviously how critical factors such as, hormone alter the determined state, or why different tissue types and comparable tissue from different species vary in their capacity to respond. For *A. salviifolium*, it is possible that positive results will come out if we have more time for the experiments on tissue culture works.

Considering about the initiation of plant tissue culture, the induction of differentiation and the regeneration of complete plants from cultured cells are governed by an integrated system of internal and external factors (Kurz and Constable, 1979). Internal

factors are usually of less interest due to the concept that the individual cells of an organism are totipotent (cell theory) and should be capable of independent development if provide with the proper external condition (White, 1954). In fact, there are also some reports about genetic instability resulting phenotypic variations arising during culter (Binns, 1981), effects of various using regions of the leaves (Wernicke and Brettel, 1980), side-depending callus initiation (Rechinger, 1893), effect of pattern of cells (Bonner, 1963), plant age depending (Robb, 1957) and so on. For *A. salviifolium* there has been no information on cell cultures before. In our experiment, internal factors were not deep involved. Only a recommendation, from experience, young leaves should be used.

External factors can be divided mainly into nutritional medium factors and other factors (such as light and temperature). Plant cell culture media are reasonably complex. Their components have been explained in many ways (Kurz and Constable,1979 ; Dodds and Roberts, 1985). In fact, we know much less about the nutritional requirements of the various organs and tissue of the plant than we do of the whole plant. However, a variety of media formulations exist, some of which have become standard (Gamborg *et al.*, 1976). Beginning from standard medium was so expedient rather than made new one. Therefore, the key point of progress in cell culture of *A. salviifolium* is modifying in plant growth regulators. Plant cell cultured *in vitro* commonly require supplement with plant growth regulators which play a dominant role in intercellular regulation of morphogenetic processes (Street, 1966), some exception (White 1934). Auxin-cytokinin supplements are instrumental in the regulation of cell division, cell elongation, cell differentiation, and organ formation (Merel and Muller, 1964). So callus cultures of *A. salviifolium* were initiated from many various auxin-cytokinin supplements among 3 basal media (MS, B5, WPM). WPM supplemented with 0.3 mg/l 2,4-D and 0.3 mg/l BA was found to be the best for callus induction and maintainance. The obtained callus on semisolid media had problems, not only slow growth rate but also non-homogenous characteristic of callus. These may be resulted from (a) necrotic tissue be transferred along with healthy tissue, (b)

metabolites secreted by the growing callus accumulate to toxic level in the medium (Dodds and Roberts, 1985). To solve the problem (a), surface clean callus were subcultured, be separated from whole amorphous mass. It was found that callus grown very slow in this way. To solve the problem (b), it became necessary to subculture before the medium turned pink.

About other external factors such as light and temperature, there have been little work studying their effects. Plant tissue cultures are generally grown under fluorescent tubes at intensities of 1000-5000 lx for 16 hours daily, temperature around 25° C but number of reports have shown that the environmental conditons can be important in some species (Murashige, 1977, Hussey and Falavigna, 1980, Fonnesbech and Fonnesbech 1980). Cell cultures of *A. salviifolium* were incubated under 2000 lx for 16 hours daily at 25 ± 2^{o} C.

For the suspension cultures of *A. salviifolium*, the cultured cells were also maintainaned in liquid WPM plus with 0.3 mg/l 2,4-D and 0.3 mg/l BA. The cell cultures looked fresh and healthy after being subcultured for many times although no alkaloid has been detected. Another problem was the presence of a number of cell aggregates in the suspension cultures. These cells aggregates turned dark easily and probably caused the slow growth of the culture.

Attempt to establish root culture of *A. salviifolium* was made using two methods. One was from isolated rootlets of the whole as explants and the other was initiated from the cell suspension culture. The first method had problems of microbial contamination in soil. Prolonged surface sterilization was tried by immersion in 15 % Clorox more than 15 min but this caused more damage to root. Alternatively, the root culture could be established from root tips of young seedlings which are produced during the axenic germination of seeds and those apical tips are then transferred to an aqueous culture

medium. We did not try this method because *A. salviifolium* seeds were not available by the time these experiments were carried out.. For the second method, the cell suspension of *A. salviifolium* were subcultured into a new medium containing various kinds of plant hormone. We found that root induction could be observed in the RM added with 2 mg/l GA_3 , 1 mg/l BA, and 0.1 mg/l Ki. However, the rate of root induction was very slow, the resulted root cultures turned brown and died eventually.

From these results of root induction, some suggestions can be made. It is generally accepted that in addition to the essential minerals, the excised root also needs the addition of certain organic compounds to the culture medium because in the whole plant the roots were provided with these compounds, which were synthesized elsewhere and transported into the root system. Although root initiation is the type of organogenesis most frequently found in cultured tissues but the potential to form roots may decline after several subcultures (Gautheret, 1966). The studies of Skoog and his colleagues (1944) led to the hypothesis that organogenesis is controlled by a balance between cytokinin and auxin. They found that the addition of auxin to he medium served to stimulate root formation, whereas shoot initiation was inhibited. A relative high auxin:cytokinin ratio induced root formation in tobacco callus, whereas a low ratio of the same hormones favored shoot production (Skoog and Miller, 1957). Gibberellins sometimes are dramatic effects on organogenesis. Gibberellic acid have been used in apical cultures(Morel and Muller, 1964). Several investigators have evidences of phenolic compounds acting in combination with auxin to promote organogenesis (Thrope, 1980). The combination of phloroglucinol and indolebutyric acid, for example, was much more effective in stimulating rooting than auxin alone (Welandeer and Huntrieser, 1981).

Plant cell culture of *A. salviifolium* should be continually developed because tissue culture have been used as an invaluable research tool in areas of phytochemistry, noticeably in biogenetic and enzymic studies. Our interest is focus on the formation of

such plant products by cell and tissue culture systems in order to understand the biosynthesis of emetine alkaloids, to isolate the involved enzymes as biocatalysts and finally to manipulate and to improve the product syntheses. Moreover, comparative studies between *A. salviifolium* with *Cephaelis ipecacuaha* in biosynthesis of isoquinoline alkaloid, emetine group, from plant cell culture is very interesting.

2. Alkaloid Detection in *A. salviifolium* Leaves and *in vitro* Cultures

Many alkaloids in the whole plant of *A. salviifolium* have been reported (Table 1). However, plant materials, generally, have some variation in the content of secondary metabolites(Evans, W.C. 1989). Therefore, detection of alkaloids from the plant that we brought to use as enzyme sources would give some valuable information in predicting biosynthetic pathway of emetine alkaloids.

During the course of experiments, selection between the leaves and roots was made in order to use as an enzyme source. Since the leaves were found to contain alangimarckine in which its biosynthesis also involves the first condensation step as emetine alkaloid. Because the leaves are much easier to collect than the roots. Therefore, the leaves were selected as the enzyme source, although the roots also found to contain the condensing enzyme.

For cell cultures, although the undifferentiated cells of a plant suspension are generally totipotent, ie they possess the complete genetic make-up of the whole plant, many genes, including those involved in secondary metabolism, are repressed with the consequence that the yields of desired compounds in such cultures are disappointing low or none (Evans, W.C. 1989). These, absolutely, involved inhibition of enzyme expression. So cell cultures which lacking in alkaloids were neglected to use for preparing enzyme.

Lacking of alkaloid production is the problem in all types of plant tissue cultures from *A. salviifolium*. The solutions for this problem involve many topics. Normally, nutrition and changes in the level of hormones as well as temperature and photoperiod may modify, induce biosynthetic activity but the effect of various component has been assessed in only a few cases (Matsumoto *et al.*, 1971). Lower nitrogen levels tend to enhance secondary product synthesis, but not a universal pattern (Yeoman, 1986). One approach used to regulate metabolic pathway flavoring the production of secondary metabolites has been to add precursors to the culture medium (Aitchison *et al.*, 1977). Elicitor-induced accumulation of secondary metabolites is recieving increasing attention in recent years (Eilert, 1987).

3. Detection of the First Enzyme of Emetine Biosynthetic Pathway in *A. salviifolium*

The first enzyme involved in emetine biosynthesis in *A. salviifolium* has been primarily detected by following its activity. The enzymatic activity was strictly observed dependent on the presence of the product and the absence of both substrates, dopamine and secologanin. The enzymatic product was detected by TLC densitometer using the wavelength of 290 nm for scanning. Although the product showed λ_{max} at both 240 nm and 290 nm (combinative wavelength of its both substrates), the λ_{240} could not be used because the enzymatic product migrated very closely to secologanin which has it λ_{max} at 240 nm on the TLC plate. More evidence to support enzymatic existences are (a) the enzymatic product was not found in the control (boiled enzyme) and (b) the formation of the enzymatic product occured in a very short time.

Unexpectedly, a couple of reaction products were formed. This propably resulted from the occurrence of two enzymes involved in the first step of dopamine and secologanin condensation. Comparing between the two enzymatic activities,

deacetylipecoside synthase seemed to be more active than deacetylisoipecoside synthase. The different levels of enzymatic products could be noticed in the early incubation time (Figure17). Both competitive enzymes probably act as rate limiting step of two separate biosynthetic pathways. While deacetylipecoside synthase leads to the pathway of lactam and alangiside formation, deacetylisoipecoside synthase push the reaction on another pathway to form emetine as the final product. More experiments should be carried out further to purify both important enzymes, deacetylipecoside synthase and deacetylisoipecoside synthase, from the proteineous extracts.

Identification of the reaction product was carried out with large scale reaction mixture (100 fold). After 2 hr incubation, the product was isolated and purified by HPLC method. From our experiments, it could be deduced that both epimers, deacetylipecoside(R) and deacetylisoipecoside(S), were formed. Deacetylipecoside (less Rf value) was produced more rapidly than deacetylisoipecoside. However, during purification of the products, their structures could be rearranged to form demethylalangiside and demethylisoalangiside (Figure 23), respectively. Therefore, HPLC chromatogram displayed the retention time of demethylalangiside at 7.21 min and of deacetylisoalangiside at 15.16 min (Figures 18 and 19). Their UV spectra, detected by Waters 991J Photodiode Array Detector, were compared to UV spectrum of demethylalangiside and demethylisoalangiside and found to have very similar patterns. Furthermore, the product was unequivocally identified as demethylalangiside and demethylisoalangiside by LC-MS since they both showed their molecular mass of 491 (Figures 20 and 21).

4. Proposed Biosynthetic Pathway of Emetine in *A. salviifolium* Plant

Although the biosynthesis of monoterpenoid isoquinoline alkaloid has essentially been clarified at the precursor feeding stage (Nagakura *et al.*, 1978), little is known about their biosynthesis at the cell-free level. Despite the fact that the ultimate proof that a biosynthetic pathway is correct can only be obtained from studies with enzymes which catalyze various steps of secondary metabolite biosynthesis, there is still not any enzyme founded in this biosynthetic pathway. However, from our experiments these results indisputably demonstrate the presence of two highly stereospecific biocatalysts in crude enzymatic extracts of *A. salviifolium.* Base on these findings the biosynthetic pathway of emetine in *A. salviifolium* could be proposed as follows (Figure 19)

Secologanin, the first precursor, are derived from primary metabolism via the mevalonic acid pathway in which geranyl diphosphate is formed from two isoprene unit. At the interface of primary and secondary metabolism geranyl diphosphate is converted into the monoterpene geraniol by a possibly specific phosphatase that has no yet been characterised. Geraniol 10-hydroxylase (G10H monoterpene hydroxylase), the enzyme catalysing the conversion of geraniol into their corresponding 10-hydroxy derivatives, was isolated from *Catharanthus roseus* G.Don seedlings by Meehan and Coscia (1973). After these, hypothetical pathways for biosynthesis of secologanin have been postulated on the basis of *in vivo* tracer studies, but most of the enzymes involved have not yet been identified (Meijer, Verpoorte and Hoge, 1993). Dopamine, the other precursor, is derived from tyrosine (Phillipson *et al.*, 1985).

Condensation of secologanin and dopamine in *A. salviifolium* is a competitive reaction between two enzyme with stereochemical control, deacetylipecoside synthase and deacetylisoipecoside synthase. More studies on both purified enzyme characteristics should be carried out. The following step that have been proven by tracer experiments,

deacetylipecoside with β-configuration is metabolically inactived by lactam formation (alangiside) whereas deacetylisoipecoside with α-configuration is further transformed to several monoterpenoid isoquinoline alkaloids including cephaeline and emetine, all possessing α-configuration.

Undoubtedly, the biosynthetic pathway of emetine is still open for studies. Many enzymes of the pathway have not yet been discovered, especially the further the next step after deacetylipecoside. In *A. salviifolium*, deacetylipecoside is converted to alangside and deposit in roots, leaves and fruits but in *Cephaelis ipecacuanha* , deacetylipecoside is turned to ipecoside and deposit only in roots. These may result the presence of more than one enzymes be concerned. As a consequence, we have to investigate in other simple process between biosynthesis, transport, storage and degradation which impact on each other work. These processes are governed by genes and, therefore, it will be necessary ultimately to understand and maybe to modulate the expression of these genes.

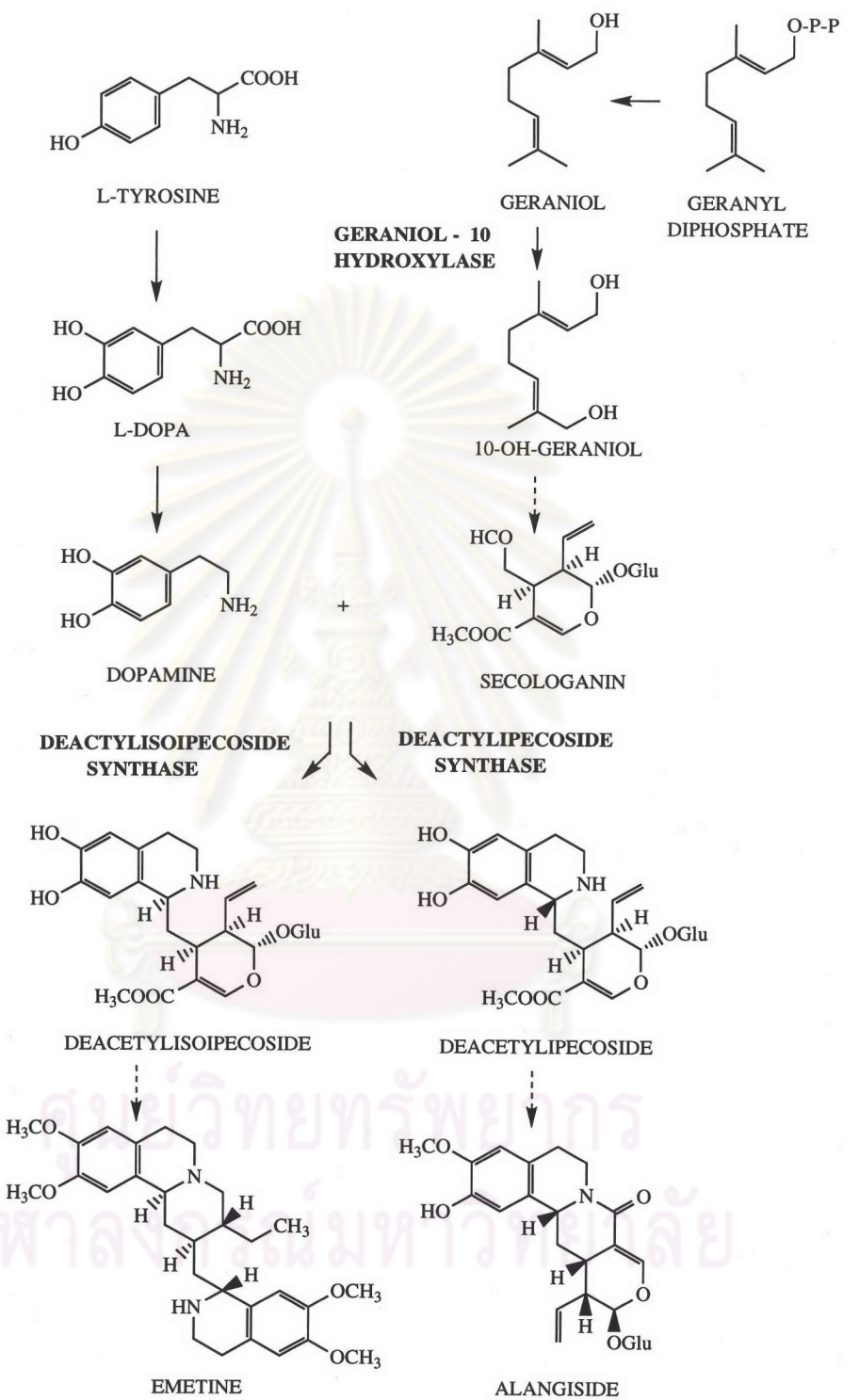

Figure 23 Proposed biosynthetic pathway of monoterpenoid isoquinoline alkaloids

CHARPTER VI

CONCLUSION

From our experiments, the following conclusions can be drawn:

1. The cell cultures of *A. salviifolium* were initiated and maintained in WPM added 0.3 mg/l BA, 0.3 mg/l 2,4-D. The root cultures could be induced by inoculate cell culture into RM containing 2.0 mg/l GA_3 , 1.0 mg/l BA and 0.1 mg/l Ki. However, the *in vitro* cultures appeared to produce no alkaloids.

2. The leaves of *A. salviifolium* contained alangimarckine as a major monoterpenoid isoquinoline alkaloid. The biosynthesis of this alkaloid is likely to occur in the leaves and, therefore, allowing to use this plant part for studying the enzymes involved in the biosynthetic pathway including the first step of dopamine and secologanin condensation.

3. Deacetylipecoside (R-configuration) and deacetylisoipecoside (S-configuration) could be detected as the enzymatic condensation products of dopamine and secologanin by TLC-densitometer. However, both product were rearranged to demethylalangiside and demethylisoalangiside, respectively, after purification and detected by HPLC-diode array detector and LC-MS. This suggested the presence of two enzymes responsible for the formation of both deacetylipecoside and deacetylisoipecoside in the leaves of *A. salviifolium*.