CHAPTER V

DISCUSSION

In the " study on isoquinoline alkaloid production in tissue cultures of *Stephania pierrei* Diels", the following steps of strategy were proceeded :

1. Establishment of tissue cultures of *S. pierrei*

2. Detection of isoquinoline alkaloids potentially accumulated in tissue cultures of *S. pierrei* that were established.

3. Quantitative analysis of the accumulated isoquinolines in tissue cultures extract of *S. pierrei* .

1. Establishment of tissue cultures of *S. pierrei*

From the results of the establishment of tissue cultures from *S. pierrei* tubers, it was found that the formation of *S. pierrei* callus can be initiated by manipulation of three factors. These include nutrient media, growth regulators and light conditions.

Nutrient media are based on the metabolites within the plant which are sufficient for the growth of explant tissues. The general composition of nutrient media consist of a salt mixture, an energy source (usually sucrose), certain amino acids, vitamins and growth factors, together with any supplementary compounds that are necessary for a particular species. The mixture salts usually contain NO_3^- and/or NH_4^+ as a nitrogen source, PO_4^{3-} as a phosphate source, SO_4^{2-} as a sulfur source, K^+, Ca^{2+} and Mg^{2+} as macronutrients (Sakuta and Komamine, 1987).

In this study, it was found that the tuber explants of *S. pierrei* are able to respond to White agar medium supplemented with various types of growth regulators. In White medium formulation (Table 3), there are higher content of SO_4^{2-} (from $MgSO_4.7H_2O$ and Na_2SO_4) than the other media, while the contents of other macronutrients (K, P, N, Ca) are the lowest. It is possible that the sulfate ion is essential for callus formation in *S. pierrei.* However, White medium appears to be unable to support further growth of the root buds initiated from the callus to form

fibrous roots, whereas Root medium does. The macronutrients in Root medium formulation consist of high potassium, phosphate, nitrate, calcium and moderate content of sulphate. This suggests that all the macronutrients are necessary for root elongation and regeneration.

Among various components in a nutrient medium, plant growth regulators play an important role in the growth and differentiation of tissue cultures. Therefore, to stimulate callus development, it is necessary to add appropriate levels of growth regulating substances to the medium. Three principal classes of the regulators used in propagation include auxins, cytokinins and gibberellins. In this experiment, by using White medium, the calli were formed rapidly by using appropriate combination of growth regulators : 0.1 mg/ml kinetin, 0.2 mg/l BA, 1.0 mg/l NAA and 1.0 mg/l GA3. These constituents made the callus twofold growth in two months, and extensive root bud forming in 3 months, although the root buds could not elongate.

In principal, rooting can be achieved by subculturing to medium lacking cytokinins, with or without a rooting hormone, or by treating the shoots as conventional cutting after removal from sterile cultures. All cytokinins inhibit rooting (Yoeman, 1986) especially BA which is particularly strong. The use of kinetin in place of BA in the final stages of multiplication often improves subsequent rooting (Webb and Street, 1977). Rooting can also be improved in many woody and herbaceous species by lowering the concentration of macro salt to a half or less, and the concentration of sucrose from 2 or 3%.

The concentration of rooting hormones required for callus is often critical to provide sufficient stimulus to initiate roots while preventing the excessive formation of callus. Root elongation may be inhibited by the levels of auxins required to initiate roots. Many species require stronger auxins IBA or NAA to stimulate root formation. NAA usually gives rise to short thick roots (Lane, 1979). Kinetin and BA which are the plant growth regulators of cytokinins have been reported to induce adventitious shoot formation, and GA_3 has been found to induce elongation of internodes and root, the growth of meristems or buds and plantlet regeneration (Piereh, 1987).

Our results showed that, White agar medium containing the hormones of 0.1 mg/l kinetin, 0.2 mg/l BA, 1.0 mg/l NAA and 1.0 mg/l GA_3 can stimulate callus and root bud formation. It is likely that this stimulation is the main effect of the

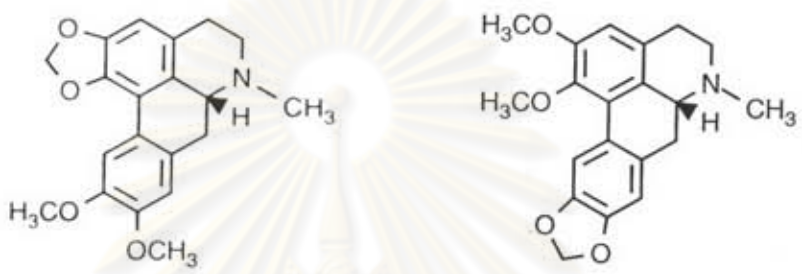
auxin, NAA and cytokinins, kinetin and BA. Although cytokinins seem to be too high and risky to inhibit root regeneration, it can be that high concentration of NAA (1.0 mg/l) can override the effect of the cytokinins. Therefore, callus and root buds of *S. pierrei* can be formed in this medium although the buds are short and thick. The hormonal combination needs low level of NAA and cytokinins but high gibberellins. In this study, it was found that the Root media which contained 0.1 mg/l kinetin, 0.2 mg/l BA, 0.1 mg/l NAA, 1.0 mg/l GA_3 and 0.3% gellan gum was the best medium for root elongation.

With respect to light requirement, plant tissue cultures are generally grown under fluorescent tubes at intensities of 1,000-5,000 lux for 16 hrs.daily (Yeoman, 1986). However, in plant tissue cultures of the species which accumulate high content of phenolic compounds, light became as an inhibitor of callus formation. Presumable, in the condition of light the large molecules of secondary metabolites were broke into many phenolic molecules which was excreted into the nutrient media to inhibit formation of callus and other regeneration. Sugimoto (1987) has reported that the production of bisbenzylisoquinoline alkaloids in the cultured roots of *Stephania cepharantha* occurs when the cultures are incubated in the drak at 26°C. For our study, it was found that the callus of the tuber explants of *S. pierrei* could be formed on White medium when being cultured in the dark at 25 - 26°C. No callus was formed in the presence of light. This can probably be explained that the light causes degradation of isoquinoline alkaloids such as aporphines, tetrahydroprotoberberines, tetrahydrobenzylisoquinolines to phenolic molecules which are then excreted to the media to inhibit callus formation of *S. pierrei* explants.

2. Detection of isoquinoline alkaloids potentially accumulated in tissue cultures of *S. pierrei* .

When the crude extract prepared from *S. pierrei* cultured roots was separated by TLC, it was found that here was an alkaloid in the extract that was chromatographed with authentic dicentrine. Further examination using three solvent systems all showed identical Rf value of the alkaloid with authentic dicentrine in each system [Rf 0.6 with toluene : ethylacetate : DEA (7:2:1), 0.28 with ethylacetate : methanol (5:1), and 0.65 with chloroform : methanol (9:1)]. Furthermore, the UV, IR and mass absorption spectra of both the unknown and dicentrine appeared to be identical with suggesting that the main alkaloid accumulated in the cultured roots of *S. pierrei* were

dicentrine or other aporphine alkaloids which had identical substituted groups but different substituted positions from dicentrine. In this case, (-)-nantenine (epidicentrine or *O*-methyldomesticine) is considered. From the dicentrine structure, thre are 1, 2 methylenedioxyl bridge and 9-and 10-methoxyl group. On the other hand, nantenine have 1-and 2-methoxyl group and 9,10 methylenedioxyl bridge as shown in Figure29.

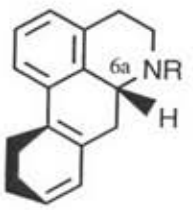


(a) (-)-Dicentrine (b) (-)-Nantenine

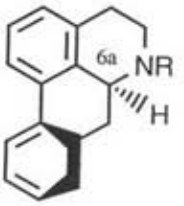
Figure 29 Structure of (-)-Dicentrine (a), and (-)-Nantenine(b).

These structures can give identical UV, IR and mass spectra . In this case the information obtained from ^{1}H-nmr spectroscopic study is invaluable in the structure elucidation.

Several charecteristics of the ^{1}H-nmr spectra of aporphine alkaloids have been noted (Bick, *et al.*, 1961; Baarschers, *et al.*, 1964). Among the aromatic methoxyl substituents, the C-1 methyl group is always the most upfield (δ 3.4-3.7), while the 2-, 9- or 10-methoxyl group is most downfield (δ 3.8–3.9). The resonance of the 11-methoxy falls in between t hese two regions. Aporphine alkaloids have a non-planar structure, due to twisted biphenyl system, as shown in Figure 30.



6a = *R*



6a = *S*

Figure 30 Twisted biphenyl system of aporphine alkaloids.

As a result of this asymetry, the 1,2-methylenedioxy protons are not magnetically equivalent because of the anisotropic effect from ring D, and therefore appear as two distinct doublets. The protons of the 9,10-methylenedioxy, on the other hand, being far from ring A, are not affected, and consequently are seen as a singlet. The aromatic proton at C-3 is almost always the furthest upfield (δ 6.5-6.7) and H-11 the most downfield (δ 7.6-8.2).

The information from ^{1}H-nmr spectra of purified sample is shown a chemical shift at 3.93 ppm which represent to 9-and10-methoxy protons, while the chemical shifts at 3.4-3.7 ppm are not found. Furthermore, the chemical shifts at 5.94 and 6.09 ppm are represented to 1,2-methylenedioxy protons. From these informations, it is indicate that main alkaloid accumulated in cultured roots of S. pierrei is only dicentrine.

Therefore, in terms of isoquinoline formation, we report here the successful production and accumulation of dicentrine by the root cultures of *S. pierrei.*
This aporphine is one of many alkaloids that have been isolated from the tubers of *S. pierrei.* (Likhitwitayawuid ,*et al.*, 1993b)

From the dicentrine structure, it is obvious that the molecule of dicentrine is relatively stable .various functional groups attached to the main aporphine skeleton include *N*-methyl, two methoxy groups, and a methylenendioxy bridge at C_1-C_2

position are chemically inactive. These make dicentrine as stable structure and difficult to be degraded or changed to other compounds. This is probably one of the reasons that dicentrine is the major isoquinoline alkaloid found in the cultured roots of *S. pierrei.* Although, at least other three alkaloids were also detected in the cultured root extract by Dragendorff's reagent, their structure could not be identified due to the very small amounts of these compounds produced in the culturrd roots. However, from the results of their Rf values in TLC plate and their UV-absorption spectra, it seems that these alkaloids do not have the characteristics of any other available authentic isoquinoline alkaloids.

Based on the knowledge of the expression of secondary metabolites of plant tissue cultures, it is generally accepted that plant tissue cultures are usually accompanied by an apparent loss of ability to accumulate secondary compounds (Charlwood and Rhodes, 1990). The reasons may be

1) the lack of expression in non-specialized cells of genes that control the essential steps in the biosynthetic pathway;

2) the diversion of substrate away from secondary product formation;

3) the non-operation of transport mechanisms by which potentially toxic end-products may be removed from the biosynthetic site;

4) the non-availability of storage sites in which secondary metabolites would normally be sequestered;

5) the unregulated catabolism of synthesized product.

In the case of *S. pierrei* only dicentrine was found to be major component in the cutlrued roots. This may be indicated that the other isoquinolines, especially aporphines, which were produced by tissue cultures were rapidly converted to dicentrine which is more stable.

3. Quantitative analysis of the accumulated isoquinolines in tissue cultures extract of *S. pierrei*

The result from the quantitative analysis of dicentrine indicated that *S. pierrei* cultured roots contain much higher content of dicentrine than the natural tuber, whereas no such alkaloid is detected in the leaves. Dicentrine content in the cultured roots is at least sixty-two times of the content found in the tuber. With respect to their chemical patterns, the crude extracts obtained from the cutltured roots and tuber also have very different chemical composition. The results suggest that the cultured roots accumulate dicentrine, as the major constituent whereas the whole tuber accumulates tetrahydro-palmatine as the major isoquinoline alkaloid (0.041% w/w fresh weight) (Likhitwitayawuid, *et al.* 1993b). In the tuber, dicentrine has been reported to contain 0.006% w/w fresh weight by using column and preparative TLC (Lithitwitayawuid, *et al.,* 1993b). These results indicate that *S. pierrei* cultured roots have a high potential to produce and accumulate dicentrine. There are three probabities to explain these results. First, dicentrine may be produced in the tuber explants and then long-distance transported to accumulate in the cultured roots. Second, the cultured roots do produce and accumulate dicentrine as a result of tissue differentiation and formation of alkaloid producing specific cells on issues which are capable of producing the alkaloid. Third, dicentrine can be produced in both the tubers and cultured roots but probably there are degradative enzymes which degrade dicentrine to other products in the tuber.

The abscence of other aporphine alkaloids in the cultured roots of *S. pierrei* makes the biosynthetic pathway of dicentrine difficult to predict particularly, the steps of conversion from *(S)* or *(R)*-reticuline to dicentrine. Nevertheless, we hope that further development for large scale of tissue cultures will lead to higher content of other isoquinoline alkaloids for determination. We also hope that the information will lead eventually to the success in elucidating the biosynthetic pathway of the aporphine alkaloids in the future.

CONCLUSION

From this reseach work of "Study on Isoquinoline Alkaloid Production in Tissue Cultures of *Stephania pierrei* Diels", the following conclusions can be drawn:

1. Callus cultures of *S. pierrei* can be established from the tuber explants on White medium containing 30 g/l sucrose, 0.1 mg/l kinetin, 0.2 mg/l BA, 1.0mg/l NAA, 1.0 mg/l GA_3, and 0.8 % w/v agar in the dark and subcultured on this medium.

2. Root buds of *S. pierrei* can be established from the callus on the explant on the same White medium in 3 months.

3. Root elongation of *S. pierrei* from the root buds can be accomplished on Root medium containing 30 g/l sucrose, 0.1 ml/l kinetin, 0.2 mg/l BA, 0.1 mg/l NAA, 1.0 mg/l GA_3 and 0.3% w/v gellan gum.

4. Dicentrine is an aporphine alkaloid detected in the cultured roots of *S. pierrei* with very high content.

5. The cultured roots of *S. pierrei* can produce and accumulate dicentrine up to 0.82 % w/w dry weight which is sixty two times of the content found in the tuber.

6. The cultured roots can be used as a source for biosynthetic studies of aporphine alkaloids.