CHAPTER V

DISCUSSION

We have shown in this study that *in vitro* cultures of *I. baisamina* can be established successfully in B5 medium. Among various nutritional and hormonal factors tested, the combination of auxins and cytokinins appears to be essential for the formation of various types of the *in vitro* cultures and their ability to produce the naphthoquinones, lawsone and 2-methoxy-1,4-naphthoquinone.

The hormonal combination of both 0.1 mg/l 2,4-D plus 1.0 mg/l kinetin and 0.1 mg/l NAA plus 1.0 mg/l BA in B5 solid medium can stimulate callus formation but result in different appearence. While the former gives friable and yellow callus, the latter gives dense and green one (Fig. 8).

As usually found in other cases, the friable callus of *I. balsamina* is easier than the dense callus in being used for establishing cell suspension cultures. This is due to the more friable callus tissues can give rise to higher degree of cell dispersion. The resulted cell suspension cultures of *I. balsamina* , nevertheless, contain mostly small aggregates rather than single cells (Fig. 9B). This is the normal characteristic of most cell suspension cultures.

In this experiment, the root cultures are formed by appropriated combination of hormones which are 0.1 mg/l NAA, 0.1 mg/l kinetin and 1.0 mg/l BA in B5 liquid media. The root cultures show to be capable of unlimited and uniformed growth in the media (Fig. 9A).

In term of naphthoquinone formation, we report here the successful production of 2-methoxy-1,4-naphthoquinone in the green callus and lawsone in the root cultures. In the cell suspension cultures, however, the formation of both naphthoquinones was not detected

under various tested conditions. The fundamental reasons of the differences in the naphtoquinone formation in these various *in vitro* cultures are still not clear. However, based on the knowledge of morphological differentiation and the expression of secondary metabolism, it is widely accepted that the unorganized tissues such as suspension cells are usually accompained by an apparent loss of their ability to accumulate secondary metabolites. The reasons may be : (1) the lack of expression in non-specialized cells or genes that control the essential steps in the biosynthetic pathway ; (2) the non-availability of storage sites in which secondary metabolites would normally be sequestered ; (3) the unregulated catabolism of synthesized product (Charlwood and Rhodes, 1990).

In the case of *I. balsamina* , only the dense and green callus produces 2-methoxy-1,4-naphthoquinone without trace amount of lawsone (Fig. 20). This indicates that lawsone, once produced by the callus, is rapidly converted to 2-methoxy-1,4 naphoquinone. It is possible that the enzyme lawsone-O-methyltransferase which is responsible for methylating lawsone to form 2-methoxy-1,4-naphthoquinone is highly active in this callus cultures. The ability of the green callus to produce the methyl ether of lawsone suggests that the expression of lawsone biosynthetic pathway and the process of morphological differentiation are closely related.

In contrast to the green callus cultures, the root cultures appear to contain no 2-methoxy-1,4-naphthoquinone, but contain high content of lawsone instead. Because of their similar morphology, it was interesting to compare the chemical patterns between the extracts obtained from the root cultures and the whole roots of *I. balsamina* plant. The patterns in Fig. 24 shows that both extracts contain quite different chemical composition. In term of naphoquinone, the root cultures can accumulate high content of lawsone without detectable amount of 2-methoxy-1,4-naphthoquinome, whereas the whole roots accumulate 2-methoxy-1,4-naphthoquinone as a major naphthoquinone and lawsone as a minor one (Table 9). Three points can be made from these results. First, the general metabolism in the root cultures is apparently different from that in the whole root. Second, the ability of the root cultures to produce lawsone indicates that in the whole plant, the root is also the

site, if not the only plant part, for lawsone biosynthesis. Third, the absence of 2-methoxy-1,4-naphthoquinone in the root cultures may be the results of the absence or non-functioning of the enzyme lawsone-O-methyltransferase in *I. balsamina* root cultures.

For the growth cycle of *I. balsamina* root cultures (Fig. 25), the period for various phases of the cycle was examined for 20 days. It appears that this root cultures has a very short lag phase probably in one day and then an exponential growth or log phase of 7 days, followed by a long linear phase of 7 days before entering stationary phase. The growth characteristic suggests that *I. balsamina* root cultures spend a very short time for its adaptation to fresh medium and a high growth rate can then be induced in the cultures. The high rate of increase in biomass goes on until presumably the nutrient depletion or a poor environment, the root cultures reach the stationary growth phase. The limiting nutrient in this system is still not known. For lawsone production, the initiation of the compound biosynthesis in *I. balsamina* root cultures is at the early exponential growth phase. There are three peaks of maximum yield during the 20-day culture (Fig. 25). The highest one is obtained at the early exponential growth phase or day 2 of the culture age, reaching 0.158% dry weight. This percent content is about 3.5 times of the amount of lawsone found in the leaves. The second peak of lawsone accumulation is in the linear phase (day 7), reaching 0.079 % dry weight. This is only one half of the content of the first peak. For the third one, it appears in the late linear phase just before entering the stationary phase. The yield of lawsone content (0.068 %) is similar to that of the early linear phase of the root cultures.

The phenomenon of the fluctuation of lawsone production in *I. balsamina* root cultures may be explained by a cooperative mechanism between a negative feed-back inhibition of lawsone biosynthesis and lawsone degradative enzyme system. When the root cultures produce lawsone and accumulate it within the cells to a certain level, the cells of the root cultures may stop producing the compound by an unknown negative feed back mechanism. Simultaneously, lawsone produced can be degraded by lawsone catabolic system in the cells. This can explain the drop of lawsone content in the root cultures.

Thereafter, the cellular lawsone concentration is lower than the minimum level, the culture root cells may be stimulated to produce lawsone again.

The abilities of both root cultures and callus of *I. balsamina* to produce the naphthoquinones suggests that they can be the materials of choice for biosynthetic studies of lawsone and 2-methoxy-1,4-naphthoquinone, respectively. Such ability imply that various enzymes involved in the pathway of lawsone and its methyl ether are operating under these control conditions. In addition to the naphthoquinones, these *in vitro* cultures of *I. balsamina* are also valuable for the biosynthetic study of scopoletin, a coumarin derivative. This is due to their high scopoletin production especially, in the cell suspension cultures of *I. balsamina*.

It should also be noted that the developed quantitative method of lawsone and 2-methoxy-1,4-naphthoquinone by TLC-densitometry is highly effective. This method allows both naphthoquinones be quantitated simultaneously and directly from the crude extracts without prior partial purification. The method is fast, accurate and reproducible. Its accuracy is reliable as confirmed by HPLC method (Table 10). Based on this TLC-densitrometric assay, the content of 2-methoxy-1,4naphthoquinone appears to be 0.13, 0.075 and 0.032 % in the leaves, stems and roots, respectively, and of lawsone is approximately 0.045 % mainly in the leaves. This is apparently the first report on the quantitative distribution of lawsone and its methyl ether in *I. balsamina* plant.

CONCLUSION

From this research work of "Study on Lawsone Derivative Formation in *in vitro* Cultures of *Impatiens balsamina* L." the following conclusions can be drawn:

1. Three types of *in vitro* cultures of *Impatiens balsamina*, including callus, suspension and root cultures, have been obtained from this study.

2. The root culture produce and accumulate lawsone up to 0.16 per cent of dry weight which is 3.5 times of the highest content found in the leaves.

3. The callus cultures can produce 2-methoxy-1,4-naphthoquinone although with a capacity of its accumulation, only 0.004% compared with 0.13% in the leaves.

4. The production of lawsone in the root cultures shows high degree of fluctuation during the culture cycle.

5. The organic solvent extracts of the root cultures and the intact roots show very different chemical patterns.

6. All the three *in vitro* cultures can also produce high content of a coumarin, scopoletin.

7. The root cultures can be used as a source for biosynthetic studies.

8. The developed TLC-densitometric method proves to be highly effective and accurate in the qualitative and quantitative analysis of lawsone and its methyl ether in the plant and culture crude extracts.

9. The quantitative distribution of both natural naphthoquinones in various *I. balsamina* plant parts is successfully worked out by this study.