CHAPTER V
DISCUSSION

An important group of natural products with industrial interest are the essential oils, they are for example being used in perfumery, as aroma products, as flavouring agents in food and beverages, in cosmetic products and as drugs. They have been a target for improving productivity by plant cell cultures.

The study of essential oil production by plant cell cultures is interesting, as it has proved to be difficult and most cultures have been unable to produce adequate yields of flavour substances. Undifferentiated cells have shown loss of ability to accumulate essential oils, and moreover, monoterpenes which are the chemical constituents in essential oil, have been proved to be cytotoxic to plant cells as described in Chapter I. The other reasons for low levels of essential oil constituents accumulating in plant cell culture are the fact that the aromas are complex mixtures, production of which is affected by the culture age and conditions, the essential oils have a high turn over or degradative capability, they are volatile and therefore can be lost from cultures. For these reasons the most cultures were unable to produce an adequate yield of essential oil.

Many strategies have been used for improving yield of essential oil produced by plant cell cultures as described in Chapter I.

The purposes of this research were to study on production of essential oil in plant cell cultures, following by chemical constituents of each plant cultures compared to that obtained from intact plants, and methods for improving yields of essential oil, particulary major chemical constituents.

In these experiments, plants containing essential oils have been selected for studying chemical constituents of essential oil. These plants are *Artemisia vulgaris* var. *indica*, *Cuminum cyminum*, *Fortunella japonica* and *Pogostemon cablin.* Essential oils obtained by hydrodistillation were identified by GC-MS. It has been found that all these essential oils contained only terpenoids of which the major components are (+)-davanone (71.59%), cuminaldehyde (36.30 %), β-pinene (47.44 %), d-limonene (87.07 %), and patchouli alcohol (60.30 %) for *Artemisia vulgaris* var. *indica*, *Cuminum cyminum*, *Fortunella japonica* leaves, *Fortunella japonica* peels and *Pogostemon cablin*, respectively.

Fruits of *Cuminum cyminum* and seeds of *Fortunella japonica* were surface sterilised by same method. They were dipping in 70% ethanol for 1 min. and then followed by in 30% hydrogen peroxide for 5 min. Sterilised explants were then germinated on sterilised petri dish containing filter papers in aseptic condition. Seedlings of *Fortunella japonica* were germinated better than that of *Cuminum cyminum*. Germination was not successful if surface sterilisation time was more than 5 min.

Leaves explants of *Artemisia vulgaris* var. *indica* were surface sterilised by shaking them in surface sterilising agent (compositions were shown in Appendix) for 1 h, followed by 7% hydrogen peroxide and then 5% hydrogen peroxide for 15 min and 7 min, respectively. Meanwhile *Pogostemon cablin* were surface sterilised in 5% Clorox® for 5 min.

These sterilised explants were then callus initiated and maintained on appropriated culture conditions. Most of these cultures were grown on MS media containing 1 mg/l 2,4-dichlorophenoxyacetic acid and 0.1 mg/l Kinetin excepted for cell cultures of *Pogostemon cablin* were grown on MS media containing 0.5 mg/l NAA and 1 mg/l BA. They were incubated at temperature of 25±2°C under different light conditions. Light used in this experiment was supply by fluorescent lamp and its onditions were also affected on growths and appearances of callus cultures. Most of these cultures were grown in 24-h light whilst cultures of *Pogostemon cablin* were grown in dark conditions. After fourth generations, healthy callus cultures were cell suspension initiated in new liquid media and maintained in the same conditions as used in callus cultures.

The essential oils of these callus and cell suspension cultures were extracted with dichloromethane and identified by GC and GC-MS. The results have shown that all these cultures, including callus and cell suspension cultures, can produce the major chemical constituents as described above, followed by a small amount of minor constituents. The major chemical constituents which have been found in dichloromethane extracts are (+)-davanone, cuminaldehyde, d-limonene, and patchouli alcohol, for *Artemisia vulgaris* var. *indica*, *Cuminum cyminum*, *Fortunella japonica* peels and *Pogostemon cablin*, respectively. However, β-pinene, which has been the major constituent found in the essential oil obtained from *Fortunella*

japonica leaves hydrodistilation, can not be found and may be caused the low boiling point, and will be lost in the air.

Unfortunately, the levels of these major chemical constituents which have been found in dichloromethane extracts are still very low compared to intact plants. So, some strategies have been used for improving yields of essential oils, particularly of the major chemical constituents. These strategies are precursor feeding and biotransformation, elicitation with chitosan or methyl jasmonate, permeabilisation, and *in situ* product removal. After applying these techniques, the level of major constituents have been improved.

Precursor feeding also leads to high yields of secondary metabolites. Precursor feeding to certain individual cell suspension cultures could improve the yield of major constituents.

Chitosan proves to be an effective elicitor which has been used for improving yield of secondary metabolites. Permeabilisation effects caused cell death and its optimum concentration should be determined before applying to cell cultures. The optimum concentration of chitosan for *Fortunella japonica* cell suspension cultures is 200 ppm, this concentration could improve yields of d-limonene successfully and are not cause cell death. Meanwhile, the optimum concentration of methyl jasmonate for *Fortunella japonica* cell suspension cultures is 50 ppm, however, it could not improve yields of d-limonene in *Fortunella japonica*. It can improve only yields of some chemical constituents e.g. β-pinene which cannot be found in the control experiment.

Tween-20 is a permeabilising agent, which has been used for the destroying permeability of cell membranes and tonoplasts. After use in cell suspension cultures of *Fortunella japonica* for 14 and 21 days, only linalool, geraniol, and nerol could be found, which has been suggested to be the precursor of d-limonene (Attaway *et al.*, 1967 and Bouwmeester *et al.*, 1995).

All concentration of chitosan, methyl jasmonate, and Tween-20 caused death of cell suspension cultures of *Cuminum cyminum*. Therefore, none of the chemical constituents were detected.

Unfortunately, use n-hexadecane for removing chemical constituents (*in situ* product removal), showed no significant different. They were still detected in low levels, however, each plant cultures look healthy compared to control experiment.

Biotransformation of exogenous terpenoids can be achieved using certain cell lines which do not accumulate any volatile constituents. Also considerable levels of enzymes could be found in some cultures compare to their intact plants, even through these cultures did not accumulate any terpenoids found in their intact plants. The biotransformation results using cell suspension cultures of *Artemisia vulgaris* var. *indica*, *Cuminum cyminum*, *Fortunella japonica* and *Pogostemon cablin* supported this review. The reactions involved in biotransformation experiments using these plant cells showed hydroxylation, oxidation, hydrolysis, reduction, and cyclisation. It might be generally accepted that the accumulation of secondary metabolites is largely dependent on enzyme activity in cell cultures, and the yield of biotransformation products were varied in a wide range of individual suspension. This kind of observation was also demonstrated by using different cultures in our work. In most cases of this work, *Fortunella japonica* cell suspension cultures possessed the highest biotransformation capacity in our screening of plant species in this work, followed by cell suspension cultures of *Cuminum cyminum*.

Use of control release systems, combined with feeding experiments was shown to benefit cell cultures. This system limited the availability of monoterpenes in the aqueous nutrient phase, hence reducing its toxic effect on plant cells, and allowing for increased amounts of substrate load, without causing any detrimental effect to the suspensions. This system tempers rapidly occurring reactions by acting as low feeders, or slow exchangers of a substrate and/or products, thus increasing their persistence. Control released polymers used in this work produce greatly extended concentration of both substrate and biotransformation products. It will also allow recycling of plant cells for the purpose pf converting monoterpenes, at concentrations that would be lethal in a one phase system.

Essential oils of these plants will be preferred to accumulate in shoots, and shoot regeneration will be established in order to be accumulation sites for these plant cell cultures. Cell suspension cultures of *Cuminum cyminum* were successfully shoot regenerated on MS containing 1 mg/l NAA and 5 mg/l BA, whilst cell suspension cultures of *Fortunella japonica* was successfully shoot regenerated on 2 mg/l TDZ followed by 0.1 mg/l NAA. Thidiazuron (TDZ) is a non-purine cytokinin-like compound, which has been shown to exhibit stronger effects than conventional cytokinins over a wide range species as described in Chapter I. However, excessive concentration of TDZ will inhibit cell growth and cause cell death. Use of low

concentrations of auxins followed by TDZ could reduce the effect of TDZ on growth inhibition.

The results show that production of essential oil by plant cell cultures can be achieved, and the prospect of producing useful secondary metabolites using undifferentiated cell cultures is feasible, with large scale production of volatile compounds realised. Meanwhile, plant cell cultures do have a large capability for biotransformation, and extended potential as useful tools for synthesis of some desirable chemical compounds which may not be easy using chemical synthesis. In attempt to improve yield of essential oil constituents, the future research would be go on with other strategies such as oxidative stress, transformed plant cultures by *Agrobacterium* spp. or genetic engineering.