DISCUSSIONS

CHAPTER FOUR

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4.1 Essential oils of selected *Citrus* species

A total of 16 out of 20 compounds from the essential oils of *C. halimii* were identified. The main components were mainly of α-terpineol (34.4%), terpinyl acetate (22.3%) and β-pinene (13.4%).

Apart from α-terpineol, other hydroxy compounds that were present as minor components were linalool (1.4%), β-terpineol (1.5%) and cedrol (0.2%). Monoterpene hydrocarbon compounds such as β-myrcene (3.6%), α-terpinene (0.1%), β-phellandrene (1.6%), limonene (1.05%), 3-Carene (4.2%), α-pinene (4.9%) and ocimene (1.5%) were also detected. Camphene occured only in trace amount (<0.1%). The only sesquiterpene hydrocarbon compound found in *C. halimii* oil was caryophyllene. The IR spectrum showed the presence of some esterified compounds which were not identified using GCMS.

Analysis of essential oils extracted from leaves of *C. halimii* was first reported in 1976 by Scora *et al.*. Most of the compounds reported are the same with those in this study. However α-terpinene, β-phellandrene, ocimene, α-terpineol, β-terpineol, cedrol and terpinyl acetate were not reported in the previous study. This could be because of the difference in geographical factor.

Out of the seventeen compounds detected, only ten were identified in *C. hystrix* essential oil. Hydroxy compounds amounted to about 73.2% forming the major constituents of the oil. The predominent components were nerol (44.1%) and citronellol (22.3%). Other compounds which were found in lesser amounts were geraniol (0.8%), linalool (3.4%) and β-terpineol (2.6%).

Monoterpene hydrocarbons identified from *C. hystrix* were β-myrcene (1.2%) and β-pinene (0.4%), whilst sesquiterpene components were α-caryophyllene (0.8%), caryophyllene (4.8%) and copaene (1.1%). Carbonyl and ester compounds were not detected in *C. hystrix* essential oil.

C. madurensis essential oil composed of seventeen detectable components. Linalool (12.1%), β-pinene (11.7%) and caryophyllene (7.3%) were the major components of *C. madurensis* oil. Other compounds found were monoterpene hydrocarbon (α-pinene) and sesquiterpenes (α-caryophyllene and elemene). *C. madurensis* oil also contained hydroxy compounds such as the terpineol group (α and β) and cineol. Ester and carbonyl compounds were absent from this oil.

Eighteen components were present in essential oil of *C. micrantha* var. *microcarpa*. Main components of the oil were nerol (20.9%), followed by citronellol (11.1%), geraniol (7.7%), caryophyllene (8.5%) and neral (6.3%). Linalool was found in a low percentage (0.7%). Among the hydrocarbon compounds, only sesquiterpenes were detected. Monoterpene compound was absent from *C. micrantha* var.

microcarpa essential oil. The former compounds identified apart from caryophyllene were α-caryophyllene (1.2%) and elemene (1.2%).

Esters were not identified in *C. micrantha* var. *microcarpa* essential oil but their presence were detected from the IR spectrum of the crude essential oil.

Nineteen components were found in citrumello leaf essential oil. Caryophyllene was the most abundant component of the oil (40.8%). Other hydrocarbon compounds observed present in low quantity were β-myrcene (6.0%), α-terpinene (1.5%), limonene (1.4%), 3-carene (2.3%), α-pinene (0.2%), β-pinene (3.8%), ocimene (1.7%), α-caryophyllene (0.5%) and elemene (0.3%). Citrumello essential oil also contained hydroxy compounds (β-terpineol and citronellol) at percentages 2.6% and 1.1% respectively.

Caryophyllene was observed present in all the species studied, with the highest percentage detected in citrumello. β-terpineol and β-pinene were commonly present except in *C. micrantha* var.*microcarpa*. α-caryophyllene was absent in the oil extracted from *C. halimii* whilst linalool was not detected in citrumello. Camphene which was not detected in most of the *Citrus* oils, was present in trace amount in *C. halimii*.

From the data presented in this study, it was observed that some of the essential oil components of certain species were similar. Most of the essential oil components of *C. halimii* were found in common with those of citrumello.

4.2 Determination of taxonomic relationships between selected *Citrus* species using isoenzymes

Isoenzymes study was carried out to determine the taxonomic relationships between selected *Citrus* species. Isoenzyme study have been reported in *Citrus* species (Torres *et al.*, 1982; Germana *et al.*, 1994). Isoenzymes provide useful genetic markers in taxonomic studies (Soost and Torres, 1981) because of their codominant inheritance (Rahman and Nito, 1994).

Differences in banding patterns were observed in three isoenzyme systems: glutamate oxaloacetae transaminase, peroxidase and esterase. No band was observed in malate dehydrogenase and polyphenol oxidase systems even after overnight incubation. This could be due to very low isoenzymes concentration, or no isoenzymes activity. Clear banding patterns were difficult to obtain possibly due to protein content. Modifications had been made, by loading different volumes of sample extracts consequently varying protein levels. New staining chemicals had been used to ensure that no oxidised chemicals were used for staining isoenzymes.

According to Rahman *et al.* (1994), the presence or absence of a certain band was considered as a diagnostic feature for a certain species. Results from this study showed that some species had a specific band in certain isoenzyme systems studied. The band which was specific for certain species could be used as a marker to distinguish from other species. No specific band was observed in glutamate oxaloacetate transaminase system. In peroxidase system band 1, 2 and 4 were specific

for *C. madurensis*. In esterase, band 5 was specific for *C. halimii* while band 4 for *C. micrantha* var. *microcarpa*.

It was observed that some species shared identical bands in the enzyme systems. According to Conklin and Smith, (1971) bands derived from two different species that migrate the same distance are considered to be produced by a gene common to both species. In the same way, bands that migrate differently are considered to be controlled by different genes. In glutamate oxaloacetate transaminae system *C. halimii* seemed to be related to *C. micrantha* var. *microcarpa* while *C. hystrix* to citrumello. In peroxidase system *C. halimii*, *C. hystrix* and *C. micrantha* var. *microcarpa* were related. In esterase system no significant correlation between species was observed.

4.3 Classification of *Citrus* taxa based on the presence or absence of browning in young shoot extracts

Results from this study showed that the taxa can be classified into two phenotypic classes: browning and nonbrowning, based on the presence or absence of browning in the young shoot extracts. Browning is the results of oxidation of phenolic substrates by enzyme polyphenol oxidases (PPO). This process involves two distinct reactions: the hydroxylation of monophenols to o-diphenols and the oxidation of o-diphenols to o-quinones (Valero *et al.*, 1988). The o-quinones which are very highly unstable undergo polymerization to yield brown pigments (polymers) of high molecular weight and react with amino acids, peptides and proteins by binding with the NH_2 and -SH groups (Rhodes, 1971; Park *et al.*,1980; Coombs and Hind, 1985; Sayavedra-Soto and Montgomery, 1986; Chilaka *et al.*, 1993).

Browning was observed in *Citrus madurensis* and *C. hystrix*. However in the previous study reported by Esen and Scora (1975), *C. hystrix* and *C. madurensis* were classified under nonbrowning phenotypes. Nonbrowning phenoypes were detected in *C. halimii* and *C. micrantha* var. *microcarpa*. Similar result was reported by Esen and Scora (1975). Citrumello, a hybrid of *C. paradisi* and *Poncirus trifoliata* was observed to be phenotypically nonbrowning. Browning was reported in *C. paradisi* while *P. trifoliata* was nonbrowning (Esen and Soost, 1974b). This result is in contrast with the fact that crosses between browning and nonbrowning parents produced hybrids of browning phenotype (Esen and Soost, 1974b). This could be because these

taxa are heterozygous for browning (Esen and Soost, 1974b). Esen and Soost (1974a) reported that single gene control the browning and nonbrowning trait.

To strengthen the present study an additional experiment should be carried out. Browning of the extract is depended upon the presence of phenolic substrate and PPO activity (Esen and Soost, 1974a). Experiment to determine if browning phenotype contained or lack of enzyme (PPO) or phenolic substrate or both, was reported by Esen and Soost (1975). In this experiment crude shoot extracts from 'willow leaf mandarin' was used as an enzyme source. 'Willow leaf mandarin' was selected as the enzyme source because it had the highest PPO activity. Substrate was obtained by boiling the extract. The boiled extract was free of the enzyme (PPO). The enzyme was mixed with the substrate and either the occurence or absence of browning was observed. The browning process which was exhibited by browning phenotypes, was between the enzyme from 'willow leaf mandarin' and the substrate. It showed that all the browning phenotypes contained the substrate which was oxidized by the enzyme. The nonbrowning phenotype was devoid of the substrate or with little or no enzyme activity. This experiment was not tried because 'willow leaf mandarin ' was not available locally and the young shoots used as the enzyme source were not easily accesible because the source was from abroad.

Variation in the intensity of browning was observed in this study. Extract colour was dark brown in *C. madurensis* and brownish green in *C. hystrix*. This could be the result of differences in PPO activity. According to Esen and Soost (1974b) the higher

the activity, the more rapid browning occured. An attempt to determine the PPO activity was unsuccesful due to the technical problems encountered in this work.

Experiments on the effect of inhibitors showed that insoluable PVP did not prevent browning. Esen and Soost (1974b) reported similar observation. PVP was reported to be effective in adsorbing tannin but was ineffective in inhibiting PPO activity (Rhodes, 1977). The enzyme was inhibited by DIECA, potassium metabisulphite and L-cystein-HCL. Similar result were reported by Esen and Soost (1974b) and Fujita *et al.* (1995). Cystein (thiol reagent) and metabisulphite (strong reducing reagent) react directly with sulfuhydryl groups or with other amino acid residues of the enzyme. This reaction occurs concurrently with the reduction of o-quinone (Lee *et al.*, 1991). The o-quinone was reduced to o-diphenol form (Baldry *et al.*, 1970) and formation of brown pigments can be prevented. DIECA, the chelating agent binds with the copper in PPO, thus preventing the formation of the brown pigments (Coombs and Hind, 1985). Metabisulphite, DIECA and L-cystein are known to be effective in inhibiting PPO activity and consequently the oxidation of phenolic compounds (Kahn, 1977; Park *et al.*, 1980; Fujita and Tono, 1988; Friedman and Bautista, 1995; Fujita *et al.*, 1995). These results indicated that the enzyme involved in the browning process was polyphenol oxidase .

From this classification, the occurence or absence of browning in young shoot extracts could be used as a criteria for taxonomic relationships between species.

4.4 Tissue culture studies in selected *Citrus* species

This study was carried out to determine the best explant and regeneration media for propagation. The four species studied showed different responses to various plant growth regulators.

Shoots regeneration were obtained by direct organogenesis, without interverning callus phase. Comparatively it seemed that the highest percentage of regeneration was observed from stem explant of citrumello in a medium supplemented with combination of 4 mg/l 6-BAP, 2 mg/l IAA and 1mg/l GA_3 (Table 3.20). The best explant for shoot regeneration of *C. hystrix* was cotyledon. Similar result was observed in *C. micrantha* var. *microcarpa*. The best shoot regeneration medium for *C. hystrix* was a medium supplemented with combination of 4 mg/l 6-BAP and 2 mg/l IAA. However in *C. micrantha* var. *microcarpa* a medium incorporated with combination of 5 mg/l 6-BAP and 1 mg/l NAA appeared to be the best medium for shoot regeneration. *C. madurensis* was not very responsive in shoot regeneration. Shoot regeneration was observed only from leaf explants.

All the explants from the species studied were responsive in shoot regeneration, in a medium which were supplemented with 4 mg/l 6-BAP and 2 mg/l of IAA. Shoots initiation were also observed in a medium incorporated with 5 mg/l 6-BAP in combination with 1 mg/l NAA. The results obtained indicated that shoots regeneration were achieved in media which contained high ratio of cytokinin (6-BAP at 4 and 5 mg/l) to low auxin (NAA (1 mg/l) or IAA (2 mg/l)). This is supported by the fact that

shoots regeneration generally are influenced by a high ratio value of cytokinin to low auxin (George and Sherrington, 1984). In *C. aurantium* shoots induction were reported from embryo derived calli in MT medium supplemented with 5 mg/l 6-BAP and 1 mg/l NAA.

Among the two auxins employed in this study in combination with 6-BAP, IAA was more effective than NAA in shoot regeneration. Most of the shoots were produced in media which contained IAA. The efficiency of IAA in promoting shoots development was reported in *Aegle marmelos* culture (Hossain *et al.*, 1994). IAA, a weak non-phenoxy auxin is favourable in shoots initiation than stronger phenoxy auxin such as 2,4-D (Bonga and Von Aderkas, 1992). Combination of IAA and 6-BAP was found to be the best for multiple shoot formation of tea (Agarwal *et al.*, 1992).

Shoots regeneration in other *Citrus* species have been described by several authors (Raj Bhansali and Arya, 1978; Kitto and Young, 1981; Barlass and Skene, 1982; Sauton *et al.*, 1982; Edriss and Burger, 1984; Burger and Hackett, 1986; Moore, 1986; Starrantino and Caruso, 1988; Tusa and Geraci, 1988; Bhat *et al.*, 1992; Singh *et al.*, 1994).

In this study shoots were also produced in a medium supplemented with 5 mg/l 6-BAP alone. Similar results were reported previously in *Poncirus trifoliata* and Carrizo citrange (Beloualy, 1991). However in some *Citrus* species optimal concentration of 6-BAP required for direct shoots regeneration was lower than that utilized in this study. Direct shoots regeneration were obtained from nodal stem

segment of *Citrus sinensis* (L.) Osb., *C. aurantifolia* (Christm) Swing. and *C. limon* (L.) Burm. f in MS medium with 1 mg/l 6-BAP (Marin and Duran-Vila, 1991). Song *et al.* (1991c) studied plant regeneration from cotyledon and hypocotyl explants of *Citrus junos* Sieb. et Tanaka (yooza) using MT medium. Shoots induction were observed from cotyledon and hypocotyl explants in a medium supplemented with 3 mg/l 6-BAP. Sim *et al.* (1989) reported shoots regeneration from cotyledon explants of *Citrus mitis* in MS medium incorporated with 2 mg/l 6-BAP. Similar response was observed in leaf explants. However higher level of 6-BAP appeared to be inhibitory.

Based on these reports, it could be concluded that 6-BAP is effective in shoot regeneration. Consequently this plant growth regulator was applied to supplement the medium. 6-BAP a cytokinin is very effective in promoting direct or indirect shoot regeneration (George and Sherrington, 1984; Bonga and Von Aderkas, 1992).

In other woody species 6-BAP was reported more effective than other cytokinins in shoot induction. The superior effect of 6-BAP was reported in apple (Kouider *et al.*, 1985), *Paeonia suffroticosa* (Bouza *et al.*, 1994) and *Aegle marmelos* (Hossain *et al.*, 1994). In *Punica granatum*, high frequency of shoots formation from leaf explants were observed in a medium incorporated with 6-BAP compared with kinetin, zeatin and 2-isopentenyladenine (Omura, 1991). Similarly in the tissue culture of *Garcinia mangostana* more shoots were formed from leaf explants in media supplemented with 6-BAP than in media with either kinetin or 2iP (Goh *et al.*, 1990).

In this study the addition of GA_3 to a culture medium did not improve shoot initiation. The reason could be because of shoot formation was prevented by GA_3 as reported by George and Sherrington (1984). According to Beloualy (1991) GA_3 is used to induce shoot elongation.

It was observed that the shoots produced from the species studied did not elongate properly. The reason could be because of the presence of 6-BAP in the medium. It was reported that, once the shoots were big enough for separation and subculture they were transferred to a cytokinin free medium (Bonga and Von Aderkas, 1992).

Shoots regeneration could be improved by adding complex organic substance such as malt extract to a culture medium. The beneficial effect of malt extract on shoot regeneration has been reported in *Citrus* species (Kochba *et al.*, 1972; Raj Bhansali and Arya, 1978; Moore, 1985 and Gill *et al.*, 1994). In culture of 'Kinnow' mandarin (*Citrus nobilis* Lour. X *Citrus deliciosa* Tenora), shoots regeneration were increased in MS medium supplemented with malt extract along with cytokinins (6-BAP or kinetin) in combination with auxin than in the medium lacking malt extract (Gill *et al.* 1994).

Rooting was observed in NAA (1 mg/l) supplemented media in all the species studied. Leaf and cotyledon explants were responsive in rhizogenesis. Similar observations were reported by Beloualy (1991). Rooting was also promoted in *Citrus aurantium, Poncirus trifoliata* and Carrizo citrange by a supplement of 1 mg/l NAA.

The role of NAA in inducing rooting at differerent concentrations has also been reported in other *Citrus* species (Barlass and Skene, 1982; Teo *et al.*, 1988; Gill *et al.*, 1994). Marin and Duran-Vila (1991) cultured excised shoots from epicotyl explants on basic nutrient solution supplemented with 3 mg/l NAA to promote rooting in *Citrus sinensis* (L.) Osb., *Poncirus trifoliata* (L.), *C. aurantifolia* (Christm.) Swing. and *C. limon* (L.) Burm. f. However root formation from stem segments of *Citrus sinensis* (L.) Osb. and *C. aurantifolia* (Christm.) Swing was reported in higher optimal concentration of NAA (10 mg/l) (Duran-Vila *et al.*, 1989).

In this study rooting was also promoted in a medium supplemented with combination of 1 mg/l NAA, 0.5 mg/l 2,4-D and kinetin. This is supported by the fact that rhizogenesis usually follows treatment with mixtures containing more auxin than cytokinin. A medium with high concentration of cytokinin generally inhibit root formation and growth. It could also prevent the promotive effects of auxins on root initiation (George and Sherrington, 1984).

Callus production was observed in all the species studied except in citrumello. The three explants of *C. madurensis* and *C. micrantha* var. *microcarpa* produced callus in most of the media tried. In *C. hystrix* callus was only produced from leaf and cotyledon explants.

In this study it was also found that callus was initiated in media supplemented with 1 mg/l 2,4-D alone and the combination of 1 mg/l NAA, 0.5 mg/l 2,4-D and 0.5 mg/l kinetin. Callus production was also observed in media which were added with 1

mg/l NAA individually or in combination with kinetin at equal concentration. However all the calli produced were non-morphogenic. It was found that, auxins (2,4-D and NAA) were essential for callus initiation in all the media tried. Theoretically, for callus induction from explant, auxin is generally required to be incorporated into the medium. Being a strong promoter for callus induction 2,4-D is most frequently used to initiate callus growth (George and Sherrington, 1984 and Bonga and Von Aderkas, 1992).

In previous studies callus was produced from *Citrus* species in media supplemented with NAA at a concentration higher than used in the present study. Teo *et al.* (1988) reported callus production from cotyledon explants of *Citrus microcarpa* in a medium supplemented with higher concentration of NAA (10 mg/l). Callus initiation in *C. sinensis, C. medica* and *C. auratifolia* was also accomplished in MS medium containing the same concentration of NAA (10 mg/l) in combination with 0.25 mg/l 6-BAP (Duran-Vila *et al.*, 1989). However high concentration of NAA (10 mg/l) seemed unfavourable to growth of callus of *C. unshiu* (Nito and Iwamasa, 1990).

Friable callus obtained in this study could be used as a source of cell suspension. Besides being an intermediate in indirect regeneration callus could be useful for secondary metabolite productions. Secondary matabolites which are commercially important such as in the production of drugs, flavours, perfumes, and pigments are often difficult to synthesize chemically. Callus culture could be an alternative method for these synthesis (Fujita, 1990). Production of secondary metabolites from callus culture of *Citrus* such as limonin (Barthe *et al.*, 1987), flavanone (Brunet and Ibrahim, 1973; Lewinsohn *et al.*, 1986), flavanoid (Vandercook and Tisserat, 1989; Berhow *et*

al., 1994) and valencene (Drawert *et al.*, 1984; del Rio *et al.*, 1991; del Rio *et al.*, 1992) has been reported. Callus has also been used in the study of Citrus diseases caused by viruslike pathogens (Navas-Castillo *et al.*, 1995). Study on fruit physiology has also been reported using callus derived from fruit explants (Unger and Feng, 1978; Gulsen and Goren, 1981; Altman *et al.*, 1982; Tisserat and Galletta, 1987; Tisserat *et al.*, 1988; Tisserat *et al.*, 1989a; Tisserat *et al.*, 1989b; Tisserat *et al.*, 1989c; Amo-Marco and Picazo, 1994).