J. Trop. Agric. and Fd. Sc. 30(2)(2002): 239–247

Analysis of flavour compounds in leech lime *(Citrus hystrix)* flower and yield improvement in callus

[Analisis bahan perisa di dalam bunga limau purut (*Citrus hystrix*) dan peningkatan penghasilannya di dalam kalus]

R. Suri*, M. Radzali**, M. Marziah** and S. Mohd. Aspollah***

Key words: *Citrus hystrix* flower, callus, major flavour compounds

Abstrak

Komponen perisa terbanyak yang dianalisis pada bunga limau purut (*Citrus hystrix)* ialah sitronelal. Anak pokok yang ditumbuhkan di atas medium Murashige dan Skoog (MS) tanpa penambahan hormon tidak berkeupayaan mensintesis sitronelal. Walau bagaimanapun kuantiti limonen didapati lebih tinggi pada batang (101.62 ± 5.24 µg/g bb.) berbanding dengan kelopak (27.30 ± 1.42 µg/g bb.), ovari (10.76 ± 0.01 µg/g bb.) dan anter serta debunga (6.64 ± 0.24 µg/g bb.). Kalus telah berjaya diaruh dari batang anak pokok, embrio dan petiol setelah eksplan tersebut dikulturkan di atas medium MS yang dibekalkan sukrosa (30 g/liter) dengan asid asetik naftalena (NAA) (2.0 mg/liter) dan kinetin (1.0 mg/liter) tetapi hanya limonen dan sikloheksanol yang dihasilkan. Rawatan kalus pada keadaan cahaya yang berbeza tidak dapat meningkatkan jumlah komponen perisa. Walau bagaimanapun rawatan kalus pada keadaan bercahaya mencatatkan penghasilan tertinggi bagi sikloheksanol (14.1 ± 1.11 µg/g bb.) dan limonen (1.48 ± 0.09 µg/g bb.) berbanding dengan rawatan lain.

Abstract

The major flavour compound obtained from *Citrus hystrix* flower was citronellal. Plantlet grown on a basal Murashige and Skoog (MS) medium without phytohormone do not produce any citronellal. However, the quantity of limonene was remarkably higher (101.62 ± 5.24 µg/g fwt.) in stem than petal (27.30 ± 1.42 µg/g fwt.), ovary (10.76 ± 0.01 µg/g fwt.) and pollen and anther (6.64 ± 0.24 µg/g fwt.). Callus was successfully induced from stem, embryo and petiole on the MS medium supplemented with sucrose (30 g/litre), naphthalene acetic acid (NAA) (2.0 mg/litre) and kinetin (1.0 mg/litre) but only limonene and cyclohexanol have been produced. Treatment of callus derived from stem under different types of light did not increase the number of flavour compounds. Treatment of callus under bright white cool fluorescent light showed the highest production of cyclohexanol (14.1 ± 1.11 µg/g fwt.) and limonene (1.48 ± 0.09 µg/g fwt.) compared to that of other treatments.

*Food Technology Research Centre, MARDI Headquarters, Serdang, P.O. Box 12301, 50774 Kuala Lumpur, Malaysia

**Department of Biochemistry and Microbiology, Faculty of Science and Environmental Studies, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

***Department of Chemistry, Faculty of Science and Environmental Studies, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

Authors' full names: Suri Roowi, Radzali Muse, Marziah Mahmood and Mohd. Aspollah Hj Sukari

E-mail: suri@mardi.my

Introduction

Citrus hystrix (leech lime or 'limau purut') from the family of *Rutaceae,* is a valuable and popular flavouring material which is mainly cultivated in Malaysia, Indonesia and Thailand (Burkill 1966). Because of its attractive and characteristic odour, many research works have been carried out on the analysis of the *C. hystrix* using gas chromatography (GC-FID) technique. For instance, Muhammad Nor and Lim (1992) reported that the total ion chromatogram (TIC) profile of *C. hystrix* leaf oil showed that the citronellal content was 93.65%, citronellol 1.01%, linalool 0.88% and α-terpinolene 0.88%. Other flavour compounds which were less than 0.5% were identified as sabinene (0.37%), β-pinene (0.32%), 2-6-dimethyl heptanal (0.14%), geranyl acetate (0.30%) and farnesol (0.31%). Similar interesting finding by Suri et al. (1998) reported that citronellal was the major compound in peel, stem and leaf of *C. hystrix* which accounted for 25.50, 45.16 and 95.52% respectively. The major flavour compound in the fruit juice was α-terpineol which was approximately 36.50% of the total flavour compounds in the essential oil.

Plant cell, tissue and organ cultures are extremely attractive for the production of structurally complicated and biologically active substances such as flavour and fragrance under controlled conditions (Nabeta and Sugisawa 1983). In addition, many related factors such as illumination, explants, phytohormones, elicitors, precursors, carbon and nitrogen are commonly involved in the production of useful plant secondary metabolites in cell or tissue cultures.

Currently, there has been no report on the production of flavour compounds from *Citrus hystrix* flowers. In order to obtain continuous supply of *C. hystrix* flavour compounds, the in vitro technique using callus cultures has been carried out. The objective of this study was to investigate the flavour compounds of *C. hystrix* flower and to enhance the production of flavour compounds in callus.

Materials and methods

Plant materials

Citrus hystrix leaves, fruits, flowers and stems were obtained from the *Citrus* orchard of MARDI, Serdang, Selangor, Malaysia. Matured fruits were first washed under running tap water for 30 min to remove all dust and dirt. Then, mature seeds were carefully removed and surface sterilised with alcohol (70%) for 1 min and then chlorox (15%) for 10 min plus 1 or 2 drops of Tween 20 (Suri et al. 1998). After washing three times with sterile distilled water, embryos were aseptically removed and transferred onto a basal Murashige and Skoog (MS) agar medium without phytohormone to regenerate complete plantlets.

Induction of callus

Callus was aseptically induced from different parts of *C. hystrix* plantlets such as leaf, stem, petiole and embryo on the Murashige and Skoog (MS) medium containing naphthalene acetic acid (NAA) (2.0 mg/litre), kinetin (1.0 mg/litre), sucrose (30 g/litre) and 2.5 g/litre gelrite (Sum 1998). Cultures were incubated in the light condition (1500-lux) at 27 ± 2 °C. The highest quantity of callus produced was subjected to further treatment.

Determination of callus growth for optimum production of flavour compounds

Callus derived from the best explant was sub-cultured on the same medium as for induction. Approximately 0.3 g fresh callus was carefully placed on the same fresh medium with five replicates for each treatment. Callus was harvested every week to 8 weeks in order to monitor the fresh weight, dry weight and quantity of flavour compounds produced. The quantity of flavour was analyzed using a gas chromatography with flame ionized detector (GC-FID). The optimum time that showed

the highest production of flavour compounds was used for further treatment.

Different types of illumination condition
Callus obtained from high producing explants was tested for different types of illumination conditions. For instance, dim, dark conditions, bright white cool fluorescent lamp (36 watt) and 'agrolite' lamp (40 watt).

Flavour extraction
The flavour compounds of *C. hystrix* plant materials and calli were extracted using simultaneous distillation extraction (SDE) (Sugisawa et al. 1987). Samples were added with cold double distilled water and extracted with 40 mL dichloromethane for 2 hours. Water was separated from the organic extract using a separating funnel. Approximately 10 g of sodium sulphate anhydrous was added to the extract and left overnight to remove the excess water. A Whatman filter paper no. 1 was used to separate sodium sulfate from the extract. The extract was concentrated at 0 °C with 20 mL/min purified nitrogen.

The flavour compounds were identified by using retention index (RI) of gas chromatography (GC).

Apparatus for gas chromatography
A Hewlett Packard 5890 series II system (USA) gas chromatography attached with a flame ionized detector (FID) was used. The column used was a HP1 (Cross linked methyl silicone gum; 60 m x 0.25 µm x 0.25 µm film thickness (USA) (Suri et al. 2000).

Gas chromatography (GC) conditions
A gas chromatography condition was carried out using a modified method previously developed by Muhammad Nor and Lim (1992). The actual conditions used were as follows [temperature programme (70–170 °C), rate; 4 °C; helium; 1.71 mL/min; injector; 250 °C; detector; 280 °C and running time; 30 min] (Suri et al. 2000).

Preparation of standard flavour compounds
The standard flavour compounds such as cyclohexanol (Aldrich, Germany), ß-pinene (Sigma, USA), p-cymene (Aldrich, Germany), limonene (Aldrich, Germany), linalool (Aldrich, Germany), terpineol (Merck, Germany), citronellal (Aldrich, Germany) and citronellol (Aldrich, Germany) were used in these experiments. Five µL of each standard were weighed, mixed and dissolved in 0.5 mL of HPLC grade dichloromethane (100%). Then, 1.0 µL of the mixed standards was injected into the gas chromatography (GC).

Statistical analysis
Data were subjected to analysis of variance (ANOVA)/Duncan using SAS.

Results and discussion
Flavour compounds in flower
Results obtained from analysis of oils of *C. hystrix* petals using retention indices (RI) of gas chromatography clearly showed that the volatile oil contained eight major compounds *(Table 1)*. Most of the identified compounds in petals were monoterpenes and alkanols. Among the monoterpene hydrocarbons that have been synthesized were limonene, citronellol, β-pinene, p-cymene, linalool and γ-terpinene. Steam distillate of the fresh petals also afforded oxygenated monoterpenes such as citronellal (1 595.03 ± 19.54 µg/g fwt.) (Suri et al. 2000) and alkanol such as cyclohexanol (20.74 ± 1.75 µg/g fwt.) *(Table 1).* Similarly, Rouseff et al. (1994) reported that the aroma of orange oil was characterized by the mixture of aldehyde, ethyl butyrate, decanal, geranial, valencene and linalool. In addition, these compounds can be separated into two categories, the oil soluble and water soluble compounds.

Analysis of volatile essential oils in ovary of *C. hystrix* showed the similar type of flavour compounds as in the petals. However, there were considerable differences in terms of quantities. The major

flavour compound was citronellal (1 759.65 ± 7.89 µg/g fwt) *(Table 1)* which was found higher than in petal (1 595.30 ± 19.54 µg/g fwt.) and anther (314.37 ± 0.41 µg/g fwt.) (Suri et al. 2000). Other flavour compounds that were found higher in ovary than in petal and anther were cyclohexanol (101.45 ± 13.69 µg/g fwt) and β-pinene (3.0 ± 0.28 µg/g fwt.) (Suri et al. 2000) *(Table 1).*

In *C. hystrix* anther, citronellal also showed the highest amounts as in ovary and petal. Other compounds such as limonene, terpinolene, citronellol, β-pinene, linalool and γ-terpinene had also been identified but in a lower quantity compared to which were present in ovary and petal *(Table 1)*. The flavour profile was shown in *Figure 1.*

In the previous experiments conducted by Sato et al. (1990), it was reported that 57 flavour constituents were identified in *C. hystrix* leaf and citronellal was the major component (81.0%) of the oil. Other volatile constituents, which are found abundantly were linalool (4.0%) and citronellol (8.0%). Analysis of the chemical compositions of *C. hystrix* leaf was also investigated by Muhammad Nor and Lim (1992) using a gas chromatography-mass spectrophototometry (GC-MS); citronellal (93.65%), citronellol (1.01%), linalool (0.88%), β-terpinolene (0.88%) and citronellyl acetate (0.86%) was found. Other compounds with less than 0.5% were sabinene (0.37%), α-pinene (0.23%), β-pinene (0.32%), 2,6-dimethyl-5-heptanal (0.14%), geranyl acetate (0.32%) and farnesol (0.31%). Similarly, Suri et al. (1998) reported that the major flavour compound in leaf, stem and peel was citronellal which accounted for 95.52, 45.16 and 25.57% respectively, whereas the major flavour compound in fruit juice was 4-methyl-cyclohexanol (36.5%).

Effect of explant material

Analysis of flavour compounds in different parts of plantlet Out of eight flavour compounds identified in *C. hystrix* flowers, only six have been synthesized in plantlets. In addition, the amounts of these compounds present in plantlets are generally low compared to the intact plant *(Table 2).* In contrast, Hirata et al. (1989) reported that the quantity of monoterpenoids especially carvone and limonene in plantlets grown on a basal Gamborg B5 medium was higher than those in the mother plants and the

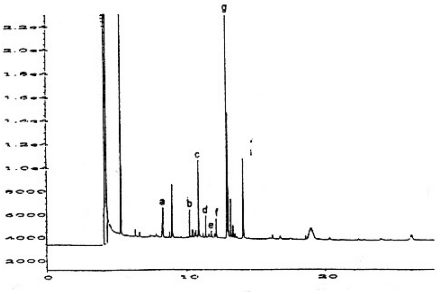

Figure 1. GC chromatogram of extracted flavour compounds in pollen and anther of ***Citrus hystrix*** *(a) cyclohexanol, (b) β-pinene, (c) p-cymene, (d) limonene, (e) γ-terpinene, (f) linalool, (g) citronellal and (i) citronellol*

Table 1. Flavour compounds in the selected parts of *Citrus hystrix* flower

Compounds	Petal (µg/g fwt.)	Ovary (µg/g fwt.)	Anther plus pollen (µg/g fwt. tissue)
Cyclohexanol	20.74 ± 1.75c	101.45 ± 13.69a	39.66 ± 0.24b
β-pinene	1.85 ± 0.08b	3.00 ± 0.28a	1.02 ± 0.11c
p-cymene	6.49 ± 0.32a	2.60 ± 0.18b	1.60 ± 0.09c
l-limonene	27.30 ± 1.42a	10.76 ± 0.01b	6.64 ± 0.24c
γ-terpinene	10.50 ± 0.50a	7.62 ± 0.08a	5.50 ± 0.38a
Linalool	40.18 ± 1.90a	27.53 ± 0.43b	9.97 ± 0.22c
Citronellal	1 595.30 ± 19.54a	1 759.65 ± 7.89a	314.37 ± 0.41b
Citronellol	180.33 ± 2.08a	66.38 ± 1.94a	42.06 ± 1.27b

Mean values with the same letter in each row are not significantly different using DMRT $p \leq 0.05$
Data indicated the mean ± standard error of mean of each determination (n = 3)

Table 2. Flavour compounds in the selected parts of *Citrus hystrix* young seedling

Compounds	Leaf (µg/g fwt.)	Stem (µg/g fwt.)	Root (µg/g fwt.)
Cyclohexanol	10.28 ± 0.62a	18.46 ± 2.62c	0.66 ± 0.02b
β-pinene	1.61 ± 0.21b	2.76 ± 0.25a	nd
l-limonene	54.96 ± 3.64b	101.62 ± 5.24a	nd
γ-terpinene	nd	1.88 ± 0.16a	nd
Linalool	nd	1.06 ± 0.14a	nd
p-cymene	nd	nd	0.24 ± 0.01a

Mean values with the same letter in each row are not significantly different using DMRT $p \leq 0.05$
Data indicated the mean ± standard error of mean of each determination (n = 3)
nd = not detected

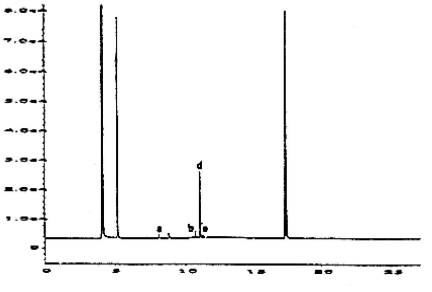

Figure 2. GC chromatogram of extracted flavour compounds in ***Citrus hystrix*** *young seedling leaf (a) cyclohexanol, (b) β-pinene, (d) limonene and (e)γ-terpinene*

production of flavour compounds was influenced by the addition of NAA and thyamine hydrochloride.

Among the three different tissues examined, the cyclohexanol content was low in root (0.66 ± 0.02 µg/g fwt.). The highest concentration of cyclohexanol (18.46 ± 2.62 µg/g fwt.) occurred in stems which was about 28 times higher than that obtained in roots. Leaf also contained cyclohexanol which was about 10.28 ± 0.62 µg/g fwt. *(Table 2)*. The flavour profile in leaf from plantlet was shown in *Figure 2*.

The presence of β-pinene and limonene were also found in stem and leaf of *C. hystrix* plantlets *(Table 2)*. The highest levels of these compounds (2.76 ± 0.25 µg/g fwt. and 101.62 ± 5.24 µg/g fwt.) were found in stem. Interestingly, limonene content was found higher than in any parts of flower. Other flavour compounds produced in plantlets were γ-terpinene and linalool which were found mainly in stems, whereas p-cymene was found only in root of *C. hystrix* (Suri et al. 2000)

Analysis of flavour compounds in callus derived from stem, petiole and embryo It was reported that callus derived from *C. hystrix* cotyledon using a basal MS medium supplemented with NAA (11.0 mg/litre) and kinetin (1.0 mg/litre) were unable to synthesize citronellal, the major compound in *C. hystrix* (Suri et al. 1998). Treatment of callus with phytohormones and bio-elicitors such as 2,4-dichlorophenoxy acetic acid (2,4-D) and kinetin, benzyl amino purine (BAP), yeast extract, casein hydrolysate and alginic acid did not produce any citronellal. Only cyclohexanol, α-pinene, β-pinene, limonene, p-cymene were produced after treatment with yeast extract, casein hydrolysate, maltose and alginic acid (Suri et al. 1999).

Another approach was carried out to induce callus from different types of explants such as peel, cotyledon, petiole, leaf, stem and embryo using a basal MS medium supplemented with NAA (2.0 mg/litre) and kinetin (1.0 mg/litre). Studies conducted earlier have shown that callus derived from the stem of *C. hystrix* was successfully optimized on a basal MS medium supplemented with NAA (2.0 mg/litre) and kinetin (1.0 mg/litre). Results showed that callus was successfully induced from stem, petiole and embryo explants after

1 week incubation at 27 ± 2 °C under the light condition *(Table 3)*. However, only leaf and peel did not form any callus. The highest callus formation was found in stem followed by petiole and embryo.

Analysis of flavour compounds in callus derived from different explants showed that only cyclohexanol and limonene have been synthesized. Callus derived from stems produced the highest cyclohexanol (14.14 ± 1.11 µg/g fwt.) as compared to embryo (0.16 ± 0.01 µg/g fwt.). Whilst, the highest production of limonene was found in callus derived from embryo ((1.84 ± 0.08 µg/g fwt.) *(Table 3)*. Therefore, the production of flavour compounds in callus might be influenced by type or source of explant used. According to Holden et al. (1988), variation found in a callus was strongly influenced by the explants material used from which it had been initiated. In addition, Nagel and Reinhard (1975) also reported that variation also arose in culture derived from explants of the same plant. For example, callus from shoot region of *Ruta graveolens* will only produce shoot-specific essential oils whereas those derived from roots will only produce root specific essential oils.

The production of flavour compounds in callus derived from different explants was still low compared to the flowers. According to Brown et al. (1987) unorganized cells showed little capacity to accumulate volatile monoterpene.

Effects of illumination

The effects of different types of light on cell growth and flavour compounds production are given in *Figures 3* and *4*. Maximum cyclohexanol (14.1 ± 1.01 µg/g fwt.) and limonene (1.48 ± 0.09 µg/g fwt.) were obtained for treatment under bright white cool fluorescent light compared to under dark condition (3.59 ± 1.11 µg/g fwt. and 0.56 ± 0.10 µg/g fwt.) and under dim condition (0.4 ± 0.01 µg/g fwt. and 0.33 ± 0.01 µg/g fwt.). Sahai (1994) reported that

Table 3. Flavour compounds in callus derived from different parts of *Citrus hystrix*

Compounds	Callus from petiole (µg/g fwt.)	Callus from embryo (µg/g fwt.)	Callus from stem (µg/g fwt.)
Cyclohexanol	nd	0.16 ± 0.01a	14.14 ± 1.11a
l-limonene	0.21 ± 0.01b	1.84 ± 0.08b	1.48 ± 0.09a

Mean values with the same letter in each row are not significantly different using DMRT $p \leq 0.05$.
Data indicated the mean ± standard error of mean of each determination (n = 3)
nd = not detected

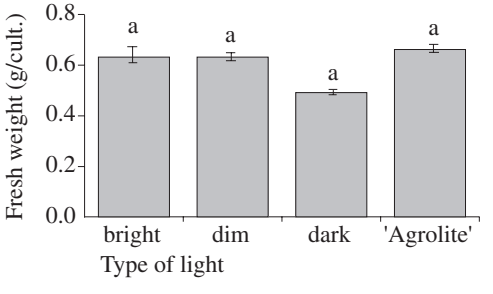

*Figure 3. Effect of different types of illumination on callus growth of **Citrus hystrix**. Mean values for the same type of light with same letter are not significantly different using DMRT p ≤0.05. Bar indicates the standard error of mean (n = 5)*

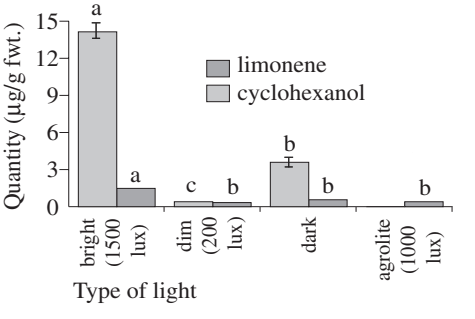

*Figure 4. Effect of different types of illumination on the production of flavour compounds from **Citrus hystrix** callus cultures. Mean values for the same type of compound with same letter are not significantly different using DMRT p ≤0.05. Bar indicates the standard error of mean (n = 3)*

the production of sesquiterpene in callus cultures of C*hamomilla matricaria* was remarkably stimulated by light. Similarly, Berger (1994) also reported that light induced cytodifferentiation and related accumulation of mono and sesquiterpenes were observed in mixotropic cell cultures of *Coleonema album* (Rutaceae). In addition, 41 volatile compounds mainly terpenes and phenylpropanoids were also detected in stem and leaf of *C. album*. According to Guenther et al. (1993), treatment under dark condition decreased the production of flavour compounds from 20% to 40% compared to light condition at the same temperature.

In this study the callus that was grown under 'Agrolite lamp' produced callus in green colour. However, the callus was unable to produce cyclohexanol, which was the major flavour compound in fruit juice of *C. hystrix* (Suri et al. 1999) These results contradicted with the statement by Schoofs et al. (1983) that accumulation of secondary metabolite in photoautotrophic cell cultures is based on the hypothesis that green callus will express the biosynthesis of secondary metabolite.

Effect of growth

The growth pattern of *C. hystrix* callus on the fresh and dry weight basis was established on a basal MS medium supplemented with NAA (2.0 mg/L) and kinetin (1.0 mg/L) *(Figure 5).* Calli were sub-cultured three times and allowed to grow for 7 weeks. The production of flavour compounds was then carefully monitored every week. The results showed that callus derived from stem reached a maximum weight (0.94 ± 0.08 g fwt. or 0.11 ± 0.01 g dwt.) after 6 weeks of incubation but declined thereafter, possibly due to cell death *(Figure 5).*

Analysis of callus using gas chromatography showed that only three flavour compounds (cyclohexanol, limonene and β-pinene) have been synthesized and the pattern of flavour accumulation in callus tissues are presented in *Figure 6*. Maximum quantity of cyclohexanol (15.1 ± 0.08 µg/g fwt.) and limonene (1.51 ± 0.10 µg/g fwt.) were obtained after 3 weeks of incubation. On the other hand, β-pinene showed the maximum production (1.11 ± 0.05 µg/g fwt) after 4 weeks of incubation *(Figure 6).* Similarly, Nabeta and Sugisawa (1983) reported that most of the flavour components from *Perilla frutescens* diminished within 2 weeks of culturing and the production of sesquiterpene gradually increased after 3–5 weeks. The results obtained however contrasted with the previous statement reported by Lindsey and Yeoman (1985) that most plant secondary metabolites accumulate late in the culture cycle after the cessation of cell division and

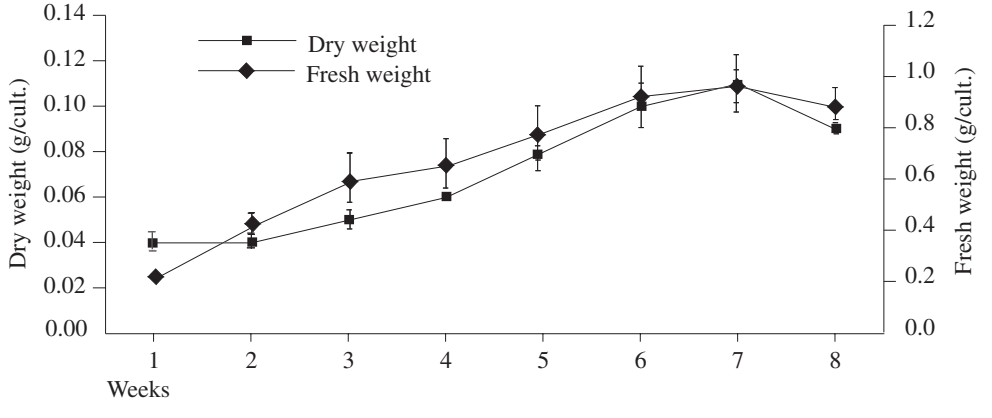

Figure 5. Growth of ***Citrus hystrix*** *callus derived from stem and incubated at 27 ± 2 °C under cool fluorescence light. Bar indicated the standard error of mean (n = 5)*

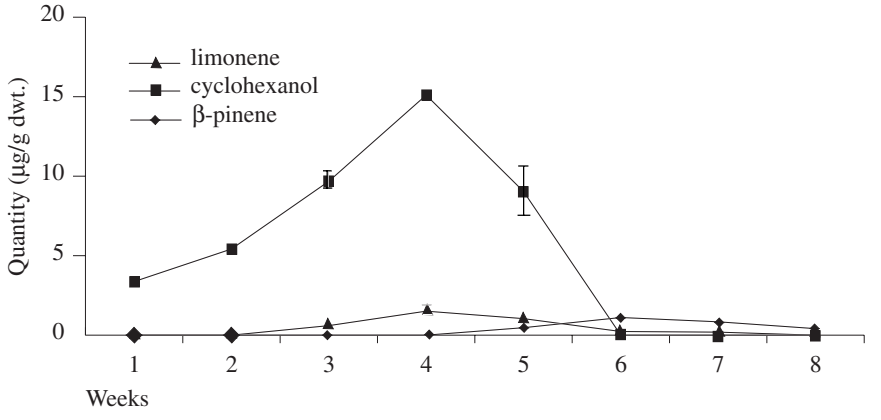

Figure 6. Production of major flavour compounds from ***Citrus hystrix*** *callus during incubation at 27 ± 2 °C under cool flourescence light. Bar indicates the standard error of mean (n = 3)*

closely associated with the process of cell differentiation.

Conclusion

Eight flavour compounds were identified in different parts of *C. hystrix* flower and citronellal was the highest compound in all tissues. Some of the compounds could be synthesized in plantlets grown in vitro. However none of any explant produced citronellal. The highest production of flavour compound was found in stem and stem derived callus.

Acknowledgement

The authors thank the Ministry of Science, Technology and the Environment and National Biotechnology Directorate, Malaysia for providing the research fund, Biochemistry and Microbiology Department, University Putra Malaysia (UPM) and Food Technology Center, Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Selangor for providing the laboratory facilities.

References

Berger, R.G. (1994). Biotechnological production of flavour- current status. In: *Understanding the Natural Flavour*. (Piggott, J.R. and Patterson, A., ed.) p. 179–93. Glasgow: Blakie Academic and Professional

Brown, J.T., Hegarty, P.K. and Charlwood, B.V. (1987). The toxicity of monoterpenes to plant cell culture. *Plant Sci*. ***48:*** 195–201

Burkill, I.H. (1966). *A Dictionary of the Economic Products of the Malay Peninsular*, vol. 1. Kuala Lumpur: Ministry of Agriculture and Cooperative, Malaysia

Guenther, A.B., Zimmerman, P., Harley, P.C., Monson, R.K. and Fall, R. (1993). Isoprene and monoterpene emmision variability: model evaluations and sensitivity analysis. *J. of Geophys. Res*. ***89:*** 12609–17

Hirata, T., Murakami, S., Ogihara, K. and Suga, T. (1989). Volatile monoterpenoid constituents of the plantlets of *Mentha spicata* produced by shoot tip culture. *Phytochem.* ***43:*** 493–5

Holden, M.A., Holden, P.R. and Yeoman, M.M. (1988). Elicitation of cell cultures. In: *Manipulating secondary metabolism in culture* (Robin, R.J. and Rhodes, M.J.C. eds.) p. 58–65. Cambridge: Cambridge University Press

Lindsey, K. and Yeoman, M.M. (1985). Cell culture and somatic genetics. In: *A comprehensive.* (Vasil, I., ed.) p. 61–101. New York: Treatise Academic Press

Muhammad Nor, O. and Tong, L.B. (1992). Development and application of *Citrus hystrix* for flavour for food. In: *Asean Workshop on the Production of Natural Flavours for Foods.* (Merican, Z., Midon, H., Tong, L.B., Yatim, A.M. and Abdullah, W.Y.W., ed.) p. 73–7. Kuala Lumpur: MARDI

Nabeta, K. and Sugisawa, H. (1983). *Volatile components produced by callus tissue from three Perilla plants*. N. York : Instrumental Analysis of Food Academic Press Inc.

Nagel, M. and Reinhard, E. (1975). Das aetherische del der callusklutur en von Ruta graveolens L. die zugammanset-zung des oeles. *Planta Med.* ***2:*** 151–8

Rouseff, R., Gmitter, F. and Grosser, J. (1994). Citrus breeding flavour. In: *Understanding Natural Flavours.* (Piggott, J.R. and Patterson, A., ed.) p. 114–27. Glasgow: Blakie Academic and Professional

Sahai, O.M. (1994). Plant Tissue Culture. In: *Bioprocess Production of Flavour and Fragrance and Color Ingredients.* (Gabelman, A., ed.). p. 239–74. Singapore: John Wiley and Sons

Sato, A., Asano, K. and Sato, T. (1990). The chemical composition of *Citrus hystrix* D.C. (Sewangi). *Journal Essen. Oil Res.* ***2:***179

Schoofs, G., Tieichmann, S., Hartman, T. and Wink, M. (1983). Lycine decarboxylase in plants and its interaction in quinolizidine alkoloid biosynthesis. *Phytochem.* ***22:*** 65 – 9

Sugisawa, H., Miwa, K., Matsuo, T. and Tamura, H. (1987). Volatile compounds produced from cultured cell of Thyme (*Thymus vulgaris* L.). *Bioflavour* ***11:*** 327–37

Sum, L.W. (1998). *Production of natural flavonoids from tissue culture of Citrus hystrix (Limau Purut)*. Thesis of Bachelor of Science (Hons), Department of Biochemistry and Microbiology, FSAS, Universiti Putra Malaysia

Suri, R., Musaalbakri, A.M., Normah, O. and Hassan, A. (1998). Effect of media and hormones on the flavour formation in 'Limau Purut' (*Citrus hystrix). Proc. 10th National Biotechnology Seminar; Biotechnology Towards the Next Millenium.* Malaysia: SIRIM

Suri, R., Radzali, M., Mohd. Aspollah, S., Marziah, M., Musaalbakri, A.M. and Normah, O. (1999). Effects of supplied elicitor on callus cultures of *Citrus hystrix* for the production of flavour compounds. In: *Medicinal Plants, Quality Herbal Products for Healthy Living*. p. 163– 8. Kepong: Forest Research Institute Malaysia

Suri, R., Radzali, M., Marziah, M. and Mohd. Aspollah, S. (2000). Increase of Certain flavour compounds in *Citrus hystrix* ('limau purut') after treatment with salicylic acid and phenylalanine. *Proc. of the 12 th National Biotechnology Seminar*. p. 308–12. Lumut, Malaysia. Kuala Lumpur: Universiti Malaya

Accepted for publication on 8 July 2002