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Micropropagation & Phytochemical Analysis of Mentha rotundifolia (L.) Huds

KEYWORDS: micropropagation, somaclonal variant, essential oil

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ABSTRACT *An efficient protocol for the micropropagation of Mentharotundifolia(L.) Huds, an aromatic medicinal plant of the family Lamiaceae was developed. Very highfrequency of shoot induction was obtained from the nodal cuttings on medium having a hormonal combination of BAP 2mg/l + IAA 2.5mg/l. Essential oil obtained from the field established plants were subjected to GC MS analysisto detect the chemical composition. A somaclonal variant plant having better essential oil profile was identified.Carvone was the major essential oil component in both the plants, but the percentage was higher in the somaclonal variant.*

INTRODUCTION

Aromatic plants have been of great interest to mankind from the beginning of human civilization. Aromatic plants and their derivatives particularly the essential oils are now becoming one of the most important export items from many developing countries. Most valuable phytochemicals are products of plant secondary metabolism. There is great potential to improve the yield and quality of these plants either by mere selection of existing species or varieties through plant breeding or by other novel methods of plant improvement.

Mints are considered today as the most important commercial essential oil bearing plant from the standpoint of worldwide production. The economic importance is due to the production of mint oil as a row material for confectionary, pharmaceutical and cosmetic industries as well as for flavouring food, beverages, tobacco, candies, medicines, tooth pastes, mouth wash and chewing gums (Bantrope, 1996).

Mentha is one of the most important taxa of the family Lamiaceae. M. rotundifolia, a perennial pubescent herb otherwise called apple mint is a delicious flavoured mint. Owing to its diversified uses the demand for mint oil is ever increasing. Decline of potential for the production of active principle or essential oil content is a common feature with cultivation of aromatic plants.In contrast to the conventional methods of vegetative propagation through rhizomes the use of invitro propagation enables the production of large number of variants. This is an alternative method for improving mint, which involve the use of tissue culture regeneration and the production of somaclonalvariants (Constable, 1990). Exploitation of possible somaclonal variation which has been observed in invitro cultures could be used to widen the genetic pool from which to select desirable traits. Variation is a ubiquitous phenomenon associated with tissue culture (Green, 1977).

MATERIALS AND METHODS

Micropropagation

M.rotundifoliaL.(Huds) cultivated in Nilgiris were collected and grown in Calicut University campus. The plant was authenticated at the herbarium of Botany Department, University of Calicut, where voucher specimens were deposited (CALI 8603). Nodal cuttings from three months old potted plants were used as explant to initiate the culture. Nodal cuttings were collected in water, expanded leaves were removed and the stem cuttings were washed with labolene detergent for 15 min. The tissue was again thoroughly washed in running water followed by a quick rinse (30 seconds) in 70% alcohol. This material was again washed in double distilled water. Surface sterilization was done using 0.1% mercuric chloride for 6 min. stem cuttings were then washed 5-8 times in double distilled water to remove the of $HgCl_2$. The tissue was trimmed again and dipped in 15% streptomycin for 5 min. to eliminate the endophytic bacteria found commonly in Mentha species (Reed, Buckley and De Wilde,1995) and implanted on to the culture medium. Small whole leaves collected from the in vitro elongated axillary buds were also used as explants.

Murashige and Skoog (1962) basal medium with 3% sucrose, 100mg/l myoinosrtol and 0.8% agar was used. MS basal medium was supplemented with different concentrations of auxins, cytokinins, different combinations of both these and also 15%-50% coconut water alone or in combination with BAP.

The pH of the medium was adjusted to 5.8. The media were sterilized at 120°C for 20 min. 10-15 replicates of each hormonal combination were tried. The cultures were grown at 25 ± 3°C with 55%-60% humidity under fluorescent day light tubes emitting 2000 lux for 16/8 h light/dark period and were subcultured every 5-7 weeks.

Four to six week old plants were subcultured in ½ MS medium for rooting. Root system with 5-10 roots was developed after 3 weeks growth in ½ MS medium for -8 days. The rooted plants were taken out and planted in a sterilized mixture of sand and soil (1:1) in plastic cups and initially covered with poythene bag to control humidity. Well established plants were transplanted to soil and tested for their essential oil composition.

Essential oil extraction

Shade dried aerial plant parts of both the parent and somaclonal variant were hydrodistilled separately in a Clevenger apparatus (Clevenger, 1928) at 100°C for 4 hrs as prolonged extraction normally increases the yield. The quantity of the essential oil was measured and the isolated oil was dried over anhydrous sodium sulphate and stored

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in small amber coloured bottles at 4°C. The percentage of essential oil was calculated on dry weight basis to avoid faulty estimation that may arise due to different water content of the tissues analysed each time.

Gas Chromatography- Mass Spectrometry (GC MS)
GCMS was carried out on a Shimadzu QP 2000 instrument at 70 eV and 250°C. GC Column: ULBON HR-1 equivalent to OV-1, fused silica capillary -0.25 mm x 50 m with film thickness 0.25 µ. The other conditions were: carrier gas – helium, flow rate 2 ml/min., temperature programme: initial temperature-100°C for 6 min. and then heated at the rate of 10°C per min. to 250°C. Mass spectral identification was bases on published spectra (de Brauw, Bowman, Tas and La Vos, 1979-1988).

Coefficient of Similitude
The data obtained from the qualitative analysis of both in vitro and in vivo developed plants were subjected to numerical analysis to understand the chemical affinity of both by arriving at a numerical constant, the coefficient of similitude (CS), following the formula proposed by Sokall and Sneath 1963 (CS = Number of similar components/ total number of components X 100).

RESULTS
Micropropagation
Murashige&Skoog (MS) basal medium with different hormonal combinations was used for micropropagation. For callus induction and multiple shoot regeneration, medium with auxins (2,4-D,IAA and NAA) alone, cytokinins (BAP and KIN) alone and different combinations of both these were used.

Direct multiple shoot induction was obtained from the nodal cuttings on medium having a hormonal combination of BAP 2mg/l + IAA 2.5mg/l after 3-4 weeks culture period. Frequency of shoot induction was very high. About 20-40 shoots arose from each node. 95% of the explants responded positively in this medium. Medium having a hormonal combination of BAP 0.2 mg/l + NAA 1 mg/l produced greenish swelling of the explant. Media with BAP 0.5 mg/l + IAA 1 mg/l and BAP 1 mg/l + IAA 1.5 mg/l produced single shoots from the explants. A combination of BAP 2 mq/l+ 2,4-D 2 mg/lresulted in rooting of 80% of the explants. White hard callus was produced on combinations like KIN 0.2 mg/l + 2,4-D 1.5 mg/l and KIN 0.2 mg/l + 2,4-D 2 mg/l. The in vitro developed white friable calli produced small roots when subcultured on to a medium with KIN 0.5 mg/l + 2,4-D 2.5 mg/l.

Small whole leaves collected from the in vitro elongated axillary buds induced direct multiple shoots in a medium with 25% coconut water, but the frequency of shoot induction was very low (3-5 shoots/leaf). CW 20% + BAP 1 mg/l combination could produce single shoot from 40% of the leaf explants.

The nodal cuttings produced white friable callus in a medium with 2,4-D 1mg/l+ KIN 0.2 mg/l. The stem-derived callus showed a low frequency of shoot regeneration when subcultured into medium withBAP 0.5 mg/l. The regeneration frequency in this case was very low (2-3 shoots). Nodal explants taken from this callus regenerated plants produced a large number of multiple shoots when inoculated in a medium with BAP 2mg/l + IAA 2.5mg/l. A few calli produced large number of small hairy roots but no shoots in medium with NAA 0.5mg/l.

A comparatively low frequency of shoot induction (5-10 shoots from each node) was obtained after 2-3 weeks when 25% coconut water was used instead of hormones. Medium with CW 35% produced only 1-2 shoots from 50% of the explants. Combinations like CW 20% + BAP 0.5 mg/l; CW 25% + BAP 1mg/l; CW 30% + BAP 1 mg/l; CW 40% + BAP 1 mg/l; CW 45% + BAP 1 mg/l and CW 50% + BAP 1 mg/l resulted in axillary bud elongation from the nodal explants. A combination of CW 15% + BAP 0.5mg/l resulted in swelling of the nodal region and callusing at the cut ends.

Nodal cultures in both the above mentioned multiple shoot induction media (BAP 2 mg/l + IAA 2.5mg/l and 25% CW) produced morphologically variant plants with varied number of leaves at the nodes, ranging from 1-4 leaves at each node. The frequency of production of morphological variants was 10-20%.

The cluster of multiple shoots was separated and subcultured for rooting on ½ MS medium. A cluster of 3-7 roots was developed after 2-3 weeks. Rooted plantlets were transferred to a sterilized mixture of sand and soil(1:1). The field survival frequency was 80%. Only those plants, which were morphologically similar to the parent plant, could establish successfully in the soil. The field-transplanted plants produced a little larger sized leaves compared to the parent plant. The plantlets obtained full maturity after a growth period of 2 months in the pots.

Since no remarkable morphological variation was noticed among the field established plants, further analyses at phytochemical level was conducted to search for the possible somaclonalvariants. In the present study GC-MS analysis revealed 18 components in the parent plant and 20 components in the somaclonal variant. Carvone was the major component in both the parent and the variant, but the percentage was higher in the variant (Fig 1-2). The complete essential oil profile of both the plants is given in Table 1.

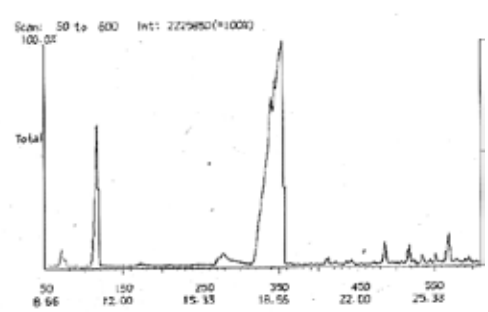

Fig 1: Gas Chromatogram of the essential oil of Mentha rotundifolia Parent plant

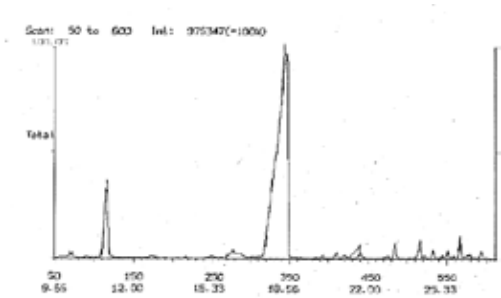

Fig 2: Gas Chromatogram of the essential oil of Mentha rotundifolia somaclonal variant

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Table. 1. Essential oil composition of the parent plant and the somaclonal variant of Mentharotundifolia

Sl no	Components (Parent plant)	Class	Composition %	
			Parent	Variant
1	α pinene	Monoterpene	Trace	-
2	Myrcene	,,	0.4	Trace
3	Limonene	,,	6.2	8.4
4	Sabinine acetate	,,	0.081	-
5	Ethyl 9,12 octadecadienoate	Ester	Trace	-
6	Trans 2 octenal	Aldehyde	Trace	-
7	Isopulegol	Monoterpene	Trace	-
8	Citronellol	,,	Trace	-
9	Carvone	,,	82	87.4
10	αterpinyl acetate	,,	0.3	0.2
11	Alloaromadendrene	Sesquiterpene	Trace	-
12	βelemene	,,	2.6	1.0
13	βcaryophyllene	,,	1.1	-
14	Trans 2 cis 6 nonadien-1-ol	Unsaturated alcohol	1.0	0.3
15	αcububene	Sesquiterpene	0.9	0.4
16	βGurgunene	,,	0.7	-
17	βterpineol	Monoterpene	Trace	Trace
18	Methyl chavicol	Phenol	-	Trace
19	Isoborneol	Monoterpdene	-	Trace
20	Cis-6-nonenal	Unsaturated aldehyde	-	0.176
21	2,3 diethyl-6-methyl pyrazine	Heterocyclic compound	-	Trace
22	p-mentha trans 2,8 dien-1-ol	Monoterpene	-	0.031
23	y caryophyllene	Sesquiterpene	-	0.4
24	aromadendrene	,,	Trace	0.056
25	β bisabolene	,,	-	1.0
26	1ethynyl-2-trimethylsilyl benzene	Benzene derivative	-	Trace
27	αamorphene	Sesquiterpene	-	0.091
28	Juniper camphor	Monoterpene	-	0.081
29	Di isobutyl phtalate	Ester	-	Trace

DISCUSSION

For the present study nodal explants were used, which is considered to be a low risk method for genetic instability. But variation among micropropagated plants was obtained at the morphological level. Phenotypic variations obtained among micropropagated plats may be due to several mechanisms of somaclonal variations like changes in chromosome number, structure etc., dominant and recessive mutations and changes in chloroplast and mitochondria (Bingham and McCoy, 1986). Stress induced by tissue culture process like hormone effects, nucleotide pool imbalance etc. can cause alterations in DNA. These alterations could affect the expression of affect specific genes (Kaepplerand Philips, 1993). Genetic variation is a common phenomenon associated with plant tissue culture. Plantlets derived from in vitro culture might exhibit somaclonal variation which is often heritable (Breiman, Rotem-Abarbanel, Karp and Shaskin1987). Shenoy and Vasil (1992) reported that micropropagation through meristem culture is generally associated with a low risk of genetic instability because the organized meristems are generally more resistant to genetic changes that might occur during cell division or differentiation under in vitro conditions. Variations may be due to several factors such as genotypes used, pathways of regeneration etc. ((Breimanet al., 1987). One of the possible mechanisms suggested explaining somaclonal variation is the activation of different classes of mobile genetic elements (Peschkeand Philips, 1991). Epigenetic variation is another important cause of somaclonal variation. This involves gene silencing or gene activation (Kaeppler, Kaeppler and Rhee, 2000).

The morphogenetic response of the explant is mainly based on the type and concentration of the hormone used.Plant tissue culture has the potential to perform biochemical reactions when organic compounds were added to the medium. The capacity of cultured plant cells to serve as catalysts for biochemical reactions such as peroxidation, esterification, glycosylation, methylation, isomerization and dehydrogenation of organic compounds has been reported earlier (Furuya 1978).

Qualitative and quantitative production of mint essential oil is controlled by simple genetic systems (Lincon, Murray-

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and Lawrence, 1986). In the present study GC-MS analysis revealed 21 components each in the parent plant and the somaclonal variant. The major component was carvone in both the cases but the percentage was higher in the variant.

The lesser value of coefficient of similitude (27.27) when the essential oils of both the plants were compared showed the dissimilar nature of the essential oil. This dissimilarity may be probably due to the variations in the biosynthetic pathways of essential oils. The nutritional component of the culture medium has been known to affect secondary product formation. High doses of growth promoters can change the content of secondary metabolites. (Misawa, 1985).

Plant cell cultures have produced (i) new compounds previously not known in the parent plant, (ii) new derivatives of the intact plant and (iii) new compounds by biotransformation. Production of new compounds may be due to altered gene function in cultured cells. The metabolism of monoterpenes is strongly influenced by environmental factors (Burbott and Loomis, 1967).

REFERENCE

1. Bantrope, D. V. (1996): Mentha species (mints): in vitro culture and production of lower terpenoinds and pigments. In: Biotechnology in Agriculture and Forestry Vol.37. Medicinal and Aromatic Plants. P.S. Bajaj (ED) Springer Verlag, Berlin, pp. 202-225. | 2. BinghamE. T.&McCoy T. J.(1986)Somaclonal variation in alfalfa. Plant Breed. Rev. 4: 123-125. | 3. BreimanA., Rotem-AbarbanelD., Karp A.&Shaskin, H. (1987) Heritable somaclonal variation in wild barley (Hordeumspontaneum). Theor. Appl. Genet. 71: 637-643. | 4. BurbottA. J.& Loomis W. D. (1967)Effects of light and temperature on the monoterpene of peppermint. Plant Physiol. 42: 20-18. | 5. Clevenger J. F. (1928) Apparatus for the determination n of volatile oil. J. Am. Pharm. Assoc. 13:346. | 6. Constable F.(1990) Medicinal Plant biotechnology. Planta Med. 56: 421-425. | 7. deBrauwMc, N., Bowman J., Tas A C& La Vos G. F. (1979-1988). Compilation of Mass Spectra of volatile components in Food. Vol. 1-16. Central Institute for Nutrition and Food Research, TNO, The Netherlands. | 8. FuruyaT. (1978) In: Frontiers of plant tissue culture. T. A. Thorpe (Ed) University of calgari, Calgary. p 191. | 9. Green E. C. (1977) Prospects for crop improvement in the field of cell cultures. Hortic. Sci. 12: 131-134. | 10. Kaeppler S. M. & Philips R. L., (1993). DNA Methylation and tissue culture induced variation in plants. In vitro Cell Dev. Biol. 29: 125-130. | 11. KaepplerS. M., Kaeppler H. F. & Rhee Y. (2000) Epigenetic aspects of somaclonal variation in plants. Plant Mol, Biol., 43: 179-188. | 12. LinconD. E., Murray M. J. & Lawrence B. M. (1986) Chemical composition and genetic basis for the isopinocamphonechemotype ofMenthacitrata hybrids Phytochemistry 25(8): 1857-1863. | 13. Misawa M.(1985) Production of careful plant metabolites. In: advances bio chemical Engineering/ Biotechnology 31. Plant cell cultures. Springer Verlag Berlin. pp 59-88. | 14. Murashige T.&Skoog F. 1962 A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497. | 15. PeschkeV. M. & Philips R. L. (1991). Activation of the maize transposable element suppressor- mutator in tissue culture Theor. Appl. Genet. 81: 90-97. | 16. Reed B. M., Buckley P. M., & De Wilde T. N. (1995) Detection and eradication of endophytic bacteria from micropropagated mint plants. In vitro Cell Dev. Biol. 31: 53-57. | 17. Shenoy, V. B. &Vasil I. K. (1992) Biochemical and molecular analysis of plant derived from embryonic tissue culture of Napier grass Pennisetumpurpurascens. Theor. Appl. Genet. 69: 575-581. | 18. SokallR. R. &SneathP. H. A. (1963) Principles of numerical taxonomy W.H. Franciscan (Ed) San Francisco. |