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In vitro micropropagation of *Mentha piperita* L. – an important medicinal plant

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ABSTRACT

In vitro propagation of *Mentha piperita* L. (Lamiaceae) by tissue culture technique was applied to multiply large populations of plants with similar characteristics. Apical and nodal segments were cultured on Murashige and Skoog solid (MS) medium supplemented with 1, 2, 3, and 4 mg/l BAP (6-benzylaminopurine) and Kinetin (Kn) alone and in different combinations. The number and length of propagules increased with the concentration of cytokinin and decreased with more concentration. The treatment containing 4.00 mg/l BAP + 1.00 mg/l Kn showed high frequency (85%) of shoot induction and maximum number (42 ± 0. 27) of propagules compared to all the concentrations tested. The high frequency (90%) and more root proliferation of shoot occurred on medium containing 2 mg/l IBA (Indole butyric acid). After hardening, the rooted plants were transferred to the greenhouse where they normally grew, matured and flowered with a survival rate of 90 %. We concluded that the present protocol can be efficiently used for mass propagation of *M. piperita*.

Key words: *Menthe piperita, Peppermint,* Lamiaceae.

INTRODUCTION

Mentha piperita L. is commonly called as Peppermint, a perennial glabrous and strongly scanted herb belonging to family Lamiaceae. It thrives well in humid and temperate climate and usually cultivated for its essential oils (Bersaghi, 1945). The oil is used commercially in herbal products, pharmaceutical products, cosmetics, for flavourings foods, toothpaste, mouthwashes, beverage and alcoholic liquors (Ohloff, 1994). It is also found in some shampoos and soaps, which give the hair a minty scent and produce a cooling sensation on the skin. The plant is stimulant, stomachic, carminative, headache, vomiting and for allaying nausea (Anonymous, 1962). This diverse utilization has promoted extensive cultivation of peppermint especially for industrial processing (Tariqul Islam *et al.,* 2005; Rech and Pires, 1986 and Ahmed *et al*., 2001).

During the last few years, *in vitro* culture techniques have been developed into a successful and rapid mean of asexually propagating a number of plant species. Clonally propagating by tissue culture is highly desirable to regenerate sufficient populations of plants with similar characteristics and reduces the risks of loss through human error and genetic instability what occurring with others methods (Bajaj *et al*., 1988 and Jullien *et al*., 1998). Also, plant tissue culture provides disease free material (Withers 1980; Chomchalow and Sahavachrin, 1981). Regeneration from axillary bud (Rech and Piresm 1986; Ravishankar and Venkataraman, 1986) leaf (Repcakova *et al*.,1986.), via organogenesis (Van Eck and Kitto, 1992), nodal (Kukreja, 1996) and shoot tip explant (Kiran *et al*., 2003) of *M. piperita* were reported with some of the serious limitations such as low frequency, inconsistent and occurrence of callus phase during organogenesis. Hence, it became imperative to establish a suitable protocol for *in vitro* propagation of this medicinally important species. Such conservation

efforts would ensure continuous and ample supply of this valuable material which is in great demand by the pharmaceutical industry. In the present study an attempt has been made to establish an experimental conditions for rapid multiplication of *M. piperita* using different concentrations and combinations of Kinetin (Kn), 6-benzyl amino purine (BAP) and alpha naphthalene acetic acid (NAA) in the nutrient medium (MS basal medium).

MATERIALS AND METHODS

Apical and nodal segments of *M. piperita* approximates 30 to 40 mm long were harvested from two months old greenhouse grown plants. In order to prevent the oxidation of phenolic compounds initially released from the material, the explants were washed in tap water for 15 minutes fallowed by aqueous solution containing 1% (v/v) soap for 3-5 minutes by constant shaking and rinsed 4-5 times with distilled water. The explants were again surface sterilized by 0.1% $HgCl_2$ for 1min. and rinsed thoroughly with sterilized double distilled water for 5 minutes (two cycles) to remove any traces of $HgCl_2$ from the surface.

The explants with 5 cm long were placed on MS medium (Murashige & Skoog, 1962) supplemented with different concentrations and combination of Kn (1-4 mg/l) and BAP (1-4 mg/l). Culture media were adjusted to pH 5.7-5.8 before adding 0.8% (wt/vol) agar, and they were sterilized by autoclaving at 121º C for 20 min. All cultures were maintained under a photoperiod regime of 16 h light, provided by Philips cool white fluorescent lamps (25 µmol.m2. s1) for 4 weeks at 26 ± 1º C. Each treatment was applied to 20 explants which were incubated vertically in 25×150 mm culture tube containing 20 ml of medium, and capped with a plastic cap. The data were submitted to analysis of variance and regression test and mean separation was tested using Duncan's test. The significance level was fixed at $P < 0.01$. The propagules were rooted on MS medium containing different concentrations of NAA(1-3 mg/l) and IBA (1-3 mg/l) after 4 weeks. Plantlets rooted were transferred to greenhouse conditions to acclimatization under nebulization intermittente water misting applied automatically.

RESULTS AND DISCUSSION

Induction of shoots from shoot tip and nodal explant: Totally 2 different cytokinins with different concentration and combinations were used for shoot induction. Explants responded within 11-17 days after inoculation and the effects of kinetin concentrations on soot multiplication from shoot tip and nodal explants are presented in Table 1 and 2. Percent frequency (Graph-1) of the shoot induction with respect to growth regulators and no of shoots from each explant versus growth regulators in each treatment were calculated and is represented in Table-1 and 2. It was observed that explant type and kinetin concentration had a significant ($P = 0.05$) influence on the number of propagules produced per explant as well as on the length of regenerated propagules.

Table - 1: Effect of cytokinins on shoot induction from shoot tip explants of *M. piperita* L.

Hormonal conc. (mg / l)	No. of tubes inoculated	No. of explants responded	Days to respond	Frequency (%)	Average No. of shoots	Average length of shoots (cm)
BAP						
1.00	20	11	12-13	55	11 ± 0.21^{h}	2.3 ± 0.27^{b}
2.00	20	13	12	65	13 ± 0.15^{g}	3.1 ± 0.12^{a}
3.00	20	13	15-16	65	27 ± 0.05^{c}	3.3 ± 0.27^{a}

4.00	20	20	12-13	75	39 ± 0. 25[b]	3.5 ± 0. 33[a]
Kn						
1.00	20	16	11-12	**80**	**14 ± 0. 34**[f]	2.9 ± 0. 20[b]
2.00	20	14	12-13	70	10 ± 0. 25[i]	**3.1 ± 0. 19**[a]
3.00	20	13	13-15	65	7 ± 0. 22[j]	2.3 ± 0. 00[b]
4.00	20	11	13-16	55	3 ± 0. 10[k]	2.7 ± 0. 13[b]
BAP + Kn						
4.00 + 0.50	20	13	11-12	65	17 ± 0. 09[e]	3.3 ± 0. 10[a]
4.00 + 1.00	20	17	10-11	**85**	**42 ± 0. 27**[a]	**3.7 ± 0. 23**[a]
4.00 + 1.50	20	14	11-12	70	23 ± 0. 05[d]	2.8 ± 0. 31[b]
4.00 + 2.00	20	12	14-15	60	11 ± 0. 14[b]	2.2 ± 0. 10[b]

NOTE: Data represents average of three replicates; each replicate consists of 10 cultures.
Mean ± standard error, Values within columns with different letters indicating significant differences according to the Duncan's test at the level of 0.05%.

Effect of BAP on *in vitro* shoot proliferation: The frequency and number of shoots/explants increased as the concentration of BAP increased from 1- 3 mg/l as the concentration increased beyond this the frequency and number of shoots/explant reduced. Amongst the concentration tested BAP at 4mg /l (75% with 39 ± 0. 25 no of shoots and 3.5 ± 0. 33 cm shoot) showed best response fallowed by 3mg /l (65% with 27 ± 0. 05 no. of shoots) from shoot tip explant (Graph-1 Table-2 and Plate-1). Whereas nodal explant responded best at 3.00 mg/l with 65% frequency 23 ± 0.04 number of shoots (Table-2); 3.7 ± 0.33 cm shoot length fallowed by 4mg /l with 60% frequency of shoot induction and 17 ± 0.15 no of shoots (Graph-I; Table – 2 and Plate-1) within 12-16 days and 9-12 days of inoculation respectively. Poovaiah *et al.* (2006 a, b) reported that nodal explants exhibited better regeneration capacity than leaf explants in *M. piperita*. Nodal explants are the best source of explants used for the multiplication of shoots. This has been suggested earlier in the case of other medicinal plants such as *Rauwolfia serpentia* (Roy *et al*., 1995), *Emblica officinails* (Rahman *et al*., 1999) and *Solanum nigrum* L. (Jabeen, *et al*., 2005) Plant growth regulators NAA and kinetin have proved to be fruitful in shoot regeneration of *Mentha piperita*. NAA and kinetin (0.40 mg/l each). Least response was of BAP 1mg/l with 11 ± 0. 21 and 8 ± 0.11 no. of shoots from shoot tip and nodal explants respectively (Table-1 and 2).

Effect of Kn *in vitro* shoot proliferation: The frequency and number of shoots/explants increased as the concentration of kinetin increased from 1- 2 mg/l as the concentration increased beyond this the frequency and number of shoots/explant reduced. Amongst the all the concentration tested Kn at 1mg /l with 80% with 14 ± 0. 34 no. of shoots showed best response fallowed by 2mg /l with 10 ± 0. 25 no. of shoots from shoot tip explant (Graph-1 Table-1). Whereas nodal explant responded best at 2.00 mg/l with 70% shooting frequency and 19 ± 0.11 no. of shoots (Graph-1; Table–2 and Plate-I) from nodal fallowed by 3mg /l with 10 ± 0.16 no of shoots within 11-13 days and 12-13 days of inoculation respectively. Maximum shoot length (3.9 ± 0.17 cm, Plate –2) was also observed on the same combination of MS medium. Tejavathi *et al*, 2001, also found that maximum numbers of plants were obtained on medium containing Kin (1mg/l) in shoot tip cultures of *Bacopa moneri*. Least response was of Kn 4.00 mg/l with 3 ± 0. 10 and 7 ± 0.11 no. of shoots from shoot tip and nodal explants respectively.

Effect of BAP in combination with Kinetin: The concentrations 4.00 mg/l BAP + 1.00 mg/l Kn showed high frequency (85%) of shoot induction and maximum number (42 ± 0. 27) of shoots formation from shoot tip

explant within 10 -12 days of inoculation fallowed by 4.00 mg/l BAP + 1.50 mg/l Kn with 70% of frequency within 11 -12 days of inoculation (Table-1; Graph-1).

Whereas the nodal explant responded best for 3.00 mg/l BAP + 1.50 mg/l Kn with 12 ± 0.32 no. of shoots within 12-13 days of inoculation with 50% of frequency fallowed by 3.00 mg/l BAP + 1.00 mg/l Kn with 45 % of frequency within 15 -16 days of inoculation (Table-2; Graph-1 and Plate -1). As the concentration of kinetin increases the frequency and number of shoots increased till 0.50 – 1.50 mg/l, beyond this concentration the frequency of shoot induction and number of shoots per explant reduced. Our results are parallel to Shrivastva and Rajani 1999, who reported that out of two cytokinins used BAP was found to be more suitable than Kn as BAP resulted in quicker and better response then the latter. Usha *et al*., 2007. Showed among cytokinins used BAP in combination with Kn showed better results in shoot induction and proliferation from shoot tip culture of *Vitex negundo* L.

Table - 2: Effect of cytokinins on shoot induction from nodal explants of *M. piperita* L.

Hormonal conc. (mg / l)	No. of tubes inoculated	No. of explants responded	Days to respond	Frequency (%)	Average No. of shoots	Average length of shoots (cm)
BAP						
1.00	20	11-13	12	60	8 ± 0.11[f]	3.1 ± 0.33[e]
2.00	20	15-16	11	55	12 ± 0.18[d]	3.3 ± 0.06[d]
3.00	20	09-11	13	**65**	**23 ± 0.04**[a]	**3.7 ± 0.33**[b]
4.00	20	11-12	12	60	17 ± 0.15[c]	2.1 ± 0.12[h]
Kn						
1.00	20	12-13	13	65	8 ± 0.45[f]	3.0 ± 0.28
2.00	20	12-13	14	**70**	**19 ± 0.11**[b]	**3.9 ± 0.17**[a]
3.00	20	13-14	10	50	10 ± 0.16[e]	2.9 ± 0.10[f]
4.00	20	16-17	10	50	7 ± 0.11[g]	2.7 ± 0.29[g]
BAP + Kn						
3.00 + 0.50	20	11-12	8	40	7 ± 0.23[g]	2.7 ± 0.21[g]
3.00 + 1.00	20	15-16	9	45	10 ± 0.13[e]	3.1 ± 0.33[e]
3.00 + 1.50	20	12-13	10	**50**	**12 ± 0.32**[d]	**3.5 ± 0.37**[c]
3.00 + 2.00	20	17	8	40	6 ± 0.20[h]	2.1 ± 0.33[h]

NOTE: Data represents average of three replicates; each replicate consists of 10 cultures. Mean ± standard error, Values within columns with different letters indicating significant differences according to the Duncan's test at the level of 0.05%.

Rhizogenesis: The regenerated shoots were subjected to different concentrations of auxins (NAA and IBA) for rhizogenesis (Table-3). The highest frequency (90%) of rooting was recorded at 2 mg/l concentration of IBA followed by 1, 3 mg/l IBA (80%) and 2 mg/l NAA (85.6%) (Graph-2). Maximum number of roots (8 ± 0.0.05) per shoot was observed in 2 mg/l IBA followed by 2 mg/l NAA and 1 mg/l IBA with 7 ± 0. 05 and 6 ± 0. 27 number of roots per explant respectively. Maximum length of root (9.3 ± 0. 23 cm) was observed in 2 mg/l IBA followed by 1 mg/l IBA (9.1 ± 0.18) and 3 mg/l IBA with 8.9 ± 0. 18 cm (Graph 2 and Plate-1). Profuse rooting was obtained in IBA (0.1 mg/l) in the case of *Morus indica* (Jagadishchandra and Sathyanarayana, 2001) whereas 4 mg/l IBA was required for profuse rooting in *Gemelina arborea* (Yang, *et al*.,1992).

Acclimatization: Totally 40 plantlets were transferred to poly cups and were kept in controlled conditions in green house (Plate-XI). Within 4h of transfer, the plantlets showed partial drooping symptoms. But after 24h, 50% of the plantlets regained turgidity and the remaining plantlets took 3 - 4 days for recovery. About 70 ± 0.02% of the plantlets survived within a period of week, the survivability reduced to 60 ± 0.02% and 56.7% in green house conditions during second and third week, respectively. After four weeks the survivability rate increased to 90.3 ± 0.02%, these survived plantlets got well established.

Survivability: Out of 100 plantlets, about 96 plantlets survived within a period of week, the survivability reduced to 94 and 92 in green house conditions during second and third week, respectively. After four weeks about 92 plants were survived and got well established.

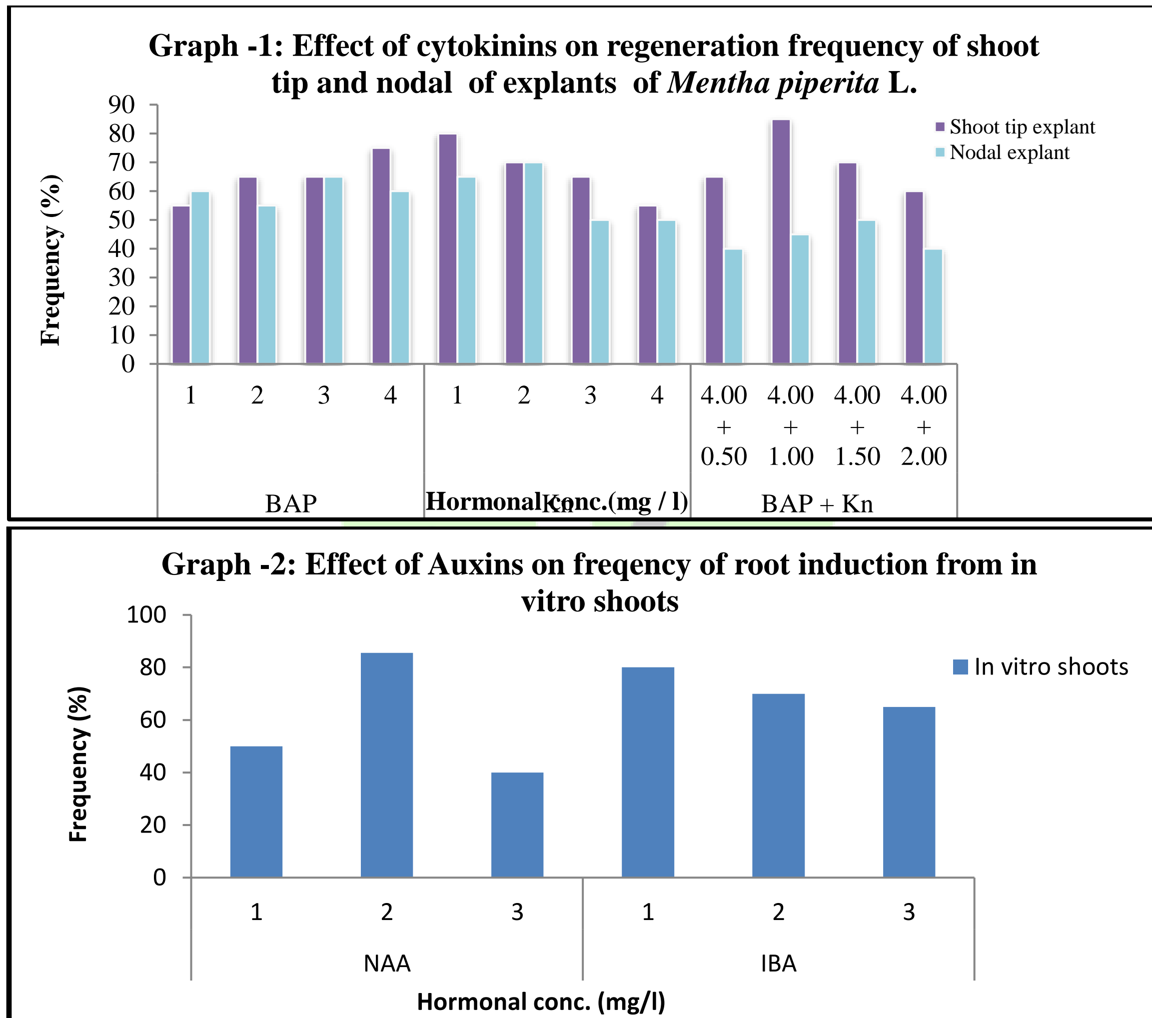
Table – 3: Response of *In vitro* grown *Mentha piperita* L. shoots for rhizogenesis

Hormonal conc. mg / l	No. of explant inoculated	No. of explant responded	Days to respond	Frequency (%)	No. of Roots/ explant	Root length/ explant (cm)
NAA						
1.00	10	5	15-16	50	3 ± 0. 04[f]	6.5 ± 0. 21[c]
2.00	10	7	12-13	70	**7 ± 0. 05**[b]	**8.5 ± 0. 27**[b]
3.00	10	4	17-18	40	5 ± 0. 15[d]	5.7 ± 0. 25[d]
IBA						
1.00	10	8	14-17	80	6 ± 0. 27[c]	9.1 ± 0.18[a]
2.00	10	9	8-10	90	**8 ± 0. 05**[a]	**9.3 ± 0. 23**[a]
3.00	10	8	13-12	80	4 ± 0. 05[e]	8.9 ± 0. 18[b]

NOTE: Data represents average of three replicates; each replicate consists of 10 cultures.
Mean ± standard error, Values within columns with different letters indicating significant differences according to the Duncan's test at the level of 0.05%.

Conclusion

In view of the medicinal properties and increased demand of *Mentha piperita* in pharmaceutical industry, a simple, clear and reliable protocol for large scale *in vitro* multiplication of *M. piperita* through direct regeneration have been achieved. The outlined procedure offers a potential system for improvement, survivability and mass propagation of this important medicinal plant.



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