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Rapid regeneration of *Mentha piperita* L. from shoot tip and nodal explants

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A high frequency and rapid regeneration protocol was developed from shoot tip and nodal explants of *Mentha piperita* L. on Murashige and Skoog's (MS) medium supplemented with either 6-benzyl amino purine (BAP; 1 mg/l)) or zeatin (0.25 mg/l). The highest number of shoots (49.8) was obtained on medium containing BAP. The regenerated dwarf shoots were further elongated on MS medium supplemented with gibberellic acid (GA_3; 1 mg/l). *In vitro* shoots were then excised from shoot clumps and transferred to rooting medium containing naphthalene acetic acid (NAA; 1 mg/l). The rooted plantlets were hardened on MS basal liquid medium and subsequently in polycups containing sterile soil and vermiculite (1:1). Plantlets, thus, developed were successfully established and finally transferred to a greenhouse. The plantlets showed high survival rate (90%) in the soil.

Keywords: BAP, GA_3, IBA, *Mentha piperita*, MS medium, NAA, zeatin

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Introduction

Peppermint (*Mentha piperita* L.) is a perennial, glabrous and strongly scented herb belonging to family *Lamiaceae*. It thrives well in humid and temperate climate and most widely cultivated in temperate region of Europe, Asia, United States, India and Mediterranean countries. However, it is sensitive to drought. The plant is aromatic, stimulant, stomachic, carminative and used for allaying nausea, flatulence, headache and vomiting[1]. Peppermint oil is one of the most popular and widely used essential oil in food products, cosmetics, pharmaceuticals, dental preparations, mouth washes, soaps, chewing gums, candies, confectionery and alcoholic liqueurs[2].

Plant regeneration from axillary bud[3,4], leaf[5] via organogenesis[6,7] and nodal explant[8] has earlier been reported in *M. piperita*. However, some of the serious limitations in the above mentioned protocols were low frequency, inconsistent and less number of shoots and occurrence of callus phase during organogenesis. Further, ruthless exploitation has resulted in drastic decrease of this natural resource. Hence, it became imperative to establish a suitable protocol for rapid *in vitro* propagation of this medicinally important species.

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Materials and Methods

M. piperita (L.) plants were obtained from Botanical garden, Gulbarga University. Shoot tips and nodes (1 cm long), excised from healthy plants, were pretreated with Teepol (5% v/v) for 5 min, surface sterilized with 0.1% mercuric chloride for 2-3 min and finally rinsed with 3-4 times in a sterile double distilled water to remove traces of mercuric chloride. The shoot tip and nodal explants were inoculated by inserting their cut-ends in the MS medium[9] supplemented with 0.5, 1, 1.5 and 2 mg/l of BAP, Kn or zeatin to induce multiple shoots. The medium contained 3% (w/v) sucrose and solidified with 0.8% (w/v) agar. It was adjusted to *p*H 5.6 and autoclaved at 121°C, 15 lb pressure for 15 min. The cultures were maintained at 25 ± 2°C under a light intensity of 3000 lux provided by cool-white fluorescent lamps.

In vitro initiated shoots from both the explants were excised after 30 days and cultured on MS medium, supplemented with 0.5, 1, 1.5 and 2 mg/l of gibberellic acid (GA_3), for shoot proliferation and elongation. The shoots (5-6 cm long) bearing at least 4-5 internodes were excised from the mass of proliferated shoots and transferred to rooting medium containing 0.5, 1, 1.5 and 2 mg/l of either indole butyric acid (IBA) or naphthalene acetic acid (NAA). Rooted plantlets were transferred to polycups containing sterile soil and vermiculite (1:1) and covered with plastic bag to maintain 85-90%

humidity. Subsequently, the plantlets were transferred to greenhouse after one month and planted in the soil.

Statistical Analysis

The experimental design was random and factorial with auxins and cytokinins as independent variables. The data pertaining to number of multiple shoots, shoot elongation and rooting were subjected to analysis of variance (ANOVA) test. Mean separation was done using Duncan's New multiple Range test (DNMRT). Thirty cultures were raised for each treatment and all experiments were repeated twice.

Results and Discussion

Emergence of multiple shoot buds from shoot tip and nodal explants on MS medium supplemented with BAP, Kn and zeatin was observed without an intervening callus phase. Shoot buds emerged on 10th and 14th day of culture from nodal (Fig. 1a) and shoot tip explants, respectively. However, shoot started proliferating (Fig. 1b) after 15-20 days. Single node explants elicited more numbers of multiple shoots as compared to shoot tip explants in medium containing BAP, Kn and zeatin (Table 1). Nodal explants as the best source of multiple shoot induction have also been suggested in case of other medicinal plants, such as *Rauwolfia serpentina*[10], *Emblica officinalis*[11], *Holarrhena antidysenterica*[12] and *Enicostemma hyssopifolium*[13].

Multiple Shoot Induction

Cytokinins, especially BAP, were reported to overcome apical dominance, release lateral buds from dormancy, and promote shoot formation[14]. Hence, different concentrations of BAP, Kn and zeatin were evaluated on shoot formation. The results presented in Table 1 show higher frequency and higher number of shoots in 0.5 and 1 mg/l of BAP (31.9-49.8 shoots) and 0.25 mg/l of zeatin (32.3-33.3 shoots) in both shoot tip and nodal explants. Further increase in the concentration of both reduced the frequency and number of shoots, indicating an upper limit in concentration of BAP and zeatin for *M. piperita*. On the other hand, Kn showed poor response to multiple shoot induction as compared to BAP and zeatin with both the explants; maximum, 14.3 and 19.7 shoots were recovered with shoot tip and nodal explants, respectively in 1 mg/l of Kn (Table 1).

Multiple shoot formation was also reported in other medicinal plants, such as *Bacopa monnieri*[15], *Phyllanthus caroliniensis*[16] and *Celastrus paniculatus*[17]. However, only 2 plantlets/explant (axillary bud) were generated in *M. piperata* on NAA and IAA+2,4-D supplemented medium[4]. Multiple shoot formation in Mentha was also reported from calli derived from mature and immature embryos, and leaves on MS medium supplemented with either BAP+NAA or BAP alone in two separate studies[6,7].

Table 1—Frequency, number of shoots and shoot length per explant obtained from shoot tip and nodal explants of *M. piperita* on MS medium containing different concentrations of BAP, KN and zeatin

Plant growth regulator	Shoot tip			Node		
	Frequency (%)	No. of shoots/explant	Shoot length/explant (cm)	Frequency (%)	No. of shoots/explant	Shoots length/explant (cm)
BAP						
0.5	98	31.90 ± 1.77^{b}	5.00 ± 0.21^{a}	96	38.40 ± 1.35^{b}	5.27 ± 0.22^{a}
1.0	100	44.50 ± 2.85^{a}	3.13 ± 0.08^{d}	100	49.80 ± 1.71^{a}	3.34 ± 0.09^{e}
1.5	95	22.10 ± 1.43^{d}	2.62 ± 0.19^{e}	94	28.80 ± 1.60^{de}	3.04 ± 0.10^{e}
2.0	92	12.10 ± 1.35^{f}	1.46 ± 0.08^{h}	88	17.40 ± 0.86^{fg}	1.72 ± 0.08^{h}
KN						
0.5	82	06.20 ± 0.49^{g}	4.23 ± 0.13^{b}	78	08.40 ± 0.43^{h}	4.69 ± 0.08^{b}
1.0	85	08.40 ± 0.52^{g}	3.92 ± 0.07^{c}	82	15.30 ± 0.60^{g}	4.15 ± 0.11^{c}
1.5	88	14.30 ± 0.60^{ef}	2.97 ± 0.08^{d}	84	19.70 ± 0.76^{f}	3.71 ± 0.08^{d}
2.0	84	06.10 ± 0.57^{g}	1.78 ± 0.26^{g}	80	09.80 ± 0.57^{h}	2.70 ± 0.05^{f}
Zeatin						
0.25	96	32.30 ± 1.08^{b}	2.16 ± 0.23^{f}	100	33.30 ± 0.87^{c}	2.36 ± 0.04^{g}
0.50	90	28.00 ± 0.92^{c}	2.51 ± 0.19^{e}	97	30.00 ± 0.76^{d}	3.22 ± 0.06^{e}
0.75	88	21.80 ± 0.76^{d}	3.28 ± 0.24^{d}	95	26.10 ± 0.74^{e}	4.30 ± 0.07^{c}
1.00	85	15.90 ± 0.59^{d}	4.30 ± 0.34^{b}	92	18.50 ± 0.76^{f}	3.35 ± 0.07^{e}

Means with different letters are significant at 0.05% level

However, neither number of shoots/explant was reported, nor nodal and shoot tip explants were used in these studies. In the present study, on the other hand, 49.8 and 33.3 shoots/explant were produced in 1 mg/l of BAP and 0.25 mg/l of zeatin, respectively with single node explants. Similar trend for zeatin was also reported in *Ensete superbum*[18]. Ravishankar and Venkataraman[4] reported induction of multiple shoots in *M. piperata* by Kn but the growth of explants was found slow in comparison with IBA; the number of shoots per explant was also not mentioned. Also, in the present study, although Kn induced the multiple shoots but response was poor as compared to BAP and zeatin.

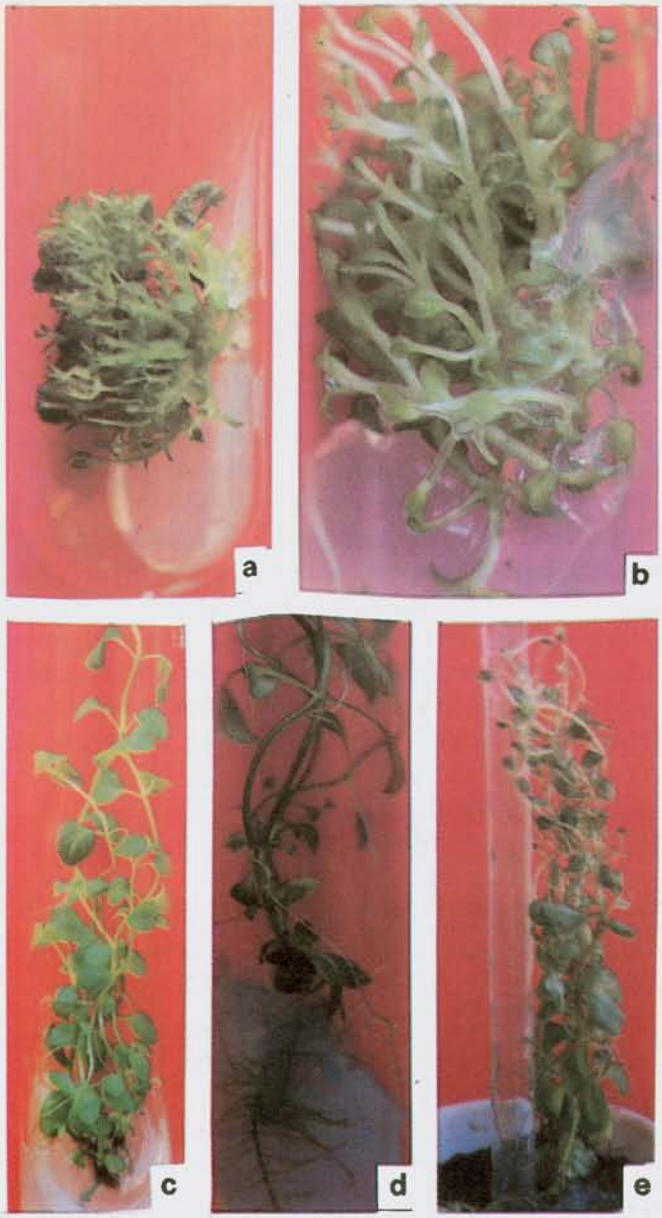

Fig. 1—*In vitro* propagation of *M. piperita*; a: Initiation of multiple shoots from nodal explant on MS medium containing 1.0 mg/l BAP after 10 days of culture; b: Proliferation of shoots on MS medium supplemented with 0.5 mg/l BAP from nodal culture after 20 days of culture; c: elongation of *in vitro* shoots on MS medium supplemented with 1.0 mg/l GA_3; d: Direct rooting from regenerated shoots on MS medium containing 0.5 mg/l IBA after 40 days of culture; and e: Hardened plant in polycups containing sterile soil and vermiculate.

The higher concentration of BAP (above 1.0 mg/l) produced hyperhydric and malformed shoots. Similarly, the shoots formed in higher concentration of Kn (2.0 mg/l) were short and thick. The leaves formed in zeatin-supplemented media were thick, dark green, large and robust and shoots had longer internodes. However, as the concentration of zeatin increased beyond 0.25 mg/l, shoot length decreased (Table 1). These observations further support the well known inhibitory influence of higher concentration of cytokinins on shoot elongation and consequent rosette type of shoot formation in *Mellissa officinalis*[19] and *Hedeoma multifolium*[20].

The combination of cytokinins and auxins was reported to stimulate *in vitro* multiplication and growth of shoots in several plant species[14]. However, addition of NAA, IAA or IBA (0.25-2.0 mg/l) to the shoot multiplication medium, in the present study, significantly reduced the number of multiple shoots per explant and did not affect shoot length in both the explants (data not shown).

Shoot Elongation

Separated individual shoots from multiple shoots were transferred to MS medium augmented with different concentrations of GA_3 (0-2.0 mg/l) for shoot elongation. The highest shoot length (12.2 cm) with 100% frequency was recorded on medium containing 1.0 mg/l GA_3 (Fig. 1c) followed by 0.5 mg/l GA_3 (9.3 cm) (Table 2). Such elongation was also reported by other workers[18,21-23]. However, shoot length and frequency considerably decreased in other higher concentrations of GA_3.

Root Initiation and Elongation

Roots were not induced during culture initiation, shoot formation and shoot multiplication in the cytokinin regime. Individual shoots when implanted in half or full strength MS medium free from growth regulators, poor and few numbers of roots were elicited with low frequency. Addition of auxins, IBA and NAA to MS medium enhanced the rate of

Table 2—Effect of GA_3 on *in vitro* shoot elongation of *M. piperata*

Plant growth regulator GA_3 (mg/l)	Frequency (%)	Shoot length/ explant (cm)
0.5	95	09.39 ± 1.13^{b}
1.0	100	12.22 ± 0.33^{a}
1.5	86	08.16 ± 0.23^{c}
2.0	72	06.65 ± 0.14^{d}

Table 3—Frequency, number of roots and root length obtained from *in vitro* excised shoots on MS medium supplemented with different concentrations of NAA and IBA

Plant growth regulator (mg/l)	Frequency (%)	No. of roots/explant	Root leanth/explant (cm)
NAA			
0.5	94	29.80 ± 1.04^{b}	3.35 ± 0.09^{c}
1.0	100	36.60 ± 1.11^{a}	5.15 ± 0.06^{a}
1.5	86	29.30 ± 1.05^{b}	4.03 ± 0.07^{b}
2.0	80	22.80 ± 0.74^{d}	3.84 ± 0.09^{bc}
IBA			
0.5	88	25.50 ± 1.52^{cd}	3.57 ± 0.39^{bc}
1.0	96	27.70 ± 0.73^{bc}	5.07 ± 0.31^{a}
1.5	82	18.00 ± 0.76^{e}	4.05 ± 0.23^{b}
2.0	74	13.30 ± 0.86^{f}	2.70 ± 0.15^{d}

Means with different letters are significant at 0.05% level

rhizogenesis in both frequency as well as number of roots. Of the two auxins tested, 1.0 mg/l NAA induced the highest number of roots per shoot (Fig. 1d) compared to other concentrations of NAA or IBA tested (Table 3). Roots formed in NAA were thick, long and dark coloured, whereas those in IBA were thin short and white coloured.

Hardening and Field Transfer

Rooted plantlets were hardened in MS basal liquid medium and subsequently transferred to polycups (Fig. 1e) containing sterile soil and vermiculate (1:1). These plantlets were acclimatized well and transferred to greenhouse and planted in the soil with 90% survivability.

In conclusion, the above protocol describes a rapid shoot regeneration from node and shoot tip explants, which can ensure a stable supply of this medicinally oil yielding plant irrespective of any seasonal variation and may serve as a better source for biological active compounds. Furthermore, *in vitro* propagules can be used for interspecific hybridization and genetic transformation studies in which relatively large number of plants is required.

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