

## Micropropagation and shoot regeneration from leaf and nodal explants of peppermint (*Mentha piperita* L.)

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### ABSTRACT

A two step procedure for *in vitro* plant regeneration of a commercial cultivar (MP-1) of peppermint *Mentha piperita* is described. Nodal explants cultured on Murashige and Skoog medium supplemented with kinetin (2-3 mg l<sup>-1</sup>) and indole-3-acetic acid (1 mg l<sup>-1</sup>) or 6-benzyl-aminopurine (2-3 mg l<sup>-1</sup>) and indole-3-acetic acid (0.25 mg l<sup>-1</sup>) produced multiple axillary and adventitious shoots which rooted on Murashige and Skoog medium containing 0.25 mg l<sup>-1</sup> indole-3-acetic acid. *In vitro* plantlets also served as source for leaf explants. Morphogenetic responses of leaf explants were growth regulator dependent and exhibited a broad maxima over a range of growth regulator levels tested. Optimal regeneration of shoot buds was observed on Murashige and Skoog media supplemented with kinetin (3 mg l<sup>-1</sup>) and indole-3-acetic acid (1 mg l<sup>-1</sup>) or 6-benzylaminopurine (2-3 mg l<sup>-1</sup>) and indole-3-acetic acid (1 mg l<sup>-1</sup>) or a-naphthaleneacetic acid (0.25 mg l<sup>-1</sup>). These plantlets exhibited a high rate (98 per cent) of survival under glass house and field conditions.

Key words : *Mentha piperita*, micropropagation, peppermint, regeneration.

### Abbreviations

- BAP : 6-Benzylaminopurine  
2,4-D : 2,4-Dichlorophenoxyacetic acid  
IAA : Indole-3-acetic acid  
Kn : Kinetin (6-furfurylaminopurine)  
MS : Murashige & Skoog medium  
NAA : a-Naphthaleneacetic acid

## Introduction

*Mentha piperita* L. (Lamiaceae) is known for its commercially valuable peppermint oil which is an important constituent of various pharmaceutical preparations and has wide applications in perfumery and confectionary items. Genetic improvement of peppermint has been largely confined to conventional and mutation breeding techniques. Attempts on improving the quality of volatile oil in micropropagated plants of *M. piperita* have proved futile (Holm *et al.* 1989; Nadaska, Erdelsky & Cupka 1990). A few reports published on tissue culture of peppermint are related to callus and cell suspension cultures (Lin & Staba 1961; Rodov & Reznikova 1982), micropropagation (Geslot *et al.* 1989; Holm *et al.* 1989; Nadaska, Erdelsky & Cupka 1990; Ravinshankar & Venkataraman 1988; Rech & Pires 1986; Repcakova *et al.* 1986) and *in vitro* essential oil biosynthesis (Rodov & Reznikova 1982). Shooty teratomas by *Agrobacterium tumefaciens* mediated transformations of *M. piperita citrata* and *M. piperita vulgaris* using stem segment tissues have also been reported (Spencer, Hamill & Rhodes 1990). However, this type of explant is not ideal for genetic transformations. Under a genetic improvement programme of mints (Kukreja *et al.* 1991; 1992) the present study aimed at standardization of optimal nutritional conditions for direct high frequency plant regeneration from nodes and leaf explants of tissue cultured peppermint, as these explants will always be in a uniform state and would be an ideal system for genetic transformation studies.

## Materials and methods

### Plant material

Runners of a commercial cultivar

(MP-1) of peppermint were planted in earthenware pots containing a mixture of sand, soil and organic manure (1:1:1). The sprouted plants were maintained under glass house conditions (80-85% RH;  $25 \pm 3^\circ\text{C}$ ) and were used as the source for explants.

### Isolation and establishment of aseptic cultures

Explants consisting of a 2-3 cm long stem piece with a node, axillary bud and petiolar base were excised from 3-month old parent plants. These were washed with distilled water containing Tween-20 (Sigma, USA) and 1% (v/v) solution of Cetrimide (IEC, India) for 1 min and surface sterilized for 20 min in 20% (v/v) commercial bleach, sodium hypochlorite. The explants were rinsed thrice with sterile deionized water before implanting vertically on the nutrient medium. Basal medium consisted of MS (Murashige & Skoog 1962), macro and micro salts, sucrose ( $30 \text{ g l}^{-1}$ ), bacteriological grade agar ( $8 \text{ g l}^{-1}$ ) and myoinositol ( $100 \text{ mg l}^{-1}$ ). Auxins (2,4-D, IAA, IBA and NAA) and cytokinins (Kn and BAP) were incorporated into the basal medium in concentrations and combinations as indicated in Table 1. The pH of the media was adjusted to 5.8 using 0.1N NaOH or HCl before autoclaving at  $120^\circ\text{C}$  for 15 min. The cultures were incubated at 16:8 h light : dark photoperiod regime under cool fluorescent light ( $40 \mu \text{ mol m}^{-2} \text{ s}^{-2}$ ) at  $25 \pm 2^\circ\text{C}$ .

### Growth regulator factorial combinations

Leaf explants positioned at 4-6th node from micropropagated plantlets maintained on rooting medium were placed in  $90 \times 15 \text{ mm}$  disposable petriplates containing 20 ml medium. Two growth regulator factorial combinations of

**Table 1. Morphogenic response of nodal explants of peppermint on MS media supplemented with auxins and cytokinins**

Cytokinin (mg l <sup>-1</sup> )		Auxin (mg l <sup>-1</sup> )												
		2,4-D							IAA			NAA		
		0	0.25	0.50	1.00	2.00	3.00	5.00	0.25	0.50	1.00	0.25	0.50	1.00
Kn	0	C <sup>0</sup> E <sup>0</sup> M <sup>0</sup> R <sup>1</sup>	C <sup>2</sup>	C <sup>1</sup>	C <sup>1</sup>	C <sup>1</sup>	-	-	E <sup>3</sup> R <sup>4</sup>	E <sup>2</sup> R <sup>3</sup>	E <sup>2</sup> R <sup>2</sup>	E <sup>1</sup> R <sup>2</sup>	E <sup>1</sup> R <sup>2</sup>	E <sup>1</sup> R <sup>2</sup>
	0.25	E <sup>1</sup> R <sup>1</sup>	E <sup>1</sup> C <sup>1</sup>	E <sup>1</sup> C <sup>1</sup>	E <sup>1</sup> C <sup>1</sup>	C <sup>3</sup>	C <sup>3</sup>	C <sup>3</sup>	E <sup>2</sup> R <sup>1</sup>	E <sup>2</sup> R <sup>1</sup>	E <sup>2</sup> R <sup>1</sup>	E <sup>2</sup> R <sup>1</sup>	E <sup>2</sup> R <sup>1</sup>	E <sup>2</sup> R <sup>1</sup>
	0.50	E <sup>1</sup> R <sup>1</sup>	-	-	-	-	-	-	E <sup>2</sup> R <sup>1</sup>	E <sup>2</sup> R <sup>1</sup>	E <sup>2</sup> R <sup>1</sup>	E <sup>2</sup> R <sup>1</sup>	E <sup>2</sup> R <sup>1</sup>	E <sup>2</sup> R <sup>1</sup>
	1.00	E <sup>1</sup> M <sup>2</sup> R <sup>1</sup>	-	-	-	-	-	-	E <sup>2</sup> R <sup>1</sup>	E <sup>2</sup> R <sup>1</sup>	E <sup>2</sup> R <sup>1</sup>	E <sup>2</sup> R <sup>1</sup>	E <sup>2</sup> R <sup>1</sup>	E <sup>2</sup> R <sup>1</sup>
	1.50	E <sup>1</sup> M <sup>2</sup> R <sup>1</sup>	-	-	-	-	-	-	E <sup>2</sup> R <sup>1</sup>	E <sup>2</sup> R <sup>1</sup>	E <sup>2</sup> R <sup>1</sup>	E <sup>2</sup> R <sup>1</sup>	E <sup>2</sup> R <sup>1</sup>	E <sup>2</sup> R <sup>1</sup>
	2.00	E <sup>3</sup> M <sup>2</sup> R <sup>1</sup>	-	-	-	-	-	-	E <sup>2</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>3</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>4</sup> R <sup>2</sup>	E <sup>1</sup> M <sup>3</sup> R <sup>1</sup>	E <sup>2</sup> M <sup>2</sup> R <sup>1</sup>	E <sup>1</sup> R <sup>2</sup>
	3.00	E <sup>3</sup> M <sup>3</sup> R <sup>1</sup>	-	-	-	-	-	-	E <sup>2</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>3</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>4</sup> R <sup>2</sup>	E <sup>1</sup> M <sup>3</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>3</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>2</sup> R <sup>2</sup>
BAP	0.50	-	-	-	-	-	-	-	E <sup>1</sup> M <sup>2</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>1</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>1</sup> R <sup>1</sup>	-	-	-
	1.00	-	-	-	-	-	-	-	E <sup>1</sup> M <sup>2</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>1</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>1</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>2</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>1</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>1</sup> R <sup>1</sup>
	2.00	-	-	-	-	-	-	-	E <sup>1</sup> M <sup>4</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>3</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>1</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>4</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>1</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>1</sup> R <sup>1</sup>
	3.00	-	-	-	-	-	-	-	E <sup>1</sup> M <sup>4</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>3</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>1</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>2</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>1</sup> R <sup>1</sup>	E <sup>1</sup> M <sup>1</sup> R <sup>1</sup>

Observations recorded after 6 weeks of culture

Numbers 0-4 represent relative degree of callusing (C), axillary shoot elongation (E), adventitious shoot multiplication (M) and rhizogenesis (R) in increasing order

Range of values for score index : 0 = No response; 1 = 1-10; 2 = 11-20; 3 = 21-30; 4 = 31-40

auxins (2,4-D, IAA and NAA) and cytokinins (Kn and BAP) were tested for their morphogenic response. The leaf explants were placed with adaxial surface towards the medium. Each growth regulator factorial combination was evaluated twice using five explants per petriplate and five petriplates per treatment. After 6 weeks of incubation, the percentage of explant regeneration and average number of shoots developed ( $> 0.15$  mm long per explant) was determined.

#### *Establishment of plants in soil*

*In vitro* regenerated plantlets were transferred to 15 cm earthenware pots containing sand, soil and organic manure and acclimatized in the glass house under high humidity (80-90% RH) for 10-15 days. The plants were initially watered with Hoagland & Arnon's (1950) nutrient solution for 1 week and were subsequently maintained under glass house conditions. The plants were later transplanted to the field.

## Results and discussion

#### *Morphogenic response of nodal explants*

Nodal explants with a dormant axillary bud did not exhibit any morphogenic response on MS basal medium even after 8 weeks of culture. Addition of 2,4-D ( $0.25$ - $2.00$   $\text{mg l}^{-1}$ ) to the medium induced callusing from the proximal cut end of explants (Table 1). Addition of Kn ( $1$ - $3$   $\text{mg l}^{-1}$ ) to the MS medium resulted in axillary shoot elongation and multiple shoot production with a few slow growing unbranched roots from the nodal region. Lower levels ( $< 1$   $\text{mg l}^{-1}$ ) did not induce multiple shoots. About 70-80 shoots per culture could be obtained after 6 weeks of culture when MS medium contained  $2$ - $3$   $\text{mg l}^{-1}$  Kn and  $1$   $\text{mg l}^{-1}$  IAA (hereafter referred to as MS-

1 medium). An equally good response was observed when Kn was replaced with BAP ( $2$ - $3$   $\text{mg l}^{-1}$ ) and IAA level was reduced to  $0.25$   $\text{mg l}^{-1}$  in the medium (Fig. 1 a), hereafter referred to as MS-2 medium. The MS-2 medium was used for maintaining stock materials. Holm *et al.* (1989) screened several media for plant regeneration and development and could obtain multiple shoots on Kn and NAA supplemented MS media using axillary shoot buds though the number of shoots per culture was very low. Rech & Pires (1986) could also obtain 15-20 shoots per culture in *M. piperita* on Kn or BAP supplemented medium while other mint species tested were less prolific. The high proliferation rate observed in the present study as compared to earlier reports of Holm *et al.* (1989) and Rech & Pires (1986) may be due to genotype specific response. The influence of genotype on initiation of plant tissue cultures and on their relative organogenic or embryogenic potential is well documented (Kurtz & Lineberger 1983; Van Eck & Kitto 1990).

Many thin branched roots originating from nodal and internodal regions of shoots were also observed on MS-2 medium. These regenerated plantlets showed poor growth and high mortality rate (50-60 per cent) when transplanted in the glass house. This necessitated a two step procedure for plant regeneration and development. *In vitro* shoots regenerated on MS-1 and MS-2 media were subcultured on MS basal or MS media fortified with IAA or NAA ( $0.1$ - $1.0$   $\text{mg l}^{-1}$ ). Though a few thin roots could be observed on MS basal medium, addition of IAA ( $0.25$   $\text{mg l}^{-1}$ ) further augmented and supported root initiation (hereafter referred as MS-3 medium) and plantlet development (Fig. 1b).

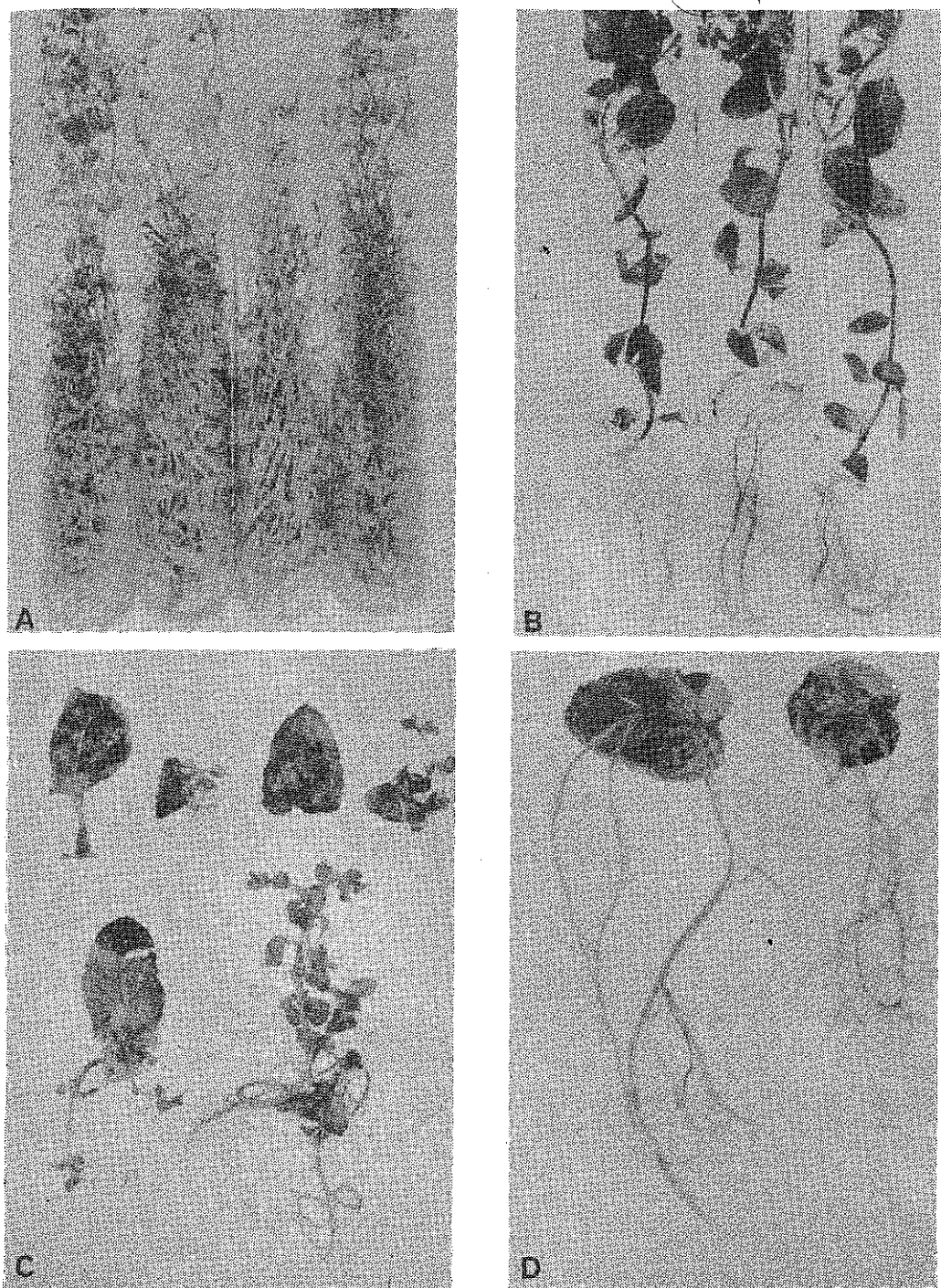


Fig. 1. Morphogenic responses of nodal and foliar explants of peppermint  
(See next page for legends)

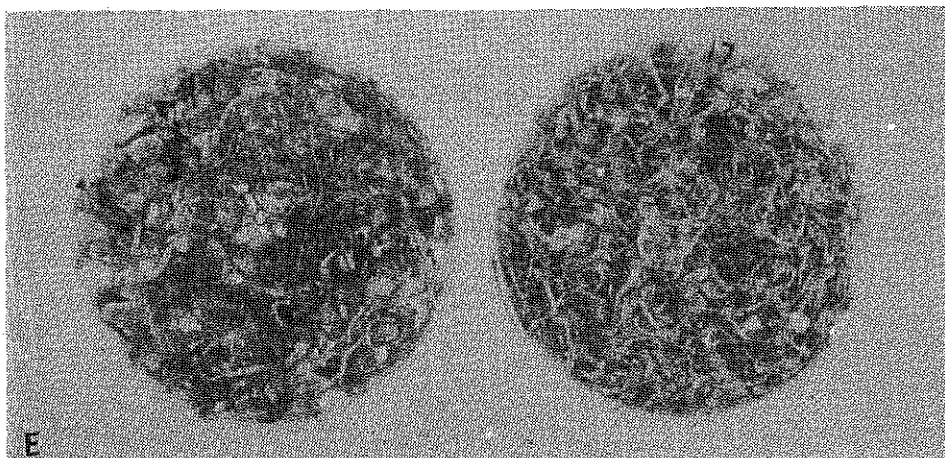


Fig. 1. (Contd.) Morphogenic responses of nodal and foliar explants of peppermint a. Multiple adventitious/axillary shoots on MS + BAP ( $2 \text{ mg l}^{-1}$ ) and IAA ( $1 \text{ mg l}^{-1}$ ) b. Root induction and plantlet development on MS + IAA ( $0.25 \text{ mg l}^{-1}$ ). c. Shoot regeneration of leaf explants on MS + Kn ( $3 \text{ mg l}^{-1}$ ) + IAA ( $1 \text{ mg l}^{-1}$ ) d. Root regeneration of leaf explants on MS + IAA ( $0.25 \text{ mg l}^{-1}$ ) e. Multiple adventitious/axillary shoots on MS + Kn ( $3 \text{ mg l}^{-1}$ ) + IAA ( $1 \text{ mg l}^{-1}$ ) after 10 weeks of culture.

Thus, a fully developed plantlet (13-15 cm in height) with normal expanded leaves and long branched roots could be obtained within 8 weeks of culture. Holm *et al.* 1989; Nadaska, Erdelsky & Cupka 1990; Ravishankar & Venkataraman 1988 and Repacakova *et al.* 1986 have also used two different media for shoot and root regeneration.

#### *Morphogenic response of cultured leaf explants*

Plantlets maintained on MS +  $0.25 \text{ mg l}^{-1}$  IAA (MS-3 medium) served as the source for obtaining sterile leaf explants. Leaves positioned at the 4-6th node from the shoot apex were used to analyse the effect of two growth regulator factorial combinations on morphogenic potential. Cultured leaf explants demonstrated variable morphogenic response to various hormonal combinations tested (Table 2). Leaf explants cultured on MS basal medium

did not exhibit any morphogenic response. The incorporation of 2,4-D ( $0.25$ - $2.00 \text{ mg l}^{-1}$ ) in the medium induced callusing. Presence of Kn ( $2$ - $3 \text{ mg l}^{-1}$ ) alone in the medium induced multiple shoot bud regeneration without any callus interphase (Fig. 1c). MS media fortified with  $3 \text{ mg l}^{-1}$  produced 30-40 shoots per culture after 6 weeks of culture. Presence of IAA ( $0.25$ - $1.00 \text{ mg l}^{-1}$ ) alone in the medium elicited rooting at  $0.25 \text{ mg l}^{-1}$  level (Fig. 1d). Similarly, lower levels of NAA ( $0.25$ - $0.50 \text{ mg l}^{-1}$ ) supported roots while the  $1 \text{ mg l}^{-1}$  level of NAA induced callusing followed by root formation.

In the experiment where the combined effect of Kn or BAP ( $0.25$ - $3.00 \text{ mg l}^{-1}$ ) and IAA or NAA ( $0.25$ - $1 \text{ mg l}^{-1}$ ) was investigated, the medium containing Kn ( $3 \text{ mg l}^{-1}$ ) + IAA ( $1 \text{ mg l}^{-1}$ ) supported multiple shoot regeneration in cultured leaf explants (Fig. 1e) without any callus interphase. One of the interesting ob-

**Table 2. Morphogenic response of leaf explants of peppermint on MS media supplemented with auxins and cytokinins**

Cytokinin (mg l <sup>-1</sup> )			Auxin (mg l <sup>-1</sup> )											
			2,4-D						IAA			NAA		
			0	0.25	0.50	1.00	2.00	3.00	5.00	0.25	0.50	1.00	0.25	0.50
Kn	0	-	C <sup>2</sup>	C <sup>1</sup>	C <sup>1</sup>	C <sup>1</sup>	-	-	R <sup>3</sup>	R <sup>2</sup>	R <sup>2</sup>	R <sup>3</sup>	R <sup>2</sup>	R <sup>1</sup> C <sup>1</sup>
	0.25	-	-	-	-	-	-	-	R <sup>3</sup>	R <sup>2</sup>	R <sup>2</sup>	R <sup>3</sup>	R <sup>1</sup>	R <sup>1</sup>
	0.50	C <sup>2</sup>	C <sup>2</sup>	C <sup>3</sup>	C <sup>2</sup>	C <sup>1</sup>	C <sup>1</sup>	-	R <sup>2</sup> C <sup>1</sup>	R <sup>2</sup>	R <sup>1</sup>	R <sup>2</sup>	R <sup>1</sup>	R <sup>1</sup>
	1.00	C <sup>1</sup>	-	-	-	-	-	-	R <sup>2</sup> C <sup>1</sup>	R <sup>2</sup> C <sup>1</sup>	R <sup>1</sup>	R <sup>2</sup> C <sup>1</sup>	R <sup>1</sup>	R <sup>1</sup>
	1.50	C <sup>1</sup>	-	-	-	-	-	-	R <sup>2</sup> C <sup>1</sup>	R <sup>2</sup> C <sup>1</sup>	R <sup>1</sup>	R <sup>2</sup> C <sup>1</sup>	R <sup>1</sup>	R <sup>1</sup>
	2.00	C <sup>1</sup> M <sup>2</sup>	-	-	-	-	-	-	R <sup>2</sup> M <sup>2</sup>	R <sup>2</sup> M <sup>2</sup>	R <sup>1</sup> M <sup>3</sup>	R <sup>2</sup> M <sup>3</sup>	R <sup>1</sup>	R <sup>1</sup> M <sup>1</sup>
	3.00	C <sup>1</sup> M <sup>3</sup>	-	-	-	-	-	-	R <sup>2</sup> M <sup>2</sup>	R <sup>2</sup> M <sup>2</sup>	R <sup>1</sup> M <sup>4</sup>	R <sup>1</sup> M <sup>2</sup>	R <sup>1</sup> M <sup>1</sup>	R <sup>1</sup> M <sup>1</sup>
BAP	0.50	-	-	-	-	-	-	-	C <sup>1</sup> R <sup>1</sup>	C <sup>1</sup> R <sup>1</sup>	C <sup>1</sup> R <sup>1</sup>	C <sup>1</sup>	C <sup>1</sup>	C <sup>1</sup>
	1.00	-	-	-	-	-	-	-	C <sup>2</sup> R <sup>1</sup>	C <sup>1</sup> R <sup>1</sup>	C <sup>1</sup> R <sup>1</sup>	C <sup>1</sup> R <sup>1</sup>	C <sup>1</sup> R <sup>1</sup>	C <sup>1</sup> R <sup>1</sup>
	2.00	-	-	-	-	-	-	-	R <sup>1</sup> M <sup>2</sup>	R <sup>1</sup> M <sup>2</sup>	R <sup>1</sup> M <sup>3</sup>	R <sup>1</sup> M <sup>3</sup>	R <sup>1</sup> M <sup>2</sup>	R <sup>1</sup> M <sup>1</sup>
	3.00	-	-	-	-	-	-	-	R <sup>1</sup> M <sup>2</sup>	R <sup>1</sup> M <sup>2</sup>	R <sup>1</sup> M <sup>3</sup>	R <sup>1</sup> M <sup>3</sup>	R <sup>1</sup> M <sup>2</sup>	R <sup>1</sup> M <sup>1</sup>

Observations recorded after 6 weeks of culture

Numbers 0-4 represent relative degree of callusing (C), axillary shoot elongation (E), adventitious shoot multiplication (M) and rhizogenesis (R) in increasing order

Range of values for score index : 0 = No response; 1 = 1-10; 2 = 11-20; 3 = 21-30; 4 = 31-40

servations in this experiment was that while shoots originated at any point on the leaf surface, roots always initiated from the mid rib region.

Regenerated shoots from leaf explants though rooted on hormone-free MS medium, showed a better rooting response on MS medium containing IAA or NAA ( $0.25 \text{ mg l}^{-1}$ ). Plants regenerated from both nodes and leaf explants were acclimatized in the glasshouse (RH 80-85%;  $25 \pm 3^\circ\text{C}$ ) for 2 weeks and ultimately transplanted to the field with a high rate of survival ( $> 95$  per cent). The standardization of procedures for micropropagation of peppermint would facilitate induction and screening for somaclonal variation to obtain new interesting mint progenies which could be used for genetic manipulation of mints.

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