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Full Length Research Paper

In vitro direct regeneration in mint from different explants on half strength MS medium

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***In vitro* shoot regeneration of *Mentha piperita* L. was investigated using shoot meristems, node, internode and petiole explants. Shoot regeneration was achieved on ½ strength MS salts and vitamins supplemented with various concentrations of BAP, Kin alone or with NAA. Shoot meristems and nodal segments showed variation in the frequency of shoot regeneration with single to multiple shoots. However, internode and petiole explants were not potent and failed to regenerate shoots at some of the tested plant growth regulators concentrations. Variable frequency of rooting was recorded at four different concentrations of auxins. The rooted plants were successfully acclimatized to *in vivo* conditions in the greenhouse.**

Key words: *Mentha piperita,* plant hormone, organogenesis, shooting.

INTRODUCTION

Medicinal plants have long been the subject of human curiosity and need. The use of medicinal plants for health reasons started thousands of years ago and is still a part of medical practice in all countries of the world (Aftab and Sial, 1999).

The genus *Mentha* belonging to family Lamiaceae includes large number of species that differ widely in their characteristics and ploidy level. Mentha species are perennial and could be multiplied both by reproductive and vegetative means. Members of this family possess great pharmacological and commercial significance. *Mentha piperita* L. is a perennial plant that is found in various countries of the world both as cultivated and wild. Pepperment oil is usually obtained from the leaves of *M. piperita* and *Menth arvensis*. Menthol is used in variety of food and medicinal products (Foster, 1996). Essential oils e.g. Limonene, cineol, polygon, piperitone in the genus mentha, have anti-feeding, insecticidal (Hori, 1999) antiviral, antibacterial, immuno modulating (Juergens et al., 1998) and anti-aging properties (Ali et al., 2002).

Plant tissue culture has the potential to introduce genetic variability in peppermint genotypes through somaclonal variants, somatic hybrids or transgenic plants. However a prerequisite to applied plant biotechnology is the development of a suitable and reproducible plant regeneration system under least cost. A number of mint species including *M. arvensis, M. piperita*, *Menth. Pulegium*, *Menth suaveolens, Menth suaveolenshybrid, Menth spicata,* and *Menth viridis* have been successfully cultured *in vitro* using leaf disc, node, inter node and shoot tip as explant either by direct organogenesis or through callogenesis (Rech and Pires, 1986; Van Eck and Kitto, 1990; Sato et al., 1993; Caissard et al., 1996; Reed, 1999).

The present study reports direct regeneration of *M. piperita* using shoot meristems, node, internode and petiole explant on ½ strength MS salts and vitamins supplemented with various concentrations of BAP or Kin alone or with NAA .

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Abbreviations: MS, Murashige and Skoog; **BAP,** N6-benzylaminopurine; **Kin**, kinetin; **IAA,** indole acetic acid; **IBA**, indole butyric acid; **NAA,** naphthaleneacetic acid.

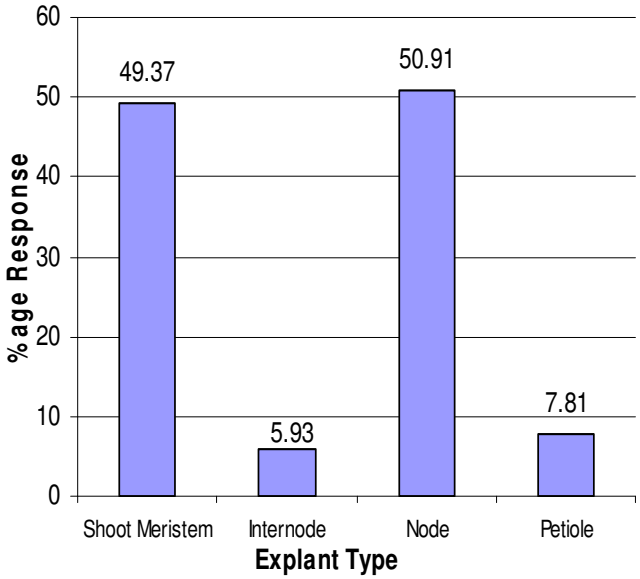

Figure 1. Percentage shooting response of different explant.

MATERIALS AND METHODS

Plant material

Young *M. piperita* plants (2-3 cm long) used as explant source were obtained from the botanical garden of the Department of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan. These plants were washed under running tap water followed by soaking in 5% Titron X100 (Sigma, USA) for 5 min. Thereafter, the plants were rinsed in distilled water for several minutes. It was followed by surface sterilization using mercuric chloride (0.1%w/v) in laminar flow cabinet and rinsing with sterilized distilled water. The first and second nodes, internodes and shoot meristems (3-4 mm) were excised from the sterilized plants while petiole margins were removed and were excised in 5 mm pieces. These explants were cultured on ½ strength of MS medium and vitamins (Murashige and Skoog, 1962) containing various concentrations of N6-benzylaminopurine (BAP), kinetin (Kin) singly or in combination with naphthaleneacetic acid (NAA), 3% (w/v) sucrose and gelled with 0.8% agar (Merck).

The pH of the medium was adjusted to 5.7 prior to addition of agar and was autoclaved at 104 KPa at 121°C for 20 min. All cultures were kept in growth chambers at 26±1°C under 16 h light photoperiod using Philips day light florescent lamps under light intensity of 10 000 lux.

Rooting and acclimatization

Scoring was done after six weeks of culture by counting all shoots on the explants. The shoots on each explant were carefully excised and rooted on half strength of MS basal salts and vitamins supplemented with various concentrations of indole acetic acid (IAA), indole butyric acid (IBA) and naphthalene acetic acid (NAA). After four weeks well rooted shoots (plantlets) were removed from the culture and rinsed with sterile distilled water to remove agar. These were transferred to plastic pots containing soil mixture composed of clay and vermiculite (1:3) and covered with transparent polythene bags to avoid evapotranspiration and maintain high humidity. After one week the polythene bags were removed gradually from the pots and the established plantlets were transferred to the growth room for next two weeks followed by transfer to the greenhouse.

Experimental design and data analysis

Each treatment had 10 replicates containing 2 explants and all experiments were repeated twice. Data were analyzed using one way ANOVA and the post hoc tests were performed using Duncans Multiple Range Test with the help of SPSS 12.00 for windows statistical software.

RESULTS

Variation in shooting response was observed due to explant type and exogenous level of growth regulator in the medium. It was found that shoot meristems and nodes were more potent for shoot regeneration as compared to internodes and petiole explants (Figure 1). The highest frequency of shoot regeneration 85% with 1.2 shoots per explant from nodal segments was recorded on 1/2 strength of MS medium supplemented with 1.5 mg/l BAP (Table 1). However, the frequency of shoot regeneration dropped to 80% with 1.6 and 1.1 shoots per explant on ½ strength of MS medium with 1.0 and 2.0 mg/l BAP, respectively. Furthermore, mean number of 1.7 shoots per shoot meristem with mean length 3.12 cm were recorded on ½ MS medium supplemented with 1.0 mg/l BAP. Internode explants were non responsive on all concentrations of BAP. At 1.5 and 2.0 mg/l BAP; 5 and 10% response was observed from internodes, respectively with only one shoot per explant with shoot length of 1.3 and 0.97 cm, respectively.

The shoot regeneration frequency of 65% with mean number 1.1 shoot per explant and shoot length of 0.79 cm was recorded on ½ strength of MS containing 1.5 mg/l Kin from node explants. Whereas, 55% frequency of shoot regeneration was recorded from shoot meristem with mean number of 1.1 shoot per explant on the same concentration. No regeneration was recorded from internodes at any concentration of Kin. The petiole explants were recalcitrant with regeneration frequency of 5-10% and maximum of one shoot per explant.

Addition of NAA in BAP and Kin containing medium was inhibitory and resulted in no to low regeneration from all four explants. At 1.0 mg/l BAP with NAA 0.5 mg/l, 75 and 70% shooting response was observed from shoot tip and nodal explants respectively (Table 1). However, mean number of shoots 2.3 per explant were recorded on nodal explants and 1.4 shoots per explant from shoot meristem explants (Figure 2). Increasing the concentration of NAA from 0.5 to 1.0 mg/l with 1 mg/l BAP shoot regeneration frequency increased to 85% and number of shoots per explant to 1.8 with mean shoot length of 2.19 cm. How-ever, low frequency of regeneration (55%) was recorded on internode explants with mean number of 2.1 shoots per explant. Decreasing the concentration of BAP (0.5 mg/l) with same concentrations of NAA (0.5 and 1.0 mg/l) was inhibitory

Table 1. Effects of various plant growth regulators on shoot regeneration from various explants of *M. piperita.*

PGR	Conc.	Shoot meristems			Inter node			Node			Petiole		
		Frequency (%) of shoot regeneration	Shoot length (cm)	Mean number of shoots per explant	Frequency (%) of shoot regeneration	Shoot length (cm)	Mean number of shoots per explant	Frequency (%) of shoot regeneration	Shoot length (cm)	Mean number of shoots per explant	Frequency (%) of shoot regeneration	Shoot length (cm)	Mean number of shoots per explant
BAP	0.5	65	2.41B*	1.3BC	-	-	-	45	3.2BC	1.3BC	-	-	-
	1.0	80	3.12A	1.7B	-	-	-	80	4.0B	1.6B	25	1.4	1.0
	1.5	70	0.94D	1.3BC	50	1.3	1.0	85	4.6A	1.2BCD	40	2.0	1.1
	2.0	35	0.41E	1.1CD	10	.97	1.0	80	3.9B	1.1CD	35	0.69	1.0
Kin	0.5	25	1.19CDE	1.0D	-	-	-	35	1.1DE	1.0D	-	-	-
	1.0	60	0.63DE	1.2C	-	-	-	40	0.83D	1.0D	-	-	-
	1.5	55	0.98D	1.4BC	-	-	-	65	0.79D	1.1CD	10	0.82	1.0
	2.0	15	0.24F	1.0D	-	-	-	20	0.8D	1.3BC	5	0.26	1.0
BAP/NAA	1.0/0.5	75	2.89AB	1.4BC	10	1.62	1.0	70	1.61CDE	2.3A	-	-	-
	1.0/1.0	85	2.19B	1.8B	25	1.47	1.1	55	1.82CD	2.1A	10	0.2	1.0
	0.5/0.5	60	2.26B	2.3A	-	-	-	40	2.1C	1.4BC	-	-	-
	0.5/1.0	70	1.71C	1.3BC	-	-	-	45	1.74CD	1.1CD	-	-	-
Kin/NAA	1.0/0.5	20	0.43E	1.1CD	-	-	-	35	1.2DE	1.0D	-	-	-
	1.0/1.0	50	0.9D	1.0D	-	-	-	65	0.88D	1.2BC	-	-	-
	0.5/0.5	10	0.48E	1.0D	-	-	-	40	0.43F	1.0D	-	-	-
	0.5/1.0	15	0.85D	1.0D	-	-	-	15	0.3F	1.0D	-	-	-

Means followed by different letters in a column are significantly different at 5% level of significance using Duncan test.

And resulted in low frequency of shoot regeneration on all explants. However, shoot meristem explants produced more number of shoots (2.3/explant Figure 2). Internode and petiole explants showed low regeneration response on BAP (1.0 mg/l) with NAA (1.0 mg/l). An average shoot length 1.47 cm was recorded from internodes with 25% shoot regeneration frequency while petiole showed only 10% shoot regeneration frequency with mean number of shoot 1.0 per explant.

The combination of Kin and NAA at levels showed maximum shoot regeneration frequency of 65% from nodal segments with average number of shoots 1.2 per explant and average shoot length of 0.88 cm (Table 1). Shoot meristems did not produce multiple shoots except on Kin 1.0 mg/l with NAA 0.5 mg/l showing 20% res-ponse. Internodes and petiole explants failed to generate shoots at any combination of Kin and NAA with necrosis of explants after few days of inoculation.

Variable rooting in range of 15-90% was recorded on all cultures containing different concentrations of IAA, IBA, and NAA (Table 2). 60% rooting was recorded on 1.5 mg/l NAA in the half MS medium. IAA was not potent and resulted in numerous small roots on 1.0, 1.5 and 2.0 mg/l with 80 to 90% rooting response.

DISCUSSION

Minimal nutrient level may be effective for callogenic response or turning of cells into organ development (morphogenesis). There are very few reports that define direct organogenesis from different explants on half MS medium (Nhut et al.,

Table 2. Effect of various concentrations of IBA; NAA and IAA on rooting of *M. piperita.*

PGR Treatments	Frequency (%) of rooting	Mean number of roots per explant
IBA (mg/l)		
0.5	40	1.0D
1	70	4.2B
1.5	70	3.8BC
2.0	85	6.4A
NAA (mg/l)		
0.5	25	1.1
1.0	45	3.1C
1.5	60	3.7BC
2.0	15	1.0D
IAA (mg/l)		
0.5	60	3.3C
1.0	85	Numerous small roots
1.5	90	Numerous small roots
2.0	80	Numerous small roots

Means followed by different letters in a column are significantly different at 5% level of significance using Duncan test.

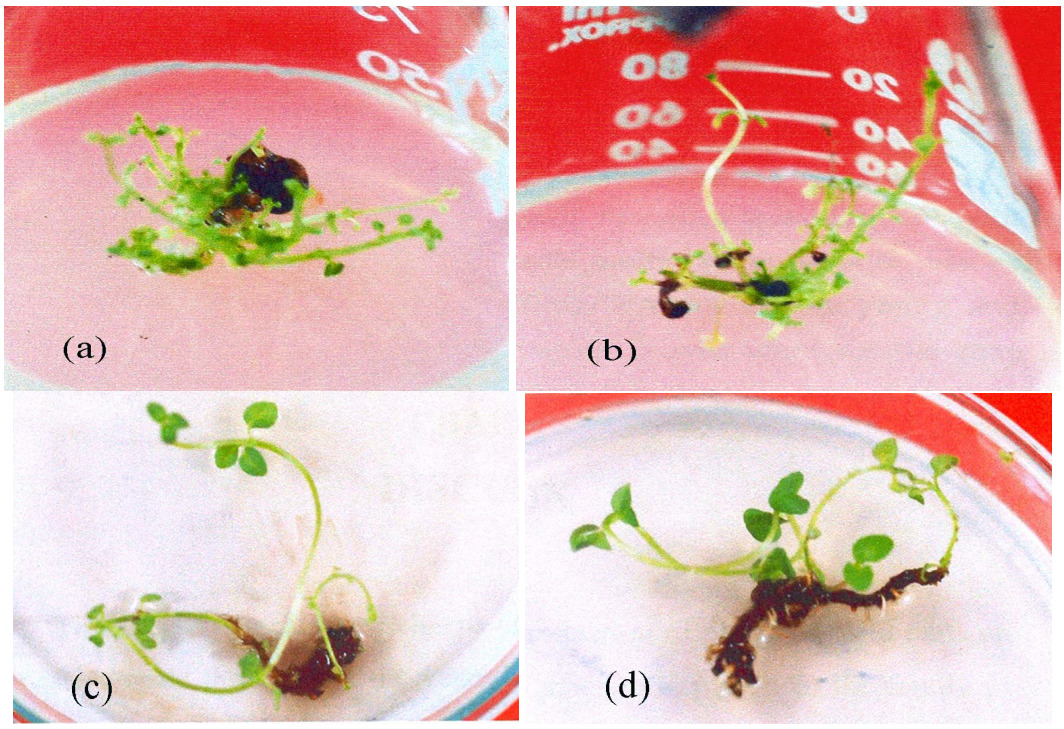

Figure 2. Axillary and adventitious shooting response of *Mentha piperita* (a) from shoot meristem with BAP / NAA (0.5 mg/l / 0.5 mg/l), (b) from node at BAP / NAA (0.5 mg/l / 0.5 mg/l), (c) from petiole at BAP (1.5 mg/l), and (d) from internode at BAP / NAA (1.0 mg/l / 1.0 mg/l).

2001; Martin, 2004). But addition of various plant growth regulators in the medium at appropriate level may influence organogenesis from any type of cells. In the present investigation, varying shoot regeneration was observed from different explants on ½ MS media but shooting response was not as high which may be due to use of half

strength MS medium. Addition of plant growth regulator especially cytokinins may turn the somatic cells into somatic embryos either present in the minimal nutritional medium (Krikorian, 1995).

Different explants, when cultured on ½ MS medium supplemented with BAP or Kin, singly or in addition with NAA, showed varying response. Shoot meristems and nodal segments either produced single or multiple shoots with 10-85% response. Higher shooting response was observed when cytokinin was added to the medium while addition of auxin as a whole reduced the percentage response and mean number of shoots per explant. BAP and Kin have been reported to be better plant growth regulators for shoot induction from axillary buds and nodal segments when cultured on MS medium (Rech and Pires, 1986; Sunandakumari et al., 2004) while Kukerja et al. (1991) reported that addition of NAA along with cytokinins resulted in more number of shoot emergence.

The process of generation of buds/shoots was observed very low from petioles and internodal segments. Both explants normally generated single shoot per explant. No shooting response was observed from internode explants on Kin alone or with NAA; however petiole explants showed 5-10% shoot regeneration when cultured on ½ MS medium supplemented with Kin. Li et al. (1999) and Van Eck and Kitto (1992) working on *M. piperita* immature leaf and leaf discs explants, respectively, reported formation of few shoots from explants. While Pooviaiah et al. (2006) reported 85% shooting response from 1st and 2nd internode with mean number of 29 shoots/explant on modified MS medium supplemented with TDZ. IAA and IBA produced more number of roots on regenerated shoots compared to NAA. Sunandakumari et al. (2004) also got rooted plants on half strength MS medium with IBA (10.3 roots/ shoot) but in our study different concentrations of IAA produced numerous roots.

The present findings show that organogenic response in *M. piperita* may be achieved on half strength MS medium by using any type of explant with appropriate level of plant growth regulator. These findings may be helpful to produce *M. piperita* transformed plants, where according to some reports low concentration of nitrogen enhances transformation efficiency.

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