Journal of Phytology 2011, 3(5): 19-22
ISSN: 2075-6240
www.scholarjournals.org

www.journal-phytology.com

JP-Tissue Culture

Indirect Plant Regeneration from Leaf Explants of *Mentha piperita* (L.) – An Important Multipurpose Medicinal Plant

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Article Info

Article History

Received : 19-12-2010
Revised : 03-03-2011
Accepted : 07-03-2011

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Summary

Peppermint (*Mentha piperita*) is a hybrid mint, a cross between the watermint (*Mentha aquatica)* and spearmint (*Mentha spicata*). The plant, indigenous to Europe, is now widespread in cultivation throughout all regions of the world. Peppermint typically occurs in moist habitats, including stream sides and drainage ditches. Being a hybrid, it is usually sterile, producing no seeds and reproducing only vegetatively, spreading by its rhizomes. If placed, it can grow anywhere, with a few exceptions. Peppermint is sometimes regarded as the world's oldest medicine, with archaeological evidence placing its use at least as far back as ten thousand years ago. Callus was obtained on MS media supplemented with different concentrations and combinations of IAA, NAA, BAP, Kn and 2,4-D from leaf explants of *Mentha piperita*. More number of shoots were differentiated from callus grown on MS medium supplemented with BAP (2mg/l) and further multiplication was achieved by repeatedly sub culturing the nodal segments. About 95% of *in vitro* shoots developed roots after they were transferred to rooting medium containing IBA (1.5 mg/l). 95% of the plantlets were successfully acclimatized and established in the field.

Key Words: Indirect regeneration, Plant growth regulators, *Mentha piperita*
Abbreviations: 2, 4 – D – 2, 4-Dichloro phenoxy acetic acid; BAP – 6-benzyl amino purine; NAA – α- naphthalene acetic acid; IAA – indole-3- acetic acid; IBA – indole butyric acid; Kn – kinetin

Introduction

Mentha piperita is believed to be a hybrid of spearmint (*Mentha spicata*) and water mint (*Mentha aquatica*), belonging to lamiaceae family. Black peppermint has violet stems and leaves, and white peppermint has light green deeply cut leaves. Peppermint raw material is used in medicine, cosmetics and food industry. Black peppermint produces a large amount of essential oils and has a better aroma than the white one, thus it is more widely grown, and especially for industrial processing [4 and 12]. The oil also contains menthone and menthyl esters, particularly menthyl acetate. It is the oldest and most popular flavour of mint-flavoured confectionery. Peppermint can also be found in some shampoos and soaps, which give the hair a minty scent and produce a cooling sensation on the skin.The growing of aromatic and medicinal plants in our country becomes more and more popular and requires selecting the most suitable plants. The essential oil is obtained from the fresh leaves of *Mentha piperita* by steam distillation and it is widely used all over the world for flavouring, cosmetic and medicinal purposes.

Materials and Methods

Culture medium

During present investigation only MS medium (Murashige and Skoog) was used [6]. The chemicals used for preparing various media were of analytical grade from Merck, Sigma and Universal chemicals. Nutrient medium was homogenized by boiling and by continuous stirring before adding agar and phytohormones. The pH of the medium was adjusted as 5.8 prior to addition of agar by using 0.1N NaOH and 0.1N HCl. After adding different concentration of growth hormones, about 15 - 20 ml of media was dispensed into each culture tube. After autoclaving the culture vials were kept inside the inoculation chamber.

Plant collection

Mentha piperita plants were obtained from Suvedha nursery, Tirupati, Andhra Pradesh, India. Leaf segments from healthy plants of *Mentha piperita* were used in the present study. The explants were collected, washed thoroughly under running tap water for 15 min. These were treated with 5% teepol (w/v) for 5 min, and again washed thoroughly in running tap water. After that leaf segments were surface sterilized with 0.1% $HgCl_2$ for 5 minutes, followed by washing with sterile double distilled water inside the laminar airflow chamber to remove traces of $HgCl_2$. The leaf segments were cultured on MS medium supplemented with different auxins like Naphthalene acetic acid (NAA), Indole acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and Benzyl amino purine (BAP) for callus induction and regeneration. For indirect shoot organogenesis one month old healthy leaf callus showing regeneration was used as a source material and transferred onto regeneration medium amended with different

concentrations and combinations of BAP (0.2 - 2.0 mg/l), Kn (0.5 – 2.0 mg/l) and NAA (0.1 – 1.0mg/l). All the cultures were maintained in a growth room with a 16 h photoperiod (cool, white fluorescent light – 3000 lux light intensity) and the temperature was maintained at 25 ± 2ºC, with 50 - 80% relative humidity. Data on shoot regeneration and mean number of shoots were recorded after four weeks. *In vitro* differentiated shoots measuring 3.0 – 4.0 cm in length were excised and cultured on rooting medium containing NAA (1.5 mg/l). Rooted plantlets were carefully washed with tap water and transferred to polycups containing sterile soil and vermiculite (1:1) and covered with plastic bag to maintain humidity. Subsequently, the plantlets were transferred to greenhouse after one month and planted in the soil. Each treatment consisted of twenty replicates and the experiment was repeated twice.

Since studies on *in vitro* multiplication on this species are almost limited, hence in the present paper we report a protocol for the indirect regeneration of *Mentha piperita* an important multipurpose medicinal plant.

Results

Indirect shoot formation

Plant propagation requires the induction of organogenic callus. Organogenic callus was observed in leaf callus only. Callus initiation was achieved from the explants within 7 days of incubation on MS basal medium supplemented with any one of the auxins like IAA, NAA and 2, 4-D in combination with either BAP or Kn. 2, 4-D along with BAP was found to be potent hormone for stimulating callus induction from leaf explants, green organogenic fragile callus was observed with (1.0 mg/l) 2, 4-D and (0.5 mg/l) BAP (Table 1 and Fig. 1).

Among the growth regulators tested, BAP (2.0 mg/l) showed maximum regeneration frequency (85%) (Table 2 and Fig.1) with maximum shoot number (5.0 ± 0.69). But the maximum shoot length was observed in BAP (1.0 mg/l). The minimum regeneration frequency was observed in combination with BAP (0.2 mg/l), Kn (0.5 mg/l) and NAA (0.1 mg/l). Least number (1.6 ± 0.33) and length of shoots (1.5 ± 0.63 cm) were observed with BAP (0.5 mg/l).

Rooting

Maximum number of roots (48.5 ± 1.45) was seen in the presence of IBA (1.5 mg/l), with the shoot length being 5.3 ± 0.27 cm (Table 3 and Fig. 1). IBA was simultaneously followed by NAA (1.5 mg/l), with root number 37.6 ± 1.68 and highest root length 6.9 ± 0.35 cm. The least number of shoots were formed with IAA (0.5 mg/l). Of all the three hormones tested IBA showed high root induction followed by NAA and IAA being the least.

Hardening and field establishment

The rooted plantlets after 30days were transplanted to mini pots containing soil and sand mixture (1:1) and placed in green house. These *in vitro* raised plants were hardened with 95% survivability and the regenerated plants did not show any detectable variation in morphological or growth characteristics when compared with the donar plants.

Table - 1: Effect of different concentrations of plant growth regulators to MS medium on induction of callus from leaf of *Mentha piperita*

Plant growth regulators (mg/l)				Intensity of callus	Nature of callus
NAA	IAA	2,4-D	BAP		
0.5	-	-	-	+ + +	White coloured, fragile
1.0	-	-	-	+ + +	White coloured, fragile
2.0	-	-	-	+ +	Light green coloured, fragile
-	0.5	-	-	+	Swelling of leaves
-	1.0	-	-	-	No callus formation
-	2.0	-	-	-	No callus formation
-	-	0.5	-	+ +	Green coloured, fragile
-	-	1.0	-	+ +	Green coloured, fragile
-	-	2.0	-	+ + +	Green coloured, organogenic, fragile
0.1	-	0.5	0.5	+ +	Yellow, fragile
-	-	1.0	1.0	+ + +	Light green, fragile
-	-	1.0	0.5	+ + +	Green coloured, organogenic, fragile
-	0.1	-	0.5	+ +	Light green, fragile

Table - 2: Indirect shoot organogenesis from callus produced from the leaf explants of *Mentha piperita.* Results are mean ± SE of 20 replicates

Plant growth regulators (mg/l)			Regeneration frequency (%)	Mean no. of shoots/callus	Mean shoot length (cm)	Nature of callus
BAP	Kn	NAA				
0.5	-	0.1	65	2.1 ± 0.40	1.9 ± 0.06	White coloured, fragile
1.0	-	0.1	70	3.5 ± 0.55	2.2 ± 0.13	Yellowish coloured, fragile
1.0	1.0	0.5	80	3.0 ± 0.54	4.1 ± 0.10	Green coloured, organogenic
1.0	-	1.0	75	4.0 ± 0.67	3.4 ± 0.05	Light green, fragile
0.2	0.5	0.1	60	2.8 ± 0.44	1.8 ± 0.07	Whitish green, fragile
0.2	1.0	0.1	75	2.2 ± 0.29	2.0 ±0.72	Light green compact
0.2	2.0	0.1	-	-	-	No callus formation
0.5	-	-	60	1.6 ± 0.33	1.5 ± 0.63	Whitish green
1.0	-	-	70	3.0 ± 0.77	4.6 ± 0.20	Yellowish green, compact
2.0	-	-	85	5.0 ± 0.69	3.2 ± 0.30	Green coloured, organogenic
-	1.0	-	-	-	-	No callus formation
-	2.0	-	-	-	-	No callus formation

Table - 3: Effect of different concentrations of NAA, IAA and IBA on root formation from *in vitro* excised shoots of *Mentha piperita* explants. Results are mean ± SE of 20 replicates

Plant growth regulators (mg/l)	Frequency (%)	No. of roots/explant	Root length (cm)
IAA			
0.5	85	4.2 ±0.27	4.7 ± 0.35
1.0	95	6.3 ± 0.42	5.8 ± 0.20
1.5	98	12.5 ± 0.25	6.5 ± 0.28
2.0	80	5.9 ± 0.33	3.0 ±0.31
NAA			
0.5	90	16.4 ± 0.65	4.2 ± 0.05
1.0	96	24.2 ± 1.19	8.8 ± 0.10
1.5	100	37.6 ± 1.68	6.9 ± 0.35
2.0	84	21.4 ± 1.98	3.9 ± 0.25
IBA			
0.5	85	9.04 ± 0.84	5.0 ± 0.20
1.0	90	20.0 ± 0.49	6.1 ± 0.02
1.5	95	48.5 ± 1.45	5.3 ± 0.27
2.0	70	18.0 ± 0.23	2.5 ± 0.11

Discussion

In the present study, leaf explants exhibited varied response for profuse callus formation to different hormonal concentrations and combinations. Green organogenic callus was observed with 2, 4 – D and BAP combination, but its presence totally suppressed shoot bud formation from callus. This was observed in other systems also [11, 9 and 3]. The effectiveness of 2, 4 – D and BAP might be due to their role in DNA synthesis and mitosis [13]. BAP was found to be desirable hormonal concentration for indirect shoot regeneration with maximum shoot number. Our results are parallel as in with *Piper longum* [1], *Origanum vulgare* [7] and *Pithecallobium saman* [2]. Kinetin when used alone did not show any shoots. In the present study BAP alone and in combination with NAA exhibited better morphogenesis. These results are in corroborated with the previous reports in *Spilanthes acmella* [10] and in *Asteracantha longifolia* [8].

Efficient rooting of *in vitro* regenerated plants and subsequent field establishment is the last and crucial stage of rapid clonal propagation. Half strength MS medium with different auxins like IAA, IBA and NAA was used for root induction since earlier studies. Root induction was best with 1.5 mg/l IBA, than the other concentrations. With the increase in the concentration of IBA, the number of roots also decreased. Profuse rooting was obtained in IBA (0.1 mg/l) in the case of *Morus indica* [3], whereas 4 mg/l IBA was required for profuse rooting in *Gemelina arborea* [14]. In contrast to our results, the earlier studies examined the effectiveness of various auxins on rooting of *Picrorhiza* micro shoots and found that NAA at 1.0 mg/l was superior to IBA and IAA [5].

Conclusion

Since studies on *in vitro* propagation of *Mentha piperita* through indirect regeneration have not been attempted, we have achieved a clear, simple and reliable protocol for large scale multiplication. In view of the medicinal properties and increased demand of this plant in the pharmaceutical industry, the outlined procedure offers a potential system for improvement, conserving and mass propagation of this important medicinal plant.

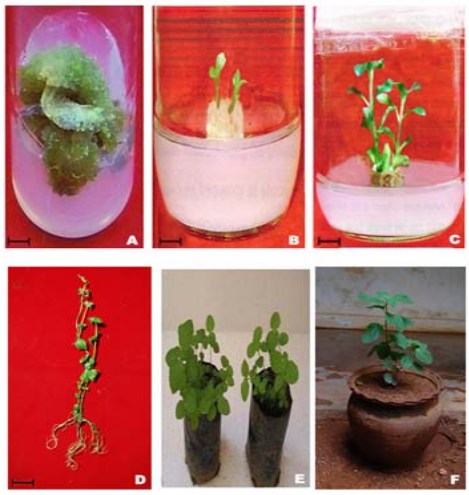

Figure – 1: Indirect shoot regeneration of *Mentha piperita*
A. Callus formation with 2,4 – D (1.0mg/l) and BAP (0.5 mg/l). B. Indirect shoot initiation from callus on MS basal medium with BAP (0.5 mg/L) + NAA (0.1 mg /L). C. Formation of multiple shoots on MS medium with BAP (2.0 mg/L). D. Rooted plantlet on MS basal medium supplemented with IBA (1.5 mg/l). E&F. Tissue cultured plantlets in field conditions.

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