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Rapid *in vitro* Clonal Propagation of Herbal Spice, *Mentha piperita* L. Using Shoot Tip and Nodal Explants

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Abstract A high frequency efficient protocol for rapid propagation of the herbal spice *Mentha piperita* L. from shoot tip and nodal explants was established by using full and half strength of Murashige and Skoog (MS) medium supplemented with various concentrations of 6-benzyl amino purine (BAP; 1.0-5.0 mg/L) and kinetin (Kn; 1.0-5.0 mg/L). The highest number of shoots (42.0) with 100% frequency was obtained from nodal explants in the full strength of medium containing 3.0 mg/L BAP. For further elongation, microshoots were transferred to MS medium containing different concentrations of gibberellic acid (GA_3; 0.5-2.0 mg/L). The highest shoot length (13.1 cm) with 100% frequency was achieved on medium containing 1.0 mg/L GA_3. *In vitro* proliferated shoots were then excised from the shoot clumps and transferred to the rooting medium containing different concentrations of indole butyric acid (IBA; 0.5-2.0 mg/L) and indole acetic acid (IAA; 0.5-2.0 mg/L) alone. Among these, the highest root proliferation was obtained in the medium containing 1.5 mg/L IBA. The rooted plantlets were hardened on MS basal liquid medium and subsequently in polycups containing sterile soil and vermiculite (1:1) and finally transferred to the field. The survival rate was 100% after 25 days.

Keywords: *in vitro, clonal propagation, Mentha piperita L., shoot tip, node, medicinal plant*

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1. Introduction

Medicinal plants have been using for health care reasons in all over the world through Ayurvedic, Unani, and the folk medicinal systems since ancient times, and still are widely used as remedies in modern therapeutic practices.

The genus *Mentha* belonging to family Lamiaceae includes large number of species that differ widely in their characteristics and ploidy level. *Mentha piperita* L. is a perennial plant that is found in various countries of the world as both cultivated and wild, and it could be multiplied in nature by reproductive and vegetative means as well [1]. Members of this family possess great pharmacological and commercial significance. Pepperment oil is usually obtained from the leaves of *M. piperita* and *M. arvensis*. Menthol is used in a variety of food and medicinal products [2]. Essential oils e.g. Limonene, cineol, polygon, piperitone in the genus *Mentha*, have anti-feeding, insecticidal [3] antiviral, antibacterial, immuno modulating [4] and anti-aging properties [5]. According to the German Commission E monographs [6], peppermint oil (as well as peppermint leaf) has been used internally as an antispasmodic (upper gastrointestinal tract and bile ducts) and to treat irritable bowel syndrome, catarrh of the respiratory tract, and inflammation of the oral mucosa and applied externally as for myalgia and neuralgia. According to Commission E, peppermint oil may also act as a carminative, cholagogue, antibacterial, and secretolytic, and it has a cooling action.

Mentha piperita is a sterile natural hybrid of *M. aquatica* ($2n = 96$) × *M. spicata* ($2n = 48$) which is allohexaploid ($2n = 72$) and produces the typical peppermint cyclic monoterpenes, menthol and menthone. Due to sterility it is not amenable to improvement by sexual crosses [7]. Moreover, due to pollen-sterility and high ploidy number, conventional breeding methods are often difficult even unsuccessful in peppermint species. In one previous report it has been demonstrated that 18,000 peppermint floral spikes containing more than 2.75 million ovules have only 6 viable seeds [8].

In vitro clonal propagation therefore could be a beneficial technique for large-scale production of fresh and disease free *M. piparita* plantlets for production of medicine and other industrial products. In addition, this technique has the potential to introduce genetic variability in peppermint genotypes through somaclonal variants, somatic hybrids and transgenic plants as well [9]. However a prerequisite to applied plant biotechnology is the development of a suitable and reproducible plant regeneration system under least cost [1]. In this case, plant tissue culture technology seems to be a very useful and promising tool to overcome this problem and can play a vital role in the rapid mass clonal multiplication,

germplasm production and conservation, secondary metabolite production and sustainable use of this plant.

A number of researcher have earlier been successfully cultured *Mentha piperita* [1,10-18], and other species of mint including *M. viridis* [19,20], *M. spicata* [22,23], *M. arvensis*, *M. pulegium*, and *M. suaveolens in vitro* using leaf disc, node, inter node, shoot tip, and other propagule as explants either by direct organogenesis or through callogenesis on different strength of MS medium [24] with or without use of plant growth regulators. However, some of the serious limitations in the above mentioned protocols were low regeneration frequency, low survival rate after acclimatization, unstable and little number of shoots and roots as well as appearance of callus phase during organogenesis.

Here, the present investigations reports direct *in vitro* rapid clonal propagation of *Mentha piperita* L. using shoot meristems and nodal explants on full and half strength MS salts and vitamins supplemented medium with various concentrations of BAP or Kn alone either in combination of both. The aims and objectives of the study was to develop a rapid, convenient regeneration and micropropagation procedures of *M. piperita* L. that could ensure high frequency of regeneration within a short time and high survival rate of plants after acclimatization.

2. Materials and Methods

2.1. Plant Material and Surface Sterilization

Healthy juvenile *Mentha piperita* L. plants were obtained from Oushodhigram (Medicinal Village), Natore district, situated in northern region of Bangladesh and raised in pots containing soil and farm yard manure (1:1) under greenhouse condition at Plant Biotechnology and Microbiology Lab., Department of Botany, University of Rajshahi, Rajshahi-6205, Bangladesh. Shoot tips and nodal segments were used as explants in the present study and collected from potted plants and processed for aseptic culture (Figure 1). Explants were washed in running tap water for 30 minutes and then in a solution of mild liquid detergent for 5 min. Disinfection was done by a quick dip in 70% alcohol and surface sterilization was done with 0.1% $HgCl_2$ solution for 3–5 min. Three washings were done with sterilized double distilled water.

2.2. Culture Medium and Culture Conditions

After surface sterilization, the explants were excised into small pieces (1cm long) and cultured individually on full and half strength MS medium [24] containing 0.8% (w/v) agar supplemented with different concentrations (0.5-3.0 mg/L) of benzyl amino purine (BAP), and kinetin (Kn) singly or in combination to induce multiple shoots. The pH of the entire medium used was adjusted to 5.8 before autoclaving at 1.06 kg/cm^2. 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 ± 2°C, with 50 - 80% relative humidity.

2.3. Sub Culturing

In vitro initiated mass of proliferated shoots from both the explants were sub cultured after 14 days and cultured on fresh MS basal medium , supplemented with 0.5, 1.0, 1.5, and 2.0 mg/L of gibberellic acid (GA_3) , only for shoot proliferation and elongation.

2.4. Rooting and Acclimation

In vitro elongated 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 0.5-2.0 mg/L of either indole butyric acid (IBA) or indole acetic acid (IAA). 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. Plantlets, thus, developed were successfully established and finally transferred to the field. The survival rate was 100 per cent after 25 days.

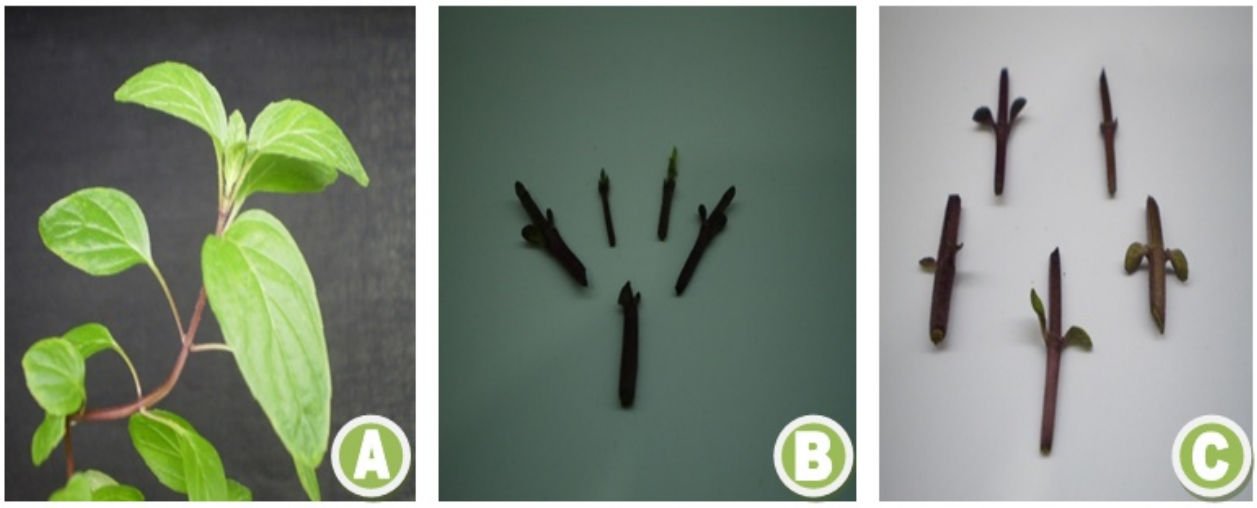

Figure 1. Materials used for *In vitro* propagation. A: *Mentha piperita* L. twig. B&C: Shoot tip and nodal explants

2.5. Experimental Design

A completely randomized experimental design was performed in this study. In all experiments, each treatment had at least three replicates, and there were 20 explants per replicate (per dish). The explants in all experiments were sub cultured at 2-week intervals. 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 Multiple Range Test (DMRT) ($P< 0.05$) and were presented as the mean ± standard error (SE).

3. Results and Discussion

3.1. Explants Superiority

Shoot regeneration was highly influenced by the explants type of *Mentha piperita*. It was observed that nodal explants were more superior for multiple shoot regeneration as compared to shoot tip explants (Figure 2). In comparison between shoot tips and nodal explants for multiple shoot regeneration, nodal explants regenerated a significantly larger average number of shoots than that of shoot tip explants (Table 1 and Table 2). The maximum number of shoot regeneration 100% with 42.00 ± 4.42 shoots per explants with an average mean length of 9.45 ± 4.13 cm from nodal segments was recorded. Whereas, 80% frequency of shoot regeneration was recorded from shoot tip explants with mean number of 37.35 ± 5.82 shoot per explants with an average mean length of 7.11 ± 4.35 cm on same environment, where all exogenous growth regulator and nutrient medium were equalized. Shoot buds emerged on 7^{th} and 13^{th} day of culture (Figure 1) from nodal and shoot tip explants, respectively. However, shoot started proliferating after 21 and 25 days respectively. Shoot meristems and nodes are more potent for shoot regeneration as compared to internodes and petiole explants was reported by Sarwar *et. al*., [1]. Single node explants elicited more numbers of multiple shoots as compared to shoot tip explants was also reported by Ghanti *et. al.*, [10]. The proliferation efficiency of nodal explants from healthy plants was significantly higher than that of shoot tip explants was also reported by Raja and Arockiasamy [19]. Nodal explants as the best source of multiple shoot induction have also been suggested in case of other medicinal plants, such as *Rauwolfia serpentina*, *Emblica officinalis*, *Holarrhena antidysenterica* and *Enicostemma hyssopifolium* [10].

3.2. Medium Strength on Shoot and Root Formation

The shoot tip and nodal explants were cultured on both full and half strength of MS medium containing with BAP and Kn at different concentrations (1.0, 2.0, 3.0, 4.0 and 5.0 mg/L) for production of multiple shoots (Table 1 and Table 2). In both explants, shoot proliferation was highly achieved in full strength MS medium supplemented with BAP either Kn as compared to half strength of MS medium. The highest frequency of shoot regeneration 100% with mean number of 42.00 ± 4.42 shoots per explants with an average mean length of 9.45 ± 4.13 cm was recorded on full strength MS medium in case of nodal explants. Whereas, only 50% shoot regeneration was recorded in case of half strength MS medium. Moreover, in half strength MS medium, explants remained vitrified with no proliferation of off-shoots and callus formation was also started at the base of the shoots. The full or half strength of MS medium without any PGR was failed to induce rooting of regenerated shoots. There is extremely few information that define direct organogenesis from various explants on half strength MS medium [25,26]. But addition of various plant growth regulators in the medium at appropriate level may influence organogenesis from any type of cells [1]. Sarwar *et. al.*, [1] reported that, varying shoot regeneration was achieved from different explants of *Mentha piperita* on half strength MS media but shooting response was not as high which may be due to use of half strength MS medium. Paques and Boxus [27] have shown in some species that media rich in mineral nutrients such as MS [24] were shown to promote vitrification, while half strength MS salts improved plant development and provide regeneration of highest number of shoots and induction of roots per explants was reported in *Mentha spicata* [23] and *Mentha piperita* [1]. Using media with lower levels of minerals or only half of the MS salts improved carnation and cucumber plant development [28,29]. In this investigation, high frequency of shoot regeneration was achieved on full strength MS medium in combination with different plant growth regulator while half strength MS medium showed less number of shoot proliferation.

Table 1. Effect of basal medium and BAP on shoot proliferation from shoot tip and nodal explants of *Mentha piperita* L.

Basal medium	Shoot tip explant				Nodal explant		
	Conc. Of BAP (mg/L)	Response (%)	No. of shoots/explants (mean ±SD)	Shoot length/explants (cm) (mean ±SD)	Response (%)	No. of shoots/explants (mean ±SD)	Shoot length/explants (cm) (mean ±SD)
Full strength MS	1.0	30	15.50 ± 9.37	2.90 ± 0.99	25	13.85 ± 8.10	3.20 ± 0.63
	2.0	55	26.80 ± 6.81	6.70 ± 4.28	35	26.40 ± 9.65	8.00 ± 4.27
	3.0	80	37.35 ± 5.82	7.11 ± 4.35	100	42.00 ± 4.42	9.45 ± 4.13
	4.0	50	27.35 ± 6.58	2.85 ± 3.82	70	19.35 ±12.41	3.95 ± 3.89
	5.0	45	16.75 ±9.61	6.85 ± 6.71	55	17.45 ±11.51	5.85 ± 3.79
Half strength MS	1.0	15	12.50 ± 1.35	3.19 ± 1.25	20	15.19± 2.13	2.85 ± 3.45
	2.0	20	13.25 ± 2.37	2.55 ± 1.82	35	19.25 ± 3.13	3.65 ± 2.45
	3.0	35	15.65 ± 5.39	5.15 ± 3.35	50	21.65 ± 4.23	6.85 ± 5.79
	4.0	25	11.35 ± 3.39	3.65 ± 1.95	30	16.29± 3.13	2.99 ± 2.45
	5.0	30	10.65 ± 5.19	3.15 ± 1.45	25	13.25± 2.29	1.99 ± 1.05

**Twenty explants were used for each treatment and data (Mean ± SD) recorded three – four weeks after culture.

Table 2. Effect of basal medium and Kn on shoot proliferation from shoot tip and nodal explants of *Mentha piperita* L.

Basal medium	Shoot tip explant				Nodal explant		
	Conc. Of Kn (mg/L)	Response (%)	No. of shoots/explants (mean ±SD)	Shoot length/explants (cm) (mean ±SD)	Response (%)	No. of shoots/explants (mean ±SD)	Shoot length/explants (cm) (mean ±SD)
Full strength MS	1.0	25	28.65 ± 6.08	3.60 ± 1.89	20	15.15 ± 8.44	2.80 ± 0.91
	2.0	45	28.75 ± 4.96	3.70 ± 3.28	30	27.65 ± 7.34	5.60 ± 4.05
	3.0	60	32.01 ± 4.70	3.10 ± 3.26	75	34.01 ± 4.02	6.50 ± 4.20
	4.0	40	20.10 ± 8.38	3.85 ± 3.02	50	19.35 ±9.41	2.50 ± 2.68
	5.0	35	15.75 ±9.51	2.75 ± 2.41	55	15.35 ±6.51	3.55 ± 2.59
Half strength MS	1.0	20	11.51 ± 2.35	3.01 ± 2.25	15	11.15 ± 6.45	2.05 ± 3.15
	2.0	35	12.55 ± 2.07	2.05 ± 1.02	25	12.15 ± 3.03	1.60 ± 1.45
	3.0	40	13.65 ± 3.39	3.25 ± 2.25	50	15.69 ± 3.25	5.15 ± 4.38
	4.0	30	11.05 ± 3.09	3.05 ± 2.98	25	13.25± 5.15	2.09 ± 2.15
	5.0	35	11.55 ± 2.09	2.25 ± 1.25	30	11.05± 3.20	2.98 ± 1.65

**Twenty explants were used for each treatment and data (Mean ± SD) recorded three – four weeks after culture.

On the other hand, roots were developed two weeks after the transfer of individual shoots on both full and half strength of MS medium containing with various concentrations of plant growth regulators. But when individual shoots were trans-cultured in half or full strength MS medium free from PGR, poor and few numbers of roots were developed with low frequency. Fadel *et. al.*, [23] was observed that there has a significant effect of the half strength of MS culture medium in combination with plant growth regulators (PGR) on root and shoot formation over the full strength of MS medium in case of *in vitro* organogenesis of spearmint (*Mentha spicata* L.). He reported that the maximum number of shoots and roots induced per explants as well as the maximum average shoot length was observed on half-strength MS medium.

3.3. Growth Regulators Promotion on Multiple Shoot Induction

Cytokinins, especially BAP, were reported to overcome apical dominance, release lateral buds from dormancy, and promote shoot formation [30]. In this investigation, different concentrations of BAP and Kn were evaluated on shoot initiation and further proliferation. For multiple shoot initiation, the nodal and shoot tip explants were inoculated on full strength and half strength MS medium containing different concentrations of BAP and Kn in the range of 1.0-5.0 mg/L and showed enhanced shoot proliferation. Comparative analysis of the results on the various cytokinins used indicated that proliferation of shoots was more effective in most of the BAP concentrations. BAP at its 3.0 mg/L concentration showed high frequency and highest number of shoot proliferation in both nodal (100%) and shoot tip (80%) explants (Table 1). Further increase in the concentration of BAP reduced the frequency and number of shoots in both explants. On the other hand, when the explants were cultured on Kn based medium only 20-75% of them responded to proliferation. In this treatment the highest number of shoots per explants and average shoot length were 34.01 ± 4.02 and 6.50 ± 4.20 cm for nodal explants, 32.01 ± 4.70 and 3.10 ± 3.26 cm for shoot tip explants, respectively. The percentage of explants showing proliferation and the number of shoots per culture increased gradually with an increase of cytokinins concentration from 1.0 to 3.0 mg/L. When the concentration of cytokinins increased to above 3 mg/L, shoot regeneration frequency decreased and vitrification occurred. Similar results were also reported in *Mentha viridis* [19] *Prosalia corylifolia* [31] and *Terminalia arjuna* Roxb. [32].The results of this experiment also indicate that 3.0 mg/L BAP was more suitable than 3.0 mg/L Kn for shoot proliferation (Table 1). Superior effect of BAP over Kn has been documented in *Mentha piperita* itself [10]. Similar results of efficacy of BAP over Kn were reported for the axillary proliferation in many medicinal plants of Lamiaceae like *M. spicata*, *M. arvensis*, and *Lavandula viridis* [33,34,35]. In contrast to, superior effect of Kn over BAP has been documented in *Mentha piperita* itself [11,36].

Besides this, incorporation of NAA or IAA in combination with BAP improved bud proliferation but the shoots remained stunted (Data not shown). After initial proliferation of shoots on medium containing 3.0 mg/L BAP were sub-cultured on same fresh medium in every 21 days later. On the other hand, Kn showed little response for multiple shoot initiation as compared to BAP in both explants. Inoculation of BAP or Kn into MS medium for multiple shoot initiation in culture, BAP showed better performance than Kn and the maximum number of shoot was obtained on its 3.0 mg/L concentration. When BAP was used in combination with Kn, a fluctuate number of responses were observed (Data not shown). But highly effective response was observed on medium containing 0.5 mg/L BAP + 2.0 mg/L Kn (Average number of shoots 3.41 +0.37, shoot length 7.56 + 0.32 cm).

3.4. Shoot Elongation

Separated single shoots from proliferated multiple shoots were transferred to MS medium containing with different concentration of GA_3 in the range of 0.5-2.0mg/L for shoot elongation. The highest shoot length (13.1cm) with 100% frequency was recorded on medium containing 1.0 mg/L GA_3. Similar results were also reported by other workers [10,37,38,39,40]. However, shoot length and frequency gradually decreased in other higher concentration of GA_3.

Table 3. Effect of GA_3 on *in vitro* shoot elongation of *Mentha piperita* L.

Plant growth regulator GA3 (mg/L)	Response (%)	Shoot length/explant (cm)
0.5	95	9.49 ± 1.15
1.0	100	13.1 ± 0.55
1.5	90	9.01 ± 1.25
2.0	85	8.99 ± 1.15

**Twenty explants were used for each treatment and data (Mean ± SD) recorded three – four weeks after culture.

3.5. Root Initiation and Elongation

Generally, roots were not initiated during the culture inoculation for shoot formation and shoot proliferation in cytokinin regime. But when individual shoots were trans-cultured in half or full strength MS medium free from PGR, poor and few numbers of roots were developed with low frequency. Root induction was enhanced in the *in vitro* regenerated well elongated shoots by culturing them on MS medium with supplementation of different concentrations of IBA and IAA separately in the range of 0.5-2.0 mg/L. However in the present study, the best rooting response was observed on medium containing 1.5 mg/L IBA (Figure 2). Incorporation of 1.5 mg/L IBA in MS medium enhanced the rate of rhizogenesis in both frequency and number of roots.

Maximum number of roots (35.01±1.99) were produced in 1.5 mg/L IBA and mean root length was found found to be 5.45±1.05 cm (Table 4). Similar results were also reported in *Mentha viridis* [19] *Ocimum amaricannum* [41] *Hybanthus enneaspermus* [42] *Tylophora asthmatica* [43]. Roots formed in IBA were thick, long and dark coloured, whereas those in IAA were thin short and white coloured.

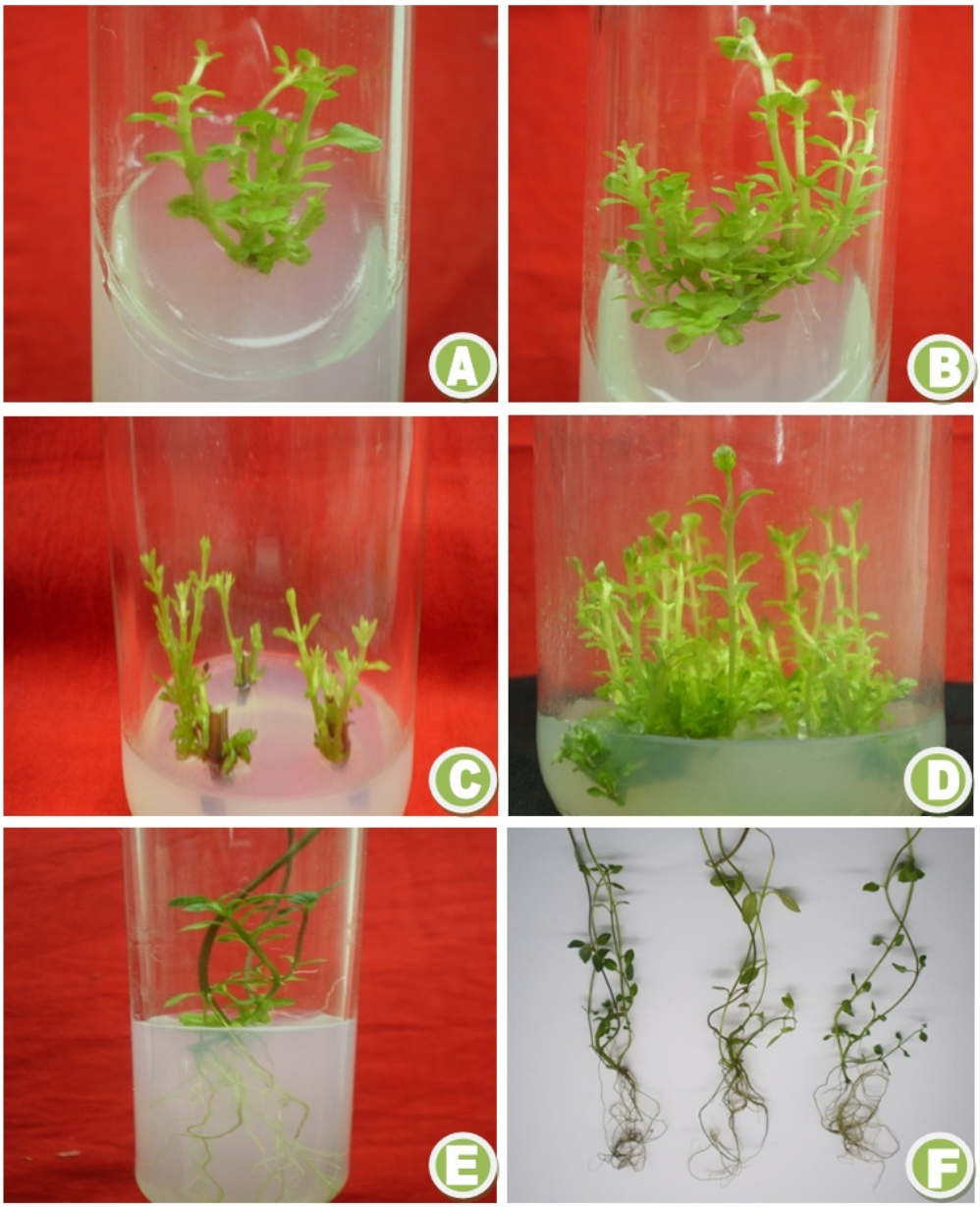

Figure 2. (A-F) – *In vitro* propagation of *Mentha piperita*. A: Initiation of multiple shoots from shoot tip explants on MS medium containing 3.0 mg/L BAP after 12 days. B: High frequency of multiple shoot formation from shoot tip explants on MS medium containing 3.0 mg/L BAP after 25 days of culture. C: Initiation of multiple shoots from nodal explants on MS medium containing 3.0 mg/L BAP after 6 days. D: High frequency of multiple shoot production from nodal explants on MS medium containing 3.0 mg/L BAP after 21 days of culture. E &F: Rooting of regenerated shoots on MS medium containing 1.5 mg/L IBA after 25 days

Table 4. Effect of different concentrations of IBA and IAA on root induction from *in vitro* grown microshoots of *Mentha piperita* L.

Plant growth regulator (mg/L)	% of response	No. of roots/explants	Root length/explants (cm)	Days to emergence of roots
IBA				
0.5	82	27.75±1.05	3.15±0.09	15-18
1.0	85	30.50±1.11	5.15±0.15	13-16
1.5	100	35.01±1.99	5.45±1.05	11-15
2.0	75	25.25±0.75	3.50±0.75	13-17
IAA				
0.5	55	11.70±1.15	2.05±0.15	14-20
1.0	70	25.75±1.50	5.01±0.35	15-18
1.5	85	27.75±0.75	5.05±.031	13-15
2.0	60	13.50±.086	2.50±0.15	14-17

**Twenty explants were used for each treatment and data (Mean ± SD) recorded three – four weeks after culture.

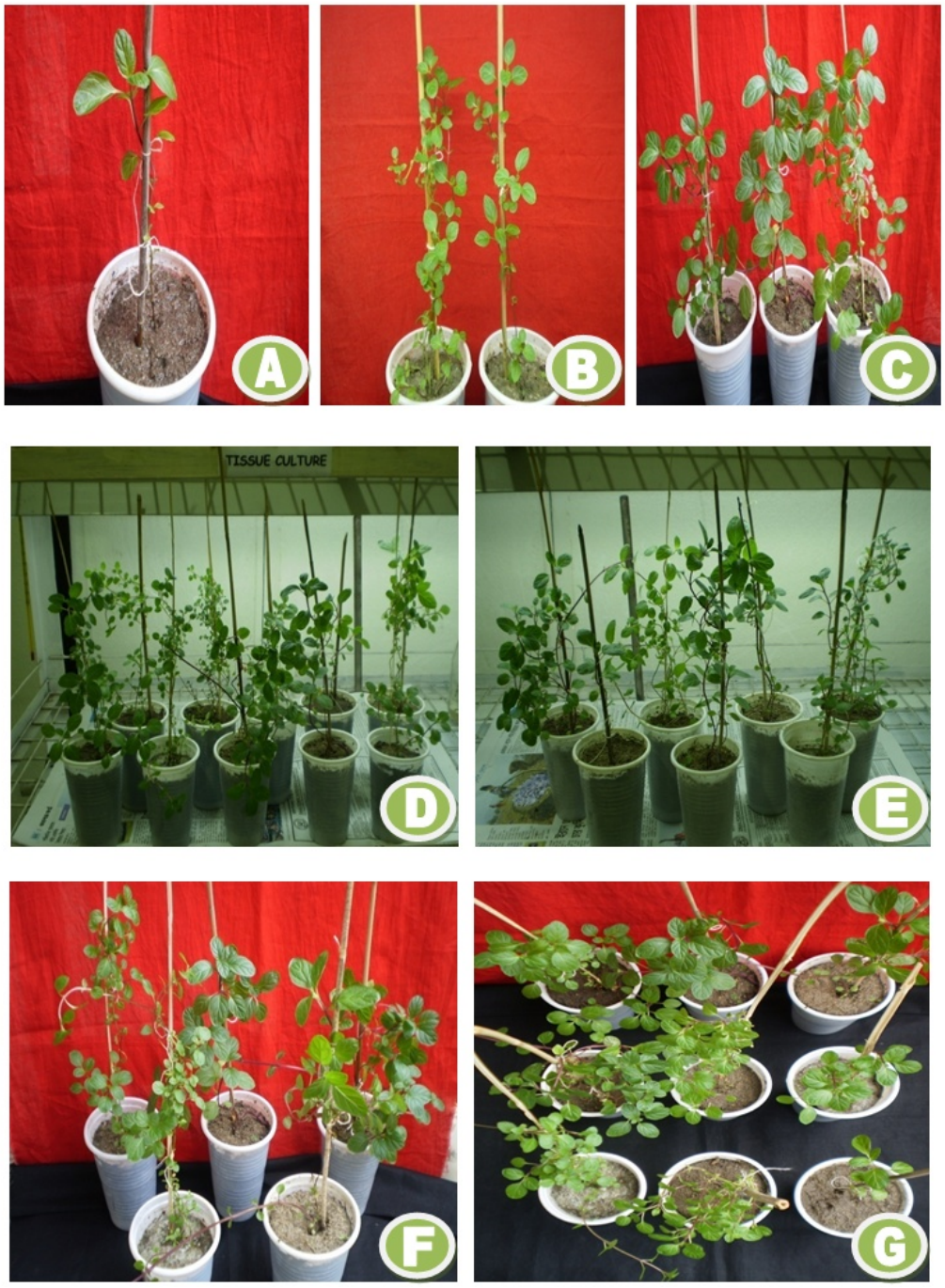

Figure 3. (A-G). A: *In vitro*- raised Peppermint plant after 2 weeks transplantation. B&C: *In vitro* grown plantlets, 4 weeks after acclimatization. D&E: Hardened plantlets in growth chamber's artificial environment. F&G: Hardened plantlets in *ex vitro* condition, showing branching-6 weeks old

3.6. Hardening and Field Transfer

After 3 week, the rooted plantlets were transferred to polycups (Figure 3) containing sterile soil and vermiculate (1:1). These plantlets were acclimatized well and transferred to green house and planted in the soil with 100% survivability. There was an increase in length of shoots and new leaves emerged which expanded quickly (Figure 3).

4. Conclusion

The above protocol describes high frequency shoot propagation along with *ex vitro* rooting enables to provide disease free planting propagules at low cost and within a short time, which will attract small scale farmers to mediculture and it can ensure a stable supply of this medicinally important oil yielding plant and may serve as a better source for biological active compounds. Furthermore, *in vitro* propagules can be used for interspecific hybridization and genetic transformation.

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