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An efficient protocol for clonal micropropagation of *Mentha piperita* L. (Pipperment)

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ABSTRACT

This review highlights the recent development and achievements made for the micropropagation of *Mentha piperita* (scented herb) in hadoti region of south east Rajasthan. Shootlets were regenerated from nodal explants of stem through auxiliary shoot proliferation. The induction of multiple shoots from nodal segments was the highest in MS medium supplemented with 0.5 mg/l BAP+3.0 mg/l Kn. For rooting different concentration of IBA were used and highest rooting was recorded on MS medium with 2.0 mg/l IBA. The rooted Plantlets were hardened initially in culture room conditions and then transferred to misthouse.

Keywords: micropropagation, shoot multiplication, Pipperment, medicinal plant.

INTRODUCTION

Plants are the key to life on the earth as they directly or indirectly supply approximately 80% of human caloric and protein intake , the remainder being derived from animal products .They are economically important to man due to their multiple applications , such as pharmaceuticals ,flavors ,fragrance ,insecticides ,dyes , food additives, toxins etc. However, it is well known that their production is frequently low and depends on the physiological and developmental stage of the plant. Micropropagation is the best alternative to overcome these hurdles and it holds tremendous potential for rapid multiplication and production of high quality medicines from them.

Mentha piperita L. (Peppermint) 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. 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 [1]. Plant regeneration from axillary bud [2, 3] Leaf [4] via organogenesis [5, 6] and nodal explant8 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. Therefore, an attempt was made to establish a suitable protocol for rapid *in vitro* propagation of this medicinal plant.

MATERIALS AND METHODS

The branches (about 5-6 cm) of shoots of *M. piperita* L. plants were collected from the Herbal Garden, Kota. The branches with node explants were washed in running tap water and then washed again thoroughly by adding a few drops of Tween-20 to remove the superficial dust particles as well as fungal and bacterial spores. They were surface sterilized with 0.1% $HgCl_2$ for 5 min followed by rinsing them five times with double distilled water inside the Laminar Air flow chamber. Nodal segments (with a single axillary bud) about 0.5-0.8 cm were prepared aseptically and were implanted vertically on MS medium prepared with specific concentrations of BAP, Kn (1.0-5.0 mg/l) singly or in combination were used for shoot proliferation. Same experiments were repeated for shoot multiplication. The medium containing 3% sucrose was solidified with 0.8% agar (Qualigens). The *p*H of the media was adjusted to 5.9±0.02 with 1 N NaOH or 1 N HCl solutions prior to autoclaving. Media poured in culture vessels were steam sterilized by autoclaving at 121°C and 15 psi for 15-20 min. The cultures were incubated under controlled conditions of temperature (25±2°C), light (2000- 2500 lux for 16 h/d provided by fluorescent tubes) and 60-70% humidity. For each experiment a minimum of 7 replicates were taken and experiments were repeated thrice. Observations were recorded after an interval of 3 wk. Once culture conditions for shoot induction from explants were established, the shoots produced *in vitro* were subcultured on fresh medium every 3 wk. The nodal and shoot tip explants were inoculated in various concentrations and combination of BAP and Kn. Among these, the maximum number of shoots (3.42±0.39) was developed on MS media fortified with 0.5 BAP+3.0 Kn. Maximum shoot length was observed as 7.54±0.31cm.of a medium supplemented with 0.5 BAP+3.0 Kn. Rooting of elongated shoots was attempted under *in vitro* conditions. Auxins (IBA) alone in different concentrations (0.5-2.5 mg/l) were incorporated in the agar (0.8%) solidified medium containing 1/4 MS salts and 1.0% sucrose. The *in vitro*-rooted plantlets were transferred to culture bottles 1/4th filled with Soilrite composition (soil: sand: peat moss) and irrigated with 1/4 MS salt solution. These bottles were kept in controlled environmental conditions of culture room. After 3 wk of growth, the plantlets were transferred to misthouse for further growth.

RESULTS AND DISCUSSION

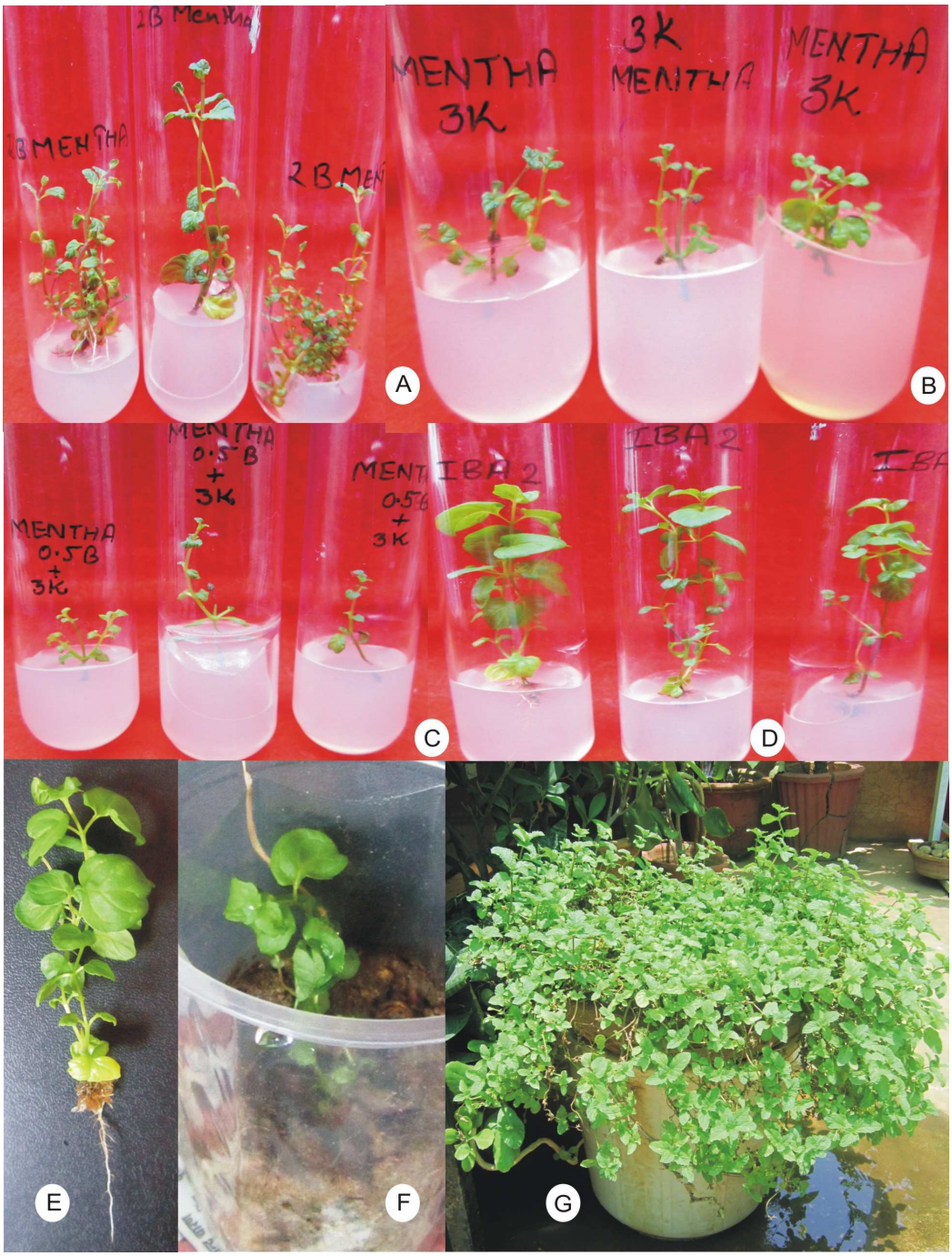
The nodal explants, when inoculated on MS medium containing BAP and Kn in the range 1.0-5.0 mg/l showed enhanced shoot proliferation. BAP at its 2.0 mg/l concentration evoked best response. Incorporation of NAA or IAA improved bud proliferation but the shoots remained stunted. Shoots after their initial proliferation on medium containing 2.0 mg/l BAP were sub-cultured on same fresh medium after every 21 days. Incorporation of BAP or Kn into MS medium supported multiplication of shoots in culture, BAP proved to be a better choice than Kn and the maximum number of shoot was obtained on its 2.0 mg/l concentration (Table 1, Fig. 1- A, B, Fig. 2). When BAP was used in combination with Kn a variety of responses were observed (Table 2, Fig. 1-C, Fig. 3). But best response was observed on medium containing 0.5 mg/l BAP + 3.0 mg/l Kn (Average number of shoots 3.42±0.39, shoot length 7.54±0.31 cm). The full or half strength of MS medium without any PGR was failed to induce rooting of regenerated shoots. However, shoots were capable to induce root when cultured on medium containing auxins.

Table-1 Effect of Cytokinin (BAP and Kn) on shoot proliferation from Nodal shoot explant of *Mentha piperita*

Hormone Con. (mg/ l) BAP	Hormone Con. (mg/ l) Kn	Response (%)	No. of Shoot/explant (mean±SD)	Shoot length (in cm) (mean±SD)
1.0	-	70	2.28±0.71	6.56±0.84
2.0	-	80	3.42±0.58	6.51±0.76
3.0	-	65	2.71±0.56	7.62±0.53
4.0	-	55	3.28±0.36	5.08±0.51
5.0	-	40	2.85±0.51	3.31±0.33
-	1.0	55	2.28±0.36	
-	2.0	60	1.85±0.27	6.47±0.29
-	3.0	75	2.42±0.39	6.15±0.24
-	4.0	40	1.57±0.40	6.30±0.26
-	5.0	30	1.28±0.36	5.70±0.41
				4.92±0.51

Medium: MS+ additives; mean± SD, n= 7 replicates
Means having the same letter in each Colum, do not different significantly at P< 0.05 (Tukey's test)

Figure 1 (A-G) Micropropagation of *Mentha piperita* from nodal shoot explants



***A.** Shoot multiplication on MS medium supplemented with 2.0 mg/l BAP, **B.** Shoot multiplicationon MS medium supplemented with 3.0 mg/l Kn, **C.** Shoot multiplication on MS medium supplemented with 0.5 mg/l BAP+3.0 mg/l Kn, **D.** In vitro root induction on ¼ of MS medium supplemented with 2.0 mg/l IBA, **E.** 4 weeks old rooted plant for hardening, **F.** hardened plant growing on soilrite moistened with basal medium, **G**. well growing plant in green house.*

Table-2 Interactive effect of Cytokinin (BAP+ Kn) on shoot multiplication by sub culture of shoot clumps of *Mentha piperita*

Hormone Con. (mg/ l)	No. of Shoot/explant	Shoot length (in cm)	Shooting Response (%)
0.5 BAP + 0.5 Kn	1.71±0.38	3.70±0.28	70
0.5 BAP + 1.0 Kn	2.14±0.51	4.71±0.29	80
0.5 BAP + 2.0 Kn	2.70±0.36	5.70±0.41	85
0.5 BAP + 3.0 Kn	3.42±0.39	7.54±0.31	90
0.5 BAP + 4.0 Kn	2.57±0.40	6.70± 0.39	82

Medium: MS+ additives; mean± SD, n= 7 replicates
Means having the same letter in each Colum, do not different significantly at P< 0.05 (Tukey's test)

Table-3 Effect of Auxin (IBA) on root induction from isolated shoot of *Mentha piperita*

Hormone Con. (mg/ l)	No. of roots/explants	Root length (in cm)	Rooting Response (%)
0.5 IBA	1.08±0.19	0.43±0.33	78
1.0 IBA	1.38±0.37	0.92±0.10	80
1.5 IBA	1.40±0.52	1.06±0.08	85
2.0 IBA	3.60±0.51	1.68±0.32	90
2.5 IBA	2.80±0.73	0.51±0.05	73

Medium: MS+ additives; mean± SD, n= 7 replicates
Means having the same letter in each Colum do not different significantly at P< 0.05 (Tukey's test)

Auxins in different concentration induced rooting when incorporated in the medium containing ¼ of MS salts. The best rooting response, however, was observed on medium containing 2.0mg/l IBA, where roots measuring 1.68±0.32 cm (average) were formed (Table 3, Fig. 1-D, Fig. 4). *In vitro* rooted plantlets were initially hardened in culture room conditions where leaves expanded. After 3 week, the plantlets were shifted to mist house. There was an increase in length of shoots and new leaves emerged which expanded quickly (Fig. E).

Figure-2 Effect of cytokine (BAP and Kn) on shoot proliferation from nodal shoot explants of *Mentha piperita*

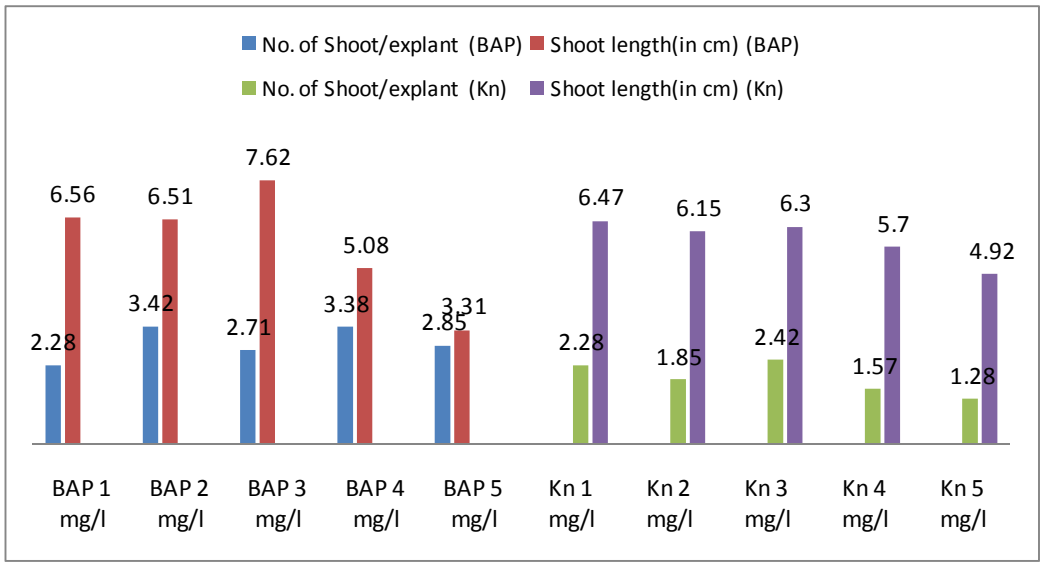


Figure-3 Interactive effect of cytokine (BAP + Kn) on shoot multiplication by subculture of shoot clumps of *Mentha piperita*

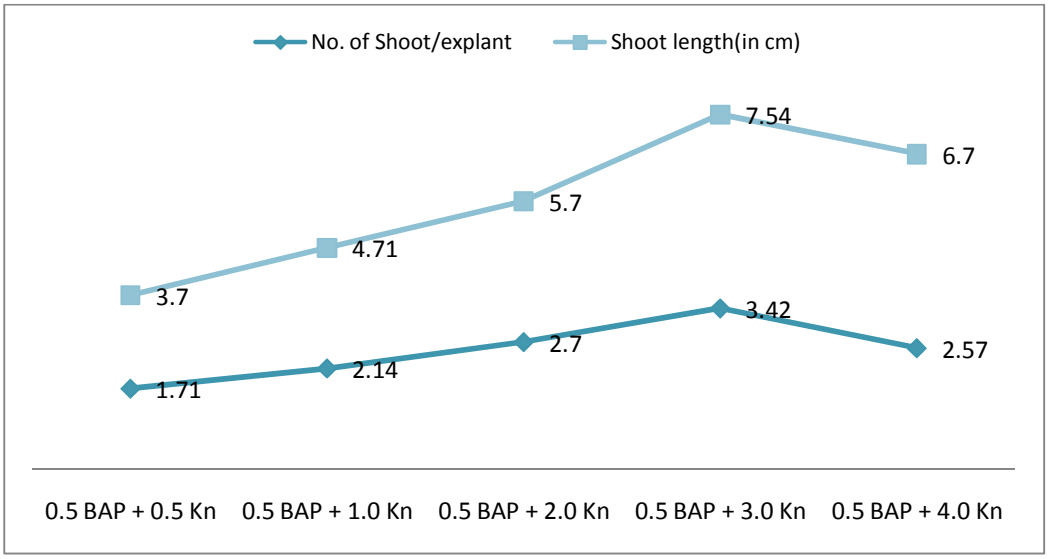
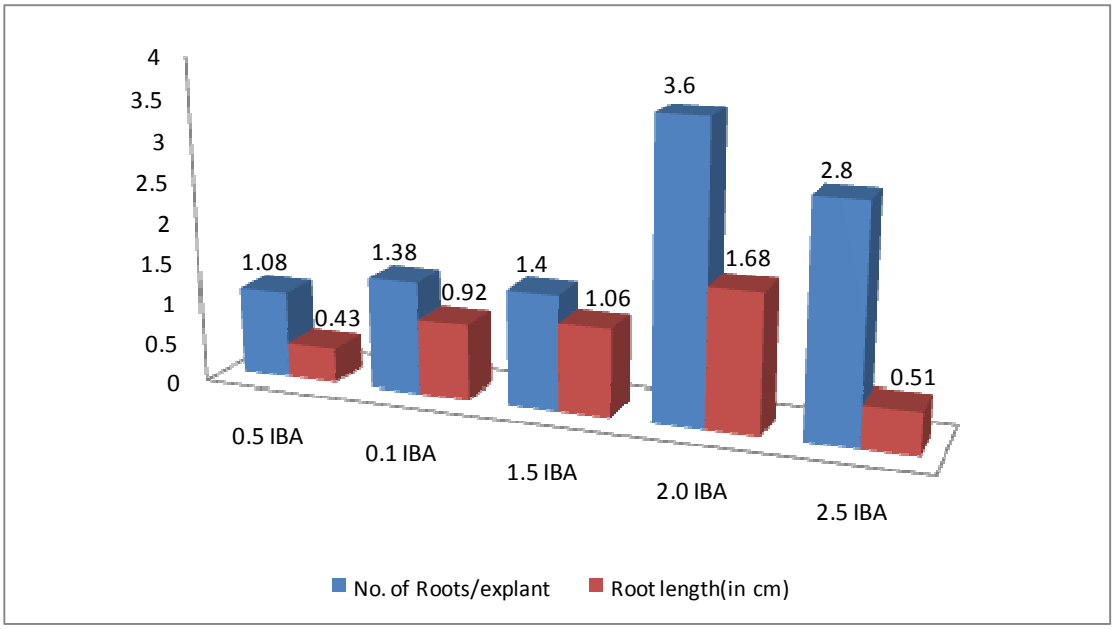


Figure-4 Effect of Auxin (IBA) on root induction from isolated shoots of *Mentha piperita*



CONCLUSION

The seedlings derived from explants, being juvenile, are frequently used for micropropagation, as they are easy to establish in culture. In *M. piperita* MS medium containing 2.0 mg/l BAP was the best for culture initiation. We have found that *M. piperita* culture grew better on MS medium in comparison to other media. In *M. piperita* 2.0 mg/l BAP was most suitable for shoot multiplication. We also observed improvement in shoot multiplication by different concentrations of Kn. (0.5-4.0 mg/l) in medium along with BAP (0.5 mg/l). Best shooting response was observed on media containing 0.5 mg/l BAP+ 3.0 mg/l Kn (Average number of shoots 3.42±0.39, Average shoot length 7.54±0.31 cm) and different concentrations of Kn. (1.0-5.0 mg/l) *M. piperita* give best shooting response in 3.0 mg/l Kn. (Average number of shoots 2.42±0.39, Average shoot length 6.30±0.26).

IBA (Auxin) has been widely used as root induction hormone under *in vitro* and *in vivo* condition. We also found positive role of IBA during *in vitro* rooting. In *M. piperita*, 2.0mg/l IBA proved to be the best for *in vitro* rooting. The *in vitro* rooted plants were hardened first under controlled conditions of culture room and then shifted to mist house where they exhibited and hence, growth and 90% survival.

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