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Development of an effective protocol for *in vitro* multiplication of peppermint (*Mentha piperita*)

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Peppermint (*Mentha piperita* L.) is a perennial, glabrous and strongly scented herb belonging to family Lamiaceae. It is an allohexaploid (2n=72) and is a natural hybrid of *M. aquatica* (2*n* = 96) × *M. spicata* (2*n* = 48) (Islam *et al*. 2017). The plant possesses a high content of flavonoids (12%), polyphenols (19%), carotenes, tocopherols, betaine and colines. The plant is stimulant, stomachic, carminative and used for allaying nausea, flatulence, headache and vomiting. Peppermint oil contains menthol, menotone, mentofuran, carvacrol, thymol, etc. *Mentha piperita* is a completely sterile hybrid due to the fact that male organs of flower fail to develop. The stamens remain abnormally short, sometimes degenerate and wither from bud. As a result it is not amenable to improvement by sexual crosses (Tucker 1992). Ruthless exploitation of peppermint due to growing interest in the commercialization of plant based medicines has lead to drastic decrease of natural resource base which will result in extinction. In order to meet pharmaceutical needs and to prevent the plants from extinction, development of rapid large scale propagation systems is a necessity. *In vitro* multiplication is a simple and reliable method for mass production of disease free planting material without seasonal constraints ensuring germplasm conservation and sustainable use of the medicinal crop.

Nodal explants collected from juvenile shoot apices of *M. piperita* maintained at Herbal Garden, SKUAST-Jammu were washed under running tap water for 30 min followed by a detergent solution (Labolenne) containing 2–3 drops of a wetting agent (Tween 20) for 15–20 min with constant shaking. These were treated with 2 g/l Dithane M-45 and 1g/l Bavistin for 15–20 min with intermittent shaking followed by washing with running tap water to remove traces of fungicides. The explants were sterilized with $HgCl_2$ (0.1%) solution for different time intervals (2–5 min) under laminar air flow chamber and were finally washed with autoclaved double distilled water. The sterilized explants were inoculated into the flasks having sterilized MS (Murashige and Skoog 1962) media supplemented with different combinations of growth hormones such as Kinetin (KN) (1.0–2.0 mg/l), Benzyl amino purine (BAP) (2.0–4.0 mg/l) and Gibberellic acid (GA_3) (1.0–2.0 mg/l) for shoot bud induction. The cultures were incubated in the culture room at 25±2°C, with a 16 h photoperiod provided with cool white fluorescent tubes under light intensity of 3 K lux to 5 K lux.

After three weeks of culture, the nodal explants with multiple number of shoot buds were sub cultured on optimal shoot induction medium, MS + 1.5 mg/l KN + 3.0 mg/l BAP. This medium was supplemented with 1.0–2.0 mg/l GA_3 for shoot elongation and proliferation. To induce *in vitro* rooting, the individual elongated shoots were separated and their lower leaves were removed. They were inoculated on either ½MS or ¼MS alone or supplemented with auxin (Naphthalene acetic acid (NAA), Indole acetic acid (IAA) and Indole butyric acid (IBA) at concentration ranging from 0.5–1.0 mg/l. *In vitro* rooted plantlets were taken out and thoroughly washed under running tap water to remove medium. The plantlets were then directly transferred to the plastic pots containing sterilized potting mixtures {Sand and Soil (1:1) and Sand, Soil and FYM (1:1:1)}. For 1 week these were covered with jars in order to maintain humidity. The pots were irrigated with liquid ½ MS treatment for initial two days for improving survival of the plants.

Murashige and Skoog medium was used throughout the study and was fortified with different concentrations and combinations of growth hormones. Sucrose (3%) was used as carbon source in all the combinations. Media were solidified by adding agar (0.8%). The *p*H of medium was adjusted between 5.6 and 5.8 using 0.1N HCl or 0.1N NaOH solutions prior to autoclaving of medium. The sterilization of medium was done at a pressure of 15 psi for 20 min. All cultures were observed at regular intervals of one week and

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Table 1 Effect of cytokinins (mg/l) on shoot induction and establishment of Menthapiperita

Treatment	Cytokinins		Average No. of shoots/ explants	Average shoot length (cm)	Average number of leaves per shoot
	BAP (mg/l)	KN (mg/l)			
T0	-	-	2.1±0.17a	0.6±0.21a	3.0±0.19a
T1	2 .0	1.0	8.5±0.20b	2.8 ±0.10b	6.8±0.11b
T2		1.5	10.5±0.25c	3.5±0.08bc	7.2±0.15b
T3		2.0	12.0±0.30d	4.2±0.18bc	8.7±0.12bc
T4	2.5	1.0	13.0±0.22d	3.8±0.20bc	7.5±0.15 b
T5		1.5	14.5±0.23d	4.8±0.12bc	8.7±0.12bc
T6		2.0	15.5±0.25d	6.5±0.09d	12.4±0.22 d
T7	3.0	1.0	17.8±0.20e	8.5±0.10e	14.8±0.28 e
T8		1.5	20.5 ±0.50f	10.8±0.20f	15.5±0.35ef
T9		2.0	19.0±0.38ef	8.5±0.18e	16.0±0.20 f
T10	3.5	1.0	18.8±0.32ef	7.8±0.20de	14.2 ±0.25 e
T11		1.5	19.0±0.30ef	7.5±0.15de	14.2±0.22 e
T12		2.0	17.5±0.25e	8.0±0.15de	14.7±0.20 e
T13	4.0	1.0	15.8±0.20d	8.2±0.14de	13.8±0.18 d
T14		1.5	14.2±0.32d	8.7±0.16de	13.4±0.20 d
T15		2.0	10.5±0.22c	9.2±0.22ef	12.8±0.16 d

In a column, different letters in superscripts indicate statistically significant difference between the means ($P < 0.05$; Tukey-Kramer Multiple Comparisons Test).

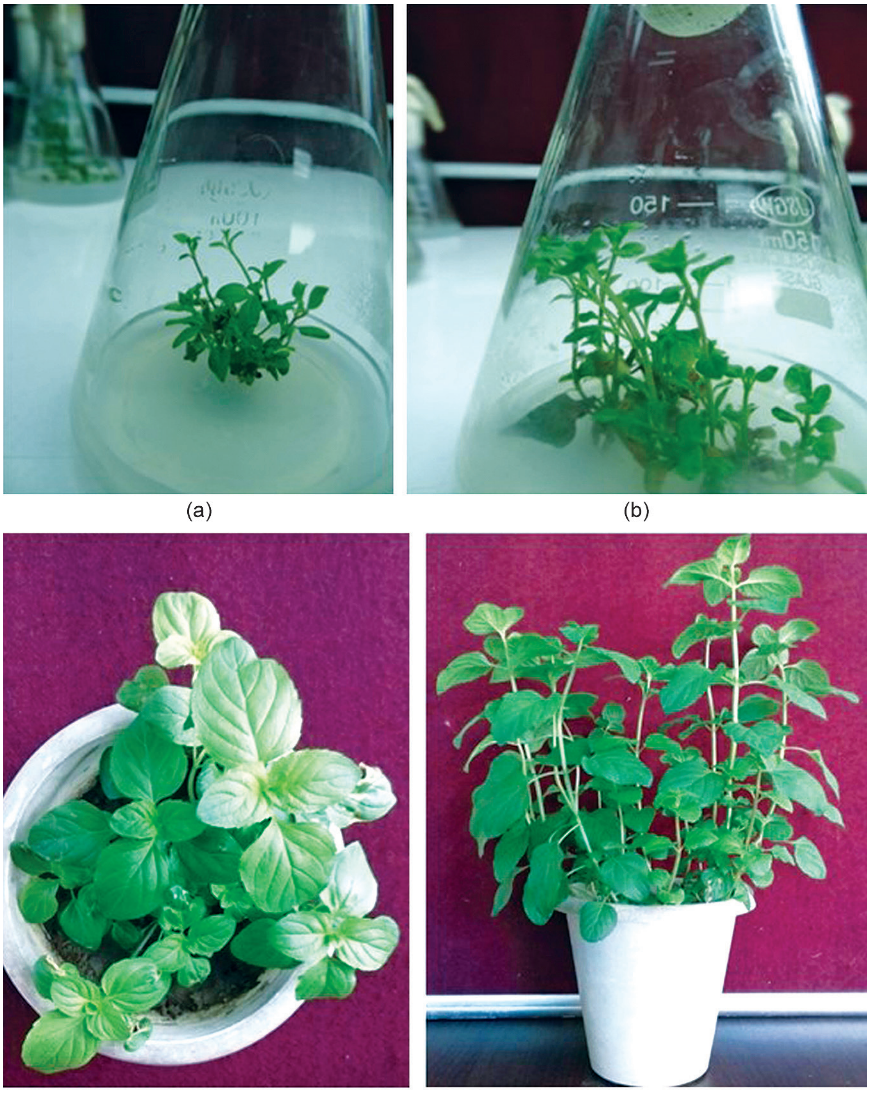
subculturing was done between 21–28 days intervals. The data were recorded after 21 days. Each treatment consisted of 15 explants and the experiment was repeated three times. All data were subjected to analysis of variance (ANOVA) in a completely randomized design (CRD). The differences among the treatment means were tested by Tukey-Kramer Multiple range test. The data were analyzed using Dell STATISTICA v.0 13.2 software.

In vitro propagation of plants is an alternative to wild plant propagation for the production of phytochemicals. Nodal explants disinfected with $HgCl_2$ (0.1%) for 4 min resulted in maximum survival of the cultures. However, the juvenile shoot tips failed to sprout and turned black indicating that the treatment proved harsh for nodal explants. Wesely *et al.* (2010) reported high percentage of explant mortality of *Mentha arvensis* when treated with $HgCl_2$ (0.15%) for 3–4 min indicating lethal effects of high concentration of mercuric chloride. The nodal explants produced more number of shoots as compared to shoot tip explants confirming numerous reports recommending the use of nodal explants for achieving high proliferation rate in herbaceous species (Laslo *et al*. 2010, Islam *et al*. 2017).

The successful establishment of cultures depends on the balance between different phytohormones (Benahmed *et al*. 2018). Bud differentiation is highly influenced by the type of cytokinin used. In the present study, maximum sprouting (100%) of the nodal buds, without an intervening callus phase, was observed on MS medium supplemented with 3.0 mg/l BAP+1.5 mg/l KN wherein, values of average number of shoots per explant (20.5 ± 0.5), average shoot length (10.8 ± 0.2) and average number of leaves per shoot (15.5 ± 0.2) were found to be significantly more in comparasion to other treatments (Table 1). Cytokinins help in overcoming apical dominance, promote shoot formation and help lateral buds to overcome dormancy, thus play an important role

Table 2 Effect of medium strength and auxin concentration (mg/l) on rooting of in vitro raised shoots of Menthapiperita after 15 days of culturing

Auxin for dipping shoots	Concentration (mg/l)	Average roots/plant	Average root length (cm)
½ strength MS medium			
CONTROL	-	0	-
NAA	0.5	6.2±0.20	7.4±0.14
NAA	1.0	7.5±0.25	8.6±0.10
IBA	0.5	10.0 ± 0.40	15.0±0.25
IBA	1.0	8.6±0.31	14.2±0.15
IAA	0.5	4.3±0.11	5.7±0.10
IAA	1.0	5.8±0.14	6.2±0.11
¼ strength MS medium			
CONTROL	-	0	-
NAA	0.5	3.2±0.09	4.4±0.08
NAA	1.0	4.3±0.04	5.2±0.10
IBA	0.5	6.1 ±0.10	5.6±0.11
IBA	1.0	6.9±0.10	6.8±0.11
IAA	0.5	2.3±0.01	3.2±0.05
IAA	1.0	3.1±0.01	3.7±0.04



(a) (b) (c) (d)

Fig 1 (a) & (b) *In vitro* cultures of *Mentha piperita,* (c) & (d) Aclimatized *in vitro* raised plants.

in induction of multiple shoot formation. Zarki and Elmtili (2012) also found BAP to be superior for micropropagation of *M. pulegium.* Similar effect of cytokinins on shoot proliferation was observed in *M. piperata* (Islam *et al*. 2017), *M. viridis* (Ghanti *et al.* 2004) and *M. spicata* (Hirata *et al*. 1990). However, the established axenic mentha cultures showed shorter intermodal distance of the proliferating shoots in medium supplemented with 3.0 mg/l BAP+1.5 mg/l KN. Mehta *et al*. (2012) observed improved bud proliferation in MS medium with BAP (2.0 mg/l) and NAA/IAA but the shoots had stunted growth. The elongation of intermodal distance is essential as it serves as base for carrying out next cycles of subculturing. Fortification of medium with GA_3 (1.0-2.0 mg/l) increased the shoot length and maximum proliferation (30.5±0.40 shoots/explant) was obtained in medium supplemented with GA_3 (1.0 mg/l), where maximum values for average shoot length (15.8±0.30 cm) and average number of leaves per shoot (20.5±0.21) were observed in cultures (Fig 1a and b). The effect of GA_3 on intermodal elongation of shoots is in close proximity with those reported earlier (Islam *et al*. 2017). Senthil and Kamraj (2012) observed maximum multiple shoot induction (100%) of *M. viridis* along with shoot elongation in MS medium with BAP (2.0 mg/l) and IAA (0.5mg/l) while 91% shoot bud proliferation was obtained in MS medium fortified with BAP (3.5 mg/l).

The maximum number of roots/explant (10.0±0.40) and root length (15±0.25 cm) was observed in *in vitro* shoots dipped in ½ strength liquid MS medium supplemented with IBA solution (0.5 mg/l) after 15 days of culturing (Table 2). These rooted plantlets were hardened under controlled conditions along with application of ½MS liquid medium for first two days of *ex vitro* acclimatization resulting in 100% survival. Effectiveness of half-strength MS salts on root induction has been reported in many medicinal plant species (Bouhouche and Ksiksi 2007). Rahman (2013) observed that *in vitro* raised shoots of *Mentha viridis* rooted in both ½ and full strength MS medium and 100% rooting was obtained in ½ strength MS medium with 1.5 mg/l IAA. Mehta *et al*. (2012) reported maximum rooting response along with root length in full strength MS medium augmented with IBA (2.0 mg/l).

Hardening and acclimatization is the most important and crucial step in the survival and successful establishment of the regenerated plants. This is mainly attributed to the inability of such plants to tolerate different types of stresses such as excessive water loss, harsh temperature, high light intensity, etc. In the present study, the plants were initially covered with jam jars for one week in order to maintain 100% humidity as it prevents water loss during the initial stages of plant growth. After acclimatization in the greenhouse (Fig 1 c and d) the regenerated plantlets were transferred to field conditions. The study reports an efficient and reproducible protocol for the *in vitro* establishment and large scale propagation of *Mentha piperita* L. which is an important medicinal plant.

SUMMARY

Mentha piperita is a completely sterile hybrid due to the fact that male organs of flower fail to develop. The stamens remain abnormally short, sometimes degenerate and wither from bud. As a result it is not amenable to improvement by sexual crosses. Ruthless exploitation of peppermint due to growing interest in the commercialization of plant

based medicines has lead to drastic decrease of natural resource base which will result in extinction. In order to meet pharmaceutical needs and to provent the plants from extinction, development of rapid large scale propagation systems is a necessity. In vitro multiplication was carried out for mass production of disease free planting material without seasonal constraints ensuring germplasm conservation and sustainable use of the medicinal crop.

REFERENCES

Benahmed A, Harfi B, Benbelkacem I, Daas A, Laouer H and Belkhiri A. 2018. *In vitro* propagated *Mentha rotundifolia* (L.) buds and antioxidant activity of its essential oil. *Current Issues in Pharmacy and Medical Sciences* **31**(4): 204–8.

Bouhouche N and Ksiksi T. 2007. An efficient *in vitro* plant regeneration system for the medicinal plant *Teucrium stocksianum* Boiss. *Plant Biotechnology Reports* **1**(4): 179–84.

Ghanti K, Kaviraj C P, Venugopal R B, Jabeen F I Z and Rao S. 2004. Rapid regeneration of *Mentha piperita* L . from shoot tip and nodal explants. *Indian Journal of Biotechnology* **3**: 594–8.

Hirata T, Murakami S, Ogihara K and Suga T. 1990. Volatile monoterpenoid constituents of the plantlets of *Mentha spicata* produced by shoot tip culture. *Phytochemistry* **29**: 493–5.

Islam A T M R, Islam M M and Alam M F. 2017. Rapid *in vitro* clonal propagation of herbal spice, *Mentha piperita* L. using shoot tip and nodal explants. *Research in Plant Sciences* **5**(1): 43–50.

Laslo V, Zaprtan and Vicaş S. 2010. *In vitro* behavior of certain varieties of *Vitis vinifera* L. grown on hormonally balanced culture medium. (In) Proceedings of International Symposium Trend In The European Agriculture Development. Timisoara May 20–21.

Mehta J, Naruka R, Sain M, Dwivedi A, Sharma D and Mirza J. 2012. An efficient protocol for clonal micropropagation of *Mentha piperita* L. (Pipperment). *Asian Journal of Plant Science and Research* **2** (4): 518–23.

Murashige T and Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**: 473–97.

Rahman M M, Ankhi U R and Biswas A. 2013. Micropropagation of *Mentha viridis* L.: An aromatic medicinal plant. *International Journal of Pharmacy and Life Science* **4**(9): 2926–30.

Senthil K and Kamraj M. 2012. Direct shoot regeneration from inter nodal explants of *Mentha viridis* L. *International Journal of Pharmaceutical Sciences and Research* **3**(4): 1101–3.

Tucker A O. 1992. The truth about mints. *Herb Companion* **4**: 51–2.

Wesely E G, Johnson M, Kavitha M S, Selvan N. 2010. Clonal propagation of *Mentha arvensis* L. through shoot tip and nodal explants. *Biotechnology- an Indian Journal* **4**(1): 48–53

Zarki K B L and Elmtili A. 2012. Micro-propagation of *Mentha pulegium* L. through high-frequency shoots-tip and nodal explants culture. *Moroccan Journal of Biology* **12**: 8–9.