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Rapid and large scale micropropagation of true to type clone of *Mentha arvensis* Linn. (Lamiaceae) -A valuable medicinal plant

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Abstract

Method for *in vitro* micropropagation of *Mentha arvensis* Linn. by axillary bud proliferation were investigated by optimizing the concentrations and combinations of different phytohormones in MS medium. Axillary bud explants could be stimulated to form multiple shoots on MS medium augmented with BAP and AdS. The best proliferation (8.81 shoots per explant) was achieved on BAP (1.0 mg/l). Shoots could be easily rooted on MS medium containing 1 mg/l IBA. Plantlets were successfully acclimatized in green house and field conditions. No detectable differences were recorded between the donor and *in vitro* generated plants with regard to biochemical and cytological parameters.

M*entha arvensis* is a valuable medicinal plant belonging to the family Lamiaceae. The plant is a perennial, glabrous, strongly scented herb. *Mentha* plant thrives well in humid and temperate climates and is sensitive to drought. It grows mainly on calcareous and rich loam soil on sunny situation. Dried leaves and flowering tops of the plant is mainly used for peppermint oil. The oil is a colourless, pale yellow or greenish yellow liquid with a strong odour. The peppermint oil contains 50-55% menthol. Besides menthol the major constituents are menthyl acetate, menthone and small amount of α-pinene, β-phellandrene, terpinene, cineole etc. The dried plant is used as an antiseptic, carminative, stomachic and refrigerant. It is also considered to be stimulant, emmenagogue and diuretic[4]. The infusion of leaves is used in rheumatism and indigestion[2]. The plant is propagated mainly by shoot cuttings, seed setting is very poor. Besides this, in dry places and during summer season, the propagation of this plant is very difficult. We report here a rapid and efficient method for *in vitro* clonal propagation of menthol producing plants through axillary bud proliferation.

The young shoot buds (both terminal and axillary) were used as explants. Shoot cuttings with the youngest two leaves were collected

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from plants grown in the experimental garden of the institute. After excision, the shoot tips (about 0.5 to 1 cm in length) were subjected to preliminary washing under running tap water for 10 to 30 minutes to remove the microflora to a substantial extent. Healthy and uniform explants were disinfected thoroughly in 5% savlon solution for 8 minutes. Explants were then rinsed under running tap water. Then they were surface sterilized with 0.1% (w/v) aqueous mercuric chloride solution for 8 minutes, followed by 4-5 rinses of 3 minutes duration in sterile distilled water.

The shoot bud explants were transferred to MS[14] medium augmented with 0.5-3.0 mg/l 6-benzylaminopurine (BAP), 0.5-3.0 mg/l Adenine sulphate (AdS), 0.5-3.0 mg/l Indole butyric acid (IBA), either individually or in various combinations. The medium pH had been adjusted to 5.6 prior to adding 7 gm/l agar (w/v, Qualigens) and was autoclaved at 121°C for 15 minutes. The cultures incubated at 24±1°C under 16 hours daily illumination with fluorescent light (12000 lux). The medium was dispensed into 25X150 mm culture tubes containing 20 ml medium or into 100 ml wide mouth conical flasks with 50 ml culture medium each.

The microshoots having the length above 4 cm regenerated from multiple shoot clusters were transferred to half or full strength MS basal medium supplemented with different concentrations of IBA for induction of roots.

Healthy rooted plantlets were taken from the rooting medium and washed several times with sterile distilled water. Plantlets were potted in sterile sand:loam:peat (in a ratio of 1:1:1) mixture for acclimatization. The pots were initially kept for two weeks under green house condition. After that the plants were moved to the field.

Isozyme (Peroxidase) analysis was performed by macerating 1 gm leaf material collected from mature plants and two months old tissue culture raised plants growing in the same environment. The leaves were excised and kept in -70°C for some days to avoid chlorophyll interference. The materials were crushed in an ice-cold mortar pastle with ice-cold PEB buffer. After centrifugation, the pellet was discarded and the supernatant was lyophilized for 10-12 hours as required. SDS-PAGE was performed using 12% polyacrylamide gels.

When the run was completed, the stacking gel part was cut off and the rest was incubated in buffer and substrate solution of Isozyme. Guaicol- H_2O_2 method by Hislop and Stahmann[7] was followed for the visualization of peroxidase isozymes.

The surface sterilization procedure followed in the present study yielded 90% of the explant free of microbial contamination (Fig 1). Multiple shoot development could not be induced from the axillary bud of *Mentha arvensis* on a growth regulator free MS medium (M 1). The present experiments revealed that addition of cytokinin to the medium was essential to induce multiple shoot formation in the explant. In a series of media (M2-M14) supplemented with BAP and AdS were tested to induce multiple shoot formation (Table 1). MS medium containing only BAP as a cytokinin at a concentration of 0.5 mg/l (M2) induced shoot multiplication within 10-15 days. Initially 1-2 shoot buds were

developed. After that number of shoot bud development was increased gradually when the cultures were maintained in the same medium upto 2-3 passages (10 days duration of each passage). In presence of BAP (0.5 mg/l) 7.82±0.3 shoots were formed per explant at the end of 4th passage. Number of shoot buds production increase after increasing the concentration of BAP upto 1.5 mg/l. The best multiplication rate was achieved (Fig. 2) in MS medium containing 1.0 mg/l BAP. The multiplication rate of the explant was 90% and each explant developed 8.81±0.5 shoots averaging 6.21±0.3 cm length within 40 days of culture (Table 1). The other media (M5, M6 and M7) containing higher concentration of BAP (2-3 mg/l) showed lower multiplication rate. The use of AdS (0.5-3.0 mg/l) also exhibited comparatively lower multiplication rate (M 9 - M14). The use of higher concentrations AdS (2.5-3.0 mg/l) did not show any advantage rather the multiplication rate became lowest. The highest numbers of shoot buds were produced in AdS (2 mg/l) containing medium is 6.2. In order to increase the rate shoot multiplication, all cultures in M3 medium were maintained upto 4th passages. After 5th passage all cultures gradually developed into dense cluster of shoots but the growth of individual shoot became stunted.

The shoot length increased in response to BAP concentration, reaching the highest growth with 1.0 mg/l treatments and declining when BAP concentration reached 3 mg/l. The best combination for *Mentha arvensis* shoot elongation consisted of 1 mg/l BAP (Table 1). The medium supported effective enhancement in shoot length and recorded a maximum length of 6.21±0.5 cm within 40 days.

Microshoots recovered from axillary bud explant cultures had no roots. Rooting could not be induced in the excised shoots in an auxin free MS medium even after 30 days. For rooting 3-4 cm long shoots were separated as minicutting, cultured on MS medium supplemented with IBA (0.5-3 mg/l) and no cytokinin for the development of a proper root system (Fig. 3). Roots were produced within 3 weeks of culture. Of all the concentrations tried for rooting on *in vitro* raised shoots, the best results were obtained in IBA at 1.0 mg/l (9.21±0.5 roots per explant).

The explants showing root initiation after 8-10 days of starting the culture were transferred to medium of the same composition to promote further proliferation (with the increase of number of root laterals, length of roots etc.) with the lapse of 25 days. The results indicate that IBA concentrations supplied to MS medium significantly influenced root proliferation and shoot growth. The best rooting treatment were 1 mg/l, since it gave the highest percentage of root induction. Higher levels of IBA (2-3 mg/l) reduced root number.

After rooting, the plants were transferred to vermicompost (Fig. 4) and acclimatized to green house condition. Regenerated plants were very sensitive to changes in the physical environment. They were grown in very high humidity and responding to decreased relative humidity too slowly to prevent desiccation of the rooted plants. Regenerated plants must be acclimated to increased light intensity in much the same manner as acclimation to decreased relative humidity. An almost 90% survival of the transplanted plantlets was observed in the soil (Fig. 5).

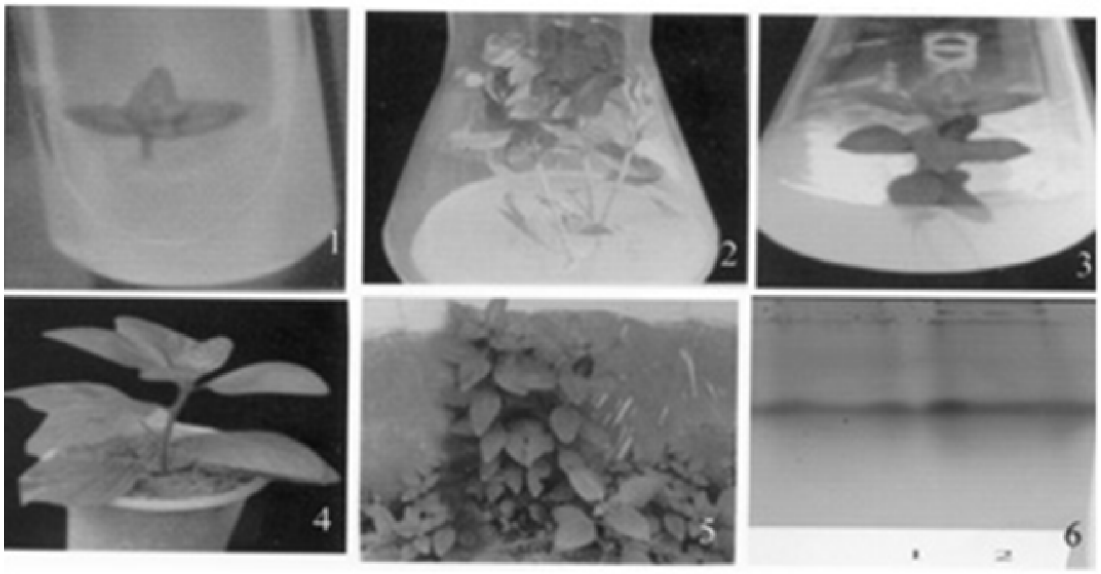

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Fig. 1. Shoot bud explant of *Mentha arvensis* cultured on MS+BAP (1.0 mg/l)
Fig. 2. Formation of multiplied shoots on MS+BAP (1.0 mg/l) after two weeks of culture
Fig. 3. Rooting of *in vitro* raised shoots on MS+IBA (1.0 mg/l).
Fig. 4. Potted *in vitro* raised plantlet.
Fig. 5. Transplanted plantlet observed in the soil.
Fig. 6. Peroxidase isozyme pattern.
1=donor plants of *Mentha arvensis*.
2= *in vitro* raised plants of *Mentha arvensis*

For detection of peroxidase, the Guaicol -H_2O_2 method has been used as the staining substrate. In both the mother plant as well as regenerated plants shows only one band in the upper (+ve) region of the gel. The Rf value of the band was 0.33. There was no difference in intensity of bands between the mother plants as well as regenerated plants (Fig. 6) of *Mentha arvensis*.

This paper provides protocols for rapid rooted shoot production in *Mentha arvensis* using axillary shoot bud explants. Axillary shoot buds have the potential for unlimited shoot proliferation because of the presence of apical meristem constantly undergoing cell division and cell differentiation. This potentiality was not expressed when the excised explants were grown in culture medium containing no growth regulators. On the contrary when the explant were grown on culture medium containing cytokinin, excised axillary shoot bud proliferated and formed 7-8 shoots. In *Mentha arvensis*, higher number of shoots per explant was

Table 1. Nutrient media used for micropropagation of axillary shoot bud of *Mentha arvensis* (MS basal medium with 3% sucrose, 0.7 % agar and pH=5.6). Results are the mean of 6 replicates±SE (after 40 days of culture).

Medium	BAP	Mean No. of shoots	Mean length of shoots(cm.)	Medium	BAP	Mean No. of shoots	Mean length of shoots(cm.)
M1	0.0	0.0	0.0	M8	0.0	0.0	0.0
M2	0.5	7.82±0.3	5.22±0.7	M9	0.5	4.21±0.4	4.12±0.2
M3	1.0	8.81±0.5	6.21±0.3	M10	1.0	4.22±0.3	4.44±0.3
M4	1.5	7.12±0.4	3.22±0.4	M11	1.5	5.32±0.2	4.82±0.2
M5	2.0	3.33±0.4	3.21±0.2	M12	2.0	6.22±0.3	4.81±0.4
M6	2.5	3.33±0.5	2.82±0.2	M13	2.5	2.51±0.2	1.26±0.4
M7	3.0	1.56±0.2	1.15±0.2	M14	3.0	2.21±0.2	1.21±0.1

recorded on a medium containing BAP. Therefore, BAP was the most effective cytokinin in shoot proliferation in the explant. The efficiency of BAP for shoot bud multiplication on explants in the present system is similar to the report on *Cymbopogon nardus*[10], *Papuacalia versteegii*[13] etc. Furthermore, increase in BAP concentration (2-3 mg/l) caused decrease in shoot bud proliferation. So, higher concentration of BAP is not effective for increasing shoot bud proliferation in case of *Mentha arvensis*. This is in agreement with the observation of banana-Lal Kela by Ganapathi *et al*.[6]. The presence of adenine in the medium is reported to promote axillary bud differentiation in many cases[3,12,17].

IBA (1 mg/l) was found to be the ideal concentration for root initiation, root length and number of root laterals. IBA is an effective auxin in root induction in a wide range of plants from herbs to tree[8]. IBA was reported to have favored root initiation in several plant species in culture[11,15]. The efficiency of auxins alone for root induction on microshoots in the present system is similar to the report on *Solanum trilobatum*[1], *Scutellaria integrifolia*[9] etc.

When other auxins like IAA and NAA were added to the medium, callus was formed from the shoot base, which did not favour root formation. Root developments were inhibited by BAP, in the presence of either IBA or NAA. This observation was at per with those of Furmanowa and Olszowska[5] and Saez *et al*.[16].

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