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Clonal propagation of *Mentha arvensis* L. through shoot tip and nodal explants

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ABSTRACT

The present study was aimed to produce an efficient direct micropropagation system for *Mentha arvensis* Linn. a medicinally important plant using shoot tip and nodal segments as explants. Adventitious proliferation was obtained from *Mentha arvensis* shoot tip and nodal segments inoculated on Murashige and Skoog's basal medium with 3% sucrose and augmented with 6 - Benzyl Amino Purine and Kinetin. Highest frequency of shoot proliferation (shoot tip 96.2 ± 0.84 and Nodal segments 94.6 ± 0.63) was observed in Murashige and Skoog's medium augmented with 1.5mg/l of 6-Benzyl Amino Purine. After 8 weeks, maximum number (81.6 ± 0.96) of multiple shoots was obtained in shoot tip cultured on MS medium supplemented with 3.0 mg/l BAP in combination with 1.5mg/l of Kin. After 8 weeks, nodal explants cultured on MS medium supplemented with 0.5 mg/l BAP in combination with 1.5mg/l Kin showed maximum number (72.3 ± 1.21) of shootlets per explants. Half strength Murashige and Skoog's medium with 3% sucrose augmented with 1.5 mg/l of Indole 3-Butyric Acid showed the maximum frequency (95.2 ± 1.24) and maximum number (8.4 ± 0.7) of rootlets per shoot lets. The micropropagated plantlets genetic uniformity was confirmed through the isozyme analysis. The in vitro raised plants were hardened then transferred to field for re-establishment. © 2010 Trade Science Inc. - INDIA

KEYWORDS

Clonal propagation;
Shoot tip;
Micropropagation;
Isozyme;
Iso-esterase;
Nodal.

ABBREVIATIONS

MS-Murashige and Skoog's medium
PGRs-Plant Growth Regulators
BAP-Benzyl Amino Purine
IAA-Indole-3-Acetic Acid

IBA-Indole-3- Butyric Acid
NAA- α Naphthalene Acetic Acid

INTRODUCTION

Medicinal plants continue to be an important

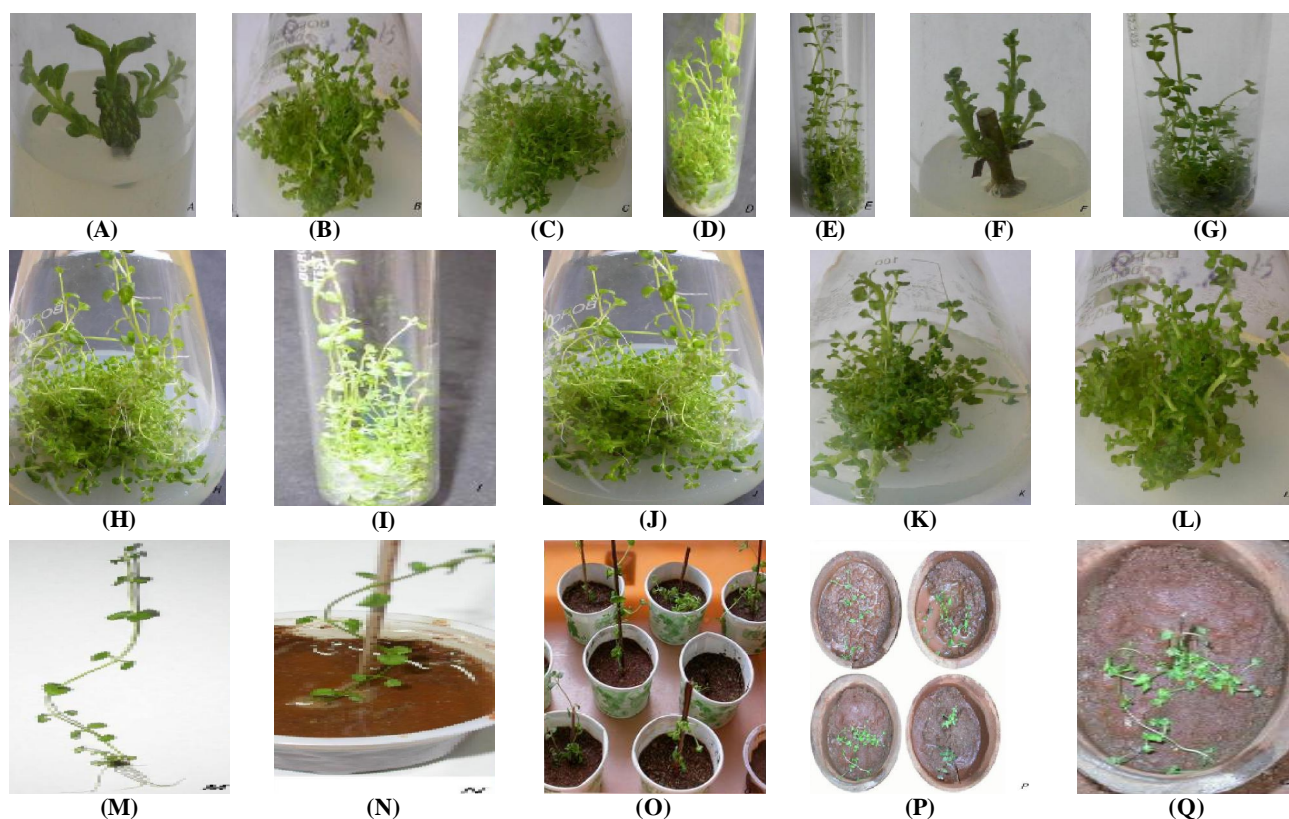


Figure 1 : *In Vitro* Multiplication and Field Establishment of *Mentha arvensis* L., A-Multiple shootlets initial stage-shoot tip. B to E-Different stages of multiple shootlets-Shoot tip. F-Multiple shootlets initial stage-Nodal segments. G to L-Different stages of multiple shootlets-nodal segments. M-*In vitro* derived plantlet with shootlet and rootlets. N & O-Different developmental stages of hardened plants in poly cups. P & Q-Different developmental stages of hardened plants in pot

therapeutic aid for alleviating the ailments of human-kind. The search for eternal health and longevity and for remedies to relieve pain and discomfort drove early man to explore his immediate natural surroundings and led to the use of many plants, animal products and minerals etc and the development of the variety of therapeutic agents. Today, there is a renewed interest in traditional medicine and an increasing demand for more drugs from plant sources. This revival of interest in plant-derived drugs is mainly due to the current widespread belief that “green medicine” is safe and more dependable than the costly synthetic drugs, many of which have adverse side effects^[1]. In India the use of medicinal herbs is as old as 1500 BC. Underlying the medicinal culture of India both folk traditions as well as codified knowledge systems is a deep understanding of the medicinal value of the plants starting with the reference in the Atharva Veda. The growing interest in the commercialization of plant based medicines has lead to over exploitation of the plants. The sad part of this situation is that medicinal plants related trade especially in India

depends on the natural populations which is not sustainable and will certainly lead to species extinction. Hence we need to develop the concept of medicinal crop and employ the modern tools to meet our domestic and export needs. In this condition, plant tissue culture is considered as promising technology to get over this hurdle and play a major role in the mass multiplication, germplasm production, conservation, secondary metabolites production and sustainable use of the “medicinal crop” concept is the only way to compete the global medicinal plant market both quantitatively and qualitatively. Tissue culture in turn is the only way to sustain the large scale farming of medicinal plants, as this is the only technique to produce plants high and uniform quality in large quantity from any part of the plant in any season^[2]. This technique provides a rapid reliable system for production of large number of genetically uniform and even disease free plantlets. *Mentha arvensis* L. is an important medicinal plant. It belongs to Lamiaceae family. It is commonly called Pudina in Tamil and Mint in English. The plant is aromatic, stimu-

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TABLE 1 : Effect of cytokinin on multiple shoots formation from shoot tip and nodal segments of *Mentha arvensis* L.

MS medium + Cytokinin concentration in mg/l		Shoot tip			Nodal segments		
BAP	KIN	Mean % Shoot formation± S.E.	Mean No. of shoots± S.E.	Average shoot length (cm)	Mean % Shoot formation± S.E.	Mean No. of shoots± S.E.	Average shoot length± S.E. (cm)
0.0	0.0	32.5 ± 0.42	10.4 ± 0.25	4.0	22.1 ± 0.36	8.4 ± 0.42	4.0
1.0	0.0	90.3 ± 0.63	55.4 ± 0.64	3.5	63.2 ± 0.49	43.4 ± 0.24	3.5
1.5	0.0	96.2 ± 0.84	53.4 ± 0.36	3.7	94.6 ± 0.52	65.6 ± 0.36	3.7
2.0	0.0	82.5 ± 0.67	42.6 ± 0.43	4.1	85.3 ± 0.65	58.4 ± 0.47	4.1
0.0	0.5	70.4 ± 0.56	33.4 ± 0.47	10.3	53.2 ± 0.42	23.4 ± 0.42	10.6
0.0	1.0	65.3 ± 0.42	42.7 ± 0.24	3.9	65.4 ± 0.61	36.7 ± 0.86	3.9
0.0	1.5	63.2 ± 0.24	45.5 ± 0.62	8.5	75.4 ± 0.47	47.4 ± 0.63	8.5
0.0	2.0	62.3 ± 0.56	35.5 ± 0.50	4.0	65.4 ± 0.34	51.4 ± 0.93	4.0
0.5	0.5	83.2 ± 0.64	37.5 ± 0.41	4.1	67.4 ± 0.46	51.2 ± 0.26	4.1
1.0	0.5	78.6 ± 0.54	43.5 ± 0.63	3.9	75.6 ± 0.94	56.8 ± 0.62	3.9
1.5	0.5	77.9 ± 0.73	60.4 ± 0.42	4.6	86.5 ± 0.43	72.3 ± 1.21	4.6
3.0	0.5	76.5 ± 0.53	65.6 ± 0.83	3.8	69.3 ± 0.86	45.8 ± 0.64	3.8
3.0	1.0	70.5 ± 0.84	67.3 ± 0.54	4.4	76.3 ± 0.78	41.4 ± 0.75	4.4
3.0	1.5	69.5 ± 0.57	81.6 ± 0.96	6.1	68.7 ± 0.76	33.6 ± 0.58	6.1

TABLE 2 : Effect of Auxins on rooting on *in vitro* derived shootlets of *Mentha arvensis* L.

S. No	Auxins conc. (mg/l)	Mean percentage of Rootlets formation	Mean No. of rootlets per shootlets	Mean length of rootlets in cm
1	NAA (1.0)	82.6 ± 0.86	2.9 ± 0.48	3.3 ± 0.43
2	NAA (1.5)	85.4 ± 0.69	4.7 ± 0.36	3.6 ± 0.49
3	IAA (1.0)	83.6 ± 0.97	3.6 ± 0.69	3.1 ± 0.68
4	IAA (1.5)	82.7 ± 0.36	5.4 ± 0.47	4.1 ± 0.48
5	IBA (1.0)	90.4 ± 0.65	3.4 ± 0.26	3.5 ± 0.46
6	IBA (1.5)	95.2 ± 1.24	8.4 ± 0.7	3.8 ± 0.24

lant and carminative^[3]. Mint is valued for its multipurpose uses in the field of pharmaceuticals, cosmetics as well as for flavoring foods, beverages and tobacco^[4]. The plant is aromatic, stimulant and carminative. The infusion of leaves affords a remedy for rheumatism and indigestion^[3]. *Mentha* (Mint) was used as medicinal herb in ancient times, but menthol crystals are used in different pharmaceutical products and cosmetics as antiseptic, stimulant and inhibitor. It gives minty flavor to various food products e.g. tooth paste and mouth fresheners due to its physiological cooling effects.

Mentha is commonly propagated by vegetative means. The technique poses considerable loss of potential productive plant stands. To alleviate this and other related problems, in many instances, the technique of micropropagation is believed to be the best alternative. It offers the potential to produce thousands, or even

millions, of plants of the desired clone per annum based on the available capacity. Besides, it also serves as a corner stone for future crop improvement by molecular biotechnology. In line with this, successful efforts have been made and encouraging results have been recorded elsewhere on micropropagation of *Mentha arvensis* has been reported by Iwao Asai et al.^[6] using shoot culture and nodal explants by Chishti et al.^[3], direct shoot regeneration from *in vitro* derived inter-nodal segments by Shasany et al.^[7]. Shoot regeneration of *Mentha viridis* using nodal and shoot tip as explants was reported by Raja and Arockiasamy^[5] and Callus production was reported for *Mentha piperita* by Amrita and Sharmila^[8], rapid multiplication protocol for six *Mentha* species using axillary buds was reported by Rech and Pires^[9]. This study describes the results of the study taken up for developing an effective, repro-

ducible and simple protocol for (a) clonal multiplication of the medicinally and economically important plant *Mentha arvensis* using shoot tip and nodal segments as explants and (b) production of isozyme marker for *Mentha arvensis* for further crop improvement by molecular biotechnology.

MATERIALS AND METHODS

Plants of *Mentha arvensis* (Lamiaceae) collected from Kolli Hills, Salem, Tamil Nadu, India was established in the Botanical garden of Muthayammal college of Arts and Science, Rasipuram, Tamil Nadu, India. Young shoots were harvested and washed with running tap water and surface sterilized in 0.05 and 0.1% mercuric chloride for 2, 3 and 5 min. After rinsing 3-4 times with sterile distilled water, shoot tip, leaves, stem nodes and internodes were cut into smaller segments (0.5 to 1.0cm) used as the explants. The explants were placed horizontally (leaves and inter-nodal segments) as well as vertically (shoot tip and nodal segments) on solid basal Murashige and Skoog^[10] (MS) medium supplemented with 3% sucrose, 0.7% (w/v) agar (Hi-Media, Mumbai) and different concentration (0.5-5.0mg/l) and combination of BAP and Kin for *in vitro* shootlets regeneration. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 15min. The cultures were incubated at 25 ± 2°C under cool fluorescent light (2250lux 12 hr/d photoperiod). For rooting, the *in vitro* raised shootlets were transferred to the ½ MS medium augmented with different concentrations and combinations of auxins (IAA, IBA and NAA). For hardening, the *in vitro* raised plantlets were removed from culture, washed thoroughly with tap water planted in small polycups filled with sterile garden soil (3:1), covered by unperforated polybags, and hardened for 4 weeks in a mist chamber before transfer to field.

For esterase, the young leaves were collected and ground with pre-chilled isolation buffer (0.1M phosphate buffer pH 9.2) and centrifuged at 12,000 rpm for 10 min. The supernatant (extract) stored in eppendorf tubes in freezer and subsequently used for isoenzyme (isoesterase) analysis^[11]. For isoesterase, the PAGE electrophoresis (with 6% stacking gel and 8% separating gel composition) was performed by Anbalagan method^[12]. After running the gel was stained with mix-

ture of 0.2g fast blue RR salts, 2.8g of sodium dihydrogen phosphate and 1.1 g of disodium hydrogen phosphate, 0.03 g of α -naphthyl acetate and 150ml of distilled water and incubated in dark for half an hour. Then the gel was fixed in 7% acetic acid for 15 min^[13]. Based on the banding profile of the enzyme systems the RF values of isoesterase were calculated.

RESULTS AND DISCUSSION

The surface sterilization of *Mentha arvensis* was carried with different concentration of mercuric chloride such as 0.05%, 0.1% and 0.15% for different time duration. Among them, 0.1% mercuric chloride for 3 min showed low percentage contamination and highest (95.6%) percentage of microbes/contaminants free explants. The explants treated with 0.05% of mercuric chloride for 3-5 min showed 50- 66% of microbes free explants. The explants treated with 0.15% for 3-4 min obtained hundred percentages of microbes free explants with high percentage of explants mortality, high concentration of mercuric chlorides leads the death of the explants (lethal effect). The medium (MS) augmented with different concentrations and combinations of cytokinin was used for multiple shoots emergence from the shoot tip and nodal segments. After 5 days of inoculation, the buds were started for shoot proliferation the effect of cytokinin on shoot multiplication from shoot tip and nodal explants is shown in TABLE 1. Among the different concentration of cytokinin tested, highest percentage (96.2 ± 0.84) of shoot induction from shoot tip was observed on MS medium supplemented with 1.5mg/l of BAP. In case of nodal segments, highest percentage (94.6 ± 0.63%) of shoot initiation was observed on MS medium fortified with 1.5mg/l BAP. After 8 weeks, maximum number (81.6 ± 0.96) of multiple shoots was obtained in shoot tip cultured on MS medium supplemented with 3.0mg/l BAP in combination with 1.5mg/l of Kin (Figure 1. A-E). After 8 weeks, nodal explants cultured on MS medium supplemented with 0.5 mg/l BAP in combination with 1.5mg/l Kin showed maximum number (72.3 ± 1.21) of shootlets per explants (TABLE 1) (Figure 1. F-L). The MS medium augmented with combinations of cytokinin induced maximum number of multiple shoots with maximum percentage. Cytokinin alone or in combinations induced

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multiple shoot formation was reported by other workers in *Rhinacanthus nasutus*^[14], *Baliospermum axillare*^[15], *Solanum surattense*^[16] and *Baliospermum montanum*^[17,18], *C. asiatica*^[19,20], *Hemidesmus indicus* and *Vitex negundo* L.^[21-23]. These results indicated cytokinin, played an important role in induction of multiple shoot formation and were very effective in shoot proliferation (Figure 1. A-L).

Of the different concentrations of IBA (0.5-1.5mg/l) tested 1.5mg/l proved to be most suitable for root induction with highest percentage (95.2 ± 1.24) and maximum number (8.4 ± 0.7) of rootlets per shoot lets (TABLE 2) (Figure 1 M). Similar effects of IBA were also observed in *Sesbania acculeata*^[24], Pigeon pea^[25] and *Vitex negundo*^[26]. This is in contrast with the results of Madhavan and Balu^[27] on *Wedelia chinensis*, wherein IBA promoted formation of multiple shoots. After 30 days of rooting, *in vitro* raised plantlets were hardened in polycups containing a mixture of sterile garden soil: sand (3:1), covered with polypropylene bags and irrigated with 10 x diluted MS liquid medium. The plants were kept in a culture room for 15 days. 90% of plants were successfully established in polycups (Figure 1. N & O). After 15 days the polycups hardened plants, were transferred to pots and kept in green house. 90% of plants were well established in the green house condition (Figure 1. P & Q). After one month, the plants were transferred to the field. About 80% of plants were established in the field.

Mass propagation of plant species through *in vitro* culture is one of the best and most successful examples of commercial application of plant tissue culture technology. Recently there has been much progress in this technology for conservation of genetic resources and clonal improvement^[28-31]. Rapid shoot regeneration has been achieved with a wide range of species with initial explants being taken from normal aerial shoots of field grown herbaceous medicinal plant species^[32-36]. In the present investigations, shoots were regenerated directly from explants of shoot tips and nodal segments of *Mentha arvensis*. Repeated subcultures of explants on fresh shoot proliferation medium helped to achieve continuous production of healthy shoot buds and shoots at least through five to ten subculture cycles.

The genetic conformity test was carried out between

the mother plant, *in vitro* raised by nodal segments and shoot tip derived plantlets. The nodal and shoot tip derived plantlets and mother plants showed similar banding profiles and no variation between the mother and daughter plants. Similar kind of analysis was performed by Mondal et al.^[36], Merce et al.^[37] and Johnson et al.^[13,16,27,38] with different plants, they also confirmed the somoclonal variations using isozyme and protein analysis. The use of isozymes as genetic markers as increased dramatically over the last decades as it has number of important advantages over more conventional morphological markers^[39]. The present study isozyme results also strengthen the application of isozymes as a marker in systematic and plant breeding programme.

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