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Rapid axillary bud proliferation and *ex vitro* rooting of herbal spice, *Mentha piperita* L.

C Sunandakumari[1], K P Martin[2], M Chithra[1], S Sini[1] and P V Madhusoodanan[1*]

[1]Department of Botany and [2]Department of Biotechnology, University of Calicut, Calicut 673 635, India

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Efficient protocol for rapid multiplication of the herbal spice *Mentha piperita* L. through axillary bud multiplication and *ex vitro* rooting was established using Murashige and Skoog (MS) medium. Media prepared with tap water and commercial sugar, and those prepared with double distilled water and tissue culture grade sucrose did not show significant difference in the *in vitro* induction of shoots/node, and roots/shoot. MS medium fortified with 4.44 μ*M* N^6-benzyladenine (BA) and 2.32 μ*M* kinetin (Kn) was the best for proliferation of shoots; induced a mean of 4.1 shoots/node explant. The shoots attained a height of more than 4.5 cm bearing more than 5 nodes within 40 days. Excision and culture of *in vitro* derived node segments on medium with 3.33 μ*M* BA and 2.32 μ*M* Kn facilitated enhanced axillary bud proliferation. Shoots developed were rooted best on half-strength MS medium with 0.49 μ*M* indole-3-butyric acid (IBA); induced a mean of 10.3 roots. *In vitro* rooted shoots exhibited 100% survival in field conditions. Dipping of the basal end of shoots harvested from multiplication medium in 0.49 μ*M* IBA solution for 10 days induced a mean of 8 roots and its transfer to small pots facilitated survival of 95% rooted shoots. *Ex vitro* rooting by direct transfer of the shoots from multiplication medium to small pots showed 72% survival. Commercial sugar and tap water and *ex vitro* rooting make the protocol economic.

Keywords: commercial grade sugar, *ex vitro* rooting, medicinal plant, tap water; *Mentha piperita*

Introduction

Mentha piperita L., an aromatic herbaceous perennial belonging to the family Lamiaceae is a native to Europe and is usually cultivated in subtropical regions of the world, and India in particular. *M. piperita*, a natural hybrid of *M. aquatica* and *M. spicata* is an allohexaploid (2n=72)[1]. The leaves and flower tops of the plant are the officinal part. The essential oils, synthesized and stored in leaf glandular trichomes are valued commercially as additives for food products, cosmetics and pharmaceuticals. This herbal spice is cultivated mainly for the essential oil, traded as peppermint oil, which contains menthol, viridiflorol, menthofuran, menthone, isomenthone, isomenthol, neomenthol, neoisomenthol, pulegone, piperitone, α-pinene, limonene, terpinene, phellandrene, cineole, carvone, menthoside, and several sesquiterpenes[2, 3]. The export of peppermint (*M. piperita*) oil from April 2000-March 2001 was about 1,000 tonnes of valued at approximately Rs. 35 crores[4]. The Indian price of peppermint oil is Rs. 5,00/kg [4](Anonymous, 2002). It has wide rage of applications in pharmaceuticals, confectionery, alcoholic drinks, candies, soaps, chewing gums, dental creams, and mouth washes [2].

Propagation of medicinal plants through axillary bud proliferation has been shown to be the simple and reliable method for rapid *en masse* production of the desired clones[6,7]. Vegetative propagation of *M. piperita* by stem cuttings is inadequate to meet the demand of disease free raw materials in time. Its propagation by seeds is also cumbersome. According to Veronese *et al*, 18,000 peppermint floral spikes containing more than 2.75 million ovules developed only 6 viable seeds[8]. Axillary bud multiplication, and subsequent *in vitro* rooting on separate medium by using tissue culture grade sucrose and distilled water has been reported in *Mentha* species such as *M. piperita*, *M. spicata* and *M. arvensis*. However, in the present study, feasibility of rapid propagation of *M. piperita* at less cost through axillary bud multiplication and *in vitro* and *ex vitro* rooting using tap water and commercial sugar was attempted.

Materials and Methods

Young shoots of *M. piperita*, collected from flowering plants as the source tissues were washed first under running tap water followed by a detergent,

*Author for correspondence:
Tel: 0494-2401144, extn 406; Fax: 0494-400269
E-mail: marttin@sify.com

Extran (5 % v/v) for 5 min. After thorough wash in water, source tissues were surface sterilized using 0.1 % (w/v) mercuric chloride solution for 7-9 min. After repeated washings with sterile water (3 times 5 min each), node segments were cut into appropriate sizes (1.0-1.5 cm) and cultured on sterile nutrient medium. The nutrient medium consisted of the salts and vitamins of Murashige & Skoog[12]. The medium was gelled with 0.7% (w/v) agar (Merck). Basal medium was supplemented with.

Different growth regulators viz. N^6-benzyladenine (BA)/kinetin (Kn)/indole-3-acetic acid (IAA)/indole-3-butyric acid (IBA)/α-naphthaleneacetic acid (NAA) at different concentrations either singly or in combination. The media (for *in vitro* rooting also) were prepared either with tap water and 3% (w/v) commercial sugar or with double distilled water and 3% (w/v) tissue culture-grade sucrose. The *p*H of all the media was adjusted to 5.8 before autoclaving at a pressure of 1.06 kg cm^{-2}. All the cultures (*in vitro* rooting also) were incubated at 25±2°C with 16/8 hr photoperiod under white fluorescent tubes (25 μmol m^{-2} s^{-1}).

Rooting was attempted both *in vitro* and *ex vitro*. *In vitro* root induction was tried on full-strength growth regulator-free or on half-strength MS solid medium with or without auxins. *Ex vitro* rooting was carried out in two ways: (1) Healthy shoots (above 4 cm height) from the shoot multiplication medium after dipping the basal cut end of shoots in IBA solution (made in tap water) for 10 days were planted in small pots containing soil and sand (1:1). Dipping was given by keeping shoots in test tubes containing 10 ml of IBA solution. The test tubes containing the shoots with dipped basal ends were kept open outside the laboratory (at room temperature, 32±2°C). (2) Well-grown shoots from the shoot multiplication medium were directly transferred to small pots containing soil and sand (1:1) and kept covered with moistened polyethylene bags at room temperature (32±2°C). Successfully established plantlets were subsequently transferred to field conditions. All the experiments were set up in a completely randomised design. Twenty cultures were raised for each treatment and all experiments were repeated twice. Mean values were compared using Duncan's multiplication range test.

Results and Discussion

Media supplemented with different growth regulators, prepared with tap water and commercial sugar, and those with double distilled water and tissue culture-grade sucrose did not show significant difference in the *in vitro* induction of shoots/node, and roots/shoot. Node explants cultured on MS basal medium facilitated initiation of both axillary buds (Table 1). MS medium supplemented with all concentrations of BA or Kn or combination of BA and Kn and also with auxins tried in the present study facilitated axillary bud initiation (Table 1). Kn was effective than BA, when used singly (Table 1). The shoots developed on medium with optimal concentration of Kn (2.32 μ*M*) were with more height (>3 cm) than that on BA alone supplemented medium. Kn supplemented medium developed 1-3 healthy roots also. However, combination of 4.44 μ*M* BA and 2.32 μ*M* Kn was superior to the individual use of BA and Kn for the proliferation of shoots (Table 1). A mean of 4.1 shoots/node explant were initiated on this medium, which attained a height above 4.5 cm bearing more than 5 nodes within 40 days (Fig. 1A). The shoots developed were healthier than those developed on MS medium containing Kn alone. Increased concentration of either BA or Kn decreased the number of shoots. Increase of Kn facilitated

Table 1—Axillary bud proliferation of *M. piperita* on MS medium with various growth regulators

Growth regulators (μ*M*)					Per cent response	Number of shoots/ node
BA	Kn	NAA	IBA	IAA		
Basal medium					45	1.4^{d}
2.22					75	1.5^{d}
4.44					90	2.2^{cd}
6.66					85	3.0^{b}
8.87					85	2.1^{cd}
	1.16				85	2.9^{bc}
	2.32				90	4.0^{a}
	4.65				75	2.0^{cd}
	6.97				70	1.4^{d}
6.66	2.32				85	3.1^{b}
4.44	0.46				85	3.0^{b}
4.44	2.32				75	4.1^{a}
4.44	4.65				80	2.5^{c}
4.44	2.32	0.54			75	3.3^{b}
4.44	2.32	2.69			90	2.6^{c}
4.44	2.32	5.37			85	1.6^{d}
4.44	2.32		0.49		75	3.2^{b}
4.44	2.32		2.46		80	2.4^{c}
4.44	2.32		4.90		85	1.3^{d}
4.44	2.32			0.57	85	2.9^{bc}
4.44	2.32			2.85	75	2.1^{cd}
4.44	2.32			5.71	70	1.2^{d}

Mean of 20 cultures. Means followed by the same letters are not significantly different at 5% level. Growth period 40 days

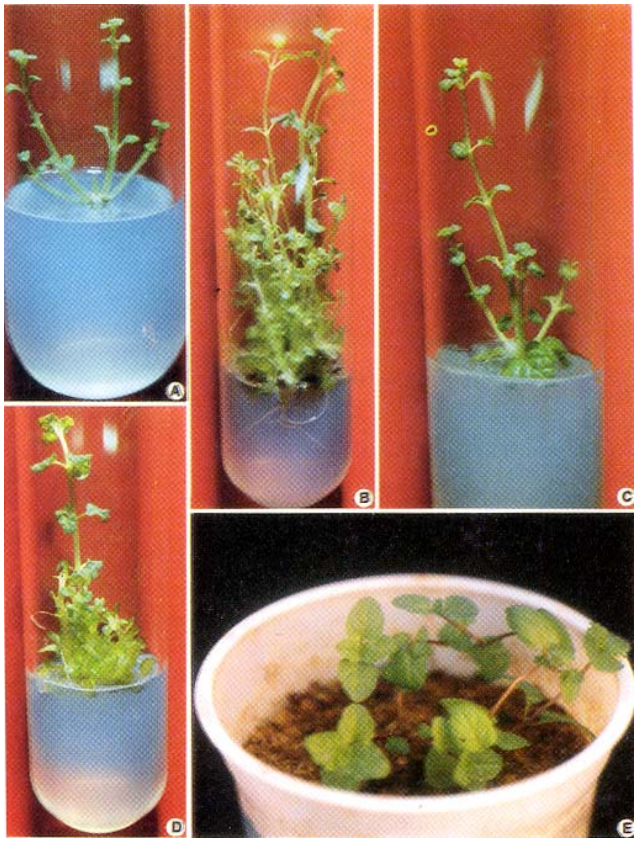

Fig. 1 (A-F)—*In vitro* propagation of *Mentha piperita.* A–Shoot proliferation on MS medium with 4.44 μ*M* BA and 2.32 μ*M* Kn; B–Enhanced shoot multiplication by the culture of node segments from the above culture on MS medium with 3.33 μ*M* BA and 2.32 μ*M* Kn; C–Initiation of three axillary buds from node and shoot with three leaves at node on MS medium with 3.33 μ*M* BA and 2.32 μ*M* Kn; D–The above culture at 40 days; and E–Plantlet established in small pot (30 days).

induction of roots. As observed on medium supplemented with either BA or Kn, the node explants cultured on various concentrations of BA and Kn developed callus from the proximal cut end. Amount of callus depended on the BA concentration. Superior effect of Kn over BA has been documented in *M. piperita* itself[9]. In contrast, efficacy of BA over Kn, when used singly, and BA in combination with auxins has been demonstrated for the axillary proliferation in many medicinal plants of Lamiaceae like *M. spicata*, *M. arvensis* and *Lavandula viridis*[10,11,13] . Superior effect of the combination of BA and Kn may be due to the synergy of cytokinins as reported in *Rollinia mucosa* and *Solanum surattense*[14,15].

During the primary culture of node explants, MS medium containing 2.32 μ*M* Kn only and both 4.44 μ*M* BA and 2.32 μ*M* Kn did not exhibit significant difference in the number of shoots (mean of 4 shoots/node). Nevertheless, excision and culture of node segments from the primary culture to Kn only and both BA and Kn containing media exhibited significant difference in the number of shoots. On 4.44 μ*M* BA and 2.32 μ*M* Kn, a mean of 10 shoots/node were developed within 40 days during the first subculture. While MS medium with 2.32 μ*M* Kn alone induced less than six shoots. More than 200 shoots were obtainable within 80 days from single initial node explant producing a mean of 4 shoots each with 5 nodes (4 × 5=20 nodes × 10 shoots=200 shoots).

Excision of node segments from the secondary culture on MS medium with 4.44 μ*M* BA and 2.32 μ*M* Kn reduced the number of shoots to a mean of 6 per node. However, culture of node segments from the secondary culture on a medium with reduced concentration of BA (3.33 μ*M*) along with the same amount of Kn (2.32 μ*M*) developed a mean of 10 shoots per node. Excision and culture of the node segments on medium having BA (3.33 μ*M*) and Kn (2.32 μ*M*) enhanced the number of shoots (Fig. 1B). Though the shoots exhibited stunted and vitrified nature, the shoots became normal as the culture period went on. Decline in the number of shoots during subsequent culture has been reported in *Hemidesmus indicus*[16]. In contrast, increase of shoot multiplication has been demonstrated in medicinal plants like *Orthosiphon spiralis* and *Wedelia chinensis*[6,7]. Increase of shoots as to the decrease of BA strengthens the inefficacy of BA alone supplemented medium during the first subculture, when compared to BA and Kn containing medium. Suppressive effect of BA with respect to the culture period has also been pointed out in *Actinidia deliciosa*[17]. However, in the present study, the shoots exhibited normal growth as the culture period went on, and this may be due to the corresponding decrease of BA in the medium.

During subculture, some of the shoots developed with three leaves at node. Excision and culture of the nodes with three axillary buds facilitated the initiation of three buds (Fig. 1C), which underwent multiplication, and increased the number. When shoot tip with a node having three leaves cultured on medium with 3.33 μ*M* BA and 2.32 μ*M* Kn, the initiated axillary buds showed 2 leaves per node, while the shoot tips grown was with three leaves per node (Fig. 1C & D). The shoots grew with three

leaves stages later changed to nodes with two leaves i.e. shoot with three leaves and two leaves per node. During subculture, the shoots developed roots from the aerial nodes. Root development in the multiplication medium depended on the number of subculture and period of culture i.e. root induction increased as the subculture and culture period goes on.

Healthy shoots having more than 4 cm height from the shoot initiation or multiplication medium were transferred to full or half strength MS basal medium for the induction of roots. Compared to full strength MS, half strength MS basal medium was superior for the induction of roots. Addition of auxins (NAA/IAA/IBA) at lower concentrations increased the number of roots. Of the different auxins, IBA at 0.49 μ*M* was the best, and which induced a mean of 10.3 roots. NAA facilitated increased callus formation and the roots were less than 1 cm. IAA was inferior to NAA (Table 2). According to Nickell[18], superior effect of IBA over other auxins may be due the slow movement and slow degradation, and the localization of it near the site of application. Root induction from the shoots by full or half-strength MS basal medium, and its augmentation by the addition of IBA has been reported in *Orthosiphon spiralis* and *Wedelia chinensis*[6,7]. All the plantlets transferred to small pots after *in vitro* rooting survived in field conditions.

Healthy shoots having more than 4 cm height with their basal end dipped in 10 ml of 0.49 μ*M* IBA solution in test tubes kept open at room temperature developed a mean of 8 roots per shoot within 10 days. The roots were longer compared to that on semisolid medium *in vitro*. Plantlets obtained through this method transferred to pots exhibited 95% survival in field conditions. Induction of rooting by basal dipping of *in vitro* developed shoots in auxin solution has been demonstrated in *Rotula aquatica* and *Wedelia chinensis*[7,19].

Table 2—*In vitro* rooting of *M. piperita* on half-strength MS medium fortified with different growth regulators

Growth regulators (μ*M*)			Per cent response	Number of roots/shoot
IBA	NAA	IAA		
	Basal medium		85	4.2[e]
0.49			100	10.3[a]
2.46			100	7.4[b]
4.9			95	4.9[d]
	0.54		100	7.6[b]
	2.69*		90	*11.1[a]
	5.37		85	3.2[f]
		0.57	100	6.4[c]
		2.85	100	4.5[de]
		5.71	85	3.4[f]

Mean of 20 cultures. Means followed by the same letters are not significantly different at 5% level. Growth period 30 days.
*High callus formation and the roots were < 1 cm

Well-developed shoots transferred to small pots filled with soil and sand (1:1) kept covered with moistened polyethylene bags at room temperature facilitated 72% survival. The shoots resumed growth within 12 days after transplantation and grew healthy with branches (Fig. 1E). Rooting *extra vitrum* has also been reported in *Camptotheca acuminata*, *Rotula aquatica* and *Wedelia chinensis*[7,19,20]. Liu and Li noticed better survival of the plantlets through *ex vitro* rooting[20]. In view of others, *ex vitro* rooting accounts 35-75 per cent reduction of the total cost of *in vitro* propagation[21]. *Ex vitro* rooting was with less per cent of survival compared to *in vitro* rooting and IBA dipped methods. Albeit, *ex vitro* rooting is promising considering the reduction in cost by avoiding the *in vitro* rooting and use of auxins, and the reduction in labour and time of establishment from laboratory to field. Considering the reduced time for establishment, auxin dipped *ex vitro* rooting was more favourable than *in vitro* rooting. In addition, rooting in liquid medium avoids removal of agar and the risk of contamination and loss during the acclimation. Plantlets established in soil were similar in morphological characters to that of mother plants.

In the present study, media prepared with tap water and commercial sugar and those prepared with double distilled water and tissue culture grade sucrose did not exhibit significant difference in the *in vitro* induction of shoots/node, and roots/shoot. Use of highly purified forms of media components (such as sucrose, double distilled water etc.) constitutes about 80% of the media cost[22]. Thus, the use of tap water and commercial sugar (costs Indian Rupees (Rs.) 15/kg; approx. Rs. 47=1 $ US) respectively in place of double distilled water and tissue culture grade sucrose (costs usually more than $ US 25/kg) reduces the cost greatly. Cost reduction by using tap water and commercial grade sugar has been reported in banana and *Rotula aquatica* [23, 19]. Rapid *in vitro* propagation of valuable medicinal plants like peppermint using commercial sugar and tap water along with *ex vitro* rooting enables to provide disease-free planting propagules at low cost which will attract small scale farmers to mediculture. This will fulfil the pilot project 'biocentres in biovillages' envisioned by Swaminathan[24].

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