

## ***In vitro* propagation of lemon balm (*Melissa officinalis* L.) through nodal bud culture of adult plant**

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### **Abstract**

A simple and efficient two step procedure for *in vitro* high frequency plant regeneration was developed for clonal propagation of lemon balm (*Melissa officinalis*) using nodal explants. Nitsch and Murashige and Skoog's medium supplemented with  $\alpha$ -naphthalene acetic acid ( $1.0 \text{ mg l}^{-1}$ ) supported axillary shoot growth and induction of roots in cultured explants. Nitsch medium showed better morphogenetic response than Murashige and Skoog's medium. Murashige and Skoog's medium fortified with  $2 \text{ mg l}^{-1}$  of either 6-benzyl aminopurine or 6-methyl aminopurine induced rapid proliferation of shoots. Rooting of the differentiated shoots was readily achieved with auxins fortified to Nitsch medium. The plantlets exhibited 80-90% success in establishment under glasshouse conditions.

**Key words:** clonal propagation, lemon balm, *Melissa officinalis*.

### **Abbreviations**

- BAP : 6-Benzyl aminopurine  
2, 4-D : 2, 4-Dichlorophenoxyacetic acid  
IAA : Indole-3-acetic acid  
IBA : Indole-3-butyric acid  
Kn : Kinetin (6-furfuryl aminopurine)  
MS : Murashige and Skoog's medium  
MAP : 6-Methyl aminopurine  
NAA :  $\alpha$ -Naphthaleneacetic acid  
NB : Nitsch & Nitsch medium

### **Introduction**

*Melissa officinalis* L. (Lamiaceae) is a perennial herb native to the eastern mediterranean region and is commonly known as lemon balm due to its citrus

aroma and has been used as a medicinal plant for more than 2000 years (Dorner 1985) and is still of topical interest.

Though much information is available on the volatile constituents of lemon

balm leaves, studies related to propagation, breeding and crop improvement aspects are scarce. Recently, some reports related to the biosynthetic potential and constituents of callus and cell suspension cultures and *in vitro* propagation of *M. officinalis* have been published (Gbolade & Lockwood 1992; Schultze *et al.* 1990, 1993; Binder *et al.* 1996; Tavares *et al.* 1996;). Under a programme initiated to exploit the biosynthetic potential of cells and tissues of *M. officinalis* under *in vitro* conditions, in the first phase, this communication sets out to present results pertaining to attempts aimed at developing a procedure conducive for direct high frequency plant regeneration of *M. officinalis* from nodal segments of adult plants.

## Materials and methods

### *Plant material*

Runners of *M. officinalis* were obtained from the field station of Central Institute of Medicinal and Aromatic Plants, Srinagar (Jammu and Kashmir) and were planted in earthen pots containing a mixture of sand, soil and farm yard manure (1:1:1). The sprouted plants were maintained under glasshouse conditions (80-85 % RH; 25±3°C) and were used as source of explants.

### *Establishment of aseptic cultures*

Explants consisting of 2-3 cm long stem piece with a node, axillary bud and petiolar base were excised from 3 month old parent plants. These were washed with distilled water containing Tween-20 (Sigma, USA) and 1 % (v/v) commercial bleach (Sodium hypochlorite). The explants were rinsed thrice in sterile distilled water before implanting vertically on the nutrient medium. Basal medium consisted of NB (Nitsch &

Nitsch 1969) and MS (Murashige & Skoog 1962) macro and micro salts, sucrose (30 gl<sup>-1</sup>), bacteriological grade agar (8 gl<sup>-1</sup>) and myoinositol (100 mg l<sup>-1</sup>). Auxins (2, 4-D, IAA, IBA and NAA) and cytokinins (Kn, BAP, MAP) were incorporated into basal media in concentrations and combinations as indicated in Table 1. The pH of all the media combinations was adjusted to 5.8 using 0.1 N NaOH or HCl before autoclaving at 120°C for 15 min. The cultures were incubated at 16:8 hours light-dark photoperiod regime under cool fluorescent light (40 µ mol m<sup>-2</sup> s<sup>-2</sup>) at 25 ± 2°C). *In vitro* responses were evaluated on 10 cultures per treatment and the experiment was repeated twice. The duration of culture passage varied from 6 to 8 weeks per cycle.

### *Establishment of plants in soil*

*In vitro* regenerated plantlets were transferred to 15 cm earthenware pots containing sand, soil and farm yard manure (1:1:1) and acclimatized in the glasshouse under high humidity conditions. The plants were initially watered with Hoagland & Arnon's (1950) solution for 1 week and were subsequently maintained in glasshouse under high humidity conditions (85 to 90 % RH) by covering the earthen pots with transparent plastic covers for the first 8-10 days. The plants were later transplanted to the field.

## Results and discussion

Nodal explants excised from adult *M. officinalis* plants maintained under glasshouse conditions were initially cultured on hormone-free MS and NB medium. During the initiation phase it was possible to achieve axillary shoot generation on a wide range of media even without growth hormones. Both

MS and NB basal media supported axillary shoot elongation. Axillary buds grew to about 1 cm in height and 4-6 roots originated from the node within 2-3 weeks of incubation. Fortification of auxins (IAA, IBA and NAA) to both NB and MS basal media ( $0.1-3.0 \text{ mg l}^{-1}$ ) not only enhanced axillary shoot growth but also supported root induction in culture explants. NAA ( $1 \text{ mg l}^{-1}$ ) was most effective among all the three auxins tested followed by IBA and IAA. NB medium supplemented with NAA ( $1 \text{ mg l}^{-1}$ ) supported profuse rhizogenesis. Plantlets (10-15 cms long) with lateral shoots and well developed roots could be obtained on NB medium (hereafter referred to as axillary shoot elongation medium, ASEM) within 6 weeks of incubation. This medium was routinely used for maintaining mother stock cultures of *M. officinalis* for obtaining nodal explants for further investigations. MS media fortified with NAA ( $0.1-3.0 \text{ mg l}^{-1}$ ) was comparatively less effective for axillary shoot elongation and root induction. In another set of experiments, for rapid mass multiplication of the regenerated shoots, nodal explants excised from mother stock cultures maintained on ASEM medium were cultured on MS and NB medium supplemented with  $0.25-5.00 \text{ mg l}^{-1}$  levels of cytokinins (Kn or BAP or MAP) individually. The axillary buds present in the subcultured units soon started elongation. Among all the media combinations tested, optimum bud elongation and regeneration of multiple axillary shoots occurred on MS basal medium supplemented with BAP ( $2 \text{ mg l}^{-1}$ ) (Fig. 1C). Shoots (50-60) of about 6-8 cm height and with 4-6 nodes could be produced from one nodal segment excised from parent shoot (Table 1). Hence this medium (hereafter abbreviated as

shoot multiplication medium, SMM) was routinely employed for large scale multiplication of axillary shoots. This medium had supported the persistence of the regeneration capacity of the system for about 6 years as tested so far. Addition of MAP to basal media instead of BAP was equally effective for shoot multiplication. Media having higher levels of MAP ( $>2 \text{ mg l}^{-1}$ ) resulted in abnormal and vitrified shoots unsuitable for *in vitro* root induction or *in vivo* hardening of the plants. Kn at all concentrations ( $0.25-5.00 \text{ mg l}^{-1}$ ) proved least effective for the multiplication of regenerated shoots. A level of  $3 \text{ mg l}^{-1}$  of Mn in the medium induced 10-15 shoots per explant within the culture passage period (Fig. 1B). Though Kn favoured shoot bud emergence from the nodal explants, it failed to support subsequent growth and elongation of shoot buds beyond 2-3 cm height. Addition of NAA ( $0.1-5.0 \text{ mg l}^{-1}$ ) to the SMM containing either of Kn, BAP or MAP ( $2 \text{ mg l}^{-1}$ ) significantly increased regeneration potential of cultured nodal segments (Table 1). NAA ( $1 \text{ mg l}^{-1}$ ) showed optimal response. Single node explant cultured on MS medium containing  $2 \text{ mg l}^{-1}$  BAP and  $0.25 \text{ mg l}^{-1}$  NAA produced as many as 82 shoots within culture passage period of 8 weeks. However, the shoots were stunted and smaller in size and thereby suggesting an inverse relationship between shoot number and shoot growth.

Lower levels of cytokinins particularly Kn induced callus formation at the proximal cut surface of the nodal explants. The callus generated in these cultures turned green and nodular and occasionally developed 1-2 adventitious shoots at the later stages of culture period (Fig. 1A). Many thin branched roots originated from nodal regions of

**Table 1.** Effect of exogenously applied hormones on shoot multiplication of *Melissa officinalis* during multiplication phase *in vitro*\*

Culture medium	Average no. of shoots/ explant	% culture with multiple shoots**	Shoot growth***
<b>A. Effect of single hormone supplementation</b>			
MS+0.25 mg <sup>l</sup> <sup>-1</sup> Kn	4.0 ± 1.02	82	+
MS+0.50 mg <sup>l</sup> <sup>-1</sup> Kn	8.0 ± 1.35	85	+
MS+1.00 mg <sup>l</sup> <sup>-1</sup> Kn	14.0 ± 1.78	100	++
MS+2.00 mg <sup>l</sup> <sup>-1</sup> Kn	18.0 ± 0.76	100	++
MS+3.00 mg <sup>l</sup> <sup>-1</sup> Kn	22.0 ± 0.67	100	++
MS+5.00 mg <sup>l</sup> <sup>-1</sup> Kn	8.0 ± 1.35	58	+
MS+0.25 mg <sup>l</sup> <sup>-1</sup> BAP	10.0 ± 1.21	45	++
MS+0.50 mg <sup>l</sup> <sup>-1</sup> BAP	14.0 ± 1.52	85	+++
MS+1.00 mg <sup>l</sup> <sup>-1</sup> BAP	27.0 ± 0.96	96	+++
MS+2.00 mg <sup>l</sup> <sup>-1</sup> BAP	53.0 ± 1.24	100	++++
MS+3.00 mg <sup>l</sup> <sup>-1</sup> BAP	42.6 ± 1.26	100	++++
MS+5.00 mg <sup>l</sup> <sup>-1</sup> BAP	38.0 ± 0.82	62	++
MS+0.25 mg <sup>l</sup> <sup>-1</sup> MAP	6.4 ± 0.67	32	++
MS+0.50 mg <sup>l</sup> <sup>-1</sup> MAP	14.2 ± 1.82	82	++
MS+1.00 mg <sup>l</sup> <sup>-1</sup> MAP	23.9 ± 1.37	85	++
MS+2.00 mg <sup>l</sup> <sup>-1</sup> MAP	48.0 ± 0.87	100	+++
MS+3.00 mg <sup>l</sup> <sup>-1</sup> MAP	44.0 ± 0.89	100	+++
MS+5.00 mg <sup>l</sup> <sup>-1</sup> MAP	14.4 ± 0.78	65	++
<b>B. Interaction of cytokinins and auxins</b>			
MS+2.00 mg <sup>l</sup> <sup>-1</sup> Kn + 0.25 mg <sup>l</sup> <sup>-1</sup> NAA	42.0 ± 0.82	100	++
MS+2.00 mg <sup>l</sup> <sup>-1</sup> BAP+ 2.25 mg <sup>l</sup> <sup>-1</sup> NAA	82.2 ± 1.32	100	++
MS+2.00 mg <sup>l</sup> <sup>-1</sup> MAP+ 0.25 mg <sup>l</sup> <sup>-1</sup> NAA	59.4 ± 1.72	100	++

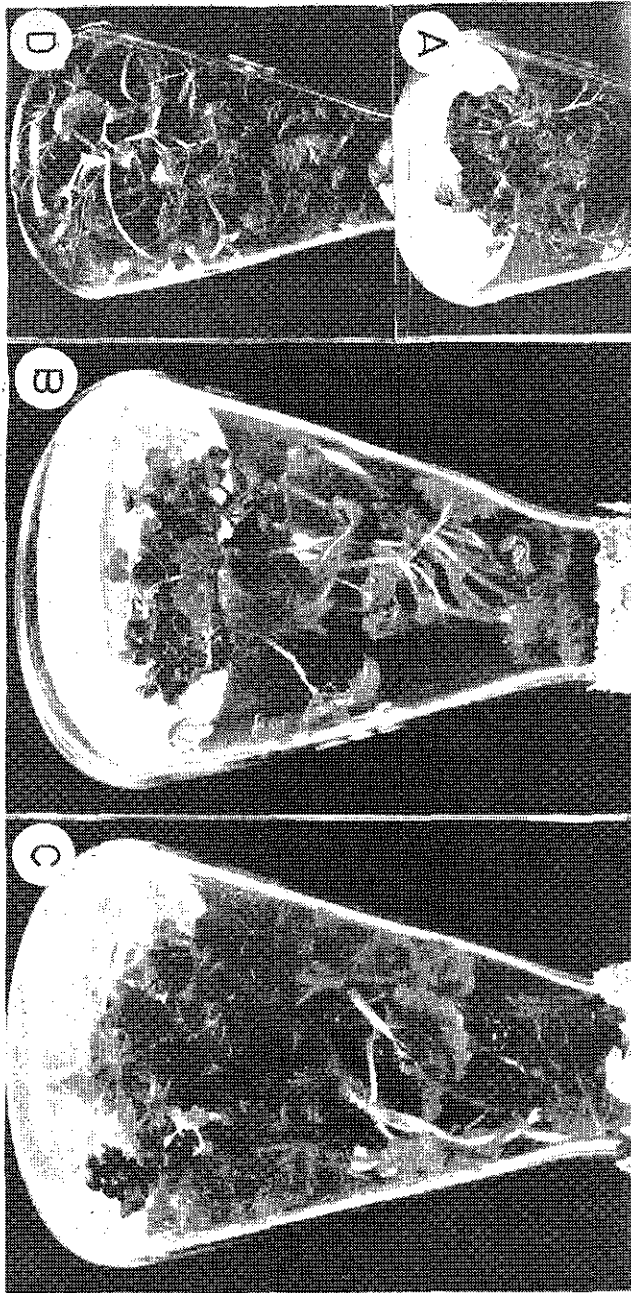
\* Data recorded after 8 weeks of culture

\*\* Mean of 10 replicates per treatment

\*\*\* + = 2 cm; ++ = 2-4 cm; +++ = 4-6 cm; ++++ = &gt; 6 cm

shoots on SMM. These regenerated plantlets showed poor growth and low survival rate when transplanted in the glasshouse. This necessitated a two step

procedure for plant regeneration and development. *In vitro* shoots generated on SMM were subcultured on MS basal medium fortified with IAA or NAA (0.1-



**Fig. 1.** *In vitro* regeneration and multiplication of *Melissa officinalis*. A. Callus formation and regeneration of multiple shoots on low levels of Kn B-C: Regeneration of multiple shoots on MS medium containing Kn ( $3 \text{ mg l}^{-1}$ ) and BAP ( $2 \text{ mg l}^{-1}$ ), respectively D. *In vitro* shoots with roots on NB medium supplemented with NAA ( $1 \text{ mg l}^{-1}$ )

$5.0 \text{ mg l}^{-1}$ ). Through a few small, thin roots could be observed on MS basal medium, addition of NAA ( $1 \text{ mg l}^{-1}$ ) further augmented and supported root initiation and plantlet development. The regenerated roots were fast growing, thin and with numerous laterals. Substituting MS with NB medium also showed the same response. NB basal with  $1 \text{ mg l}^{-1}$  NAA (hereafter referred to as root induction medium, RIM) induced profuse rhizogenesis in excised shoots. This medium also supported the growth of shoots.

Once a vigorous root system was established on RIM, the plantlets thus formed were transferred to pots. Maintenance of high humidity (80-90%) during the early hardening phase in the glasshouse was essential for a high rate of survival. The plantlets grew into normal adults in terms of leaf morphology and branching pattern. Studies on essential leaf oil of *in vitro* raised plants of *M. officinalis* are in progress.

The present work demonstrates a reproducible procedure for mass clonal propagation of *M. officinalis* from cultured nodal segments of adult plants. The pattern of morphogenesis in cultured nodal explants in this species as regards to responses to various

phytohormonal regimes largely conform to that of other species of Lamiaceae (Rech & Pires 1986; Holm *et al.* 1989; Sen & Sharma 1991; Kukreja *et al.* 1991, 1996; Binder *et al.* 1996). In *M. officinalis*, multiple shoots appeared directly from the cultured nodal explants without any intervening callus phase. BAP alone in the medium supported multiple shoots in cultured nodal segments while presence of NAA in the NB medium induced rooting in the cultured excised shoots. NAA was more effective than IAA for root induction and subsequent growth and development of plantlets. Thus, *M. officinalis* cultures showed superiority of a synthetic auxin in comparison to the natural (IAA) one for root induction. This procedure for successful regeneration of *M. officinalis* plants by *in vitro* culture of nodal segments will be useful for rapid cloning of elite strains of the plant in areas where conventional cultivation is restricted due to poor seed germination and seedling establishment.

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