



IN VITRO CLONAL PROPAGATION OF MENTHA ARVENSIS THROUGH CALLUS CULTURE

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Abstract: A rapid and reliable high fidelity micropropagation technique was established for *in vitro* mass propagation of a valuable medicinal herb, *Mentha arvensis* Linn. (Lamiaceae) through callus culture. MS medium containing BAP (0.5 mg/l) and NAA (0.2 mg/l) in callus induction medium induced green compact and nodular callus within four weeks of culture. The maximum number of globular embryoids was recorded (268 ± 0.4) on the MS medium containing 0.2 mg/l BAP. Calli when transferred from callus induction medium to germination medium containing growth regulator free MS resulted in high frequency of shoot and root induction (96.43 ± 0.21 shoots/culture) and with an average shoot length of 5.61 ± 0.45 cm after 4 weeks of subculture. The regenerated plantlets were acclimatized by transferring them to soil.

Keywords: Micropropagation, callus culture, *Mentha arvensis*.

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INTRODUCTION:

Micropropagation techniques offer great potential not only for rapid multiplication of existing stock of plant species but also for conservation of important and elite ones such as *Mentha arvensis*, is a valuable medicinal herb. The aerial parts of adult plants are commonly used in folk medicine for the treatment of cold, cough, asthma, and chest inflammations, including pulmonary tuberculosis. It is also used externally to treat wounds and swollen glands (Mimica-Dukic *et al.*, 2003). The majority of *Mentha* species contain piperitenone oxide, piperitone oxide, carvone, menthone, and 1,8-cineol as the main constituents, even though major variation in the dominating compounds has been found in wild or cultivated plant material grown in different habitats (Mathela *et al.*, 2005; Oyedeji & Afolayan, 2006; Gulluce *et al.*, 2007).

Dried leaves and 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, flowering stomachic and refrigerant. It is also considered to be stimulant, emmenagogue and diuretic (Chopra *et al.*, 1986). The infusion of leaves is used in rheumatism and indigestion (Chadha, 1976). 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. Therefore, micropropagation technique is an effective approach for conserved such plants. *In vitro* propagation has proven as a potential technology for mass scale production of plant species (Wawrosch *et al.* 2001; Martin 2003; Azad *et al.* 2005; Hassan and Roy 2005; Hassan *et al.* 2009). The improvement of *in vitro* clonal propagation efficiency of *Mentha* plant is very important for its medicinal uses. The present study was, therefore, undertaken to develop a suitable protocol for *in vitro* propagation of this important medicinal herb through callus culture.

MATERIAL AND METHODS:

1. Explants preparation:

Nodes of *Mentha arvensis* were taken as explants collected from the garden plants. After excision, the explants were subjected to preliminary washing under running tap water to remove the microflora to a substantial extent. Healthy and uniform explants were



disinfected thoroughly in 4% savlon solution for 8 minutes. Explants were then rinsed under running tap water. Then they were sectioned into a suitable size (0.5-1 cm) and surface sterilized with 0.1% mercuric chloride solution for 8 minutes, followed by 6 washes in sterile distilled water. For induction, growth and maintenance of callus tissues, Murashige and Skoog's (1962) basal medium (MS) was used.

2. Media preparation:

The explants were cultured in the MS medium supplemented with different combination and concentrations of growth regulators such as 6-benzyl amino purine (BAP), α -naphthalene acetic acid (NAA) and indole butyric acid (IBA). The MS medium also contained 3% sucrose and 0.5% (w/v) agar. The medium pH had been adjusted to 5.6 before it was autoclaved at 121°C for 15 minutes. Cultures were grown at 22-24°C with a relative humidity of 50-60% and a photoperiod for 16h per day provided by fluorescent tube (12000 lux).

3. Acclimatization:

Healthy rooted plantlets were taken from the rooting medium and washed several times with sterile distilled water to remove the medium. Plantlets were potted in soil and were kept under controlled temperature at 22-26°C and light conditions in the culture room. The bags were removed periodically for gradual hardening. After 2 weeks when new leaves emerged from such plantlets, they were taken outside the culture room and kept in a shady place under normal temperature and light.

OBSERVATIONS:

Induction of callus:

The nodal explants were used for the induction of callogenesis of *Mentha arvensis*. Initially callus formation was observed from the cut ends and the epidermal surfaces of the nodal explants as protuberances within 10-12 days. The whole explants were gradually covered by callus within 30 days of culture (Fig 1). BAP in the range of 0.2-1.0 mg/l and NAA 0.2-1.0 mg/l in different combinations were tested for callus induction. Of all the combinations and concentrations of growth regulators tested, MS medium supplemented with BAP (0.5 mg/l) and NAA (0.2 mg/l) proved to be the best with regard to callus formation and their further development into embryoids (Fig 1). In this combination deep green compact and nodular callus developed after five weeks of culture. The explants cultured in higher concentrations



of BAP (more than 2.0 mg/l) and NAA (more than 0.5 mg/l) did not initiate callus and the explants began to become brown within 25 days.

Growth of callus:

The explants were inoculated in MS medium with various concentrations of BAP: (0.2- 1.0) mg/l and NAA: 0.2-1.0 mg/l for callus initiation and subsequent differentiation. The calli were maintained by subculturing at an interval of 2 weeks. After 4 weeks the differentiated calli were again subcultured on MS medium supplemented with lower concentrations of BAP (0.2 mg/l) for the development of the embryoid. The calli containing embryoids were then transferred to growth regulator free MS medium for the maturation of embryoids and plant regeneration. Embryos developed into plantlets on growth regulator free MS medium. The globular structures of embryos was appeared on the surface of the pro-embryogenic callus gradually became enlarged to a detectable size within 9-10 weeks of culture.

Visual identification and selection of embryogenic sectors and removal of non-embryogenic portions maintained the embryoids developed from friable callus during subcultures at two weeks interval. The maximum number of globular embryoids was recorded (268 ± 0.4) on the MS medium containing 0.2 mg/l BAP after 10 weeks of culture (Table 1).

Germination of embryos:

Maximum numbers of germinated embryos were observed at the end of second week, after transferring the embryos on growth regulator free MS medium. Of the various medium tested, growth regulator free MS medium was more effective over singly or in combination of BAP-NAA for germination of embryos (Table 2). Shoot differentiation and profuse shoot formation were found to be best from callus on MS medium and the highest number of shoots was 96.43 ± 0.21 per culture and with an average shoot length of 5.61 ± 0.45 cm after 4 weeks of culture (Table 2). If the MS medium containing growth regulators either singly or in combination of BAP-NAA for germination of embryos which are adversely affected the shoot development, as the regenerated shoots became stunted and dense. The elongated shoots were rooted simultaneously on the same growth regulator free MS medium. Hundred percent cultures exhibited rooting on MS medium, producing maximum of 10.23 ± 0.24 roots per shoot with average root length of 2.45 ± 0.11 cm. The regenerated plantlets with well developed root systems were removed from the culture medium. They were subsequently hardened and acclimatized. The *in vitro* raised shoots grew vigorously and developed



without any visible deformities (Fig 4). An almost 90% survival of the transplanted plantlets of *Mentha arvensis* was observed in field.

DISCUSSION:

The MS medium containing BAP (0.5 mg/l) and NAA (0.2 mg/l) proved to be the best with regard to callus formation and their further development into embryoids, the concentrations of cytokinin and auxin are also known to be critical in the induction of differentiating callus and their subsequent proliferation. BAP, which has been used as a source of cytokinin in the present study, has also been used successfully to induce embryogenesis in several plants (Chand & Singh, 2001; Anand *et al.*, 2001; Prakash *et al.*, 2001; Faisal *et al.*, 2006; Aastha *et al.*, 2010). In the present study, it has been found that the higher concentration of cytokinin (0.5 mg/l BAP) was necessary for the initiation of the callus although low concentration (0.2 mg/l BAP) was needed for the proliferation and differentiation of the calli.

Mostly auxins or substances having auxin like activity were reported to be effective for inducing embryogenesis in different plants (Carman, 1990; Eapen & George, 1992; Craig *et al.*, 1997; Maity *et al.*, 2010). The auxin-cytokinin combination, which was found to be effective in inducing embryogenesis in the present investigation, was also used in some species like *Mussaenda philippica* var. *aurorae* for the induction of embryogenesis (Eapen & George, 1992) and *Mucuna pruriens* var. *utilis* (Sathyanarayana *et al.*, 2008). According to Zimmerman (1993), removal of auxin results in the inactivation of a number of genes so that the embryogenesis program cannot proceed further.

Three weeks old germinating embryoids when transferred to germinating medium consisting of MS basal medium were converted to plantlets within five weeks of transfer. The same was reported earlier by Jayanti and Mandal (2001), where embryoids transferred to basal medium developed into complete plantlets. This is probably due to restoration of endogenous hormone balance necessary for normal plantlet development.

The method of callogenesis and their development were shown in the present investigation demonstrates the potentiality of the nodal explants for generating highly proliferative, embryogenic cultures for *Mentha arvensis*. Since *Mentha* is a medicinal plant, this approach can be useful in maintaining the germplasm of the genus for future conservation.



Table 1. Effect of BAP and NAA on embryogenesis via nodal explant derived callus culture.

Results are the mean of 6 replicates \pm SE.

MS basal medium		% of explants producing callus	Total no. of embryoids
BAP(mg/l)	NAA(mg/l)		
0.2	0.2	85	122.33 \pm 0.1
0.5	0.2	88	268.33\pm0.4
1.0	0.2	59	62.33 \pm 0.2
0.2	0.5	66	53.83 \pm 0.2
0.5	0.5	61	32.67 \pm 0.4
1.0	0.5	61	20.33 \pm 0.4
0.2	1.0	72	23.67 \pm 0.8
0.5	1.0	47	26.33 \pm 0.5
1.0	1.0	47	23.33 \pm 0.5

Table 2. Effect of various concentrations and combinations of growth regulators on schizogenesis from the callus. Results are the mean of 6 replicates \pm SE.

Medium	% of response for germination of embryos	Mean no. of shoots/culture (Mean \pm SE)	Mean length of shoots (cm) (Mean \pm SE)
MS	94.23\pm0.11	96.43\pm0.21	5.61\pm0.45
MS+BAP(0.1mg/l)	61.44 \pm 0.35	56.78 \pm 0.13	5.11 \pm 0.33
MS+BAP(0.2mg/l)	60.38 \pm 0.33	26.49 \pm 0.13	3.78 \pm 0.54
MS+BAP(0.5mg/l)	48.56 \pm 0.67	15.56 \pm 0.43	3.12 \pm 0.88
MS+BAP(1.0mg/l)	32.54 \pm 0.34	13.43 \pm 0.56	2.98 \pm 0.54
MS+BAP(0.1mg/l)+NAA(0.2mg/l)	19.54 \pm 0.44	13.33 \pm 0.21	2.43 \pm 0.81
MS+BAP(0.2mg/l)+NAA(0.2mg/l)	19.36 \pm 0.76	11.54 \pm 0.32	2.65 \pm 0.11
MS+BAP(0.5mg/l)+NAA(0.2mg/l)	12.11 \pm 0.43	11.21 \pm 0.65	2.23 \pm 0.21
MS+BAP(1.0mg/l)+NAA(0.2mg/l)	09.21 \pm 0.67	03.12 \pm 0.32	2.12 \pm 0.37

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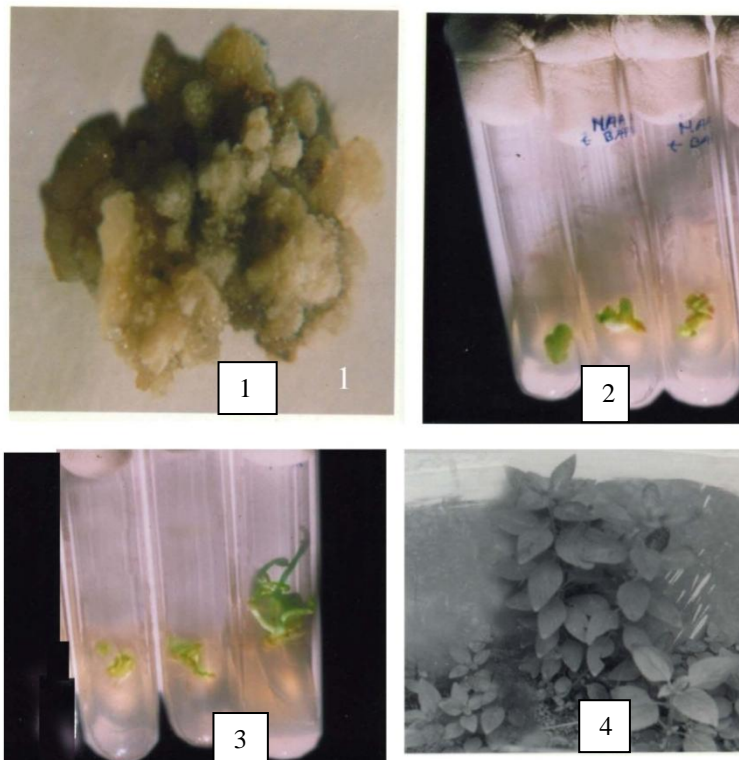


Figure captions:

Fig. 1. Profuse growth of callus on MS medium containing BAP (0.5 mg/l)+ NAA (0.2 mg/l) within 8 weeks of culture.

Fig. 2 &3. Germinating embryos in growth regulator free MS medium.

Fig. 4. Acclimatized plantlets on the soil.