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# AN *IN VITRO* STUDY OF REGENERATION AND MICROPROPAGATION OF *MENTHA ARVENSIS*

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## ABSTRACT

Shoot tip and nodes were used as explant source and were cultured on MS media containing different concentrations of auxins and cytokinins. Shoots were induced on MS media containing 1.0 mg/l BAP. The newly formed shoots were transferred on MS medium containing different concentrations of hormones for shoot multiplication. Maximum 35 shoots were obtained on MS media containing 1.0 mg/l BAP + 0.5 mg/l NAA. Microcuttings of 3-3.5 cm in length were harvested from proliferating cultures and were shifted for rooting in MS media containing different concentrations of NAA and IBA. Well rooted plants were successfully established in sterile sand following hardening phase of 20 days.

**Key-words:** Micropropagation, *Mentha arvensis*, mint, tissue culture, plant biotechnology

## INTRODUCTION

Mint is a group of perennial herbs belongings to family Lamiaceae (Labiateae). These ever green herbs on distillation yield essential oils containing a large variety of aroma chemicals in varying composition. These oils and their aroma chemicals in pure forms have worldwide demand in trade. Japanese mint (*Mentha arvensis*) is a perennial herb of this family. It has been widely grown in the world for its high medicinal value and use in pharmaceutical, food, flavour, condiments and allied industries. It is a very important herbaceous plant because menthol is extracted from its leaves. Menthol is the chief constituent of peppermint oil and is responsible for its odor, taste and cooling sensation when applied to skin. *M. arvensis* contains 70%-80% menthol (Siegel, 1998).

*Mentha arvensis* can be grown in all tropical and subtropical areas under irrigation and is generally propagated through vegetative methods by creeping stolons, suckers or shoot cuttings. Stolons are obtained from previous year planting. However, such methods of vegetative propagation require considerable time and therefore limits the speed of plant propagation. Moreover, the method of propagation by cutting is not a reliable method for production of good quality planting material because these are prone to attack by a variety of pathogens which cause many fungal (rust, powdery mildew and stolon rot) and viral (spot wilt) diseases, which reduce the yield and also cause the gradual degeneration of cultivar.

In recent years plant tissue culture and application of micropropagation technique to plant breeding and disease eradication has been recognized as potentially valuable tool in crop improvement program and from last two decades this technique has gained greater momentum on commercial application in the field of plant propagation. The main objective of present study was to establish protocols for mass scale propagation and faster development of *Mentha* plants using *in vitro* propagation techniques.

## MATERIAL AND METHODS

Apical and nodal explants (1.0 mm size) were used as explant source for this study. Explants were obtained from field grown plants. Before surface sterilization all the leaves were removed to avoid contamination. Explants were washed thoroughly with tap water and then with house hold detergent to remove all the traces of dust particles. Surface sterilization was carried out by immersing the axillary and terminal buds in 15% Sodium hypochlorite solution for 15 minutes. Three washings with autoclaves distilled water were carried out to remove all the traces of sterilant. Laminar flow was used for inoculation. It was sprayed with ethanol before use. The media was autoclaved at 121°C and at 15 lbs pressure for 15 minutes. Explants were inoculated in test tubes containing 10ml of media and were capped before autoclaving. MS media (Murashiage and Skoog, 1962) supplemented with different doses of Auxins and cytokinins along with 3% sucrose was used. The pH of the medium was adjusted to 5.7 ± 0.5. The medium was gelled with 0.7% agar.

For shoot induction and poliferation different concentration of BAP and Kinetin were used either alone or in combination with each other. For rooting of induced shoots different concentrations of two different auxins i.e.NAA and IBA were used. Ten test tubes were used for each treatment. Single explant was placed in each test tube. All cultures were maintained under white fluorescent light having 2000 lux light intensity. The incubation temperature

was adjusted to 27 °C ± 1 °C and photoperiod was 16 h light and 8 hours dark period in every 24 h cycle. Sub-culturing was done after every four week.

## RESULTS

### Shoot Induction

### Effect of different concentrations of BAP on Shoot Induction:

All the explants induced shoot primordial within 8-15 days of inoculation on MS media supplemented with 0.5 to 2.0 mg/l of BAP. However Bugara and Bugara (2002) reported the poor response of MS media for in vitro shoot induction in *M. arvensis*, while modified Gamborg Eveleigh medium gave good results for *in vitro* morphogenesis. In present study the concentration of BAP in MS media affects the shoot induction response. The shoot induction response was 90% in both apical and nodal explants at 1.0 mg/l of BAP. There was a gradual decrease in shoot induction response with increasing the concentration of BAP (Table 1). Shoot induction response was suppressed to 40% in apical and 50% in nodal explants were observed at 2.0 mg/l of BAP. Time taken for shoot induction was not same in both kinds of explants. It was 8 days in apical and 10 days in nodal explants. Mostly single shoot emerged from both explants (Plate **I**). Some explants also induced multiple shoots (Plate **II**).

When combination of BAP with NAA was used for shoot induction, no good results were obtained in both kind of explants. Best combination obtained was 0.5 mg/l NAA and 1.0 mg/l BAP in MS media which showed 80% shoot induction response within 9 days of inoculation in apical meristem and 10 days on nodal explants. Other combinations of BAP with NAA showed non satisfactory results (Table 2).

Position of nodal explants also influenced the growth response. Nodal explants excised from middle and close to the base of stem exhibited rapid development as compared to nodal explants excised from terminal position. It was also noticed that the explants harvested in the spring season responded vigorously as compared to explants excised in hot summer.

Table 1. Effect of different concentrations of BAP on shoot induction.

| Sr. No. | Media | Composition | No of test tubes cultured | No of cultures showing shoot induction | | Days for shoot induction | | Rate of shoot induction (%) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Apical | Nodal | Apical | Nodal | Apical | Nodal |
| 1 | MS 1 | MS Basal | 10 | 2 | 2 | 15 | 15 | 20 | 20 |
| 2 | MS 2 | MS+ BAP 0.5 mg/l | 10 | 5 | 4 | 12 | 14 | 50 | 40 |
| 3 | MS 3 | MS+BAP 0.75 mg/l | 10 | 7 | 6 | 10 | 13 | 70 | 60 |
| 4 | MS 4 | MS+ BAP 1.0 mg/l | 10 | 9 | 9 | 9 | 10 | 90 | 90 |
| 5 | MS 5 | MS+BAP 1.25 mg/l | 10 | 8 | 7 | 11 | 12 | 80 | 70 |
| 6 | MS 6 | MS+ BAP 1.5 mg/l | 10 | 6 | 7 | 14 | 15 | 60 | 70 |
| 7 | MS 7 | MS+ BAP 2.0 mg/l | 10 | 4 | 5 | 18 | 18 | 40 | 50 |

### Multiple Shoot Formation:

For multiplication of induced shoots different concentration of BAP either alone or in combination with NAA was used. When BAP was used alone it was observed that at the concentration of 1.0 mg/l of BAP 22 shoots were formed. By decreasing the concentration of BAP the shoot multiplication response was also decreased and at 0.5

mg/l very poor results for shoot multiplication were obtained (Table 3). Similarly the concentration of BAP higher than 1.5 mg/l did not give good results for shoot multiplication. When the combination of BAP with different concentration of NAA was tested, comparatively better results for shoot multiplication were obtained than those obtained with BAP alone. Best results for shoot multiplication were obtained in MS medium supplemented with 1.0 mg/l BAP and 0.5 mg/l NAA. At this concentration 35 shoots were formed within 26 days of inoculation. By further increase or decrease in the concentration of NAA with same concentration of BAP the rate of shoot multiplication was decreased (Table 4) (Plate **III**)

Table 2. Effect of different concentrations of BAP and NAA on shoot induction.

| Sr. No. | Media | Composition | No of test tubes cultured | No of cultures showing shoot induction | | Days for shoot induction | | Rate of shoot induction (%) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Apical | Nodal | Apical | Nodal | Apical | Nodal |
| 1 | MS 1 | MS+NAA0.5mg/l + BAP 0.5 mg/l | 10 | 6 | 6 | 14 | 12 | 60 | 60 |
| 2 | MS 2 | MS+NAA0.5mg/l + BAP 1.0 mg/l | 10 | 8 | 8 | 9 | 10 | 80 | 80 |
| 3 | MS 3 | MS+NAA0.5mg/l + BAP 1.5 mg/l | 10 | 7 | 7 | 12 | 13 | 70 | 70 |
| 4 | MS 4 | MS+NAA0.5mg/l + BAP 2.0 mg/l | 10 | 5 | 6 | 15 | 17 | 50 | 60 |
| 5 | MS 5 | MS+NAA0.5mg/l + BAP 2.5 mg/l | 10 | 4 | 4 | 20 | 20 | 40 | 40 |

Table 3. Effect of different concentrations of BAP on shoot multiplication in *Mentha arvernsis.*

| Sr. No. | Media | Composition | No of test tubes cultured | No of cultures showing multiplication | No of shoots formed | Time for multiple shoot formation (week) |
|---|---|---|---|---|---|---|
| 1 | MS 1 | MS+BAP 0.5 mg/l | 10 | 6 | 04 | 6 |
| 2 | MS 2 | MS+BAP 0.75 mg/l | 10 | 8 | 14 | 4.5 |
| 3 | MS 3 | MS+BAP 1.0 mg/l | 10 | 9 | 22 | 4 |
| 4 | MS 4 | MS+BAP 1.25 mg/l | 10 | 7 | 19 | 5.5 |
| 5 | MS 5 | MS+BAP 1.5 mg/l | 10 | 5 | 18 | 6 |
| 6 | MS 6 | MS+BAP 2.0 mg/l | 10 | 4 | 10 | 7 |

**Rooting:**

For rooting of well developed *in vitro* plants two different auxins NAA and IBA were used either alone or in combination with each other in MS media. No good rooting response was observed in hormone free MS media. Among different concentration of NAA, used 1.0 mg/l of NAA gave good results i.e., 90% for root induction after 5

Table 4. Effect of different concentrations of BAP and NAA on shoot multiplication in *Mentha arvernsis*.

| Sr. No. | Media | Composition | No of test tubes cultured | No of cultures showing multiplication | No of shoots formed | Time for multiple shoot formation (week) |
|---|---|---|---|---|---|---|
| 1 | MS 1 | MS+BAP 1.0mg/l + NAA0.25 mg/l | 10 | 9 | 32 | 4 |
| 2 | MS 2 | MS+BAP 1.0mg/l + NAA0.5 mg/l | 10 | 10 | 35 | 3.5 |
| 3 | MS 3 | MS+BAP 1.0mg/l + NAA0.75 mg/l | 10 | 9 | 30 | 5 |
| 4 | MS 4 | MS+BAP 1.0mg/l + NAA1.0 mg/l | 10 | 8 | 27 | 5 |
| 5 | MS 5 | MS+BAP 1.0mg/l + NAA1.5 mg/l | 10 | 5 | 19 | 6 |

Table 5. Effect of different concentrations of NAA on *in vitro* root induction in *Mentha arvensis*.

| Media | Composition | No of test tubes cultured | No of cultures showing Root induction | Days for root induction | %age of root induction |
|---|---|---|---|---|---|
| MS 1 | MS Basal | 10 | 3 | 20 | 30 |
| MS 2 | MS + 0.5 mg/l NAA | 10 | 5 | 15 | 50 |
| MS 3 | MS + 0.75 mg/l NAA | 10 | 6 | 8 | 60 |
| MS 4 | MS +1.0 mg/l NAA | 10 | 9 | 5 | 90 |
| MS 5 | MS +1.25 mg/l NAA | 10 | 8 | 7 | 80 |
| MS 6 | MS +2.0 mg/l NAA | 10 | 4 | 9 | 40 |

Table 6. Effect of different concentrations of IBA on *in vitro* root induction in *Mentha arvensis.*

| Media | Composition | No of test tubes cultured | No of cultures showing Root induction | Days for root induction | %age of root induction |
|---|---|---|---|---|---|
| MS 1 | MS + 0.5 mg/l IBA | 10 | 4 | 15 | 40 |
| MS 2 | MS + 0.75 mg/l IBA | 10 | 5 | 13 | 50 |
| MS 3 | MS +1.0 mg/l IBA | 10 | 6 | 11 | 60 |
| MS 4 | MS +1.25 mg/l IBA | 10 | 6 | 11 | 60 |
| MS 5 | MS +1.50 mg/l IBA | 10 | 7 | 10 | 70 |
| MS 6 | MS +2.0 mg/l IBA | 10 | 8 | 8 | 80 |

Table 7. Effect of different concentrations of IBA with NAA on *in vitro* root induction in *Mentha arvensis*.

| Media | Composition | No of test tubes cultured | No of cultures showing Root induction | Days for root induction | %age of root induction |
|---|---|---|---|---|---|
| MS 1 | MS +0.5 mg/l IBA+1.0mg/l NAA | 10 | 4 | 14 | 40 |
| MS 2 | MS +1.0 mg/l IBA+1.0mg/l NAA | 10 | 5 | 12 | 50 |
| MS 3 | MS +1.5 mg/l IBA+1.0mg/l NAA | 10 | 8 | 10 | 80 |
| MS 4 | MS +2.0 mg/l IBA+1.0mg/l NAA | 10 | 10 | 5 | 100 |
| MS 5 | MS +2.5 mg/l IBA+1.0mg/l NAA | 10 | 7 | 7 | 70 |
| MS 6 | MS +3.0 mg/l IBA+1.0mg/l NAA | 10 | 6 | 10 | 60 |

days of inoculation. By increase or decrease in the concentration of NAA the rate of root induction was decreased. In case of IBA supplemented media the maximum rooting response was 80% when 2.0 mg/l of IBA was used after 8 days of inoculation.

The best response of *in vitro* rooting was observed in MS media containing both NAA & IBA (Plate **IV**) 100 % root induction was obtained when MS media was supplemented with 2.0 mg/l IBA and 1.0 mg/l NAA within five days of inoculation. This concentration induced roots successfully in almost all shoots (Table 7).

As far as hardening is concerned it was observed that the hardening response was excellent in a mixture containing sand + sand + peat (1:1:1) AT 95% humidity level (Plate **V** and **VI**).

## DISCUSSION

*In vitro* techniques offer new possibilities in commercial clonal propagation (Short *et al.*, 1981; Robert, 1991). The present investigation aims for standardization of protocols for the mass scale production of disease free plants of *M. arvensis* through *in vitro* propagation. The results of present study revealed that explants cultured in MS medium with different concentrations and combinations of BAP and NAA greatly influence shoot induction response. Best shoot induction response was observed in media containing 1.0 mg/l of BAP. It was observed that by increasing the concentration of BAP the shoot induction response was decreased. Naz *et al.* (1993) and Anjali *et al.* (2000) also reported the promotive effect of lower concentration of BAP in *in vitro* cultures in Rose and *Withania somnifera* respectively.

In the present study shoot induction response was less satisfactory when the combination of BAP with different concentrations of NAA was used. However, BAP and NAA combination proved good for multiplication of induced shoots. Best response for shoot multiplication was obtained in media containing 1.0 mg/l BAP and 0.5 mg/l of NAA. Promotive response of the combination of BAP and NAA for *in vitro* shoot multiplication in *M. arvensis* has also been reported by many workers. Pathak and Heble (2002) also reported the best shoot multiplication response in MS media supplemented with BAP and NAA but they used higher concentration of BAP i.e. 5.0 mg/l BAP with 0.5 mg/ l NAA. Xue (1998) also used the combination of BAP and NAA for in vitro shoot multiplication but he preferred B5 media over MS media. However, Chisti and Siddiqui.(2003) reported best shoot multiplication response in MS media containing BAP + Kin + IAA.

Position of excised nodal explants is also important in expressing successful growth response. Nodal explant excised from terminal portion did not respond like explant from middle or basal position of the stem. It is thought that this inhibitory effect is due to change auxin and abscissic acid concentration gradient which ultimately determine growth response (Zieslin *et al.*, 1978)

After the formation of well developed multiple shoots, all the plants attaining the height of 3-3.5 cm were transferred to MS media supplemented with different concentrations and combinations of NAA and IBA for in vitro root induction. It was observed that among both the auxins NAA gave better results as compared to IBA at the

concentration of 1.0 mg/l. At this concentration 90% root induction response was obtained with 5 days of culturing. Shasany *et al.* (1998) also reported the rooting in *M. arvensis* in MS medium containing 0.2 mg/l NAA.

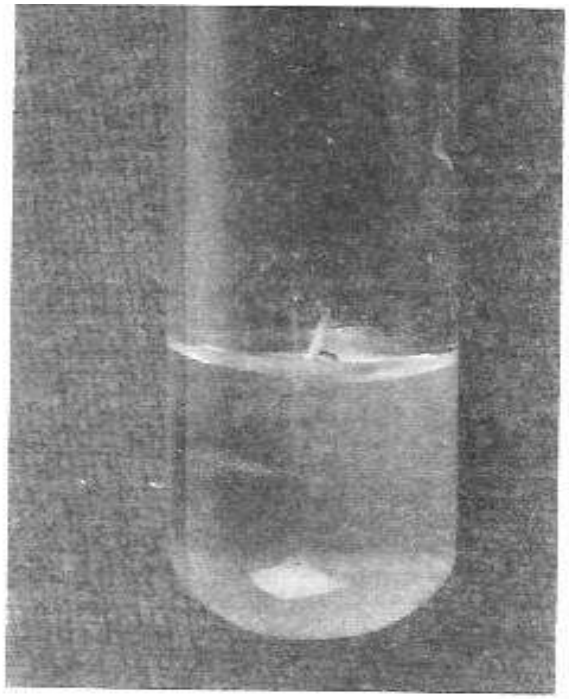

**Plate I.** *In vitro* induction from meristem explant of *Mentha arvensis* in MS media containing 1.0 mg /l BAP

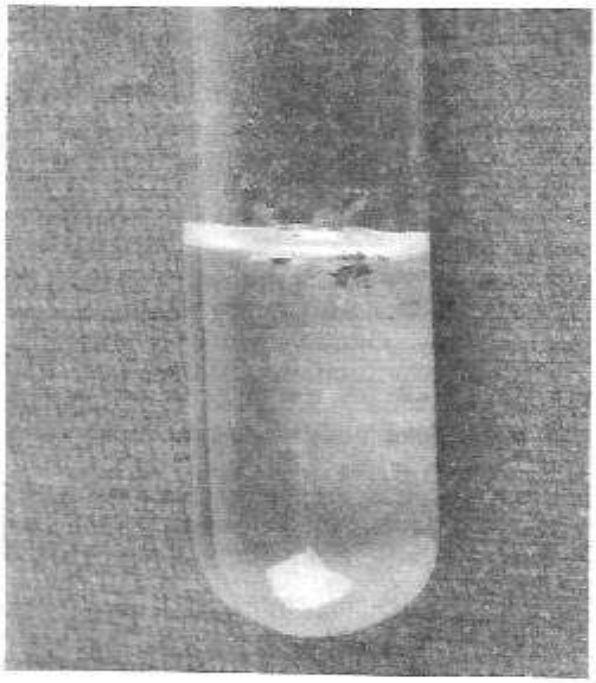

**Plate II.** Explants inducing multiple shoots.

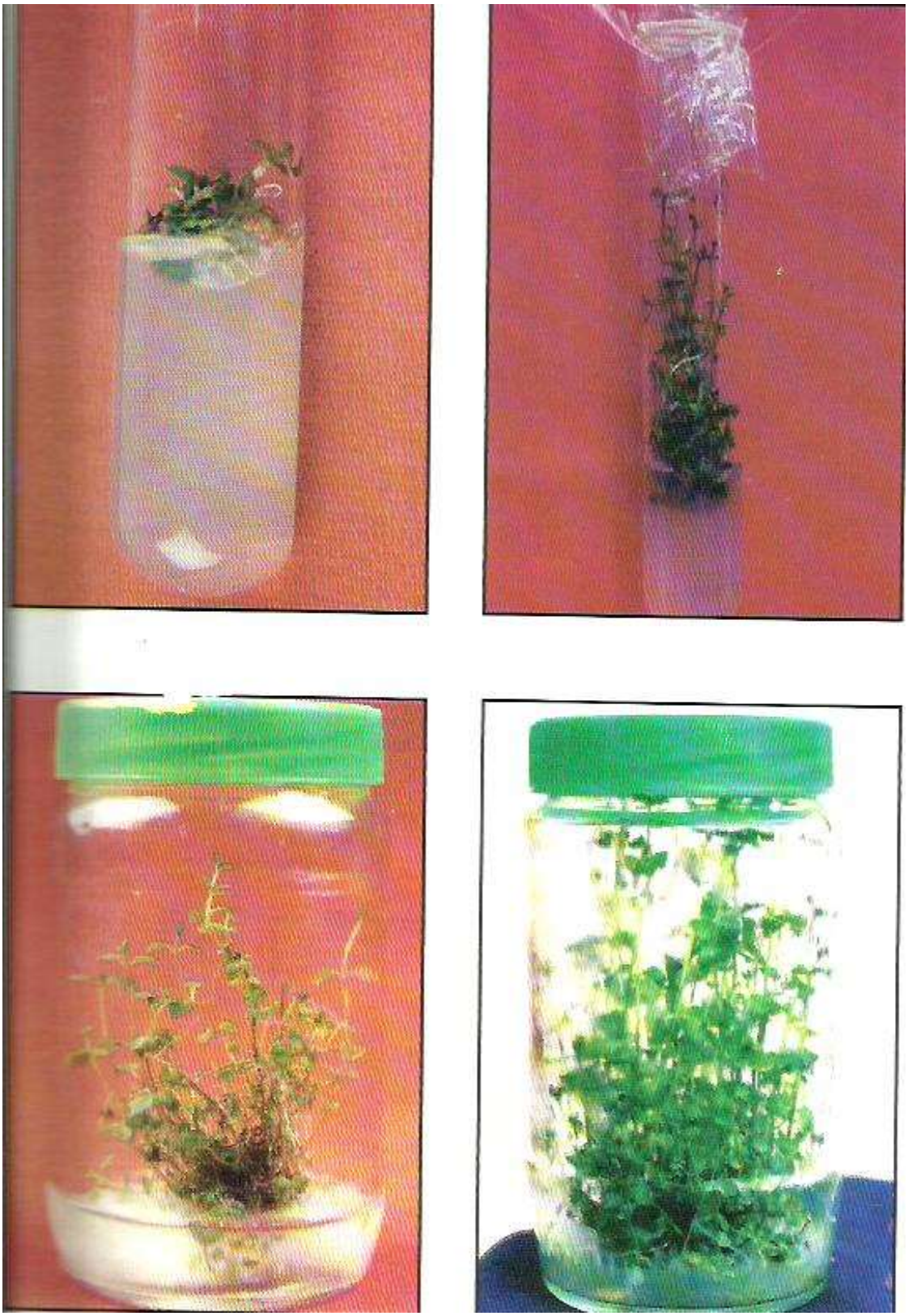

**Plate III.** Different stages of multiple shoot formation after 9, 18, 27 and 36 days of inoculation in MS media containing 1 mg/l BAP + 0.5 mg/l NAA.

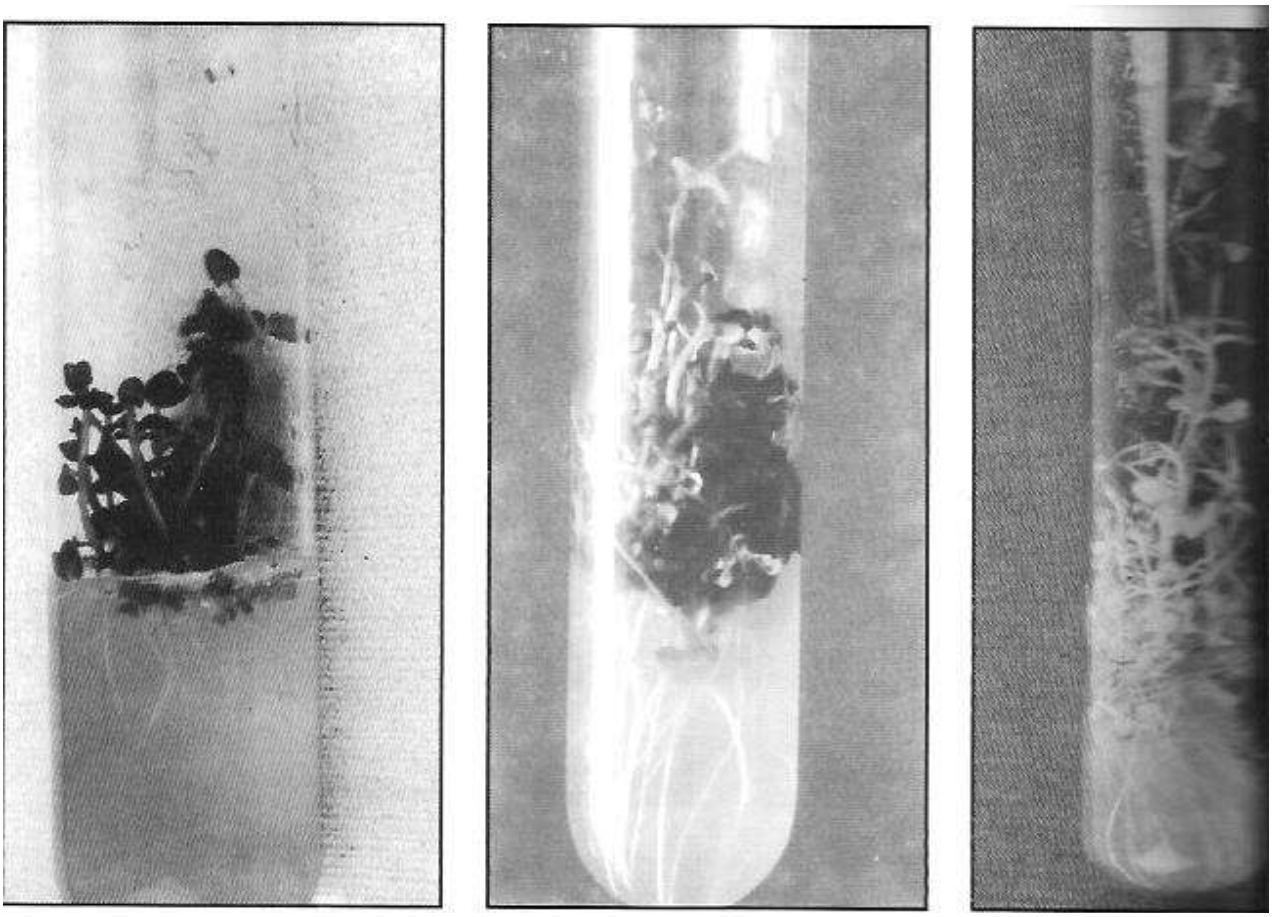

**Plate III.** Different stages of multiple shoot formation after 9, 18, 27 and 36 days of inoculation in MS media containing 1 mg/l BAP + 0.5 mg/l NAA.

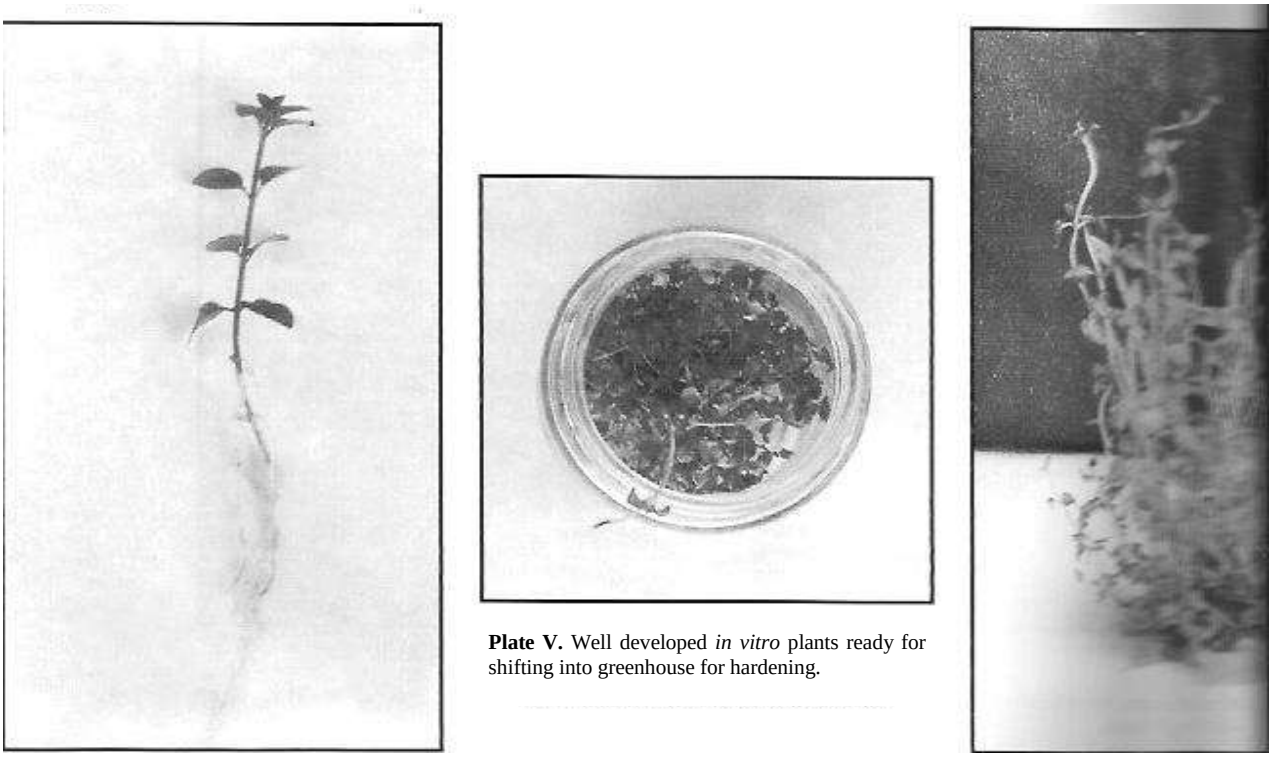

**Plate V.** Well developed *in vitro* plants ready for shifting into greenhouse for hardening.

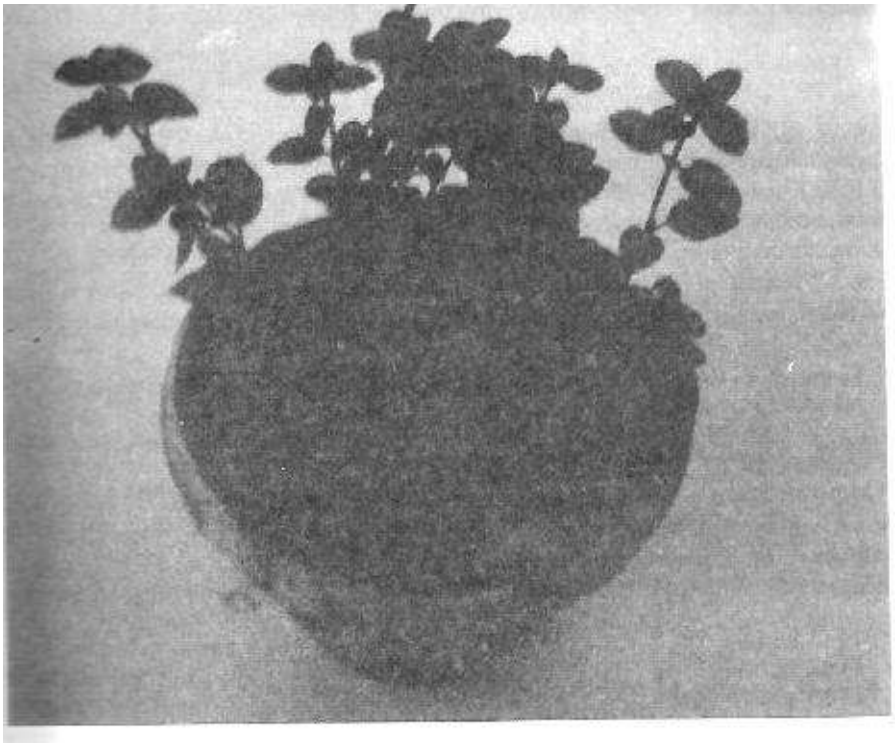

**Plate VI.** Well developed plant of *Mentha arvensis*.

All other combinations of NAA or IBA did not give satisfactory root induction response. However, best results for root induction were obtained when NAA and IBA at the concentration of 1.0 mg/l NAA + 2.0 mg/l IBA were used. At this concentration 100% shoot induction response was obtained with in 5 days of inoculation. However, Pathak and Heble (2002) used half strength MS media supplemented with 2.0 mg/l of IAA for *in vitro* root induction in *M. arvensis*.

Well developed *in vitro* plants were shifted to different compositions (sand, soil, peat, peat + sand (1:!), sand+peat+soil (1:1:1)) for hardening and plants were kept under cover or uncovered conditions. It was observed that rate of survival of uncovered plants was very low as compared to covered plants. As far as media for hardening is concerned rate of survival of plants was high in sand (i.e. 90%). Other compositions i.e. peat 75%; soil 10%; sand+peat (1:1) 70% and sand+peat+soil (1:1:1) 50%. Chisti and Siddiqui (2003), Phatak and Heble (2003), Savithri *et al* (2001) while working on *M. arvensis* also hardened the regenerated plants and successfully transferred to soil.

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