

**IN VITRO SHOOT REGENERATION OF MINT (*Mentha sp. L.*) USING
DIFFERENT TYPES OF EXPLANTS AND LEVELS OF
BENZYLAMINOPURINE**

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Abstract

An experiment was conducted in the Tissue Culture Laboratory of the Department of Horticulture, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Salna, Gazipur-1706 from March 2013 to February 2014 for *in vitro* shoot regeneration of mint using different *explants* and levels of benzylaminopurine (BAP) in full strength MS media. Three different types of *explants viz.* nodal segment, shoot tip and leaf were evaluated using three levels of BAP (1.0, 2.0 and 3.0 mg/l) along with control for shoot regeneration. Results revealed that shoot tip and nodal segments performed better than leaf as *explants* in almost all the characters studied. Shoot tip and nodal segments initiated shoot within the shortest time of 9.6 and 10.6 days, respectively with 1.0 mg/l of BAP. Regarding number of shoot per *explant* and number of node/shoot, shoot tip and nodal segments along with 1.0 mg/l of BAP performed superior at almost all days after inoculation. In case of interaction of *explants* and BAP, better performance was recorded in most of the studied parameters from shoot tip and nodal segments alongwith 1.0 mg/l BAP. Therefore, for *in vitro* shoot regeneration of mint, shoot tip or nodal segment may be used with 1.0 mg/l of BAP.

Keywords: *In vitro* shoot, BAP, *Explants*, Mint., *Mentha*.

Introduction

Mint (*Mentha sp*) is an aromatic plant that contains volatile essential oils, used fresh or dried as flavoring agent in a wide variety of foods. Mint oils are used in making different dental and medicinal products as well as in cosmetic industries. It is commonly known as *pudina* having dark green leaves and is usually found near ponds and other humid places of homestead. This perennial plant belongs to the family Lamiaceae and has approximately 25 species (Bhat *et al.*, 2002). Mint cultivation is widely distributed around the world. The United States of America is the main producer of peppermint and spearmint followed by India. The average yield range of mint is 5.35-11.40 MT/ha where maximum yield is 13.75 MT/ha in some cultivars (Anon., 2008).

Mints are mainly propagated vegetatively rather than by seeds (Safaeikhorram *et al.*, 2008). Programs for crop improvement via conventional breeding have been

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unsuccessful in mint because commercial cultivars are pollen-sterile and have a high ploidy number. Only some mints, such as *M. arvensis* L., *M. pulegonium* L. and *M. spicata* L., are propagated by seed (Heidari *et al.*, 2012). In our climatic conditions, poor overwintering may occasionally produce an insufficient number of seedlings. However, this conventional process of vegetative propagation through stolon is a slow process and they are susceptible to many diseases (Safaeikhorram *et al.*, 2008). To overcome these problems, *in vitro* propagation of mint may be beneficial. It also offers year-round production, precise crop production scheduling and reduce stock plant space of crops. The main advantage of *in vitro* propagation lies not only in the mass scale production but also in the production of high quality and uniform planting material that can be multiplied on a year-round basis under disease-free conditions anywhere irrespective of the season and weather.

As an important medicinal plant, mint may be a source of income for domestic use as well as for export. Therefore, attempt was made to develop protocols for large-scale *in vitro* propagation of locally available *Mentha sp.* So far, several works have been done in Bangladesh scatteredly on *in vitro* propagation of this plant. Considering the above facts, the present investigation has been undertaken to find out the performance of different *explants* with the optimum level of BAP for *in vitro* shoot regeneration of mint.

Materials and Methods

The experiment was conducted in the Tissue Culture Laboratory of the Department of Horticulture, Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Salna, Gazipur-1706, from March 2013 to February 2014. *Explants* of mint were taken from previously collected genotype (MP-12) grown in the Research Field of Department of Horticulture, BSMRAU, Gazipur. The genotype was collected from Mymensingh under a completed project of Ministry of Science and Technology, GoB. Tender and actively growing nodal segments (E_1), shoot tips (E_2) and leaves (E_3) were used as *explants*. Three levels of BAP (mg/l) viz. B_2 -1.0, B_3 -2.0 and B_4 -3.0 alongwith control (B_1 - no BAP), were included in the experiment. Two factors experiment was laid out in Completely Randomized Design (CRD) with ten replications, where one (1) culture tube represented one replication.

Preparation of stock solution of BAP (6- Benzyl-aminopurine)

For preparing stock solution, 10 mg of BAP was weighed by an electric balance and placed on a clean beaker and then dissolved by few drops of 0.1 N HCl. The mixture was then collected in a 100 ml measuring cylinder and volume was made up to 100 ml by addition of distilled water. The strength of prepared stock solution was 100.0 mg/l. The prepared solution was then poured into a glass bottle, labeled properly and then stored in a refrigerator at 4°C.

Preparation of culture media

The medium contained full strength (4405.19 mg/l) Murashige and Skoog's (MS) inorganic salts and vitamins. To prepare 400 ml of the MS medium; 1.76 g MS powder was weighed and dissolved in 300 ml of distilled water in a 1.0 L beaker. Therefore, 24 g sucrose along with treatment wise different concentrations of hormonal supplements of BAP were added to the solution in the beaker and the mixture was mixed properly by using magnetic stirrer. The pH of the medium was adjusted to 5.7 ± 1 with a pH meter by using 0.1 N sodium hydroxide (NaOH) or 0.1 N HCl and finally the volume of the solution was made to 400 ml with further addition of distilled water. In order to solidify the media, high brand agar of 2.8 g (@ 7%) was added to the solution and then thoroughly mixed and gently boiled in a microwave oven for 10–12 min. until the agar was dissolved completely. After that, about 10 ml prepared melted media was dispensed into each culture tube while the medium was still hot. The culture tubes were sealed with aluminum foil and marked with glass marker pen to indicate specific treatment. The culture tubes were then sterilized at 1.06 kg/cm^2 pressure at 121°C for 25 min. in an autoclave. After autoclaving, the culture media was left overnight within the autoclave to become cool and solid and were taken out. Inoculation was done in the following day.

Preparation of explants and inoculation

For establishing the plant in media, the tender actively growing mint shoots were collected and used in this study. The collected *explants* were taken in a beaker and washed in running tap water for 15 min. The *explants* were then sterilized with 1% osbam for 15 min and were again washed with tap water three times. Finally, sterilization was carried out in the aseptic condition under a laminar flow cabinet. Previously prepared materials were taken in a sterile pot and suspended in 0.1% HgCl_2 solution for 10 min. to ensure contamination free *explants*. Then the *explants* were washed for 3–4 times with double distilled water to remove all traces of HgCl_2 . Therefore, the leaves around the shoot tips were carefully removed and shoot tips were placed inside the test tubes carefully. After placing the *explants* in the test tubes, they were kept inside the growth chamber where, physical condition for growth and development of cultures were maintained. The temperature was set to $22 \pm 1^\circ\text{C}$ with a light intensity of 2000–3000 lux from fluorescent tubular lamps and the photoperiod maintained generally 16 h light and 8 h dark (16L/8D) with 60–70% relative humidity.

Successful shoot formation becomes evident when small green fresh leaves began to emerge. It was the first sign of regeneration. So, each test tube was observed regularly and the days to shoot initiation was recorded for the respective test tube. Other data were collected from 25 to 65 days after inoculation (DAI) at 10 days interval on number of shoot per *explant*, length of the longest shoot and number of node per shoot. Collected data were analyzed

using MSTAT-C statistical package programme. Differences among the means were compared following Duncan's Multiple Range Tests (DMRT) at 1% level of significance.

Results and Discussion

Results regarding response of types of *explants*, levels of BAP and their interaction on different aspects of *in vitro* shoot regeneration of mint are presented in the following heads.

Days to shoot initiation

Response of *explants* regarding days required for *in vitro* regeneration of shoot showed significant variation in mint. The highest number of days (46.15) required for shoot initiation in leaf whereas, shoot tip and node required relatively shorter period of 18.85 and 17.10 days, respectively (Fig. 1.) which were statistically similar. This finding corroborated with Ghanti *et al.* (2004) who also reported a non-significant difference regarding days required for shoot initiation in mint from shoot tip and nodal segment *in vitro*. The lowest number of days required for *in vitro* shoot regeneration in shoot tip and node might be due to higher totipotency of shoot tip and node having dormant bud which is absent in leaf, or might be due to different cytokinin complements of the shoot tip, nodal segment and leaves (Van, 2009). Heidari *et al.* (2012) opined that shoot meristems and nodes were more potent for shoot regeneration compared to leaf disk *explants* which agreed the present findings. Range of days required for shoot initiation from shoot tip, nodal segment and leaf was 17.10- 46.15 in the present study which differed from the findings of Samantaray *et al.* (2012) who reported a range of 22.6-26.0 days required for shoot initiation in mint.

Different concentration of BAP showed significant variation in mint regarding days required for *in vitro* shoot initiation (Fig. 2). The highest days (35.53) required in control followed by BAP at 3.0 (27.33 days), 2.0 (24.73 days) and 1.0 mg/l (21.87 days) (Fig. 2). It was observed that days required for shoot initiation increased with the increase of BAP concentration. It might be due to inhibition characteristic of BAP on shoot initiation (Van, 2009). The highest days required in control reflected the positive effect of BAP on shoot initiation.

The interaction effect of *explants* and levels of BAP on days required for shoot regeneration *in vitro* also showed significant variation (Fig. 3). The highest number of days required (47.80 days) in the combination of leaf without BAP (E_1B_1) followed by E_3B_4 (46.80 days), E_3B_2 (45.40 days) and E_3B_3 (44.60 days). The lowest number of days required for *in vitro* shoot regeneration was in E_2B_2 (9.60 days) followed by E_1B_2 (10.60 days) where shoot tip and nodal segments were used with 1.0 mg/l of BAP, respectively. It might be due to higher rate of growth and regeneration of shoot tip and nodal segment and best performance of BAP at 1.0 mg/l in mint. Present findings provide evidence that a further increase

in concentration of BAP showed some adverse effect and subsequently increased number of days required for shoot development which corroborated with the findings of Samantary *et al.* (2012), Xiao *et al.* (2007), Shawl *et al.* (2006) and Godoy *et al.* (2005). It is also revealed from Fig. 3 that days required for *in vitro* shoot regeneration increased with the increased concentration of BAP. Inhibiting criteria of cytokinin at higher concentration on shoot initiation and shoot elongation might be the reason behind this (Van, 2009).

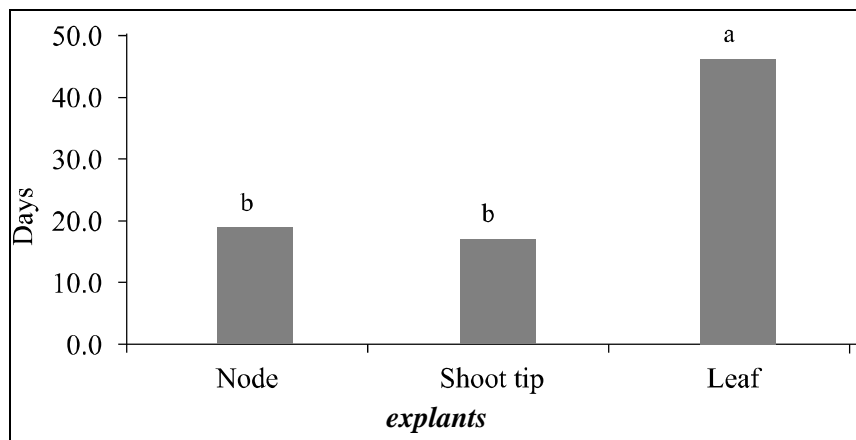


Fig. 1. Response of explants on days required for shoot initiation in mint after inoculation. Significant differences ($p < 0.01$) among the explants are indicated by different letters according to DMRT.

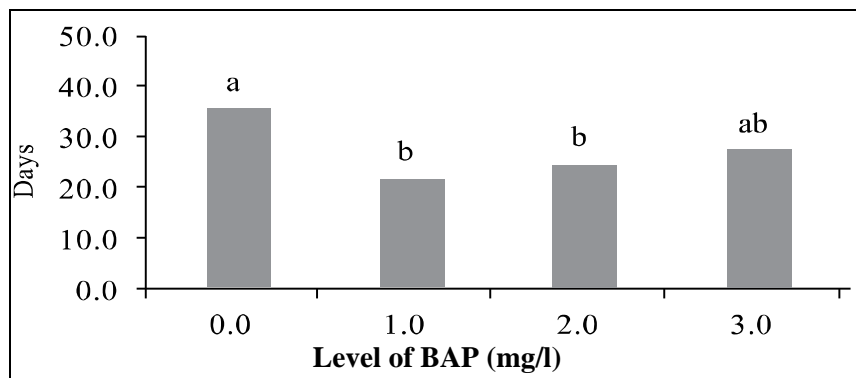


Fig. 2. Effect of BAP on days required for shoot initiation in mint after inoculation. Significant differences ($p < 0.01$) among the explants are indicated by different letters according to DMRT.

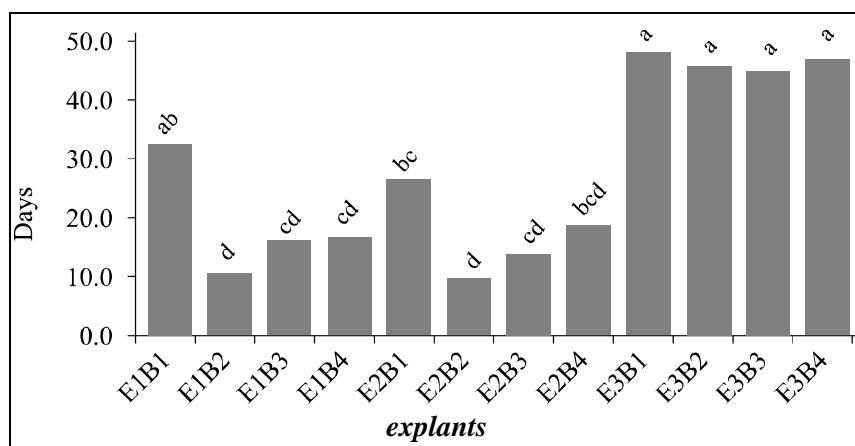


Fig. 3. Effect of BAP on days required for shoot initiation in mint after inoculation. Significant differences ($p < 0.01$) among the *explants* are indicated by different letters according to DMRT.

E₁= Node, E₂= Shoot tip and E₃= Leaf; B₁= 0.0, B₂= 1.0, B₃= 2.0 and B₄= 3.0 mg/l of BAP.

Number of shoots per *explant*

Results presented in Fig. 4 revealed that the number of shoots increased among the *explants* with the increase of days after inoculation (DAI). Shoot tip was found to be more responsive regarding number of shoots followed by node and leaf. At 25 days after inoculation, number of shoots produced by shoot tip and node were 0.95 and 0.80, respectively produced shoot but leaf started to produce shoot (0.10) at 45 DAI. Statistically similar numbers of shoots were produced by shoot tip and nodal segment of mint till 55 DAI in *in vitro* condition. However, at 65 DAI, shoot tip produced the highest number of shoots (3.35) per *explant* and this was statistically different from other two *explants*. Node produced the second highest number of shoots (2.45) per *explant*, which differed significantly from the leaf (Fig. 4). The better performance of shoot tip and node in producing the highest number of shoots might be due to presence of apical bud in those *explants*. Dhawan *et al.* (2003), Chaturvedi *et al.* (2007), Sujana and Naidu (2011a, 2011b) and Heidari *et al.* (2012) also reported the better performance of node and shoot tip as *explant* for *in vitro* shoot regeneration in mint. On the other hand, Eck and Kitto (1992) successfully regenerated shoots from peppermint and orange mint using leaf as *explants* which differed from the present findings.

BAP exhibited significant influence on *in vitro* shoot regeneration in mint in respect of number of shoots/*explant* at different DAI (Fig. 5). At 25 DAI, the highest number of shoots (1.27) was recorded from BAP at 1.0 mg/l followed by 0.67 and 0.40 at 2.0 and 3.0 mg/l, respectively. Similar trend was also observed at 35, 45, 55 and 65 DAI. No shoot was found up to 35 DAI in control.

Number of shoots was found to be decreased with the increase of BAP concentration at all the studied days after inoculation. However, the best performance was observed with the BAP at 1.0 mg/l concentration in all the studied days after inoculation. This result is consistent with the findings of Ghanti *et al.* (2004) who reported that the highest number of shoots per *explant* in mint using 1.0 mg/l of BAP. They also found the number of shoots to be decreased with the increase of BAP concentration from 1.0 mg/l. This indicated the upper limit of the concentration of BAP for mint. This result slightly differed from Heidari *et al.* (2012), who recorded the highest number of shoots with 1.5 mg/l of BAP in *Mentha longifolia explants* under 24 hours photoperiod in culture condition. This discrepancy might be due to the difference between *explants* and culture condition. Many other researchers also documented the response of BAP in shoot regeneration of mint (Chishti *et al.*, 2006; Bolouk *et al.*, 2013; Bariya and Pandya, 2014 and Shasany *et al.*, 1998). George (1993) stated that cytokinins, especially BAP are reported to overcome apical dominance, release lateral buds from dormancy and promote shoot formation. The result differed from Bolouk *et al.* (2013) who reported that using *Mentha piperita explants* produced the highest number of shoots in those receiving a treatment of 2.0 mg/l of BAP. This disparity might be due to species or *explant* or culture condition differences between two experiments.

Regarding interaction effect of *explants* and different concentrations of BAP, it was observed that shoot number per *explant* was increased gradually from 25 to 65 DAI in all the treatment combinations. However, no shoot was found to be produced upto 35 DAI in the treatment combinations E₁B₁, E₂B₁, E₃B₁, E₃B₂, E₃B₃ and E₃B₄ (Table 1). No shoot formation in E₁B₁, E₂B₁ and E₃B₁ treatment combinations might be due to the absence of BAP in the medium, as BAP have effective role in DNA synthesis and mitosis (Sujana and Naidu, 2011b). While, no shoot in E₃B₂, E₃B₃ and E₃B₄ treatment combinations might be due to the absence of any apical buds in leaves. Shoot tip with 1.0 mg/l of BAP (E₂B₂) produced the highest number of shoots in all the recorded DAI, which were statistically similar with E₁B₂ upto 55 DAI. At 65 DAI, although, the treatment combination E₂B₂ produced significantly the highest number of shoot per *explant* (5.20) but the treatment combination E₁B₂ produced the 2nd highest number of shoots (3.80) per *explant*. This results authenticated the superiority of shoot tip and node as *explant* with 1.0 mg/l of BAP for *in vitro* regeneration of shoot in mint, which corroborate the findings of Ghanti *et al.* (2004) and Heidari *et al.* (2012), who recorded the highest number of shoots using 1.0 mg/l of BAP in both shoot tip and nodal *explant* of mint *in vitro*.

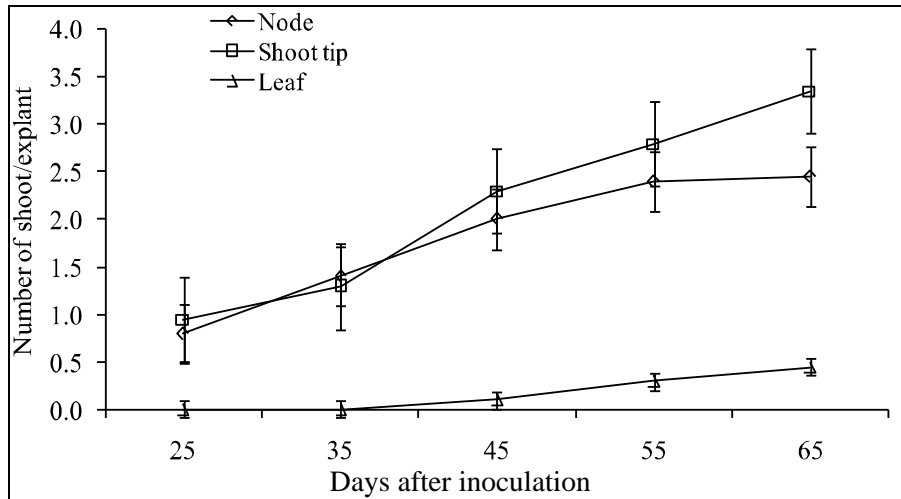


Fig. 4. Response of explants on number of shoot regeneration in mint at different days after inoculation. Vertical bars indicate \pm SE.

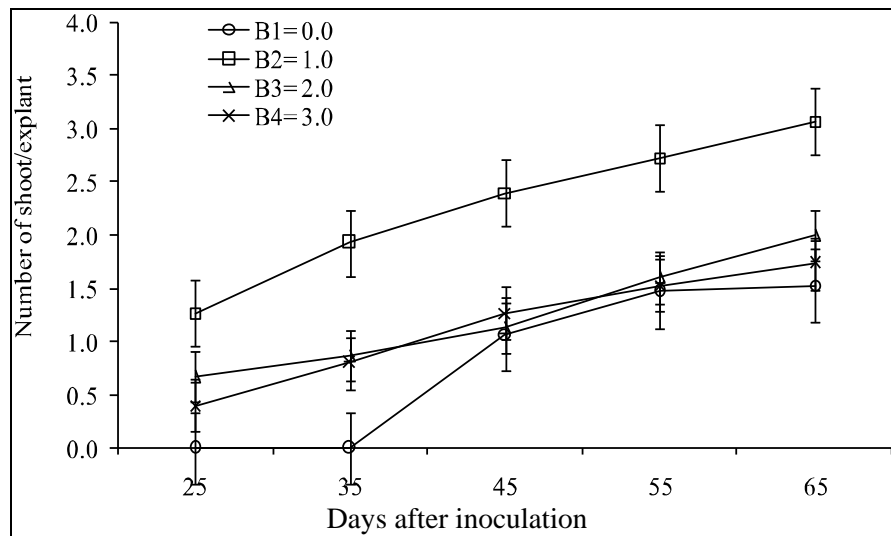


Fig. 5. Effect of BAP on number of shoot regeneration in mint at different days after inoculation. Vertical bars indicate \pm SE.

Number of node per shoot

While studying the effect of *explant* on number of node, it was found that the number of node increased among the *explants* with the increase of days after inoculation (Fig. 8). Shoot tip performed better regarding the number of node per shoot followed by nodal segment and leaf. At 25 DAI, shoot tip (1.85) and nodal segment (1.25) produced node but leaf started to produce node at 45 DAI (0.20)

as it started to produce shoot at 45 DAI. The highest number of node produced by shoot tip and nodal segment of mint showing statistically similar result from 25 to 65 DAI in *in vitro* condition. However, at 65 DAI, nodal segment produced the highest number of nodes (9.15) per shoot, which was statistically similar with shoot tip (8.90) and significantly different from leaf (1.30). More number of node is important for *in vitro* culture, as it may be used as *explants* in further multiplication of plant through subculture, which is one of the main objectives of *in vitro* propagation from a single plant at the shortest time. So, from this point of view, shoot tip and node performed better.

Table 1. Interaction effect of *explants* and BAP on number of shoot per *explant* in mint at different days after inoculation

Treatment combination	Number of shoots/ <i>explant</i> at				
	25 DAI	35 DAI	45 DAI	55 DAI	65 DAI
E ₁ B ₁	0.00 d	0.00 c	1.20 bcd	1.80 bc	1.80 de
E ₁ B ₂	1.60 ab	2.80 a	3.40 a	3.80 a	3.80 b
E ₁ B ₃	1.00 bc	1.40 b	1.80 bc	2.00 b	2.00 d
E ₁ B ₄	0.60 cd	1.40 b	1.60 bc	2.00 b	2.20 d
E ₂ B ₁	0.00 d	0.00 c	2.00 b	2.40 b	2.60 cd
E ₂ B ₂	2.20 a	3.00 a	3.80 a	4.40 a	5.20 a
E ₂ B ₃	1.00 bc	1.20 b	1.60 bc	2.40 b	3.40 bc
E ₂ B ₄	0.60 cd	1.00 bc	1.80 bc	2.00 b	2.20 d
E ₃ B ₁	0.00 d	0.00 c	0.00 d	0.20 d	0.20 f
E ₃ B ₂	0.00 d	0.00 c	0.00 d	0.00 d	0.20 f
E ₃ B ₃	0.00 d	0.00 c	0.00 d	0.40 d	0.60 f
E ₃ B ₄	0.00 d	0.00 c	0.40 d	0.60 cd	0.80 ef
Mean	0.58	0.90	1.47	1.83	2.08

Means followed by same letter(s) in a column do not differ significantly at 1% level by DMRT.

E₁= Node, E₂= Shoot tip and E₃= Leaf; B₁= 0.0, B₂= 1.0, B₃= 2.0 and B₄= 3.0 mg/l of BAP.

Results presented in Fig. 9 revealed that the effect of BAP on *in vitro* regeneration of mint regarding the number of node was significant in different concentrations at each day of data recorded (25, 35, 45, 55 and 65 DAI). While studying the effect of BAP on *in vitro* regenerated mint at 25 DAI statistically similar rank was found regarding the number of node per shoot at 1.0 mg/l (1.80) and 2.0 mg/l (1.40) which was significantly different from 0.93 at 3.0 mg/l of BAP. However, no node was produced at 25 DAI without BAP. Similar trend was observed at 35, 45, 55 and 65 DAI where number of node per shoot produced using 1.0, 2.0 and 3.0 mg/l of BAP occupied the statistically similar rank and that was significantly different from control. However, the highest

number of node per shoot produced when BAP was used @ 1.0 mg/l at all the studied days.

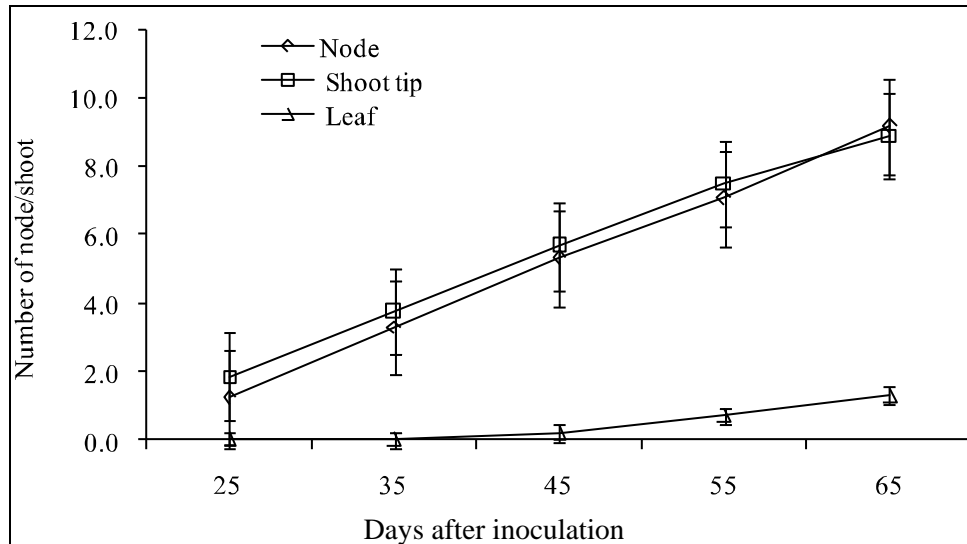


Fig. 8. Response of *explant* on number of node in mint at different days after inoculation. Vertical bars indicate \pm SE.

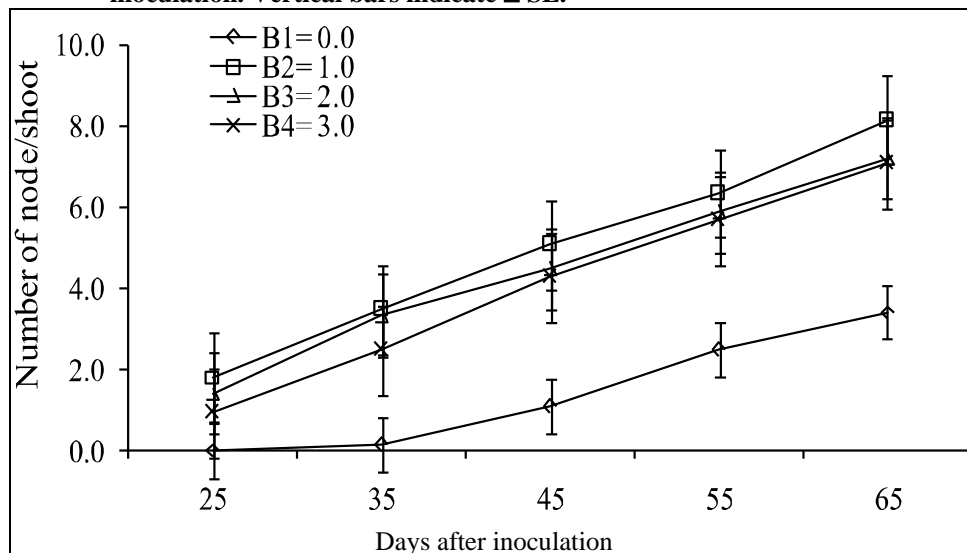


Fig. 9. Effect of BAP on number of node in mint at different days after inoculation. Vertical bars indicate \pm SE.

In case of interaction of *explants* with different concentrations of BAP on number of node per shoot, it was observed that node number increased gradually from 25 to 65 DAI in all the treatment combinations (Table 3). However, no nodes were

found to be produced upto 25 DAI in the treatment combinations E₁B₁, E₂B₁, E₃B₁, E₃B₂, E₃B₃ and E₃B₄; upto 35 DAI in the treatment combinations E₂B₁, E₃B₁, E₃B₂, E₃B₃ and E₃B₄; upto 45 DAI in the treatments E₃B₁, E₃B₂ and E₃B₃; and upto 55 DAI in the treatment combination E₃B₂. As in these days, no shoot was produced and therefore, no node was observed in these treatments. Shoot tip with 1.0 mg/l of BAP (E₂B₂) produced the highest number of nodes per shoot at all recorded days (3.40 at 25 DAI, 5.60 at 35 DAI, 7.60 at 45 DAI, 9.60 at 55 DAI and 12.00 at 65 DAI), which were statistically similar with some other combinations of shoot tip and nodal segment with different concentrations of BAP. A similar trend of decreasing number of node with higher concentration of BAP (2.0 and 3.0 mg/l) than at 1.0 mg/l was observed in shoot tip and leaf. But in case of leaf, it performed better regarding number of node per shoot at higher concentration of BAP at 3.0 mg/l than other concentrations of BAP at each day of data recorded. It might be due to lack of cytokinin in leaf showing the higher requirement of BAP for better performance of leaf in *in vitro* condition.

Table 3. Interaction effect of explants and BAP on number of node in mint at different days after inoculation

Treatment combination	Number of node/shoot at				
	25 DAI	35 DAI	45 DAI	55 DAI	65 DAI
E ₁ B ₁	0.00 c	0.40 c	1.60 b	3.20 bc	5.00 b
E ₁ B ₂	2.00 ab	4.80 ab	7.60 a	9.40 a	11.80 a
E ₁ B ₃	1.80 b	4.80 ab	6.80 a	8.40 a	10.80 a
E ₁ B ₄	1.20 bc	3.20 b	5.20 a	7.20 a	9.00 a
E ₂ B ₁	0.00 c	0.00 c	1.60 b	3.80 b	4.60 b
E ₂ B ₂	3.40 a	5.60 a	7.60 a	9.60 a	12.00 a
E ₂ B ₃	2.40 ab	5.20 ab	6.60 a	8.40 a	9.20 a
E ₂ B ₄	1.60 b	4.20 ab	6.80 a	8.20 a	9.80 a
E ₃ B ₁	0.00 c	0.00 c	0.00 b	0.40 cd	0.60 c
E ₃ B ₂	0.00 c	0.00 c	0.00 b	0.00 d	0.60 c
E ₃ B ₃	0.00 c	0.00 c	0.00 b	0.80 bcd	1.60 bc
E ₃ B ₄	0.00 c	0.00 c	0.80 b	1.60 bcd	2.40 bc
Mean	1.03	2.35	3.72	5.08	6.45

Means followed by same letter(s) in a column do not differ significantly at 1% level by DMRT.

E₁= Node, E₂= Shoot tip and E₃= Leaf; B₁= 0.0, B₂= 1.0, B₃= 2.0 and B₄= 3.0 mg/l of BAP.

Conclusions

Full strength MS media along with 1.0 mg/l of BAP can be used for *in vitro* shoot regeneration of mint; where, the *explants* may be shoot tip or nodal segment.

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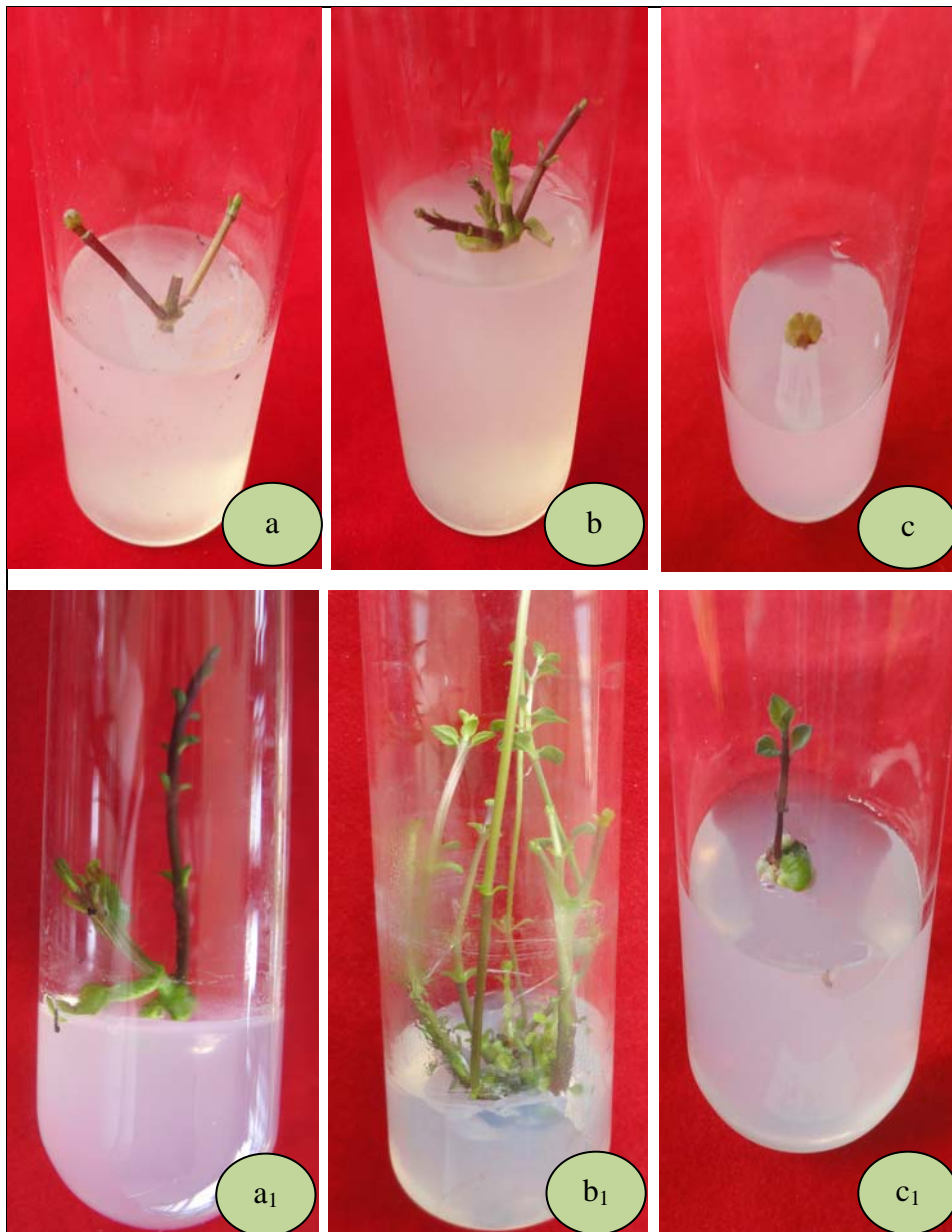


Fig. 10. *In vitro* regeneration of shoot in mint (a, b and c= *explant* from nodal segment, shoot tip and leaf, respectively; a₁, b₁ and c₁ is regenerated shoot from nodal segment, shoot tip and leaf, respectively).

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