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Influence of Bavistin, Cefotaxime, Kanamycin and Silver Thiosulphate on Plant Regeneration of *Mentha piperita* (L.) – An Important Multipurpose Medicinal Plant

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Summary

Effect of different concentrations of bavistin, cefotaxime and kanamycin along wih the ethylene inhibitor STS were examined for *in vitro* plant regeneration using axillary bud explants of *Mentha piperita*. Filter sterilized bavistin, cefotaxime and kanamycin were added separately in the range of 0 – 300 mg (bavistin); 10, 20, 30, 40, 50, 100, or 120 µM/L (cefotaxime or kanamycin) to growth regulator free MS medium containing 3% sucrose. In a separate experiment bavistin (100 mg/l) along with growth regulators such as BAP and TDZ were added to MS medium containing 3% sucrose. Similarly the regeneration medium was supplemented with 10, 20, 30, 40, 50, 80, 100 or 120 µM/L of STS. Maximum number of shoots 6.85 ± 0.18 was seen with 150 mg/l bavistin with 100% regeneration. These plantlets were further maintained for root emergence on a rooting medium supplemented with growth regulators such as IAA, IBA and NAA. The rooted plants were acclimatized and transferred to field plots with 95% of plants surviving in the field.

Key Words: *Mentha piperita*, Bavistin, Cefotaxime, Kanamycin, Silver thiosulphate, Plant growth regulators

Abbreviations: BAP – 6-benzyl amino purine; NAA – α- naphthalene acetic acid; IAA – indole-3- acetic acid; IBA – indole butyric acid; TDZ – thidiazuron; STS - silver thio sulphate

Introduction

Peppermint, a perennial herb of the lamiaceae, is an aromatic and medicinal plant widely cultivated for its essential oils. Components of the oils are used commercially in herbal products, as well as in flavorings for mouthwashes, toothpaste, and medicinal products. This diverse utilization has promoted extensive cultivation of peppermint in the USA as well as in Asia and Europe. Many of these species are used for ornamental purposes and ground cover. The family comprises more than 6500 species in at least 240 genera of cosmopolitan distribution and highly varied morphological characters. In fact peppermint is particularly well represented in the temperate regions of the world, such as the Mediterranean region and in tropical upland savannas. The leaves are often hairy, with epidemial glands, which secrete volatile oils giving characteristic scents to many species. In addition these herbs are used for many other purposes including beverages such as tea, dying, repellents, fragrances, cosmetics, charms, smoking and industrial use. *Mentha piperita* is one of the most widely cultivated peppermint species for oil production. The essential oil extracted from peppermint is used worldwide in the confectionary and pharmaceutical industries, especially for gum and oral hygiene products [9]. Peppermint oil is one of the most popular and widely used essential oil in food products, cosmetics, pharmaceuticals, dental preparations, mouth washes, soaps, chewing gums, candies, confectionery and alcoholic liquors [20]. Tissue culture in turn is the only way to sustain the large scale farming of medicinal plants, as this is the only technique to produce plants of high and uniform quality in large quantity from any part of the plant in any season [11]. Historically plants of the mint family have played an important role in herbal remedies also for drinking as hot tisane (herbal tea) or in aromatherapy. Some varieties are added to the bath, for their aromatic qualities and processed and used in soaps, toothpaste and cosmetics. Since studies on *in vitro* multiplication on this species using different antibacterial agents and an ethylene inhibitor STS are almost limited, hence in the present paper we report a protocol for the direct regeneration of *Mentha piperita* an important multipurpose medicinal plant.

Materials and Methods

Culture medium

During present investigation only MS medium [18] was used. The chemicals used for preparing various media were of analytical grade from Merck, Sigma and Universal chemicals. Nutrient medium was homogenized by boiling and by continuous stirring before adding agar and phytohormones. The pH of the medium was adjusted as 5.8 prior to addition of

agar by using 0.1N NaOH and 0.1N HCl. After adding different concentration of growth hormones, about 15 - 20 ml of media was dispensed into each culture tube. After autoclaving the culture vials were kept inside the inoculation chamber.

Plant collection

Mentha piperita plants were obtained from Suvedha Nursery, Tirupati, Andhra Pradesh, India. Axillary bud segments from healthy plants of *Mentha piperita* were used in the present study. The explants were collected, washed thoroughly under running tap water for 15 min. These were treated with 5% teepol (w/v) for 5 min, and again washed thoroughly in running tap water. After that nodal segments were surface sterilized with 0.1% $HgCl_2$ for 5 minutes, followed by washing with sterile double distilled water inside the laminar airflow chamber to remove traces of $HgCl_2$. Axillary buds were cultured individually on MS medium containing different concentrations (50 - 200 mg/l) of bavistin singly and in combination of BAP and TDZ to induce multiple shoots. In the same way, axillary bud explants were also inoculated on MS medium containing different concentrations of cefotaxime and kanamycin (10 – 120 µM/L). Both proliferation and rooting media contained 3% sucrose and gelled with 0.8% agar (Hi - Media, India). The pH was adjusted to 5.8 and autoclaved at 121°C, 15 lbs pressure for 15 min. All the cultures were maintained in a growth room with a 16 h photoperiod (cool, white fluorescent light – 3000 lux light intensity) and the temperature was maintained at 25 ± 2ºC, with 50 - 80% relative humidity. After regeneration and sufficient elongation, the micro shoots were carefully excised and rooted on MS medium with IBA, IAA and NAA separately. Rooted plantlets were transferred to polycups containing sterile soil and vermiculite (1:1) and covered with plastic bag to maintain humidity. Subsequently, the plantlets were transferred to greenhouse after one month and planted in the soil. Each treatment consisted of twenty replicates and the experiment was repeated twice.

Results

Effect of bavistin, cefotaxime and kanamycin on axillary bud explants

Axillary bud explants responded well with bavistin. Maximum frequency of regeneration (100%), shoot number (6.85 ± 0.18) and maximum shoot length (8.40 ± 0.35 cm) was obtained in 150 mg/l of bavistin. (Table 1 and Fig. 1). Bavistin (100 mg/l) in combination with BAP (1.0 mg/l) showed the decreased frequency of regeneration (95%), shoot length (6.60 ± 0.81 cm) and increased shoot number (8.3 ± 0.54) when compared with the cultures having only bavistin. Bavistin with TDZ also showed less regeneration (85%), shoot length (6.30 ± 0.84 cm) and shoot number (4.9 ± 0.51).

The present study and obtained results reveal the influence of cefotaxime on the regeneration of axillary buds of *Mentha piperita.* Cefotaxime does not decrease the shooting response but it induced the maximum shoot formation (5.25 ± 0.45) with a maximum shoot length (7.5 ± 1.4 cm) and a high frequency of regeneration (85%) at a concentration of 80 µM/L. At highest concentration, cefotaxime (150 µM/L) showed decreased frequency of regeneration (40%), shoot number (1.2 ± 0.27) and shoot length (2.48 ± 0.29 cm). A concentration of 80 µM/L of cefotaxime was chosen as the optimum concentration for the *Mentha piperita* cultures (Table 2 and Fig. 1).

The influence of kanamycin on regeneration of *Mentha piperita* was evaluated. Shoot initiation was observed after one week of inoculation. MS medium supplemented with 80 µM/L kanamycin induced highest number of shoots (2.85 ± 0.40) and highest mean shoot regeneration frequency (95%) with maximum shoot length (7.5 ± 1.5 cm). Above 40 µM/L concentration, the regeneration frequencies gradually decreased with increase in concentrations up to 150 µM/L. Higher concentrations (above 150 µM/L) tested completely inhibited regeneration (Table 3 and Fig. 1)

Effect of ethylene inhibitor silver thiosulphate on axillary bud explants

Bud break from nodal explants was observed *in vitro* one week after concentrations of silver thiosulphate. Silver thiosulphate, at any concentration tested, had a positive effect and showed shoot formation. Highest frequency of regeneration (90%) was observed at 50 µM/L STS, with maximum number of shoots (4.75 ± 0.39) and highest shoot length (3.36 ± 0.55 cm). Lower concentrations of STS favoured the high frequency of regeneration and highest number of shoots. The optimum range of STS concentrations recorded was between 10 – 50 µM/L (Table 4 and Fig. 1).

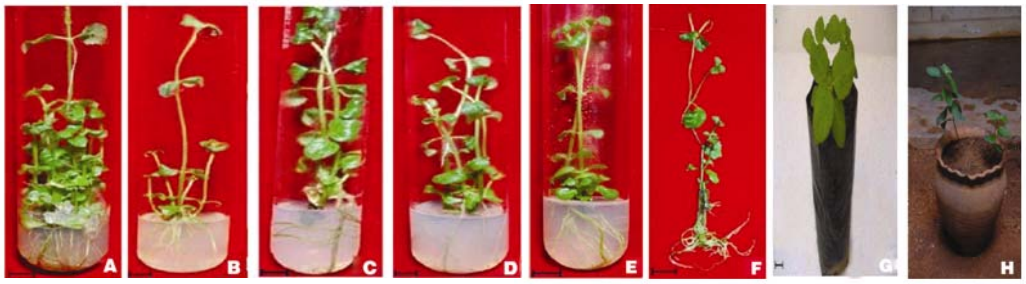

Figure – 1: A. Multiple shoots regenerated from axillary bud explant with MS + bavistin (100 mg/L) + BAP (1.0 mg /L). B. Shoot regeneration from axillary bud explants on MS + cefotaxime (80 µM/L). C. Maximum shoots formed on MS + Kanamycin (80 µM/L). D. MS + sliver thiosulphate (50 µM/L). E&F. Rooting from regenerated shoots on MS medium (IBA 1.5 mg/l). G&H. Hardened plants in polycups and pots containing sterile soil and vermiculate.

Table – 1: Effect of bavistin singly and in combination with plant growth regulators on *in vitro* shoot organogenesis from axillary bud explants of field grown plants of *Mentha piperita*. Observations: after 4 weeks; Values are mean ± SE of 20 independent determinations

Bavistin (mg/L)	BA	TDZ	Frequency of shooting response (%)	No. of shoots/explant	Mean shoot length (cm)
-	-	-	-	-	-
50	-	-	75	2.5 ± 0.37	3.6 ± 0.1
100	-	-	85	3.9 ± 0.42	4.2 ± 0.1
150	-	-	100	6.85 ± 0.18	8.40 ± 0.35
200	-	-	80	4.4 ± 0.55	6.0 ± 0.50
300	-	-	65	2.4 ± 0.43	5.0 ± 0.90
100	1.0	-	95	8.3 ± 0.54	6.60 ± 0.81
100	-	1.0	85	4.9 ± 0.51	6.30 ± 0.84
-	1.0	-	90	7.2 ± 0.59	4.0 ± 0.1
-	-	1.0	70	2.0 ± 0.38	3.5 ± 0.1

Table – 2: Influence of cefotaxime added to MS medium on regeneration of plantlets from axillary bud explants of *Mentha piperita.* Observations: after 4 weeks; Values are mean ± SE of 20 independent determinations

Cefotaxime (µM/L)	Frequency of regeneration (%)	Number of shoots/explant	Mean shoot Length (cm)
-	-	-	-
10	45	1.2 ± 0.31	3.62 ± 0.06
20	55	1.4 ± 0.30	4.55 ± 0.36
30	60	1.85 ± 0.37	4.68 ± 0.39
40	65	2.0 ± 0.36	5.7 ± 0.8
50	75	3.7 ± 0.42	5.36 ± 0.55
80	85	5.25 ± 0.45	7.5 ± 1.4
100	60	2.55 ± 0.31	4.0 ± 0.8
150	40	1.2 ± 0.27	2.48 ± 0.29

Table – 3: Influence of kanamycin added to MS medium on regeneration of plantlets from axillary bud explants of *Mentha piperita.* Observations: after 4 weeks; Values are mean ± SE of 20 independent determinations.

Kanamycin (µM/L)	Frequency of regeneration (%)	Number of shoots/explant	Mean shoot length (cm)
10	60	1.5 ± 0.29	4.2 ± 0.1
20	65	1.95 ± 0.35	4.9 ± 0.3
30	75	2.0 ± 0.41	5.0 ± 2.2
40	80	2.2 ± 0.37	5.3 ± 0.6
50	90	2.8 ± 0.39	6.8 ± 0.9
80	95	2.85 ± 0.40	7.5 ± 1.5
100	65	1.25 ± 0.15	2.6 ± 0.1
150	-	-	-

Table – 4: Effect of different concentrations of silver thiosulphate supplemented in MS medium, on regeneration of plantlets from axillary bud explants of *Mentha piperita*. Observations: after 4 weeks; Values are mean ± SE of 20 independent determinations

STS (µM/L)	Frequency of regeneration (%)	Number of shoots/explant	Mean shoot length (cm)
-	-	-	-
10	65	1.6 ± 0.28	2.01 ± 0.12
20	70	1.9 ± 0.32	2.2 ± 0.18
30	80	2.9 ± 0.33	3.62 ± 0.06
40	85	3.25 ± 0.35	4.45 ± 0.36
50	90	4.75 ± 0.39	3.36 ± 0.55
80	70	1.4 ± 0.23	5.4 ± 0.20
100	55	1.2 ± 0.30	2.96 ± 0.0
150	40	1.0 ± 0.10	2.81 ± 0.21

In vitro rooting

The new shoots formed were excised and transferred onto half strength MS media with IAA, IBA and NAA of varied concentrations (Table 5 and Fig. 1). IBA (1.5 mg/l) produced the highest number of roots (30.4±1.26) with root length 5.0±0.37 cm; whereas the least number of roots (5.4±0.37) were seen in IAA (0.5 mg/l). NAA (1.5 mg/l) also gave the second highest number of roots (22.6±1.38) with root length 6.2±0.36 cm along with 100% regeneration frequency; whereas the highest root length 7.4±0.28 cm was observed with IBA (1.0 mg/l). Prolific rooting of *in vitro* grown micro shoots is critical for the successful establishment of these shoots in the greenhouse and field.

Table – 5: Effect of different concentrations of NAA, IAA and IBA on root formation from *in vitro* excised shoots of *Mentha piperita* explants. Results are mean±SE of 20 replicates.

Plant growth regulators (mg/l)	Frequency (%)	No. of roots/explant	Root length (cm)
IAA			
0.5	70	5.4 ±0.37	3.0 ± 0.14
1.0	85	6.0 ± 0.40	5.2 ± 0.21
1.5	95	10.5 ± 0.28	3.6 ± 0.38
2.0	80	4.6 ± 0.12	4.1 ±0.31
NAA			
0.5	80	7.2 ± 0.25	2.7 ± 0.18
1.0	90	15.2 ± 0.17	4.3 ± 0.29
1.5	100	22.6 ± 1.38	6.2 ± 0.36
2.0	85	10.4 ± 1.29	4.5 ± 0.27
IBA			
0.5	85	10.02 ±0.62	3.8 ± 0.12
1.0	90	21.0 ± 0.40	7.4 ± 0.28
1.5	95	30.4 ± 1.26	5.0 ± 0.37
2.0	75	12.0 ± 0.23	2.4 ± 0.15

Acclimatization and field transfer

Plantlets with well developed roots were successfully acclimatized and eventually established in green house. Acclimatization of these *in vitro* plants was maintained with high humidity which gave the better survival percentage (95%). Among the various hardening media used for acclimatization of rooted plants, a mixture of sterile soil and vermiculate (1:1) supported maximum percentage of survival. Gradual acclimatization was done with decreasing humid conditions and transition to the field condition.

Discussion

Bavistin appeared to have much stronger cytokinins-like activities. This is evident from a protomotory effect of bavistin on shoot bud regeneration. The shoot regeneration promoting activities of bavistin are results of increased biosynthesis of endogenous cytokinins within the cultures [25]. The explanation that bavistin induces shoot regeneration in *Mentha piperita* cultures is possibly due to its cytokinins-like activity. Similar results were also reported in *Bacopa monniera* cultures. One study has shown that bavistin induces differentiation of roots and shoots in calli derived from carrot segment [24]. Bavistin was found to be least toxic to plant cells and has a broad-spectrum fungicidal activity [23]. It has also been demonstrated that these compounds can have beneficial effects on the physiology of the plant [8]. Bavistin is a systemic fungicide that belongs to benzimidazole family. Benzimidazoles are a group of organic fungicides with systemic action that are extensively used in agriculture [6]. It has been reported that the molecular structure of methyl benzimidazole carbamate or carbendazim (bavistin) has some resemblance to kinetin, adenine and to many other cytokinins based on adenine [24]. From the present study it has been known that the usage of bavistin to control the fungal contamination does not show any negative effect on *Mentha piperita* cultures and further promotes the shoot regeneration.

Cefotaxime alone stimulated the shoot organogenesis in the axillary bud explants of *Mentha piperita,* whereas in case of apricot cefotaxime alone did not induce the shoot organogenesis but in combination with the other antibiotics such as timentin or vancomycin, stimulated the shoot organogenesis [2]. The chemical structure of this antibiotic does not readily suggest a breakdown product with auxin-like properties and a different mode of action may have to be sought [12].

With kanamycin above 40 µM/L concentration, the regeneration frequencies gradually decreased with increase in concentrations up to 150 µM/L. Higher concentrations (above 150 µM/L) tested completely inhibited regeneration. Similar results were also found with quite tolerant species such as pear [3], olive [16] or citrus [7], that required 171.6 µM/L to inhibit regeneration. Kanamycin sensitivity seems to be very dependent on the explant and species, and a wide range of organogenesis inhibiting concentrations can be found in the literature. Very sensitive species such as almond [17], grape [4], apple [19] or apricot [2], where 8.6 – 17.1, 12, 8.6 and 8.6 µM/L, respectively, inhibited regeneration. Further the use of kanamycin along with the nutrient media is also useful to inhibit the growth of a wide range of unwanted bacteria in tissue culture vessels.

Studies conducted in other plant systems have also shown that antibiotics [5] and fungicides [23] promote shoot regeneration. The possible mechanism of stimulatory effect of antibiotics on regeneration may involve generation of stress

that makes cells competent for regeneration; alternatively, the antibiotics may mimic plant growth regulators [12, 21]. Earlier reports demonstrated that certain antibiotics like chloramphenicol exhibited stimulatory effect on shoot organogenesis in callus cultures of *Hyoscyamus muticus* [15]. The tripeptide antibiotic bialaphos which is produced by *Streptomyces hygroscopicus* has been reported to induce shoot regeneration from hairy roots of *Antirrhinum majus* transformed by *Agarobacterium rhizogenes* [13]. In addition, the role of antibiotics on somatic embryogenesis is also well documented [22]. Previous studies proved that vancomycin was not toxic and significantly enhanced regeneration and shoot development on *Pinus pinea* cotyledons [14].

Ethylene inhibits the shoot morphogenesis and also affects the root formation. Ag+ ions inhibit ethylene action in a wide variety of ethylene induced responses in plants by reducing the receptor capacity to bind ethylene [1, 26]. These findings are in agreement with the reports in *Decalepis hamiltonii,* where silver nitrate favoured the shoot morphogenesis [10]. The addition of STS to the regeneration media increased regeneration in both cultivars, Canino and Helena [2]. Thus, silver thiosulphate may be useful as a media supplement to develop efficient protocols for *in vitro* propagation of *Mentha piperita* as it favours the shoot formation.

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