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Micro-propagation and biochemical analysis of Spear Mint (*Mentha spicata*)

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Abstract

The investigation on "Micropropagation and biochemical analysis of Spear Mint (*Mentha spicata*)" was carried out during 2008-09 in the department of Agricultural Biotechnology, OUAT, and Bhubaneswar. The objectives were to standardize the protocols for the surface sterilization of explants and micro propagation and biochemical analysis in spear mint (*M. spicata*). The best surface sterilants for the mint leaf, nodes and inter nodal explants was the treatment, comprising of washing with double distilled water, deeping in the solution of 0.1% bavistin and 0.1% tetracycline for 20 minutes and then in 70% alcohol (0.5 minutes) followed by 0.1 % Mercuric Chloride (5 minutes). In the concentration of 2.5 mg/l 2, 4-D, the weight of callus (0.199g) and callus induction per cent (92.0%) was high, whereas days to callus induction (12) was low in spear mint. The concentration of 2.5mg/l BAP produced maximum number of shoots from embryogenic callus (10), maximum numbers of leaves (38) and less number of days required for shoot regeneration (62). The concentration of 4.0mg/l NAA along with normal MS medium produced maximum number of roots (54.0), whereas, 4.0mg/l IAA produced 53.2 roots which is less than that of NAA. But the combination of 2.5 mg/l NAA and 2.5 mg/l IAA produced 56 roots. The quantity of solvent extracted was 226.2ml. The range of days taken for evaporation of extract was from 20 days to 21 days. The weight of methanol extracted per four gram of sample was 0.0822g. Per cent of oil extracted in spear mint was 2.1%. Tissue culture in spear mint is a viable novel method for rapid rate of multiplication and production of true to the type and disease free planting material within a shorter period of time.

***Key words*:** Mentha spicata; Callus; Shoot regeneration; Root initiation; In-vitro multiplication.

Abbreviation: RBD - Randomized block design.

Introduction

Mentha spicata (Spear mint or Spearmint) is a species of mint native to Europe and South East Asia. It grows in wet soils. It is an invasive species in the Great Lakes region where it was first sighted in 1843. It is a herbaceous rhizomatous perennial plant growing 30–100 cm tall, with variably hairless to hairy stems and foliage and a wide-spreading fleshy underground rhizome. The leaves are 5–9 cm long and 1.5–3 cm broad, with a serrated margin. Spearmint produces flowers in slender spikes, each flower pink or white, 2.5–3.0 mm long and broad.

It is an industrial crop which is widely cultivated for its essential oil from which menthol is crystallized. The essential oil, menthol and terpenes of the dementholated oil, are variously used in the food, perfumery and pharmaceutical industries. Improvement in the pest and disease tolerance and other adaptive characters determining the yield and quality of essential oil, will make spear mint cultivation more economical. Since *Mentha spicata* is largely cultivated by the use of vegetatively produced root suckers as planting materials, successive use of the same material carries with it any associated bacterial, fungal and/or viral infections to the next crop. Rapid and convenient regeneration and micro propagation procedures are required for cleaning of planting material free of infection and modification of monoterpene pathway towards more economic production of essential oil of better quality. So, the

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present study was designed for standardization of efficient protocols for explant sterilization, callus induction regeneration and proliferation of shoots and roots from the explants of *Mentha spicata* (Spear mint).

Methods

The present investigation was carried out in the department of Agricultural Biotechnology, College of Agriculture, OUAT, and Bhubaneswar during the session 2008-09 with a motto to find out a suitable method for producing quality planting material in spear mint in commercial scale. The Planting material of *M. spicata* (Spear mint) were collected from Krishi Vignyan Kendra, Semiliguda, Koraput, Orissa and were raised in the polyhouse with suitable package and practices . The explants like nodes, internodes and leaves of Spear mint were surface sterilized taking six treatments (T_1= Washing with tape water twice, T_2= Bavistin 0.1% (20min), T_3= Tetracyclin 0.1% (20min), T_4 = Bavistin 0.1% + Tetracyclin0.1% (20minute), T_5= T_4+70% alcohol (30sec), T_6= T_5+ 0.1% $HgCl_2$ (4 minutes)) to find out suitable sterilants. Excised leaf, nodal and inter nodal segments were cultured on Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with different concentrations of growth regulators. The cultured explants on MS medium supplemented with different growth regulators at various concentration (2-4D-0.5,1.0,1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg/l ; 2-4D-2.0mg/l & BAP0.5mg/l and 2-4D-2.0mg/l & BAP1.0mg/l) in the dark produced callus, which was subsequently proliferate at a faster rate on plane MS medium. Embryogenic callus was subsequently transferred to shoot regeneration medium *i.e* MS medium with different concentration of BAP (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 mg/l) for shoot initiation in spear mint. Healthy *in vitro* regenerated mint plants of about 3-5 cm height were placed in a root induction media. Different concentrations of phyto hormones like NAA (2.0, 3.0 and 4.0mg/l), IAA (2.0, 3.0 and 4.0 mg/l) and NAA (2.5mg/l) + IAA (2.5 mg/l) were applied along with normal MS medium for the root initiation. *In vitro* cultured plants having well developed shoot and root systems were plugged off one day before pre-hardening inside the culture room with controlled temperature, light and humidity conditions. Then they were taken from the culture room and kept in sterile distilled water for 2-3 days. Soil mixture was prepared with a view to harden regenerated plants by taking soil: sand: FYM= 1:1:1 ratio. Then sterilization of soil was done by saturating the soil mixture kept in the gunny / polythene bag at dark with 0.4% formaldehyde solution for 2-3 days. Then the sterilized soil was dried in the sun and was used for planting the pre- hardened plants in the green house under controlled temperature and humidity. The survival per cent of the plants was recorded.

Biochemical analysis was done by taking four gram of air dried samples from each regenerated mint plants and was ground in mortar and pestle. Powdered plant sample was dipped in the 250ml 95% ethyl alcohol kept in the flasks of Soxhelet Apparatus. Then, the apparatus was kept in hot water bath at 78.2˚C. After 9 hour, the first cycle was completed and then after 5 hours, the second cycle was completed. The apparatus was taken away from the water bath for cooling. The plant extract along with alcohol was poured into a beaker

Table 1. Effect of different surface sterilants on the level of contamination and survival of spear mint

Treatments	Fungal infection	Bacterial infection	Death (%)	Aseptic culture (%)	Survival
T_1 (washing in double distilled water)	100	100	100	0	0
T_2 (Bavistin) O.1%)	0	100	87	13	13
T_3 (Tetracyclin O.1%)	100%	0	86%	14%	14%
T_4 (Bavistin 0.1%+Tetracyclin 0.1%)	0	0	62%	38%	38%
T_5 (T_4 + 70% Alcohol for 30 sec)	0	0	47%	53%	53%
T_6 (T_5 +0.1% $Hgcl_2$ for 5 min)	0	0	0	100%	100%
SE (m) ±	0.02	0.03	0.05	0.68	0.07
CD (P=0.05)	0.1	0.12	0.18	1.44	0.28

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Table 2. Effect of growth regulators on callus induction in spear mint

Treatments	Concentration of growth regulators (mg/l)	Days to Callus induction	Callus induction (%)	Fresh weight (g)
T_1	2-4D(0.5mg/l)	19.0	55.9	0.020
T_2	2-4D (1.0mg/l)	18.0	60.0	0.037
T_3	2-4D (1.5mg/l)	15.0	64.5	0.089
T_4	2-4D (2.0mg/l)	14.0	76.9	0.092
T_5	2-4D (2.5mg/l)	12.0	92.0	0.199
T_6	2-4D (3.0mg/l)	15.0	83.4	0.085
T_7	2-4D (3.5mg/l)	16.0	76.4	0.055
T_8	2-4D(4.0mg/l)	26.0(rudimentary)	29.9	0.006
T_9	2-4D (2.0mg/l)+BAP (0.5mg/l)	18.0	72.2	0.084
T_{10}	2-4D (2.0mg/l) +BAP (1.0mg/l)	19.0	84.6	0.014
SE (m) ±	-	0.05	0.11	0.06
CD (P=0.05)	-	0.16	0.48	0.18

and Fig.1. The explants simply washed with double distilled water (T_1) were completely dead due to fungal and bacterial infection when cultured in MS media. The contamination percent was reduced when the explants were treated with different surface sterilants. The statistically analyzed data revealed that survival percentage of explants after

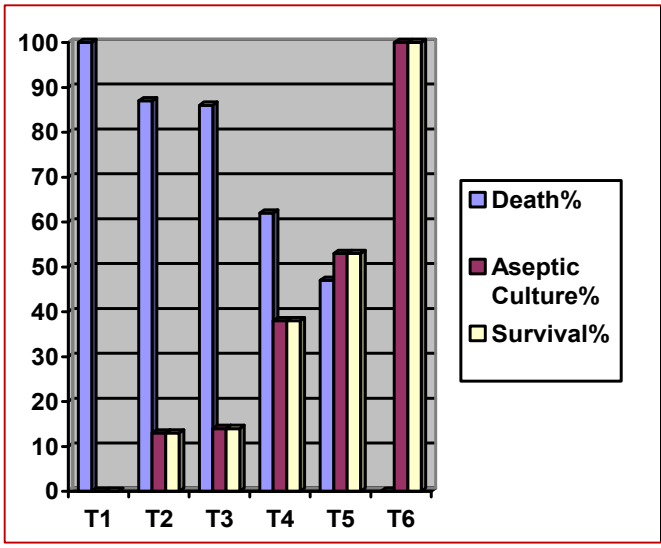

Fig.1. Effect of different surface sterilants on the level of contamination and survival of spear mint

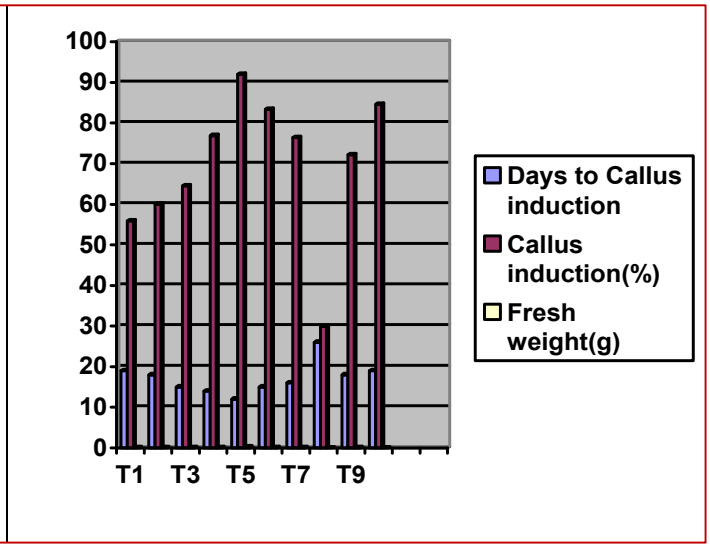

Fig.2. Effect of growth regulators on callus inductionin spear mint

and volume was measured. The extract was kept for few days until the evaporation of alcohol. Finally the weight of oil was taken. The observations for various characters were picked out by taking 10 culture tubes at a time. The data recorded from the experiment were analyzed following the method of Singh and Chowdhury (1985) considering each culture tubes as a replication in each experiment by Randomized block design (RBD).

Results and Discussion

The result of the experiments on surface sterilization of explants of spear mint, using different surface sterilants, is elucidated in Table 1 treatment with several sterilants for different duration was highest (100%) in case of T_6 comprising washing with double distilled water, treatment with 0.1% bavistin and 0.1% tetracycline for 20 minutes and then 70% alcohol for 0.5 minutes followed by 0.1 % Mercuric Chloride for 5 minutes. Total aseptic culture was maintained at this treatment. These findings are in agreement with Wang *et al.* (2007).

Sterilized leaves, inter nodes and nodal explants of *M. Spicata* (spear mint) were inoculated in the MS medium supplemented with various concentrations of 2, 4-D and other growth regulators for induction of callus. The effect of

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different hormones on callus induction is presented in Table 2 and Fig.2. Callus induction and profuse proliferation was obtained in case of the MS medium supplemented with 2,4-D @2.5 mg/l. It has been generally observed that the callus initiation and outgrowth requires auxin elimination is often necessary to obtain morphogenesis and production of organ. In the concentration of 2.5 mg/l 2,4-D , the weight of callus(0.199g) and callus induction per cent (92.0%) was high, whereas days to callus induction (12 days) was low. The range of weight of proliferated callus was 0.006g to 0.199g in spear mint. Induced callus percent was ranged from 29.9 to 92.0% and the days to callus induction was varied from 12 to 26. These findings are in agreement with Jullien *et al.* (1998) and Chaput *et al.* (1996).

Table 3. Effect of BAP on shoot proliferation in spear mint

Treatments	Concentration of BAP(mg/l)	Days to shoot proliferation	No of Shoots	No of leaves
T1	0.5	93.0	6.0	26.0
T2	1.0	90.2	6.2	26.4
T3	1.5	82.4	8.0	32.6
T4	2.0	74.2	8.0	30.4
T5	2.5	62.0	10.0	38.0
T6	3.0	72.4	6.2	26.0
SE (m) ±	-	0.04	0.08	0.21
CD (P=0.05)	-	0.11	0.43	0.69

Table 4. Effect of Phyto hormones on root initiation in spear mint

Treatments	Concentration	Days to root initiation	No of roots	Length of root (cm)
T_1	NAA(2.0mg/l)	26.0	51.8	5.1
T_2	NAA(3.0mg/l)	24.0	52.9	5.8
T_3	NAA(4.0mg/l)	22.6	54.0	6.0
T_4	NAA(5.0mg/l)	25.8	51.6	5.0
T_5	IAA (2.0mg/l)	28.0	48.0	5.2
T_6	IAA (3.0mg/l)	27.0	50.0	5.4
T_7	IAA (4.0mg/l)	24.8	53.2	6.2
T_8	IAA (5.0mg/l)	26.0	51.0	5.2
T_9	2.5mg/l NAA +2.5mg/l IAA	24.0	56.0	5.8
SE (m) ±	-	0.01	0.08	0.21
CD (P=0.05)	-	0.01	0.22	0.62

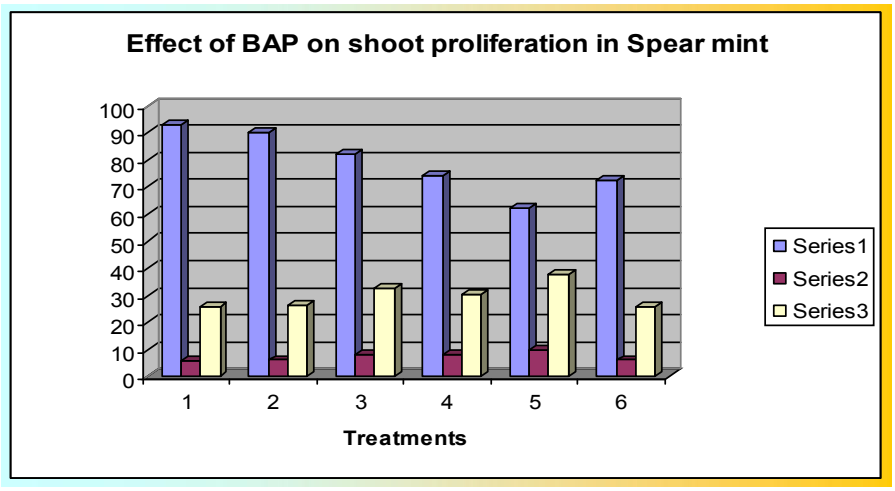

Fig.3. Effect of BAP on shoot proliferation in spear mint

Embryogenic callus was subsequently transferred to shoot regeneration medium. Efficient regeneration of shoot buds and their conversion into shoots was recorded on MS medium supplemented with 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l BAP. Effect of BAP on proliferation of shoots is presented in Table 3 and Fig.3. The concentration of 2.5mg/l BAP along with normal MS medium produced significantly maximum number of shoots per explants (10), maximum numbers of leaves (38) and less number of days required for shoot regeneration (62). The number of regenerated shoots per explant was ranged from 6-10. The range of regenerated leaves was 26-38. The number of days required for shoot regeneration was ranged from 62-93. A further increase in concentration of BAP showed some adverse effect and subsequently reduced the number of shoot development from callus. The above findings are in agreement with Xiao *et al.* (2007), Wang *et al.* (2006), Shawl *et al.* (2006) and Godoy *et al.* (2005).

Effect of IAA and NAA in rooting of elongated shoot derived from callus and nodal explant in spear mint is presented in Table 4. The concentration of 4.0mg/l NAA along with normal MS medium produced maximum number of roots (54.0) of 6cm in 22.6days, whereas, 4.0mg/l IAA produced 53.2 roots of 6.2cm in 24.8days which is less than that of NAA. But the combination of 2.5 mg/l NAA and 2.5 mg/l IAA produced 56

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roots of 5.8cm in 24days. The number of roots of regenerated shoots was ranged from 48.0-56.0. The range of root length was 5.0-6.2cm. The number of days required for root initiation was ranged from 22.6 – 28.0. A further increase in concentration in NAA or IAA showed some adverse effect and subsequently reduced the number of initiated roots and root length and more days required for root initiation in explants. The above findings are in agreement with Xiao *et al*. (2007), Shawl *et al.* (2006) and Godoy *et al.* (2005).

Table 5. Extraction of methanol from regenerated spear mint plants

Plant no	Test Sample (gm)	Quantity of Solvent extracted (ml)	Time taken for evaporation of extract (days)	Weight of solvent extracted (gm)	Per cent of solvent extracted
1	4	226	20	0.092	2.3
2	4	230	20	0.108	2.7
3	4	225	21	0.104	2.6
4	4	220	21	0.097	2.4
5	4	230	20	0.067	1.7
Mean	4	226.2	20.4	0.0822	2.1

Extraction of methanol from the regenerated plants of is presented in the Table 5. The weight of the dried sample taken was 4g and extraction solvent ethyl alcohol used for each sample was 250ml. The quantity of solvent extracted was 226.2ml. The range of days taken for evaporation of extract was from 20 days to 21 days. The weight of methanol extracted per four gram of sample was 0.0822g. Per cent of oil extracted was 2.1%. These findings are similar with Alvi *et al.* (2004).

Conclusion

Micropropagation and biochemical analysis of Spear Mint (*Mentha spicata*) was carried out and investigated during 2008-09 in the department of Agricultural Biotechnology, OUAT, Bhubaneswar. The protocols for the surface sterilization of explants and micro propagation and biochemical analysis in spear mint (*M. spicata*) were standardized.

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