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High Frequency Rapid Plant Regeneration from Shoot Tip and Nodal Explants of *Mentha piperita* (L.) – An Important Multipurpose Medicinal Plant

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Summary

Mentha piperita L. (Peppermint) is a perennial glabrous and strongly scented herb belonging to the family lamiaceae. The plant is aromatic, stimulant, and used for allaying nausea, headache and vomiting. Peppermint raw material is used in medicine, cosmetics and food industry, therefore this plant is widely grown around the world. Peppermint produces a large amount of essential oils and has a good aroma, thus it is more widely grown, especially for industrial processing. An *in vitro* regeneration system with a maximum efficiency rate was developed in *Mentha piperita* using shoot tip and nodal segments of three week old stock plants on Murashige and Skoog's (MS) medium supplemented with combination of 6- benzyl amino purine and naphthalene acetic acid (BAP - 1.5mg/L and NAA - 0.1 mg/L). Shoots developed at sites of excision directly from the cells. The highest numbers of shoots (53) were obtained on medium containing BAP and NAA. *In vitro* shoots were then excised from the shoot clumps and transferred to rooting medium containing indole butyric acid (IBA - 1.5 mg/L). The rooted plantlets were hardened in polythene cups containing sterile soil and vermiculite (1:1). Plantlets thus developed were successfully established and finally transferred to a greenhouse. The present regeneration system is highly reproducible and the regenerated plants developed normally and were phenotypically similar to the parent plant.

Key Words: *Mentha piperita*, Nodal and shoot tip explants, Plant growth regulators
Abbreviations: BAP – 6-benzyl amino purine; NAA – α- naphthalene acetic acid; IAA – indole-3- acetic acid; IBA – indole butyric acid

Introduction

Mentha is a genus of aromatic perennial herbs belonging to the lamiaceae family. It is distributed mostly in the temperate and sub-temperate regions of the world. Several species of the genus are considered as industrial crops because they are the sources of essential oils enriched in certain monoterpenes, widely used in food, flavor, cosmetic and pharmaceutical industries. *Mentha* has a large number of species that differ widely in their characteristics and polyploidy level [22]. Most of the commercially important mints are hybrids or amphiploids. *Mentha piperita* (peppermint) is a sterile first generation hybrid between *M. spicata* and *M. aquatica* [29]. Mints are extensively cultivated for their oils and terpenoid components of the oil such as menthol, carvone, linalyl acetate and linalool, for use in pharmaceutical, cosmetic, food, flavor, beverage and allied industries [16].

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 [7]. 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 [17]. 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 [8]. This technique provides a rapid reliable system for production of large number of genetically uniform and even disease free plantlets. The perspective objective of the present study is to develop a rapid system for regenerating *in vitro* clonal tissue proliferation and finally the process of proliferation has proved to be the most reliable method for large-scale biomass production of this medicinal plant. Earlier studies showed that many factors are crucial in successful responsiveness of plant tissues to plant regeneration, including the culture system components and explants tissue selected [30]. Since studies on *in vitro* multiplication on this species 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 [15] 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. Nodal and shoot tip 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 and shoot tip 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 $HgCl_2$. Explants were cultured individually on MS medium containing different concentrations (0.5 - 2.0 mg/l) of BAP and NAA to induce multiple shoots. 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

Multiple shoot induction from nodal explants

Nodal explants from field grown plants of *Mentha piperita* were inoculated on MS medium supplemented with BAP and NAA at different concentrations (0.5, 1.0, 1.5 and 2.0 mg/l) for production of multiple shoots. Emergence of multiple shoot buds from shoot tip and nodal explants on MS media supplemented with BAP and NAA was observed without an intervening callus phase. Shoot buds emerged on 10^{th} and 14^{th} day of culture from nodal and shoot tip explants respectively. However, shoots started proliferating after 15-20 days. The results obtained showed higher frequency and high number of shoots in most of the combinations tested, although the frequency, number and length of multiple shoots varied with different concentrations and combinations of plant growth regulators (Table 1 and Fig. 1). Maximum shooting frequency in the nodal explants (100%) and highest number of shoots (53.3±1.60) was observed in BAP (1.5 mg/l) in combination with NAA (0.1 mg/l). The maximum shoot length (4.8±0.40) was observed in BAP (0.5 mg/l).

Table - 1: Effect of different concentrations of BAP and NAA singly and in combination on multiple shoot regeneration from shoot tip and nodal explants. Results are mean ± SE of 20 replicates.

Plant growth regulators (mg/l)	Shoot tip explants			Nodal explants		
	Frequency (%)	No. of shoots/ explant	Length of shoot(cm)	Frequency (%)	No. of shoots/explant	Length of shoot (cm)
BAP						
0.5	80	4.9 ± 0.22	3.1 ± 0.37	85	5.2 ± 0.41	4.8 ± 0.40
1.0	90	5.8 ± 0.40	2.2 ± 0.31	90	17.7 ± 0.53	3.7 ± 0.06
1.5	95	28 ± 0.92	2.5 ± 0.19	98	32.30 ± 1.08	1.7 ± 0.27
2.0	85	17.3 ± 1.66	4.6 ± 0.40	80	12.3 ± 1.66	2.1 ± 0.32
NAA						
0.5	65	3.8 ± 0.21	2.6 ± 0.19	75	13.6 ± 0.34	4.2 ± 1.00
1.0	70	4.3 ± 0.35	3.3 ± 0.35	80	17.9 ± 0.47	3.0 ± 0.22
1.5	92	25.8 ± 1.04	2.5 ± 0.31	95	38.8 ± 1.35	2.2 ± 0.33
2.0	90	22.1 ± 1.35	3.5 ± 0.24	90	26.1 ± 0.74	3.22 ±0.04
BAP+NAA						
0.5+0.1	75	14.3 ± 1.30	4.5 ± 0.22	90	15.9 ± 1.48	4.2 ± 0.32
1.0+0.1	85	38.9 ± 2.40	3.5 ± 0.63	95	30.2 ± 2.40	3.2 ± 0.24
1.5+0.1	95	48.5 ± 1.05	2.3 ± 0.22	100	53.3 ± 1.60	1.2 ± 0.27
2.0+0.1	90	27.6 ± 2.35	2.9 ± 0.08	80	21.0 ± 0.64	2.3 ± 0.30

Multiple shoot induction from shoot tip explants

The data obtained for shoot tip explants revealed that the maximum regeneration frequency (95%) and highest shoot number (48.5±1.05) was observed in BAP (1.5 mg/l) in combination with NAA (0.1mg/l). Maximum shoot length (4.6±0.40) was seen in BAP (2.0mg/l), followed by (BAP 0.5 mg/l + NAA 0.1 mg/l) with shoot length (4.5±0.22) (Table 1 and Fig. 1).

Root initiation and elongation

Of all the strengths of media tried, exogenous supply of auxins favoured the root formation. Addition of auxins, IAA, IBA and NAA to MS medium enhanced the rate of rhizogenesis in both frequency as well as number of roots. In all the concentrations, root primordial appeared between 7 -10 days of inoculation. According to our data half strength MS salt fortified with NAA (1.5 mg/l) showed highest frequency (100%) and the maximum number of roots (42.4 ± 1.45) was recorded with IBA (1.5 mg/l). Of the three auxins tested 1.5 mg/l IBA

proved slightly superior to NAA and IAA in terms of root induction and produced the highest number of roots per shoot (Table 2 and Fig. 1). The least number of roots (4.0 ± 0.23) were seen in IBA (2.0 mg/l) followed by IAA (2.0 mg/l).

Table - 2: 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	85	6.2 ±0.47	3.7 ± 0.37
1.0	95	9.5 ± 0.60	4.8 ± 0.18
1.5	98	14.5 ± 0.20	5.1 ± 0.28
2.0	80	5.9 ± 0.33	3.0 ±0.31
NAA			
0.5	90	18.6 ± 0.65	3.2 ± 0.04
1.0	96	21.2 ± 1.17	4.8 ± 0.10
1.5	100	33.6 ± 1.63	6.9 ± 0.35
2.0	84	15.4 ± 1.98	3.9 ± 0.25
IBA			
0.5	85	9.04 ± 0.94	4.0 ± 0.20
1.0	90	16.0 ± 0.39	5.1 ± 0.02
1.5	94	42.4 ± 1.45	6.3 ± 0.27
2.0	70	4.0 ± 0.23	2.3 ± 0.11

Figure - 1

A &B. Initation of multiple shoots from shoot tip and nodal explants on MS medium after 4 days of culture (MS + BAP 1.0 mg/l). (bar 1cm = 0.63 cm). C. Proliferation of multiple shoots from shoot tip explants on MS medium after 20 days culture (BAP1.5 mg/l) and (NAA 0.1 mg/l). (bar 1cm = 0.63 cm). D. Proliferation of multiple shoots from nodal explants on MS medium after 20 days of culture (BAP1.5 mg/l) and NAA (0.1 mg/l). (bar 1 cm = 0.6 cm).

E&F. Direct rooting from regenerated shoots on MS medium after 40 days of culture (IBA 1.5 mg/l). (bar 1 cm = 0.7 cm) (bar 1 cm = 0.8 cm). G&H. Hardened plants in polycups and pots containing sterile soil and vermiculate.

Hardening and field transfer

Rooted plantlets were hardened in MS basal liquid medium and subsequently transferred to polycups containing sterile soil and vermiculate (1:1). These plantlets were acclimatized well and transferred to green house and planted in the soil with 98% survivability.

Discussion

Different types and concentrations of hormones were tried to study their effect on *in vitro* growth of *Mentha piperita.* In accordance to our study, the earlier findings also reported that addition of BAP alone to MS medium is responsible for shoot induction and multiplication of *in vitro* cultures of *Feronia limonia* [9]. Similar findings were also reported in *Acmella calva*, where multiple shoots induced from nodal explants

using BAP alone supplemented MS medium [24]. BAP at higher concentrations more than 2.0 mg/l showed the formation of vitrified shoots and later necrosis appeared. Further increase in the concentration of both BAP and NAA reduced the frequency and number of shoots, indicating an upper limit in concentration of BAP. These observations further support the well known inhibitory influence of higher concentrations of cytokinins on shoot elongation and consequent rosette type of shoot formation in *Mellissa officinalis* [26] and *Hedeoma multifolium* [12].

The increase in frequency and number of shoot formation was observed when BAP was used in combination with NAA. The combination of cytokinins and auxins was reported to stimulate *in vitro* multiplication and growth of shoots in several plant species. Thick and highest number of shoots (53.3±1.60) was regenerated after sub culturing to the MS media containing BAP (1.5 mg/l) along with NAA (0.1 mg/l). The obtained results are in line with the earlier reports, where the addition of NAA in the culture medium improved the response in shoot induction from nodal segments of various other plants, such as *Vitex negundo* [10], *Syzygium travancoricum* [2], *Ancistrocladus abbreviates* [4] and *Coleus blumeni* [20]. The response of axillary bud and shoot tip explants in the presence of various concentrations of plant growth regulators are represented in figure 1. Similar to our results, multiple shoot formation was reported in other medicinal plants such as *Bacopa monnieri* [25], *Phyllanthus caroliniensis* [5] and *Celastrus paniculatus* [13]. Recently, Poovaiah (2006 b) reported that internodal explants exhibited better regeneration capacity than leaf explants in two *Mentha* species (native spearmint and scotch spearmint), suggesting a new approach for optimization of peppermint regeneration [18]. Nodal explants as the best source of multiple shoot induction have also been suggested in case of other medicinal plants such as *Rauwolfia serpentine* [21], *Emblica officinalis* [19], *Holarrhena antidysenterica* [1] and *Enicostemma hyssopifolium* [23].

Roots were not induced during the culture initiation, shoot formation and shoot multiplication in the cytokinin regime. Individual shoots when implanted in half or full strength MS medium free from growth regulators, poor and few numbers of roots were elicited with low frequency. Parallel to our results the earlier studies used half strength MS medium for root initiation in *Spilanthes acmella* [14]. The promotory effect of IBA in root formation with our data has shown comparable results in case of *Centella asiatica* [3 and 27] and *Acacia sinuata* [28], where as higher concentrations of IBA, IAA, and NAA did not show the root formation. Only NAA (2.0 mg/l) showed the maximum root formation at the higher concentration. Similar to our data, in *Tagetes erecta* [6], *Acmella calva* [24] and *Mentha piperita* [11] NAA alone showed the maximum root formation as the auxin concentration increased.

Conclusion

In conclusion, the above protocol describes rapid shoot regeneration from node and shoot tip explants, which can assure a stable supply of this medicinally important oil yielding plant irrespective of any seasonal variations and may serve as a better source for biological active compounds. Furthermore, regeneration system can be used for interspecific hybridization and genetic transformation studies for large-scale production of a robust genetically engineered peppermint plants which are in need of development.

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