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Impact of Different Carbohydrates on High Frequency Plant Regeneration from Axillary Buds of *Mentha piperita* (L.) – An Important Multipurpose Medicinal Plant

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Summary

Mints are a group of plants belonging to family lamiaceae, which yield essential oils on distillation. *Mentha piperita* is a native of the Mediterranean region and has naturalized in Europe, Asia, North America and Australia. The plant is a perennial, glabrous herb with strong pepper-like pungent odour and hence the specific name "*piperita*". Peppermint generally grows in shade and moist areas with a good water supply. The ideal way of planting this mint is in part-sun to shade areas, well drained soils with good water – holding capacity are better suited for mint cultivation as it avoids frequent irrigation during summer and problems in rainy seasons. Mint does not tolerate water stagnation. Its oil is one of the most popular widely used essential oil in food products, cosmetics, pharmaceuticals, dental preparations, mouthwashes, soaps and alcoholic liquors. The influence of different sugars (sucrose, maltose, glucose, and fructose) and plant growth regulator BAP 2.0 mg/l and MS media were investigated for the development of multiple shoots from axillary bud or nodal explants of *Mentha piperita.* Among the different sugars tested, 4mg/ml fructose was the best for plant regeneration. Maximum number of shoots (27.6) was induced on the medium containing 4mg/ml fructose. Observations of the shoot cultures developed on media containing one of these carbohydrates indicated that fructose was the preferential carbon source for the proliferation of multiple shoots. *In vitro* shoots were then excised from the shoot clumps and transferred to rooting medium containing indole butyric acid (IBA - 1.5 mg/L). These shoots when removed from the culture tubes and transferred into sterile soil in green house, most of the plantlets survived (98%) and they were suitable for field planting after 1 month.

Key Words: *Mentha piperita*, Nodal explants, Carbohydrates, Plant regeneration
Abbreviations: NAA – α- naphthalene acetic acid; IAA – indole-3- acetic acid; IBA – indole butyric acid

Introduction

Several mints of *Mentha* species are industrial crops, a source of essential oils enriched in certain monoterpenes that are widely used in food, flavour, cosmetic and pharmaceutical industries. The genus *Mentha* has a large number of species that differ widely in their characteristics and ploidy level. *Mentha* is a genus of aromatic perennial herbs belonging to the family lamiaceae, distributed mostly in temperate and sub-temperate regions of the world. *Mentha piperita*, the peppermint, is a sterile first generation hybrid between *Mentha spicata* and *Mentha aquatica* [5]. Mints have been used and valued as aromatic herbs for thousands of years [10]. It is an herbaceous rhizomatous perennial plant growing to 30–90 cm tall, with smooth stems, square in cross section. The rhizomes are wide-spreading, fleshy, and bare fibrous roots. The leaves are from 4–9 cm long and 1.5–4 cm broad, dark green with reddish veins, and with an acute apex and coarsely toothed margins. The leaves and stems are usually slightly hairy. The flowers are purple, 6–8 mm long, with a four-lobed corolla about 5 mm diameter; they are produced in whorls (verticillasters) around the stem, forming thick, blunt spikes. Flowering is from June to October. Peppermint has high menthol content, and is often used as flavouring in tea, ice cream, confectionery, chewing gum, and toothpaste. The oil also contains menthone and menthyl esters, particularly menthyl acetate. It is the oldest and most popular flavour of mint-flavoured confectionery. Peppermint can also be found in some shampoos and soaps, which give the hair a minty scent and produce a cooling sensation on the skin.

Materials and Methods

Culture medium

During present investigation only MS medium [11] 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 or axillary buds 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 $HgCl_2$. Explants were cultured individually on MS medium containing different carbohydrates (sucrose, maltose, glucose, and fructose) with concentrations (1 – 6%) and BAP (2mg/l) to induce multiple shoots. 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. The 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 on different carbohydrates

The experiments were performed to determine the effect of different carbon sources at different concentrations on shoot multiplication from axillary bud explants of *Mentha piperita*. It was observed from the results that, among the different carbohydrate sources used, fructose performed well followed by sucrose, maltose and glucose in keeping shoot number constant. The maximum shoot number (27.6 ± 0.79) was recorded at MS medium supplemented with 4% of fructose with a maximum mean shoot length of (6.8 ± 0.10). Highest frequency of shoot regeneration was observed both at 4% of fructose (95%) and 4% 0f sucrose (98%) (Table 1 and Fig. 1).

Least mean number of shoots (1.5 ± 0.30) was observed in MS medium supplemented with glucose 6% followed by maltose 6% with a mean shoot number (3.0 ± 0.22), with the mean shoot lengths being (1.22 ± 0.25) and (1.21 ± 0.15) respectively. In all the concentrations tested and observed the second highest mean number of shoot was noticed at 4% of sucrose (15.7 ± 1.05) with a mean shoot length (5.3 ± 0.50).

Rooting

The new shoots formed were excised and transferred onto half strength MS media with IAA, IBA and NAA of varied concentrations (Table 2 and Fig. 1). IBA (1.5 mg/l) produced the highest number of roots (35.4±1.36) with root length 6.0±0.17 cm; whereas the least number of roots (4.4±0.57) were seen in IAA (0.5 mg/l). NAA (1.5 mg/l) also gave the second highest number of roots (25.6±1.33) with root length 5.9±0.30 cm along with 100% regeneration frequency; whereas the highest root length 7.1±0.09 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.

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 (98%). 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

Although sucrose has been the carbohydrate of choice in the vast majority of work on *in vitro* shoot induction and shoot development, it is not always the most effective carbon source for these purposed [18]. Sucrose has been proved to be better for shoot regeneration than the other carbon sources in micro propagation of cork oak [15]. Similar to our results two to six percent sucrose was found suitable for regeneration of green plantlets. These findings are consistent with the previous reports [14, 1, 2, 16 and 13].

Sucrose was followed by fructose with next highest frequency of shoot regeneration (95%) and the results obtained by us are in line with the results obtained in mulberry, where addition of fructose instead of sucrose in the multiplication medium increased the shoot number and also the growth of the shoots [19].

The least number and length of shoots were noticed with glucose followed by maltose. In contrast to our results, the beneficial effect of glucose on direct shoot formation was observed in *Prunus mume* [6]. Several reports indicate that the carbohydrate source can influence the degree and type of differentiation and thus the efficiency of plant regeneration [17, 3, 4, 12 and 8]. Although carbohydrates are of prime importance for *in vitro* organogenesis, carbon metabolism *in vitro* is still not clearly understood [9]. It is well established that carbohydrate requirements depend upon the stage of culture and may show differences according to the species [18].

Of all the three hormones IBA 1.5 mg/l was most effective for root induction and survival in the field of three auxin tested. As the concentration of IBA was increased the number of roots were decreased. These results are in line with *Aegle marmelos*, which showed that with increase in the concentration of IBA, root formation was inhibited [7]. Similarly when the concentration of NAA was raised the rooting rate was decreased. These results indicated that NAA at a higher concentration has obvious inhibitory impact on root production. The same result was reported in *Robinia pseudoacacia* [20].

Conclusion

It can be concluded that various carbon sources used in our experiment affected the growth of *Mentha piperita* plants. Our results offer better performance in terms of number of shoots/explant, shoot length, rooting percentage (100%), plant survival (98%) and rapid growth. However further research is required to explore the possible growth promoting factors in these carbon sources.

Table - 1: Effect of different carbohydrate sources on multiple shoot regeneration from nodal explants of *Mentha piperita.* Results are mean ± SE of 20 replicates

Carbohydrate source	Concentration (%)	Regeneration frequency (%)	Mean no. of shoots	Mean shoot length (cm)
Glucose	1	60	3.5 ± 0.30	1.97 ± 0.66
	2	65	4.15 ± 0.39	2.10 ± 1.28
	3	70	4.45 ± 0.41	2.15 ± 1.50
	4	75	4.95 ± 0.41	2.23 ± 0.18
	5	85	3.7 ± 0.40	2.68 ± 0.07
	6	65	1.5 ± 0.30	1.22 ± 0.25
Fructose	1	70	2.45 ± 0.40	2.12 ± 0.10
	2	75	6.15 ± 0.44	2.20 ± 0.80
	3	85	15.35 ± 0.57	2.85 ± 0.30
	4	95	27.6 ± 0.79	6.8 ± 0.10
	5	80	12.6 ± 0.64	3.67 ± 0.07
	6	65	10.5 ± 0.35	1.50 ± 0.22
Maltose	1	55	1.15 ± 0.25	1.00 ± 0.22
	2	60	3.25 ± 0.42	2.70 ± 0.29
	3	65	3.55 ± 0.46	3.30± 0.08
	4	75	4.65 ± 0.50	4.75 ± 0.15
	5	80	6.15 ± 0.73	5.45 ± 0.15
	6	60	3.0 ± 0.22	1.21 ± 0.15
Sucrose	1	65	2.3 ± 0.25	1.10 ± 0.25
	2	75	4.85 ± 0.41	2.40 ± 1.50
	3	90	11.65 ± 0.99	4.50 ± 0.90
	4	98	15.75 ± 1.05	5.30 ± 0.50
	5	80	5.15 ± 0.64	2.26 ± 0.11
	6	65	4.55 ± 0.44	2.00 ± 0.60

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	4.4 ±0.57	5.2 ± 0.34
1.0	95	8.2 ± 0.65	6.8 ± 0.20
1.5	98	12.0 ± 0.28	3.5 ± 0.38
2.0	80	7.6 ± 0.42	4.0 ±0.41
NAA			
0.5	90	14.2 ± 0.45	2.1 ± 0.08
1.0	96	19.2 ± 0.27	3.5 ± 0.19
1.5	100	25.6 ± 1.33	5.9 ± 0.30
2.0	84	12.4 ± 1.58	3.0 ± 0.22
IBA			
0.5	85	11.04 ± 0.82	4.8 ± 0.32
1.0	90	18.2 ± 0.59	7.1 ± 0.09
1.5	94	35.4 ± 1.36	6.0 ± 0.17
2.0	70	14.0 ± 0.15	3.7 ± 0.25

Figure – 1: Effect of different carbon sources on multiple shoot regeneration
A. Fructose (4%), B. Sucrose (4%), C. Glucose (5%) , D. Maltose (5%), E&F. Direct rooting from regenerated shoots on MS medium after 40 days of culture (IBA 1.5 mg/l), G&H. Tissue cultured plants in polycups and pots.

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