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In vitro Conservation of Four Mint (*Mentha* spp.) Accessions

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Abstract

This is a part of long term study of slow growth culture (*in vitro* storage) of four mints (*Mentha* spp.) accessions and had been initiated between September 1998 and November 1999. This long term experiment had been assessed in two consecutive phases with four accessions of mint (*Mentha* spp.), namely MEN 204, MEN 148, MEN 186, and MEN 166 which were diploid, tetraploid and octoploid, respectively. The explants were cultured on MS without hormones for six months. Before culturing the explants in a +2_C cell, they were cultured at 20 and 10_C for one week and three weeks, respectively. In phase I, apical and nodal explants were used. The highest numbers of leaves (12) were obtained from MEN 148 and the lowest (8.62) from MEN 204 for apical explants, but in case of nodal explants MEN 148 was also the highest (15.79) score followed by MEN 186 (15.31). The highest number of branches both for apical and nodal explants were counted in MEN 186. All the accessions showed maximum leaves and branches from nodal explants. Root development was comparatively better in MEN 148 (87%) and MEN 186 (53%) for nodal explants. In phase II, only apical explants were used. The highest number of leaves was counted in MEN 186 (12.43) and the lowest (7.87) in MEN 204. The highest number of branch (1.20) was counted in MEN 148. Root development was similar as of the first phase. The *in vitro* plantlets were transplanted into the soil of the greenhouse from phase I. After 19 and 32 days of transplanting, MEN 148 and MEN 186 exhibited the maximum plant height both for apical and nodal explants. But MEN 166 and MEN 186 showed the maximum number of branches for both explants after 32 days of transplanting.

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Introduction

Mint (*Mentha* spp.) plants belong to the Labiatae family and account for more than 25 species, not including the numerous varieties obtained by spontaneous hybridization (Banthorpe 1996). It was originally cultivated in Eastern Asia, mainly in Japan and China (Bersaghi 1945). It is an aromatic and medicinal plant widely cultivated for its essential oils. Components of the oil are used commercially in herbal products, as well as in flavorings for foods, beverage, tobacco, mouthwashes, toothpaste and medicinal products. This diverse utilization has promoted extensive cultivation of peppermint in the USA as well as in Asia and Europe. Plant tissue culture has the potential to introduce genetic variability in peppermint genotypes through somaclonal varaints, somatic hybrids or transgenic plants (Jullien et al. 1998). *In vitro* conservation techniques can facilitate the application of genetic manipulation procedures by providing a simple way of storing experimental material in the form of *in vitro* cultures. More importantly, these techniques can relieve the burden placed on all *in vitro* based procedures imposed by the need to maintain stock cultures. Applications can be envisaged in aspects ranging from physiological studies through to secondary synthesis on an industrial scale, which potential savings on costs, and reduced risks of loss through human error and genetic instability. Moreover, it provides an easy and inexpensive way for international exchange of disease free material (Withers 1980; Chomchalow and Sahavachrin 1981). Many authors work on micropropagation (Rech and Pires 1986), plant regeneration (Caissart et al. 1996, Van Eck and Kitto 1990) and cryopreservation (Withers 1987, Towill and Roos 1989, Towill 1990). Galzy (1969) stored grape shoots in solid media at 9_C for ten months. In liquid medium with a filter paper bridge under dark conditions at a temperature of 1 or 4_C, Mullin and Schlegel (1976) succeeded in storing plantlets derived from meristems of strawberry for six years with the addition of one to two drops of culture media when required. Miedema (1982) was able to maintain plantlets of sugar beet at 5 - 10_C with low light intensity for one year. Cheyne and Dale (1980) reported successful storage of temperate forage legume plantlets at 2 - 6_C with 300 lux light intensity or 4 - 6_C in complete darkness for 15 - 18 months, but there is lack of information on slow growth storage of mint. The reduction of incubation temperature has been shown to be very effective in prolonging the subculturing cycle by reducing the growth rate. The reduction of incubation light intensity or total darkness in conjunction with low incubation temperature can effectively slow down the growth rate. The recommended temperature regimes differ from crop to crop. Some crops are more cold tolerant than others, and the cultures can be maintained at very low temperatures. For

gene bank purposes, it is essential to standardise the culture conditions. In preparation to these measures, it was interesting to find out any differences in the performance of four standard mint accessions that can be preserved under conditions of slow growth at low temperature.

Materials and Methods

The experiment was conducted at the Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany during September 1998 to November 1999. This is a part of long term study of slow growth culture (*in vitro* storage) on four mint (*Mentha* spp.) accessions. This long term experiment had been assessed in two consecutive phases. The accessions MEN 204 (*Mentha* ∇ *villosa* Huds.), MEN 148 (*Mentha* ∇ *villosa* Huds.), and MEN 186 (*Mentha* ∇ *piperita* L.), and MEN 166 (*Mentha* ∇ *piperita* L.) were diploid, tetraploid and octoploid, respectively. The accessions MEN 148, MEN 166, MEN 186 and MEN 204 were obtained from Iraq, Germany, Cuba and Canada, respectively.

Phase I : Apical and nodal explants, having one node and one leaf pair, were collected from glasshouse grown plants. The explants were surface sterilised with 70% ethanol, sodium hypocholorite (effective chlorine concentration 3%) and Tween 20 for a period of 10 minutes. MS medium (Murashige and Skoog 1962) supplemented with 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine-HCl, 0.1 mg/l thiamine-HCl, 100 mg/l myo-inositol, 2 mg/l glycine, 3% sucrose and without any hormones were used. Apical and nodal explants of about 10 mm length were excised aseptically and inoculated into 10 ∇ 6 cm Magenta boxes containing 80 ml of medium, five explants in each box. Before culturing the explants in a +2_C cell, they were cultured at 20 and 10_C for one week and three weeks, respectively. After six months, the following data were taken from each accession. (i) Number of leaves per plantlet, the leaf size was at least 2 mm in length, (ii) number of branches per plantlet, the branch having one leaf pair of at least 2 mm length and (iii) root development scored in absent, few and profuse.

Phase II : Apical explants of about 10 mm length, taken from phase I plantlets, were excised aseptically and inoculated into similar Magenta boxes containing 80 ml of medium. All other procedures and data collections were the same as of phase I. The explants were cultured for a period of six months at 2_C.

A part of the *in vitro* plantlets of phase I was transferred into well-amended soil in a greenhouse. Temperature, light and relative humidity were ocntrolled in the greenhouse. Plant height and number of branches per plant

were recorded 18 and 32 days after planting, respectively. Data on plant height, number of leaves and branches were recorded and mean separation was done using LSD by Gomez and Gomez (1983).

Results and Discussion

Phase I : Number of leaves and branches developed from apical and nodal explants from different accessions of mints are shown in Table 1. After six months culture of the apical explants in MS at +2_C, significantly higher number of leaves were obtained from MEN 148, which was statistically similar with MEN 166 and MEN 186 but they differed significantly with MEN 204 ($p = 0.01$). MEN 148 yielded the highest number of leaves (12.00), which was followed by MEN 166 (10.06), MEN 186 (9.33) and MEN 204 (8.62). Regarding the number of leaves produced from nodal explants, MEN 148 appeared to be the best one producing 15.79 leaves but the difference to the other accessions was statistically insignificant. The lowest number of leaves were produced by MEN 204 (12.20).

Table 1. Number of leaves and branches developed from apical and nodal explants of mint accessions in phase I.

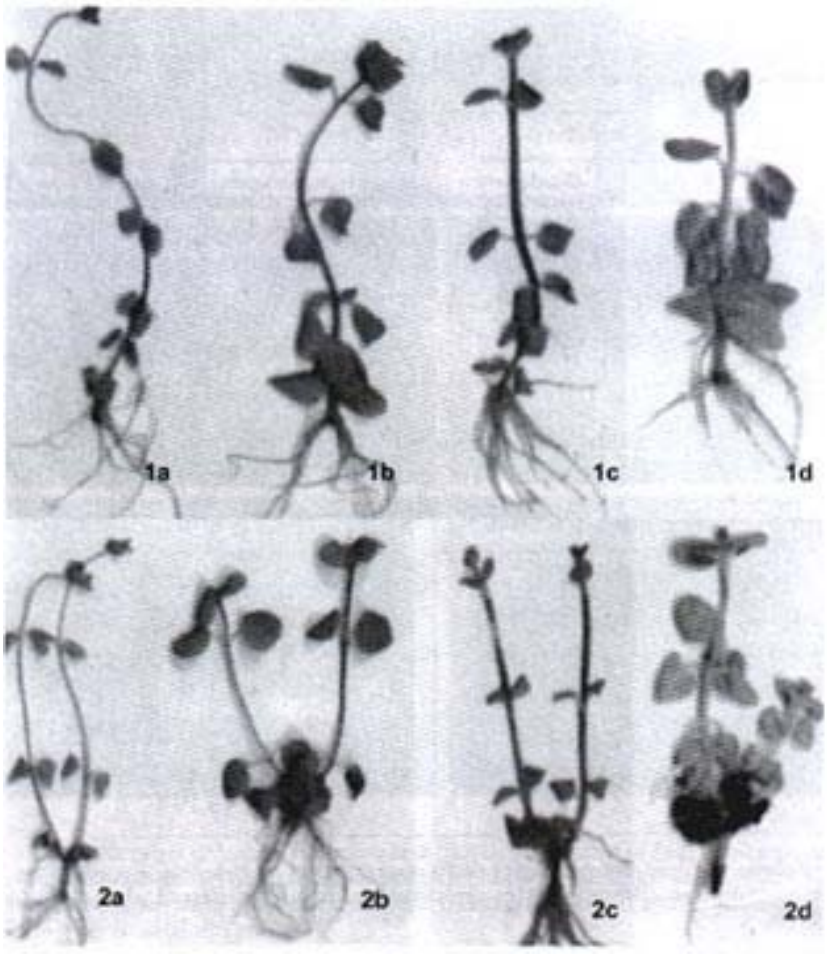
Accession	Number of leaves[1]		Number of branches[1]	
	Apical[2]	Nodal[2]	Apical[2]	Nodal[2]
MEN 148	12.00[a]	15.79[a]	1.07[a]	1.90[a]
MEN 166	10.06[a]	12.31[a]	1.03[a]	1.63[b]
MEN 186	9.33[a]	15.31[a]	1.14[a]	1.95[a]
MEN 204	8.62[b]	12.20[a]	1.10[a]	1.88[a]
Mean	10.00	13.90	1.09	1.84
LSD (1%)	3.37	2.74	0.21	0.16
CV (%)	12.28	10.50	10.47	4.61

[1]Mean of three replications and mean of 45 observations represent one replication. [2]Any two means having a common letter are not significantly different at the 1% level of significance.

The accession MEN 186 produced the highest number of branches (1.14) from apical explants. MEN 204 ranked next to MEN 186 followed by MEN 148 and MEN 166 (1.03) but these differences were statistically insignificant (Table 1). In case of nodal explants, the significantly higher branch number was

recorded from MEN 186 (1.95), followed by MEN 148 (1.90) and MEN 204 (1.88). The branch number of MEN 166 (1.63) was significantly lower.

Among the mint accessions, MEN 148 appeared to be the best one for the initiation of roots from both apical and nodal explants (100%). All the accessions showed more or less a few roots from both apical and nodal explants (Table 2). In case of profuse root initiation, MEN 148 scored the best one to yield maximum root initiation (87%) from apical explants followed by MEN 166 (65%), MEN 186 (53%) and MEN 204 (28%). In descending order of root production in case of nodal explants, the accessions can be arranged as : MEN 148 (86%), MEN 186 (52%), MEN 166 (29%) and MEN 204 (16%) (Figs. 1 and 2).



Figs. 1 - 2: 1a - d). Number of leaves, branches and root development of four mint accessions after six months conserved the apical explant at 2_C in phase I. (Where a = MEN 148, b = MEN 166, c = MEN 186 and d = MEN 204). Figs. 2a - d. Number of leaves, branches and root development of four mint accessions after six months conserved the nodal explant at 2_C in phase I.

Phase II : The highest and the lowest number of leaves from apical explants were recorded in MEN 186 (12.43) and MEN 204 (7.87) (Table 3), respectively. Statistical analysis revealed that MEN 186 produced the highest number of leaves compared to the remaining accessions, but it was not significantly different to MEN 148 and MEN 166. In case of branch numbers, there were no remarkable differences among the treatments and they were statiscially at par. Rooting of *in vitro* plantlets from apical explants was specially profuse in accessions MEN 148 and MEN 186, where 100% of plantlets initiated roots (Table 4, Fig. 3).

Table 2. Percentage of roots developed from apical and nodal explants of mint accessions from phase I.

Accession	Absent (%)		Few (%)		Profuse (%)	
	Apical	Nodal	Apical	Nodal	Apical	Nodal
MEN 148	0	0	13	14	87	86
MEN 166	13	37	22	34	65	29
MEN 186	4	2	43	46	53	52
MEN 204	17	27	55	57	28	16
Mean	8.5	16.5	33.25	37.75	58.25	45.75

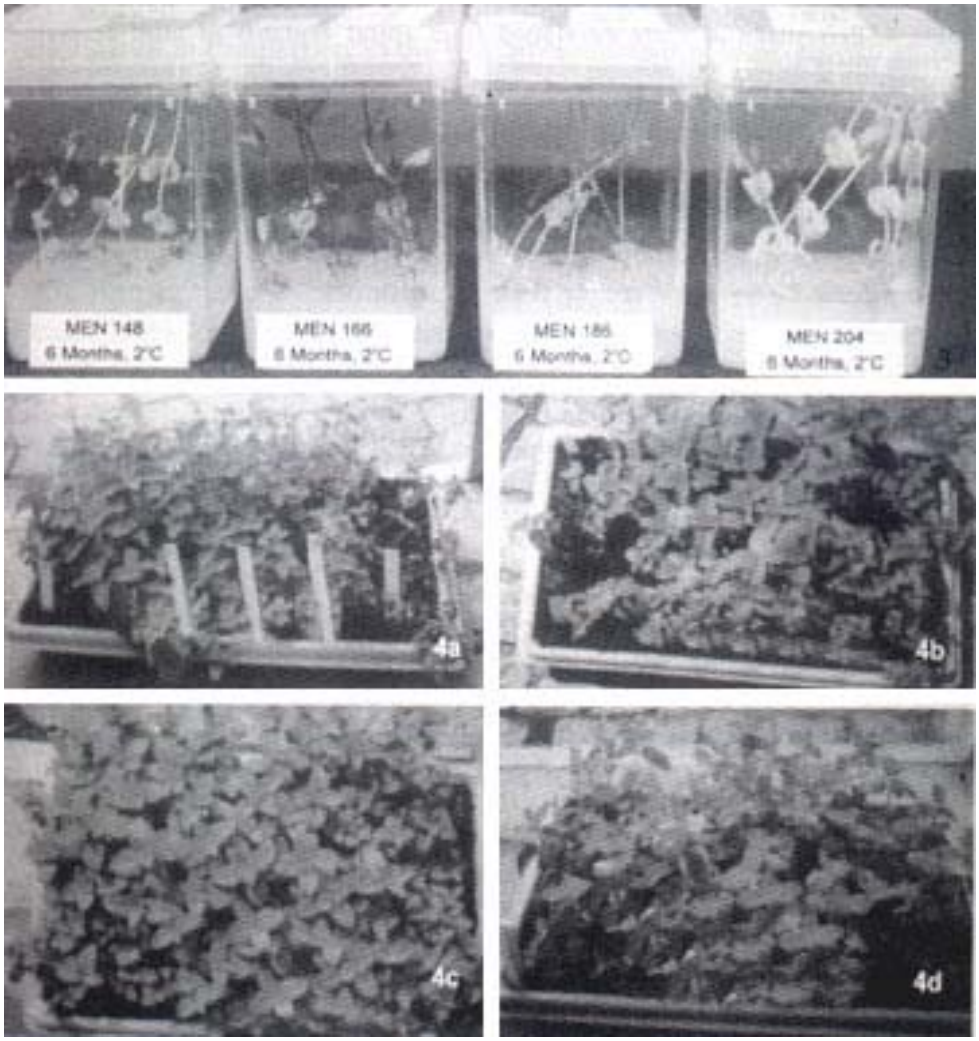
Table 3. Number of leaves and branches developed from apical explants of mint accessions in phase II.

Accession	Number of leaves[1]	Number of branches[1]
MEN 186	12.43[a]	1.02[a]
MEN 148	11.99[a]	1.20[a]
MEN 166	10.05[a]	1.01[a]
MEN 204	7.87[b]	1.06[a]
Mean	10.59	1.07
LSD (!%)	2.87	0.47
CV (%)	9.91	16.19

[1]Mean of three replications and mean of 70 observations represent one replication. [2]Any two means having a common letter are not significantly different at the 1% level of significance.

Under greenhouse conditions, the maximum and the minimum plant heights from apical explants after 19 days were measured in MEN 148 (15.93 cm) and in MEN 204 (3.77 cm), respectively. Significantly higher plant height was found in MEN 148. It differed significantly with only MEN 204 ($p = 0.01$). In case of plant heights from nodal explants, the accessions may be arranged in

ascending order as: MEN 204 (3.71 cm), MEN 166 (10.11 cm), MEN 186 (14.29 cm) and MEN 148 (15.84 cm). When plant heights of 32 days old apical explants were compared, it was found that the maximum height was recorded in MEN 148 (17.4 cm) but it was statistically at par with MEN 186 and MEN 166. In 32



Figs. 3 - 4: 3. Number of leaves, branches and root development of four mint accessions after six months conserved the apical explant at 2_C in phase II. Figs. 4a - d. Plant height and branches of four mint accessions after six months conserved at 2_C and then six weeks in the greenhouse from phase I.

days old nodal explants, the maximum plant height was measured in MEN 186 (18.41 cm) (Table 5). It differed significantly to the remaining accessions except MEN 148. The accession MEN 204 had the lowest plant height (7.21 cm), but it was statistically insignificant with only MEN 166. The highest branch number

from 32 days old apical explants was counted in MEN 166 (9.23) followed by MEN 186 (7.55), MEN 148 (6.25) and MEN 204 (4.65), and they were statistically insignificant. The significantly highest branch number from nodal explants was obtained in MEN 186 (8.53). It differed significantly to MEN 148 (5.23) and MEN 204 (5.20) (Fig. 4).

Table 4. Percentage of roots developed from apical explants of mint accessions from phase II.

Accession	Absent (%)	Few (%)	Profuse (%)
MEN 148	0	2	98
MEN 166	15	25	60
MEN 186	0	10	90
MEN 204	12	50	38
Mean	6.75	21.75	71.5

Table 5. Plant heights and branch numbers 19 and 32 days after transplanting the *in vitro* plantlets to the greenhouse.

Accession	Plant height (cm)				Branch number	
	After 19 days		After 32 days	after 32 days		
	Apical	Nodal	Apical	Nodal	Apical	Nodal
MEN 148	15.93^{a}	15.84^{a}	17.40^{a}	17.40^{ab}	6.25^{a}	5.23^{b}
MEN 186	13.65^{a}	14.29^{ab}	16.63^{a}	18.41^{a}	7.55^{a}	8.53^{a}
MEN 166	12.24^{a}	10.11^{b}	13.66^{a}	12.17^{bc}	9.23^{a}	7.92^{ab}
MEN 204	3.77^{b}	3.71^{c}	7.25^{b}	7.21^{c}	4.65^{a}	5.20^{b}
Mean	11.40	10.99	13.74	13.80	6.92	6.72
CV (%)	26.24	23.18	26.51	22.22	39.34	23.34

LSD (0.01): Between MEN 148 and MEN 186 is 5.04, between MEN 148 or MEN 186 with MEN 166 or MEN 204 is 5.64 and between MEN 166 and MEN 204 is 6.18. [1]Mean of six or four replications and mean of 30 observations represent one replication. [2]Any two means having a common letter are not significantly different at the 1% level of significance.

Results of the comparison of *in vitro* phases I and II indicate that there is no remarkable variation among the accessions in initiating leaf numbers from apical explants. The trend of the accessions was more or less similar also in case of branch numbers between phases I and II. In this study, the most profuse root formation from apical explants was found in MEN 148, where more than 85% rooting was evident in both phases I and II. Under greenhouse conditions, MEN 148 appeared to be the superior one in reaching the maximum plant height at 19

days from both apical and nodal explants sources followed by MEN 186, MEN 166 and MEN 204. A similar trend was also observed 32 days after transplanting. In respect of branch numbers from apical explants, the accessions were statistically insignificant 32 days after planting, but in nodal explants MEN 186 and MEN 166 appeared suitable than remaining two accessions. From this study it appears that MEN 148 and MEN 186 are better in their performance in comparison to the two other accessions. Slow growth storage techniques by reducing temperature have been successfully applied in many crops. Pre growth of the cultures under normal incubation conditions to allow the explants to establish before being transferred to lower temperatures for storage was also reported to be important for apple (Lundergan and Janick 1979), *Prunus* spp. (Marino et al. 1985) and sweet potato, yams and cocoyams (Ng and Hahn 1985). Apple (*Malus domestica*) and *Prunus* shoots survived 52 weeks at 2_C (Druart 1985). Zandvoort and Staritsky (1986) reported that they were able to store *Colocasia* at 9_C for three years and *Xanthosoma* spp. at 13_C. Garlic explants can be stored at 4_C for at least six months (El-Gizawy and Ford-LIoyd 1987). *In vitro* bulblets of onion are storable at –1_C for more than one year (Kastner et al. 2001). It may be concluded that mint can also well be conserved *in vitro* at low temperature.

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