High regenerative nature of *Mentha arvensis* internodes

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Media and incubation conditions have been defined for highly efficient regeneration of shoots from internode explants of slow and fast growing cultivars of *Mentha arvensis*. Internodal segments excised from the *in vitro* raised shoots were inoculated on the MS medium supplemented with combinations of 5 concentrations of 1-napthalene acetic acid (NAA) and 3 concentrations of 6-benzyl amino purine (BAP). The media containing 2 μg ml^{-1} NAA, 10 μg ml^{-1} BAP and 1 μg ml^{-1} NAA, 5 μg ml^{-1} BAP proved best for shoot regeneration and growth responses on cv Himalaya and cv Kalka explants, respectively. In 12 weeks time, on average one explant of cv Himalaya produced about 200 shoots and that of cv Kalka produced about 180 shoots. The Himalaya explants required higher concentrations of NAA and BAP for high efficiency proliferation as compared to the Kalka explants. The experiments demonstrated that internodal tissue in *Mentha arvensis* can be induced to obtain direct shoot regenerants with high efficiency. The analysis of the RAPD profiles of 100 regenerated plantlets each of cv Himalaya and Kalka showed more than 99·9% homogeneity in bands with respect to the parents.

1. Introduction

Mentha arvensis Linn. var *piperascens* Holmes (menthol or Japanese mint) is an industrial crop that is widely cultivated for its essential oil from which menthol is crystallized. The essential oil, menthol and terpenes of the dementholated oil of *M. arvensis* 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 mint cultivation more economical. Construction of the desired *M. arvensis* genotypes will require transfer of specific foreign genes into the crop. Efficient procedures are required to regenerate plants from the transformed cells and for rapid micro-propagation of plant(s) of selected genotype(s).

In vitro high efficiency procedures for cell and callus cultures and shoot regeneration from axillary buds and leaf explants have been reported in some species of the genus *Mentha*, especially the commercially important species *M. piperita* and *M. spicata* (Lin and Staba 1961; Cellarova 1992). However, in *M. arvensis* the media used have permitted shoot proliferation from explants such as nodes, terminal and axillary buds and distal segments of leaf petiole at relatively low levels of efficiency (Karasawa and Shimizu 1980; Kukreja *et al* 1992). This work reports culture conditions under which the internodal segments of *M. arvensis* regenerated shoots efficiently.

2. Materials and methods

2. *Plant material and preparation of explants*

The cultivars Kalka (CIMAP/HY77) and Himalaya (CIMAP/MAS92-1) were the source of explants. The cultivar Kalka is grown for one harvest of herbage due to poor response to ratooning of its shoots. The hybrid

Keywords. *Mentha arvensis*; RAPD; regeneration

Abbreviations used: BAP, 6-benzyl amino purine; CRD, completely randomized design; NAA, -napthalene acetic acid; RAPD, randomly amplified polymorphic DNA

cultivar Himalaya combines the essential oil quality and disease resistance characteristics of Kalka and good ratooning character of its other parent cultivar Gomti (Sushil Kumar *et al* 1997). One hundred plants from each cultivar were analysed initially through randomly amplified polymorphic DNA (RAPD) using 12 random primes to check the field variation and one sucker from each of the two cultivars was planted in pots containing soil and organic manure (1 : 1) and maintained in glass house. Apical stem portions carrying 4–5 nodes were surface sterilized by dipping sequentially in 2% detergent (Teepol), distilled water containing a few drops of Savlon (Johnson and Johnson) and 0·1% acidified mercuric chloride for 1 min each followed by thorough washing in sterile distilled water. The stem was cut into pieces of about 2 cm, such that each piece carried a node. The explants so derived were planted vertically in MS medium with 1·5% wv^{-1} agar (Murashige and Skoog 1962) and incubated for four weeks. The shoots formed on them were the source of the experimental explant material.

2.2 *Media and establishment of cultures*

The explants used were 0·5 cm long pieces of the second and third internodes of the shoots formed from axillary buds in culture. The internode segments were inoculated in MS based media containing vitamins, 100 µg ml^{-1} myo-inositol, 3% wv^{-1} sucrose, 1·5% wv^{-1} agar (Difco) and different concentrations of auxin and cytokinin. Five different concentrations of (0, 0·5, 1·0, 1·5 and 2·0 µg ml^{-1}) 1-napthalene acetic acid (NAA) were used in combination with three different concentrations of (0, 5, 10 µg ml^{-1}) 6-benzyl aminopurine (BAP). On each kind of medium, 10 replicates of the explants were inoculated into 10 independent flasks with each flask containing one explant. The experiment was arranged in the form of a completely randomized design (CRD). Cultures were maintained at 25 ± 2°C and 400–600 lux light intensity with 16 h photoperiod. The response of explants was recorded every 24 h over a 4-week period. The media on which initiation of shoot primordia on the internodal explants occurred earliest were selected for further experimentation. A set of 20 pre-weighed internode explants of 0·5 cm size were inoculated on each of the most optimal media (table 1). Each explant was observed at 4 weeks interval and subcultured on the same medium. The proportional increase in biomass was recorded by taking the fresh weight of the growing tissue during subculture and dividing the increase with the initial weight. At the end of 12 weeks from inoculation the shoots were separated and individually transferred to MS basal medium containing vitamins for rooting. The rooted plantlets were subsequently transferred to pots in a greenhouse.

2.3 *DNA isolation and PCR amplification reactions*

DNA was isolated from 40 mg of leaf tissue according to the protocol described by Doyle and Doyle (1987). Polymerase chain reactions (PCRs) were carried out in 25 µl volume. A reaction tube contained 25 ng of DNA, 0·2 unit of Taq DNA polymerase, 100 µl each of dNTPs, 1·5 mM $MgCl_2$ and 5 pmol of decanucleotide primers. The amplifications were carried out using a thermal

Table 1. Types of early growth responses observed on the internode segment explants of *M. arvensis* inoculated on the MS medium containing varying concentrations of NAA and BAP.

	Concentration of growth regulators in the basal MS medium (µg ml^{-1})		Type of growth responses observed in explants of the cultivar		Average number of days after inoculation at which the initial earliest growth response was observed on the explants of the cultivar	
Medium	NAA	BAP	Himalaya	Kalka	Himalaya	Kalka
A	0	0	N	N	NA	NA
B	0	5	N	N	NA	NA
C	0	10	S	S	24·2 (0·4)	19·3 (0·4)
D	0·5	0	C	C, R	15·4 (0·4)	17·1 (0·4)
E	0·5	5	S	C, S	13·3 (0·3)	14·2 (0·4)
F	0·5	10	S	S	14·5 (0·5)	16·3 (0·4)
G	1·0	0	R	C	18·6 (0·5)	16·4 (0·6)
H	1·0	5	S, R	S	16·2 (0·5)	12·0 (0·7)
I	1·0	10	S	S	15·9 (0·6)	14·7 (0·5)
J	1·5	0	C, R	C, R	20·5 (0·4)	23·8 (0·5)
K	1·5	5	C, S, R	C, S, R	19·1 (0·4)	18·9 (0·6)
	1·5	10	C, S, R	C, S, R	15·8 (0·3)	16·1 (0·4)
M	2·0	0	C, R	C	20·8 (0·6)	21·2 (0·4)
N	2·0	5	C, S, R	S, R	20·0 (0·5)	20·1 (0·3)
O	2·0	10	S	S	12·0 (0·4)	13·8 (0·5)

N, Response not detected; C, callusing; S, shoot primordium formation; R, root primordium formation; NA, not applicable. Values in parentheses are standard errors.

cycler (MJ Research, USA) following the protocol of Shoyama *et al* (1997). The amplified products were loaded in 1·2% agarose gel containing 0·5 μg ml^{-1} of ethidium bromide and photographed by Polaroid system. Custom-made decanucleotide primers were obtained from M/s Bangalore Genie, India. Twelve decamer primers having the sequences AAATCGGAGC, GTCCTACTCG, GTCCTTAGCG, TGCGCGATCG, AACGTACGCG, GCACGCCGGA, CACCCTGCGC, CTATCGCCGC, CGGGATCCGC, GCGAATTCCG, CCCTGCAGGC, CCAAGCTTGC were used to analyse 100 *in vitro* regenerated plantlets from each cultivar.

3. Results and discussion

3. *Field variation*

To check the inherent variability (if any) of the mother plant material at the population level, leaf samples of 100 plants were collected and the DNA isolated was subjected to RAPD analysis using the random primers. The vegetatively propagated plant *M. arvensis* is fairly stable at population level as no polymorphic profile was observed in any plant.

3.2 *Regeneration from internodal tissue of two mint cultivars*

To find out the media that promoted early differentiation of shoot primordia on the explants of *M. arvensis*, small segments (0·5 cm) of the internodal region of the plants of cvs Kalka and Himalaya were inoculated on MS agar medium supplemented with 15 combinations of the growth regulators NAA and BAP. The growth responses of the explants were observed after 4 weeks of inoculation on different media (table 1). No callus, shoot or root primordia were formed by the explants inoculated on media without NAA and containing 0 or 5 μg ml^{-1} of BAP. On media containing 0·5 to 2 μg ml^{-1} of NAA but no BAP, explants formed callus and/or root primordia. Callusing and/or shoot and root differentiation was observed on the explants inoculated on the media containing 1·5 μg ml^{-1} NAA and 5 or 10 μg ml^{-1} BAP or 2 μg ml^{-1} NAA and 5 μg ml^{-1} BAP. The earliest shoot differentiation response from the explants of both the cultivars, Kalka and Himalaya, was observed on media containing either 1 μg ml^{-1} NAA with 5 μg ml^{-1} BAP (medium H) or 2 μg ml^{-1} NAA with 10 μg ml^{-1} BAP (medium O). The sequence of shoot primordia formation, proliferation, regeneration and rooting responses for both the cultivars Himalaya and Kalka is shown in figure 1.

It was found that medium H was more suitable for shoot regeneration from the explants of cv Kalka and medium O for the explants of cv Himalaya (table 2). Internode explants of cv Himalaya required relatively higher concentrations of phytohormones in the medium for the rapid differentiation and growth of shoots as compared to the explants of cv Kalka. The supplementary requirements of the phytohormones for *in vitro* regeneration may be related to the inherent phytohormone status of the stem/shoot in the two cultivars. In 12 weeks, while the cv Kalka explants on an average underwent 1552-fold increase in biomass by giving rise to 179 shoots on medium H, the cv Himalaya explants increased 2166-fold in biomass by producing 205 shoots on medium O. The biomass of *in vitro* regenerated shoots of cv Himalaya was about 20% more than cv Kalka. Thus the shoot proliferation from cv Kalka internode explants on the medium H was about 10 to 25% lower than that from cv Himalaya explants on the medium O. This difference may be related to the inherent lower regeneration ability of cv Kalka observed also under field conditions after the first harvest.

3.3 *Survival of the plants in pots and RAPD analysis*

As shown in table 3, in the 125 shoots for each of the two cultivars that were separated and transferred to MS basal medium, rooting was observed to the extent of 96% and 94% in cv Himalaya and cv Kalka, respectively. Out of 100 plantlets for each cultivar transferred to the pots, 94 in the case of cv Himalaya and 91 in case of cv Kalka survived. The genetic homogeneity of regenerants of each cultivar was ascertained with the use of RAPD analysis. There were only two exceptions in terms of the polymorphic bands with respect to the mother plant RAPD pattern in cv Himalaya (table 4). The total number of bands produced with the 12 primers by the 100 regenerants in cv Himalaya was 5498. The frequency of polymorphic bands in the regenerants was estimated as 0.036. This level of variation has been considered as tolerable by Munthali *et al* (1996) in their evaluation of RAPD patterns in a population of *in vitro* raised plants of beet. The RAPD profiles of all the *in vitro* raised plantlets of cv Kalka were identical to the parent for all the 12 primers.

The approach of using molecular markers including RAPD profiles is a powerful tool not only for the identification of genotypes but also to quantify the extent of genetic variation in any given population. While on one hand the approach of RAPD profiling has been useful in tissue culture methods for detection and selection of somaclonal variants (Munthali *et al* 1996), this molecular technique at the same time with the same logic is directly utilizable for assessing the population of micro-propagated clones from any given explant for genetic uniformity. Among the reports on use of RAPD analysis for molecular analysis in the micro-propagated clones, the emphasis has been laid on the confirmation of genetic homogeneity of the raised plantlets. Wallner

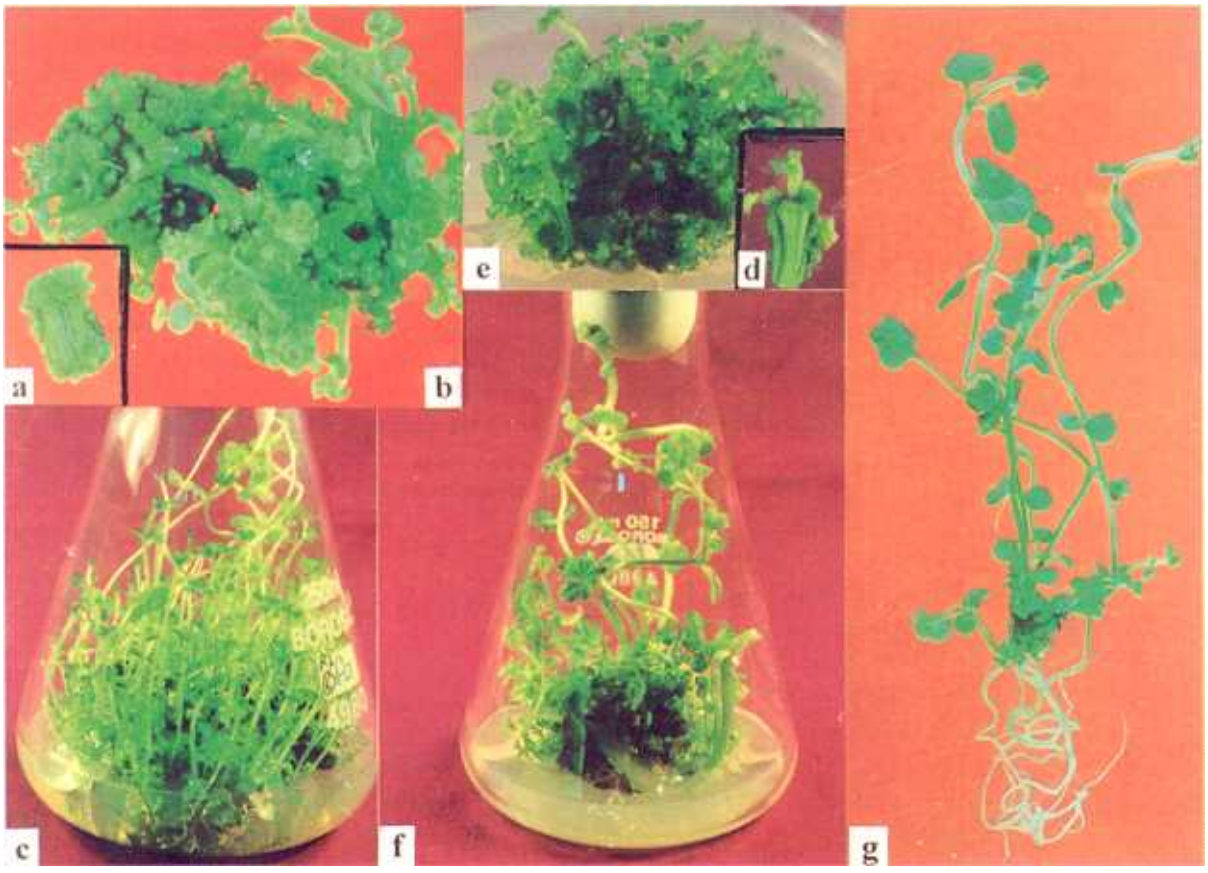

Figure 1. Internodal segment explants of *M. arvensis* cultivars Himalaya and Kalka at different stages of regeneration. (**a**) Explant of the cultivar Himalaya at 2 weeks from inoculation. (**b**) Shoot proliferation seen at 6 weeks in an explant of Himalaya. (**c**) Growing shoots seen at 10 weeks on an explant of Himalaya. (**d**) Internodal segment explant of the cultivar Kalka, at 2 weeks from inoculation. (**e**) Shoot proliferation seen at 6 weeks in an explant of Kalka. (**f**) Growing shoots seen at 10 weeks on an explant of Kalka. (**g**) A plantlet of Himalaya obtained after transfer of 12 weeks old shoot to the rooting medium for 4 weeks.

Table 2. Biomass increase in the form of shoots on the internode segment explants of *M. arvensis* at 4, 8 and 12 weeks from inoculation on the MS medium containing 1 μg ml^{-1} NAA + 5 μg ml^{-1} BAP (medium H) or 2 μg ml^{-1} NAA + 10 μg ml^{-1} BAP (medium O).

		Number of shoots			Increase in biomass		
Cultivar	Medium	4th week	8th week	12th week	4th week	8th week	12th week
Kalka	H	10·0 ± 0·3	97·0 ± 0·7	177·7 ± 1·0	62·5 ± 0·9	410·0 ± 4·9	1552·5 ± 9·6
	O	2·0 ± 0·29	20·5 ± 0·4	60·5 ± 0·7	4·7 ± 0·1	100·7 ± 0·8	243·3 ± 1·1
Himalaya	H	8·0 ± 0·3	35·0 ± 0·7	102·3 ± 1·1	9·1 ± 0·3	108·5 ± 1·1	986·2 ± 6·7
	O	13·9 ± 0·9	37·6 ± 1·1	204·6 ± 13·0	86·0 ± 3·0	295·8 ± 21·4	2166·1 ± 74·7
CD at 5%		1·4	2·2	18·5	4·5	31·2	107·0
CD at 1%		1·9	2·9	24·6	6·0	41·4	142·3

Values represents means ± standard error

et al (1996) have applied this technique to compare the field grown and *in vitro* micro-propagated plants of *Achillea* sp., while Shoyama *et al* (1997) have used the approach to assess the micro-propagated clones in *Panax notoginseng*. Similarly, from the conservation point of view, Parani *et al* (1997) used the RAPD approach to compare the mother plant and micro-propagated progenies to maintain the fidelity of elite genotypes that were to be conserved.

Table 3. Rooting and *in vivo* establishment and characteristics of the plants regenerated *in vitro* from the internode explants of *M. arvensis* cv Himalaya and Kalka.

Cultivar	Number of shoots separated and inoculated for rooting	Rooting frequency of shoots (%)	Number of *in vitro* plants transferred to pots	Survival frequency (%)	Number of plants whose RAPD profiles were ascertained	Frequency of plants of which RAPD profiles were congruent with parent (%)
Kalka	125	93·6	100	94	100	98
Himalaya	125	96·0	100	91	100	100

Table 4. RAPD analysis of the plant populations regenerated from the explants of the cultivars Himalaya and Kalka of *M. arvensis*.

Sequence	Total number of amplified fragments in the RAPD profiles of the *in vitro* regenerated plantlets of the cultivars		Total number of polymorphic bands detected in the RAPD profiles of the *in vitro* regenerated plantlets of the cultivars	
	Himalaya	Kalka	Himalaya	Kalka
AAATCGGAGC	400 (4)	400 (4)	0	0
GTCCTACTCG	100 (1)	100 (1)	0	0
GTCCTTAGCG	500 (5)	500 (5)	0	0
TGCGCGATCG	800 (8)	900 (9)	0	0
AACGTACGCG	499 (5)	500 (5)		0
GCACGCCGGA	400 (4)	300 (3)	0	0
CACCCTGCGC	100 (1)	100 (1)	0	0
CTATCGCCGC	400 (4)	500 (5)	0	0
CGGGATCCGC	600 (6)	600 (6)	0	0
GCGAATTCCG	899 (9)	900 (9)		0
CCCTGCAGGC	600 (6)	600 (6)	0	0
CCAAGCTTGC	200 (2)	200 (2)	0	0
All sequences	5498 (55)	5500 (55)	2	0

Number in parentheses indicate number of bands in the profile of the mother plant.

Among the *Mentha* species only limited amount of information has been documented on the micro-propagation and specific morphogenetic responses of individual genotypes to the exogeneous promoting factors. Interest has mainly been focused on peppermint (*M. piperita*) wherein, the explants used for clonal propagation have been terminal or axillary buds and young leaves (Cellarova 1992). Rech and Pires (1986) have reported *in vitro* clonal propagation from stem node(s) in *M. arvensis*, *M. piperita*, *M. spicata*, *M. pulegium* and *M. viridis*. While they showed extensive shoot proliferation in *M. viridis*, *M. pulegium* and *M. piperita*, they reported limited success in the *in vitro* regeneration of shoots in the remaining species, including *M. arvensis*. Subsequently, Kukreja *et al* (1991) using stem pieces containing 2 to 3 nodes as explants of *M. arvensis* obtained 30 to 40 shoots per explant in 6 to 8 weeks. The observations reported here have identified conditions under which stem explants derived from internodes of *M. arvensis* produce 4–6-fold more shoots than reported earlier. Genotypic differences could be identified for the regeneration responses. The cultivar Himalaya has been found to regenerate with about 12% higher efficiency than the cultivar Kalka.

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