Positive correlation between menthol content and *in vitro* menthol tolerance in *Mentha arvensis* L. cultivars

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Menthol is a highly valued monoterpene produced by Japanese mint (*Mentha arvensis*) as a natural product with wide applications in cosmetics, confectionery, flavours, beverages and therapeutics. Selection of high menthol yielding genotypes is therefore the ultimate objective of all genetic improvement programmes in *Mentha arvensis*. A positive correlation was observed in the present study between menthol content in oils of evaluated genotypes and the level of tolerance to externally supplied menthol of explants of these genotypes in culture medium. The easy use of this relationship as a selectable biochemical marker opens the practical applicability of large-scale *in vitro* screening of the germplasm, clones and breeders' material for selection of elite genotypes.

1. Introduction

Mints are highly popular industrial crops cultivated in several countries for the high value monoterpenes present in their essential oils. The oil from *Mentha arvensis* L. var. *piperascens* is a rich source of the monoterpene 'menthol', which is industrially used in the cosmetics, pharmaceuticals, food, confectionery and beverages. Therefore, in the genetic improvement of *M. arvensis*, high menthol content is a desirable trait. A biochemical marker, which can facilitate selection of high menthol line *in vitro*, therefore, can become an important tool for biotechnological improvement of mints. The biosynthesis of menthol in the plant is regulated in a cascade fashion during plant differentiation and the oil containing menthol and related monoterpenes are stored in specialized oil glands called trichomes, which are formed on the surface of the leaves. Thus trichomes separate the toxic monoterpenoid products from normal cells. The cytotoxic effect of monoterpenes on plant tissues, by inhibiting respiration and photosynthesis by drastically affecting the mitochondria, golgi bodies etc. and decreasing cell membrane permeability has been demonstrated experimentally (Brown *et al* 1987). For higher menthol yields in mints, besides the efficient rate of menthol synthesis, the level of menthol that may be detrimental or inhibitory to its further synthesis is also an equally important aspect. With this rationale, we screened the known cultivars of *M. arvensis* L. var. *piperascens* available at CIMAP, to explore whether there is any correlation between the menthol content of these genotypes and their *in vitro* tolerance level against menthol in the culture medium, to serve as a selectable marker in genetic improvement.

2. Materials and methods

2.1 *Plant variety, ex-plants and culture conditions*

The cultivars of *M. arvensis* (2n = 96) used were Himalaya, Shivalik, Gomti, MAS-1, Kosi and Kalka. The suckers of these cultivars were obtained from CIMAP's gene bank. The explant materials were collected from the field grown plants and surface sterilized by washing sequentially with 2% detergent, distilled water containing a few drops of Savlon (Johnson and Johnson, India), 0·1% acidified mercuric chloride and, finally, autoclaved distilled water before inoculation. For *in vitro* multiplication by direct regeneration, about 1 cm long internodal pieces were inoculated in the MS medium (Murashige and Skoog 1962) containing vitamins, 100 mg l^{-1} myo-inositol, 3% w v^{-1} sucrose, 1·5% w v^{-1} agar (Difco), 0·5 μg ml^{-1} of an auxin,

Keywords. *Mentha arvensis* L.; menthol content; menthol tolerance

(1-naphthalene acetic acid, NAA) and 5 μg ml^{-1} of a cytokinin (benzyl aminopurine, BAP) (Shasany *et al* 1998). The cultures were grown at 25 ± 2°C, and under 400 to 600 lux light intensity with 16 h photoperiod. The regenerated shoots were separated 12 weeks after the explant inoculation for rooting and/or screening.

2.2 *Evaluation for* in vitro *menthol tolerance*

The regenerated shoots (3–4 cm long, with the apical bud and 2–3 nodes) were separated after 12 weeks and inoculated into the MS basal agar medium with or without menthol (0 to 100 μg ml^{-1}; concentration stepped up by 10 μg ml^{-1} in each treatment). Menthol was added after melting and filter-sterilizing. On each treatment, 5 shoots of each variety were inoculated per flask; 4 flasks were inoculated per replication and each treatment was replicated 5 times, making the number of flasks to 20 and number of explants to 100 for each concentration of menthol. Control flasks contained only MS basal medium and no menthol. The experiment was laid out in a completely randomized design (CRD). Cultures were incubated and maintained at 25 ± 2°C and 400–600 lux light intensity with 16 h photoperiod. The initial response and survival of the explants was recorded every 24 h over a period of one week.

2.3 *Oil extraction and gas-liquid chromatography analysis*

Oil samples from fresh leaves of the field grown clones were extracted by hydrodistillation using Clevenger's apparatus and weighed to record the yield. Gas-liquid chromatography (GLC) analysis was accomplished on Varian CX-3400 using a 30 m × 0·25 mm (0·25 μ) Supelcowax-10 column. The injector and detector temperature were maintained at 200 and 225°C, respectively, with oven temperature programmed from 60 to 200°C at the rate of 7°C min^{-1} increase, with initial and final holds of 2 and 5 min, respectively. Hydrogen gas was used as carrier at the rate of 1 ml min^{-1} and 0·1 μl of sample was injected with a split ratio of 1 : 50. Data were processed in the electronic integrator Varian 4400 and the identification was based on retention time of authentic samples of l-menthol (Takasago, Japan) and retention indices calculations (Jennings and Shibamoto 1980).

3. Results and discussion

Toxicity of monoterpenes to the eukaryotic cells is known for affecting cellular viability and growth in suspension cultures (Brown *et al* 1987). This type of end product toxicity by menthol in *M. arvensis*, which produces this monoterpene in large amount, is likely to play a role in determining the level of menthol accumulation. Since there are a number of *M. arvensis* varieties available with varying potential to accumulate menthol in trichomes, we attempted to study the correlation between the level of menthol tolerance *in vitro* in these varieties and their menthol yields. When menthol was supplied exogeneously, through the medium, to short cuttings of these varieties, it was observed that a higher dose of the monoterpenes (100 ppm) was cytotoxic, causing chlorosis and ultimately death of the shoots. At this concentration menthol also suppressed regeneration from all kinds of explants. After this observation on the lethal effect of 100 μg ml^{-1} menthol, relative tolerance level of the varieties to various levels of menthol (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μg ml^{-1}) was examined. The survival of the regenerated shoots decreased, with increase in menthol concentration in the medium (table 1). The toxic

Table 1. Survival of regenerant shoots from internodal explants at different concentrations of menthol.

Concentration of menthol (μg ml^{-1})	Per cent survival after 24 h					
	Himalaya	Shivalik	Gomti	MAS-1	Kosi	Kalka
0	100	100	100	100	100	100
10	100	100	100	100	100	100
20	100	100	100	100	100	100
30	100	100	100	100	100	100
40	100	100	100	100	100	100
50	85	90	80	95	85	97
60	70	75	60	80	75	85
70	45	40	30	50	35	55
80	20	20	8	25	15	30
90	10	10	5	15	10	15
100	0	2	1	2	1	2
Critical difference at 1%				4.658		

effect was visible even after 7 days (figure 1). Shoots turned chlorotic after 24 h in menthol and did not revive even upon transfer to menthol-free medium.

Comparatively, MAS-1 and Kalka showed highest survival frequency at all the menthol concentrations. Similarly, after 7 days of incubation at 40 ppm and above concentration of menthol, shoot lethality was observed for all the cultivars. At 80 ppm menthol, 100% mortality of shoots was observed for all varieties except in case of MAS-1 which still showed about 2% survival.

Interestingly, although lowest essential oil content was observed for MAS-1 (0·50%) followed by Kalka (0·57%) the menthol concentration in the oil was highest for MAS-1 followed by Kalka as compared to all other cultivars studied (table 2).

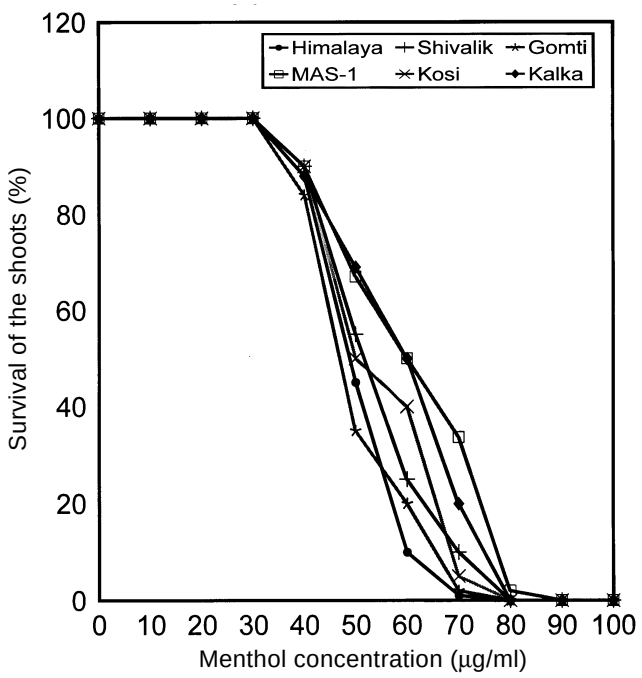

Figure 1. Survival of regenerants after 7 days at different concentrations of menthol in medium.

Considering the importance of menthol in commerce, it is desirable to develop variety(ies) with higher menthol content. Here, we explored the possibility of using a correlation between the menthol content in oil and the *in vitro* menthol tolerance of the genotypes for employing it as a selection strategy for evaluating the breeding material for menthol yield. The experiments showed a positive relationship between the menthol content in the oil of the genotypes and the survival frequencies of the regenerated shoots of different varieties on medium containing menthol as selection pressure. Pearson correlation coefficients were calculated between the menthol tolerance levels (survival frequencies) at different concentrations of menthol and the menthol yields in the essential oil of the tested cultivars. The survival kinetics in relation to menthol tolerance level of regenerated shoots for all the cultivars showed a definite positive corelation with the menthol content in the essential oils of the cultivars (table 3). Thus, it was obvious that the cultivars producing more amount of menthol in oil could tolerate higher amount of menthol under *in vitro* selection pressure meaning thereby that a definite corresponding relationship existed between the survival rate (menthol tolerance) and menthol content. In Japanese mint, the highly demanded monoterpene menthol is produced by the conversion of pulegone into menthone and menthone into menthol which is further convertible into menthyl acetate similar to the peppermint (Murray *et al* 1972). Earlier negative correlation between menthol and menthone on one hand, and menthol and menthyl acetate on the other has been reported (Kukreja *et al* 1991, 1992).

Plants are known to evolve mechanisms either to escape or detoxify the toxic substances whether produced by it or supplied externally into the system. In this case, menthol the major monoterpene component of the essential oil, which is known to be cytotoxic to the plant at higher concentrations, is expected to be produced up to a level that the cellular system can tolerate and thus limiting the yield levels. As a dependable mechanism for the utilization/ catabolism of the stored product upon need, it gets isolated in specialized tissue (trichomes). Thus, it is logical

Table 2. Oil and menthol yield of cultivars.

	Oil (%)			Menthol (%)		
	1st harvest	2nd harvest	Average	1st harvest	2nd harvest	Average
Himalaya	0·64 ± 0·06	0·70 ± 0·06	0·67	77·1 ± 1·4	78·0 ± 1·1	77·5
Shivalik	0·66 ± 0·09	0·53 ± 0·06	0·59	71·0 ± 3·8	80·1 ± 1·7	75·5
Gomti	0·65 ± 0·03	0·53 ± 0·03	0·59	67·6 ± 4·7	75·9 ± 4·2	71·7
MAS-1	0·491 ± 0·06	0·52 ± 0·041	0·50	77·4 ± 0·5	83·2 ± 1·0	80·3
Kosi	0·74 ± 0·04	0·57 ± 0·13	0·65	77·7 ± 1·3	75·9 ± 4·2	76·8
Kalka	0·60 ± 0·00	0·54 ± 0·05	0·57	77·0 ± 2·1	82·0 ± 2·1	79·5

Table 3. Correlation between menthol content and survival in the presence of menthol in poison agar medium.

Concentrations of menthol (μg ml^{-1})	Correlation coefficient	
	24 h	7 d
50	0·838	0·871
60	0·887	0·646
70	0·891	0·680
80	0·895	0·542

to anticipate that a genotype which can tolerate higher level of menthol may in turn be able to accumulate higher menthol by bypassing the feed back or end-product toxicity through the changed biochemical pathway(s) resulting from a corresponding genetic change at DNA level. This may possibly result from genomic rearrangements leading to lifting of feedback inhibition in combination with efficient mechanism for detoxification by excluding the toxic monoterpene to specialized tissues like trichomes. Thus, the level of menthol accumulated could be linked to the tolerance levels of different cultivars. The correlation established in this study has opened up the possibility to generate high menthol yielding line(s) from the well adapted variety(ies) by *in vitro* selection of clones tolerant to high menthol supplied exogeneously in the culture medium. This approach may be highly useful in the large-scale evaluation of breeder's material or germplasm in the genetic improvement programs.

Acknowledgements

We thankfully acknowledge the Department of Biotechnology and Council of Scientific and Industrial Research, New Delhi for the financial support.

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MS received 27 March 2000; accepted 17 July 2000

Corresponding editor: DEEPAK PENTAL