Original Papers Plant Tissue Culture Letters, **11**(3), 218-225 (1994)

Growth and Monoterpene Production in Shoot Culture and Regenerates of *Mentha arvensis*

Iwao ASAI*, Kunitoshi YOSHIHIRA*, Toshio OMOTO**,
Norihiro SAKUI** and Koichiro SHIMOMURA***

* *Division of Applied Life Sciences, Graduate School of Integrated Science and Art, University of East Asia*, 2-1 Ichinomiya-Gakuen, Shimonoseki 751, Japan
** *San-Ei Gen F·F·I. Inc.*, 1-1-11 Sanwa, Toyonaka, Osaka 561, Japan
*** *Tsukuba Medicinal Plant Research Station, National Institute of Health Sciences*, 1 Hachimandai, Tsukuba, Ibaraki 305, Japan

(Received June 8, 1994)
(Accepted June 23, 1994)

Axenic shoot culture of *Mentha arvensis* was established from young shoots of plants cultivated in a field. In the shoots cultured on Murashige-Skoog solid and liquid media for 3-4 weeks, pulegone was the main constituent in both cases, and the content of pulegone in liquid medium was much more than that on solid medium. The shoots cultured in the dark for 3 weeks produced only pulegone. However the shoots placed under 16 h/day light condition started to produce menthone and menthol together with pulegone which was the main compound. The plantlets obtained by culturing on hormone-free Murashige-Skoog solid medium at 25°C in 16 h/day light were transplanted to pots, and formed pulegone at the early cultivation stage, then menthol was detected as the main compound in the later cultivation stage at week 8.

Introduction

About 20 species of the genus *Mentha* (Lamiaceae family) are cultivated in the world, including well-known plants such as *Mentha arvensis*, indigenous to East Asia, *M. piperita*, indigenous to the Mediterranean coastal areas, and *M. spicata*, indigenous to Europe. *M. arvensis* grows to 30-60 cm in height, having 2. 5-3. 8 cm ovoid dentate leaves with a slightly acute tip[1]. This plant provides a starting material for perfumes and other essential oil products.

Monoterpenes produced by plants of the genus *Mentha*, specifically menthol, pulegone and menthone which are important flavoring substances, are commonly used in the food, cosmetic and pharmaceutical industries (**Fig. 1**). Menthol, in particular, is known to have analgesic, antipruritic and preservative action.

Callus proliferation and plantlet regeneration from adventitious roots and buds have been reported[2]. In addition to these findings, the effects of BAP concentration on the monoterpene production in the shoots of *M. arvensis* has been reported (Kawabe *et al.*[2,3]). Kim and Lee also demonstrated the difference of monoterpene production in the *M. piperita* callus cultured under various culture conditions[4]. M. J. Bricout and C. Paupardin reported the effects of liquid culture medium and light conditions on monoterpene production in the shoots regenerated from callus[5]. However, there are no reports on the productivity of monoterpenes in the shoots cultured on solid and in liquid media.

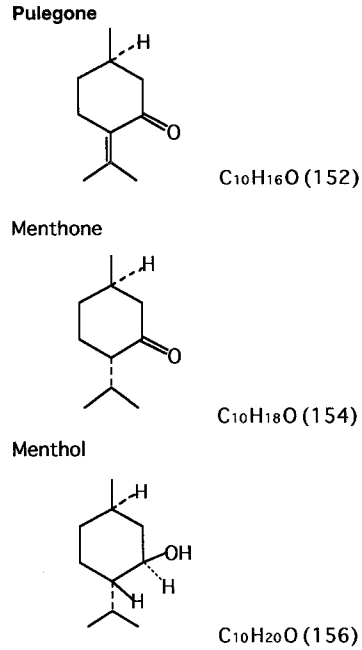

Fig. 1 Structures of monoterpenes (Molecular weight).

In this study, we describe the contents of menthol and the precursors[6] pulegone and menthone in the shoots of *M. arvensis* cultured on hormone-free solid and in liquid Murashige-Skoog[7] (MS) media and under different light conditions. In addition, we discuss the growth of the plants regenerated from shoots and cultivated in pots, and the change of the monoterpene yield in potted plants with correlation to chlorophyll formation.

Materials and Methods

1. *Plant materials*

About 2 cm apical buds of *Mentha arvensis* cultivated in a field was immersed in 75% ethanol for 30 seconds and washed once with sterile distilled water. Then they were put in NaClO (Tween 20, 1 drop/40 m*l*) for 10 min. and washed with sterile distilled water three times. Apical buds (*ca.* 5 mm) excised from the disinfected shoots were cultured on hormone-free Murashige-Skoog (MS) solid medium to obtain sufficient shoots. Apical and lateral buds (about 2 cm) of the axenic plantlets were subcultured at about 8 week intervals for about 7 years. About 7 mm apical buds of plantlets cultured on hormone-free MS medium were used for experiments.

2. *Preparation of media*

MS medium was employed to prepare solid and liquid media for experiments. The solid medium was prepared by adding Gelrite (0.2% w/v gellan gum for plant tissue culture) to MS medium, previously adjusted to pH 5.8, dispensing the mixture to test tubes (25 mm i.d. × 120 mm length, 20 m*l* medium), and autoclaved at 120°C for 15 min. The liquid medium was prepared by dispensing MS medium to 100 m*l* Erlenmeyer flasks (30 m*l*), and autoclaved at 120°C for 15 min.

3. *Culture conditions*

Shoots cultured either on solid or in liquid media at 25°C under different light conditions: 16 h/day light (4000 lux) or in the dark.

4. *Transplantation to soil and cultivation*

Plants obtained *in vitro* were transplanted to pots containing a mixture of soil, sand and leaf mold

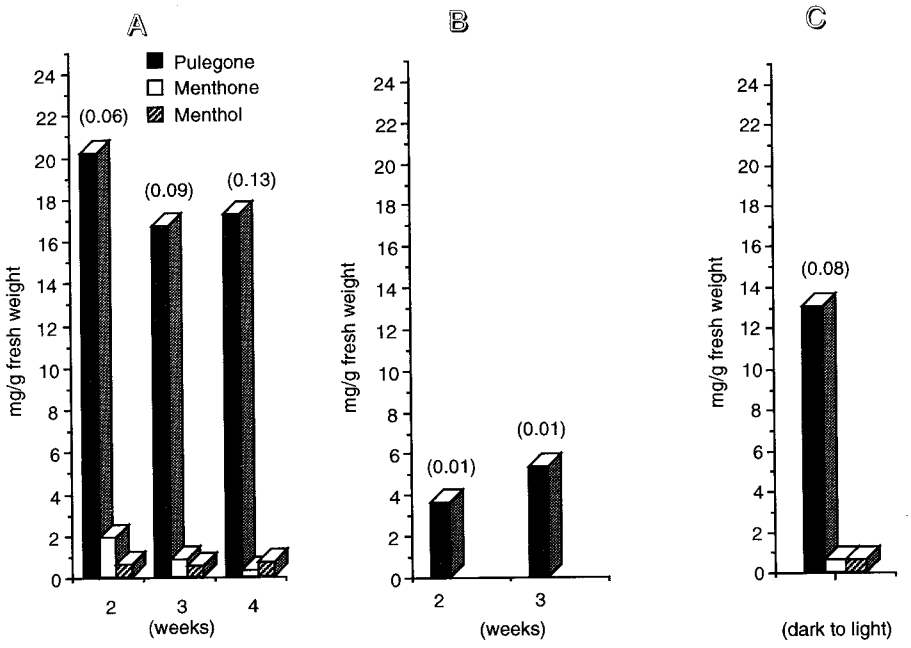

Fig. 2 Growth and monoterpene production in *Mentha arvensis* shoots cultured on hormone-free MS solid medium at 25°C.
A : Shoots were cultured in 16 h/day light. B : Shoots were cultured in the dark. C : After shoots were cultured in the dark for 3 weeks, they were placed under 16 h/day light for 1 week. Numbers in parentheses show fresh weight (g).

(5 : 1 : 1), and cultivated at 25°C (16 h/day light, 4000 lux).

5. *Monoterpene analysis*

After fresh weight measurement of cultured shoots (leaves and stems) and cultivated plants (leaves and stems), they were immediately frozen by liquid nitrogen and stored in a deep freezer (−20°C). Monoterpenes in diethylether extracts of these samples were determined by GC-MS. Ethyl heptanoate was used as an internal standard. The analytical conditions are as follows:

Gas chromatograph: HEWLETT-PACKARD 5890 A
Column: J & W DB-WAX (0.32 mm i.d.×60 m)
Column temperature: 60°C (5 min., hold) to 220°C, 3°C/min.
Injection port temperature: 250°C
Detection temperature: 250°C (hydrogen ionization-detector)
Injection volume: 1 μl (split ratio 70 : 1)
Mass analyzer: HITACHI M-80 B
Ionization: Electron bombardment (20 eV)

6. *Chlorophyll quantification*

Chlorophyll was determind spectrophotometrically by the method of Porra and Thompson[8].

Results and Discussion

1. *Shoot growth and monoterpene production on solid medium*

The shoots (*ca.* 7 mm length) cultured on hormone-free MS solid medium under 16 h/day light grew plantlets with emerged roots after 3 weeks of culture (93 mg fresh weight at week 3 and 133 mg fresh weight at week 4) (**Fig. 2-A**). On the other hand, in the dark, very thin and etiolated plantlets with no leaf development was observed, (10 mg fresh weight at week 3) (**Fig. 2-B**). The buds

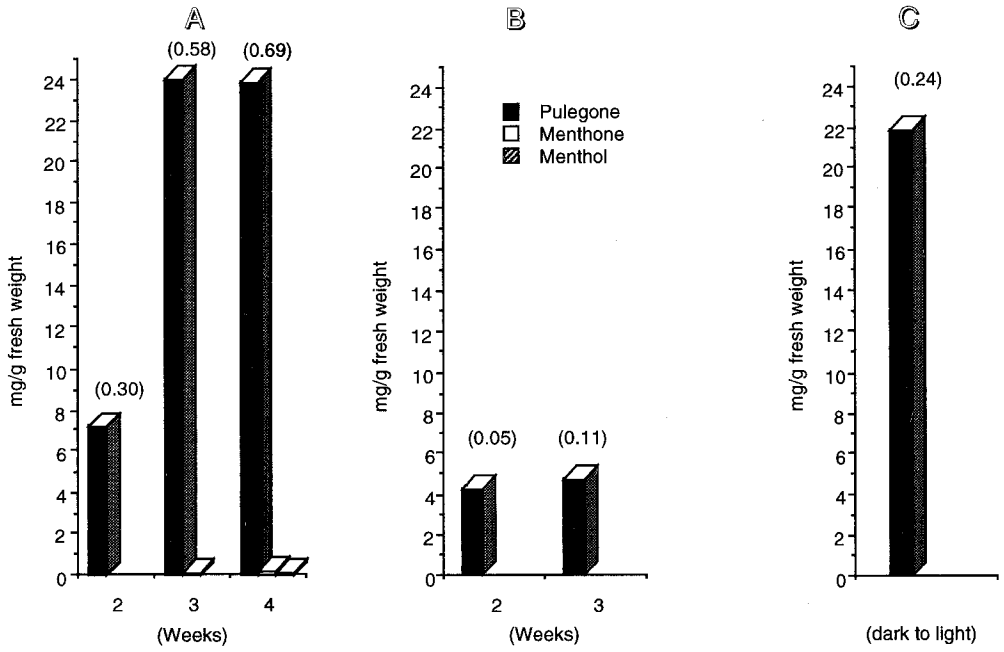

Fig. 3 Growth and monoterpene production in *Mentha arvensis* shoots cultured in hormone-free MS liquid medium at 25°C.
The explanation is the same as in **Fig. 2**.

cultured in the dark for 3 weeks first and then placed 16 h/day light for 1 week grew with leaf proliferation (76 mg fresh weight) (**Fig. 2-C**).

In the shoots cultured in 16 h/day light, about 1 mg/g fresh weight of menthol was produced, and the content of menthone decreased gradually (**Fig. 2-A**). In contrast over 17 mg/g fresh weight of pulegone was detected during the culture period. In the dark, only pulegone was produced, no menthone and menthol were detected by GC-MS analysis (**Fig. 2-B**). When the apical buds were cultured in the dark and then placed under 16 h/day light conditions, the leaf proliferation and its greening were observed. In addition small amounts of menthone and menthol were found in the green shoots at week 1 (**Fig. 2-C**). These results suggest that light irradiation is one of the critical factors for menthone and menthol production.

2. *Shoot growth and monoterpene production in liquid medium*

Growth and production of monoterpenes of *M. arvensis* shoots cultured in MS liquid medium were investigated. As shown in **Fig. 3**, under same conditions mention above, the shoot growth in MS liquid medium was remarkably greater than that on the solid medium. On the other hand, monoterpenes were detected in the shoots cultured in liquid medium and pulegone was the main constituent. Although the contents of pulegone in the shoots cultured on solid and in liquid media in the dark were almost the same, the shoots cultured in 16 h/day light showed the highest pulegone content (23. 8 mg/g fresh weight). In addition pulegone content in shoots cultured first in the dark and then in 16 h/day light was about 2-fold higher than that in the solid medium. However, the production of menthone and menthol decreased though the growth of shoots was improved in liquid medium.

3. *Time course of monoterpene production in liquid medium*

Since relatively large amounts of pulegone were produced in the liquid medium, growth of shoots and monoterpene production in the liquid medium were examined. Shoots were cultured in the

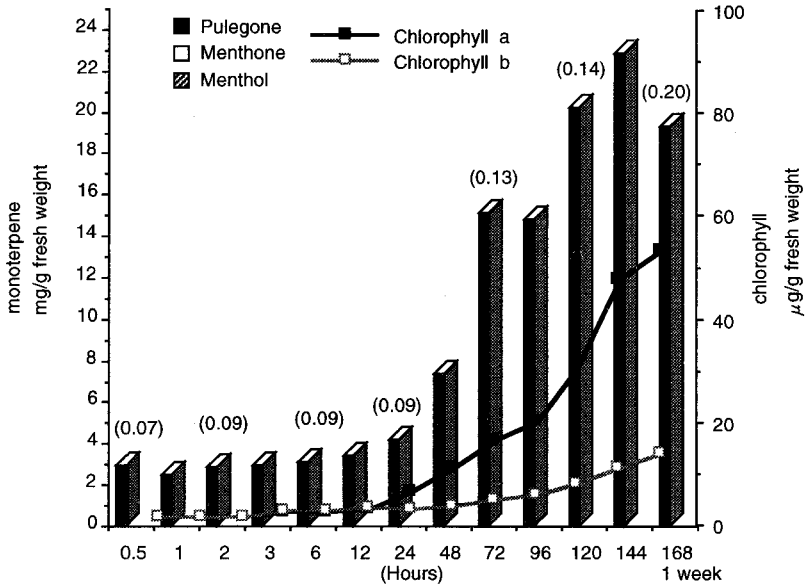

Fig. 4 Growth and monoterpene production and chlorophyll production in *Mentha arvensis* shoots cultured in hormone-free MS liquid medium at 25°C.
After shoots were cultured in the dark for 3 weeks, they were placed under 16 h/day for 1 week. Numbers in parentheses show fresh weight (g).

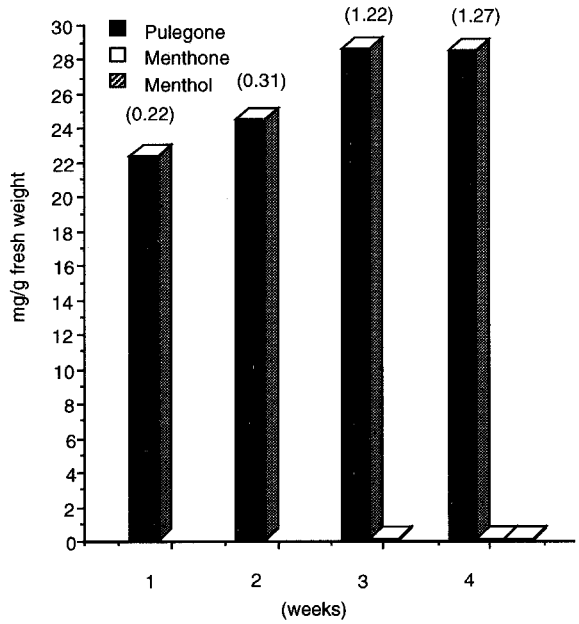

Fig. 5 Growth and monoterpene production in *Mentha arvensis* shoots cultured in hormone-free MS liquid medium at 25°C.
After shoots were cultured in the dark for 3 weeks, they were placed under 16 h/day light for 4 weeks. Numbers in parentheses show fresh weight (g).

dark for 3 weeks and then cultured under 16 h/day light, and contents of three monoterpenes were measured periodically. Menthone and menthol were not detected by GC-MS analysis even after 168 hours (**Fig. 4**). These results agreed well with the previous results (**Fig. 3**).

As shown in **Fig. 5**, further culture under 16 h/day light until the 4th week induced the production

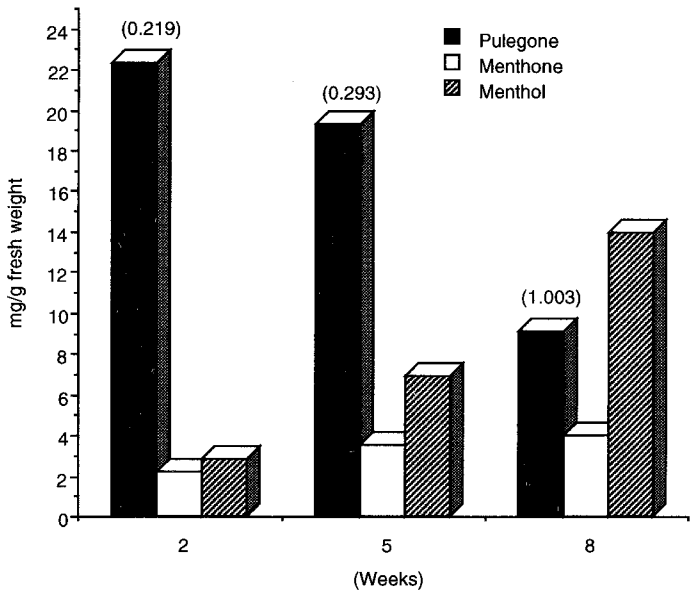

Fig. 6 Growth and monoterpene production in regenerates of *Mentha arvensis* cultivated in pots.
Plantlets were cultured on hormone free MS solid medium at 25°C under 16 h/day light for 3 weeks. Numbers in parentheses show fresh weight (g).

of small amounts of menthone and menthol, 0. 13 mg/g and 0. 02 mg/g fresh weight respectively, at week 3.

4. *Monoterpenes in plants cultivated in soil*

Despite the fact that the main component of *M. arvensis* cultivated in a field is normally menthol, pulegone was produced in much greater amounts than menthol in its shoots even when they were cultured 16 h/day light. Since this finding suggested the differences in monoterpene production between the cultures *in vitro* and the plants cultivated in soil, *M. arvensis* plantlets obtained by culturing *in vitro* for 3 weeks were transplanted to soil, and the monoterpenes examined. The fresh weight at week 2 after transplantation was 219 mg fresh weight, and it increased gradually until 8 weeks of cultivation (**Fig. 6**). The composition of monoterpenes in the plants cultivated in pots was determined by GC-MS. Pulegone was the predominant component until 5 weeks after transplantation, while menthol was produced gradually and became the main compounds at week 8. This results suggest that monoterpene production is significantly affected by differences in environment, such as culture *in vitro* and cultivation in soil. It can therefore be speculated that the stress due to pressure on roots, sterile conditions and cultivation conditions might affect on monoterpene production.

5. *Simultaneouse analysis of monoterpenes*

Table 1 shows the results of a simultaneous analysis of 13 monoterpenes (percent ratios to the total monoterpene content) in the shoots of plantlets cultured in liquid medium and regenerated plants cultivated in soil.

In the shoots of plantlets cultured on liquid media in the dark for 3 weeks, only pulegone was detected by GC-MS analysis, but no other monoterpenes were detected. When the plantlets were cultured for 3 weeks in the dark and then placed under 16 h/day light, the pulegone content was over 95% until 4th weeks, but menthone and menthol were detected at 0. 41% and 0. 33%, respectively. When the plantlets were cultivated in soil, the pulegone content remained as high as 80. 7% at week

Table 1. Monoterpenes in *Mentha arvensis* shoots and plantlets cultivated in MS liquid medium and in pots.

	Total reconstructed ion current (%)				
	Shoots cultured in liquid medium			Plantlets cuitivated in pots*2	
	dark for 3 wks	light for 1 wk*1	light for 4 wks*1	2 wks	8 wks
α-pinene	N. D.	0. 320	0. 364	0. 484	0. 402
β-pinene	N. D.	0. 320	0. 407	0. 522	0. 444
Sabinene	N. D.	0. 133	0. 192	0. 239	0. 217
Limonene	N. D.	2. 056	1. 160	1. 278	1. 098
Menthone	N. D.	N. D.	0. 414	6. 253	11. 35
Isomenthone	N. D.	N. D.	N. D.	0. 811	3. 285
Methyl acetate	N. D.	N. D.	0. 171	0. 322	3. 534
Isopulegone	N. D.	0. 643	1. 208	1. 139	0. 660
Neomenthol	N. D.	N. D.	0. 060	0. 322	1. 379
Neoisomenthol	N. D.	N. D.	N. D.	N. D.	N. D.
Menthol	N. D.	N. D.	0. 332	7. 487	50. 82
Pulegone	100. 0	96. 53	95. 33	80. 65	26. 17
Piperitone	N. D.	N. D.	0. 366	0. 496	0. 641

Ether extract of the leaves and stems of plant was analyzed by GC-MS.

*1 Shoots were cultured in hormone-free MS liquid medium in the dark at 25°C for 3 weeks and then placed under 16 h/day light.

*2 Shoots were cultured on hormone-free MS solid medium at 25°C in 16 h/day light for 3 weeks.

2 after transplantation, followed by menthol (7. 49%) and menthone (6. 25%), together with isomenthone detected in a small amount. When the plantlets were further cultivated until the 8 th week, the pulegone content declined rapidly to 26. 2%, while the menthol content increased to 50. 8%, with increase of menthone and isomenthone contents.

Of 13 monoterpenes analyzed, pulegone, menthone, menthol and isomenthone showed a significant change in content by changing the cultivation condition from *in vitro* to soil. This result demonstrates a definite difference in the production of monoterpenes, especially pulegone, menthone and menthol, between culture *in vitro* and cultivation in soil. This result agrees with the biosynthetic pathway from pulegone to menthone and menthol suggested by Kawabe *et al.*[3]. Since the amount of isomenthone was very low in leaves of cultivated plants, and no neoisomenthol was detected in them, the enzymes converting these monoterpenes from pulegone might not express in these plants. Further experiments will be required.

Conclusion

There are significant differences in monoterpene production in *M. arvensis* between culture *in vitro* and cultivation in soil. In the former case, the synthesis of pulegone, menthone and menthol might be regulated by some factors. The present results demonstrated that light is one of the essential factors for menthol production in *M. arvensis* shoot cultures. In addition pulegone, normally present in a trace amount in *M. arvensis* grown in a field, was produced in large amounts in cultures *in vitro*, especially in the dark. Taking into account the consideration of a great amount of pulegone in plantlets cultured in the dark, it is noteworthy that culture of *M. arvensis* may have a great potential for industrial production of pulegone, an important component of perfumes.

Acknowledgement

This work was supported by Ministry of Health and Welfare, Science Research Fund Subsidy granted to the Japan Health Science Foundation.

References

1) Evertt, T. H., 1981. The New York Botanical Garden Illustrated Encyclopedia of Horticulture, **7**: 2184-2186.
2) Kawabe, S., A. Watanabe, K. Watase, K. Murakami, K. Hosomi, 1993. Plant Tissue Culture, **10**: 184-187.
3) Kawabe, S., H. Fujiwara, K. Murakami, K. Hosomi, 1993. Biosci. Biotech. Biochem., **57**: 657-658.
4) Kim, J.-H. H.-J. Lee, 1992. J. Korean. Agric. Chem. Sci., **35**: 443-448.
5) Bricout, M. J., C. Paupardin, 1975. C. R. Acad. Sc. Parls., t 281(4, 11, 18, 25): 383-386.
6) Fujita, S., Y. Fujita, 1970. J. Agric. Chem. Soc. Japan., **44**: 293-298.
7) Murashige, T., F. Skoog, 1962. Physiol. Plant., **15**: 473-497.
8) Porra, R. J., W. A. Thompson, 1989. Biochim. Biophy. Acta., **975**: 384-394.

《和文要約》

Mentha arvensis のシュート培養及び再生植物体における生育とモノテルペン類生産

浅井以和夫*・義平邦利*・大本俊郎**・佐久井徳広**・下村講一郎***

* 東亜大学大学院 総合学術研究 応用生命化学
** 三栄源エフ・エフ・アイ株式会社
*** 厚生省 国立衛生試験所 筑波薬用植物栽培試験場 育種生理研究室

圃場栽培している *Mentha arvensis* の頂芽より，シュート培養系を確立した．得られたシュートを固形及び液体培地で3～4週間培養すると，これら培地における主成分はプレゴンであった．プレゴン生産量は，液体培養の場合が固形培養に比較して，数倍多かった．

一方，暗黒下で3週間培養した場合は，プレゴンのみが生成されたが，その後照明下で更に培養を続けると，プレゴンより少量であるが，メントン及びメントールの生成も認められた．増殖した幼苗を土壌に移植した結果，初期はプレゴンが主成分であったが，8週目にはメントールが主成分となった．