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Growth and Essential Oil Production in Shoot Culture and Regenerates of *Anthemis nobilis* L.

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Axenic shoot culture of *Anthemis nobilis* L. was established from young shoots of plants cultivated in a field. The shoots grew rapidly in hormone-free Murashige-Skoog liquid medium although they proliferated on the solid medium. In the shoots cultured in Murashige-Skoog liquid medium at 25°C, GC-MS analysis revealed that geranyl isovalerate was produced in greater amounts than angelates. In the roots of plantlets obtained from shoot cultures, angelates were not detectable by GC-MS, but higher content of geranyl isovalerate were detected. The plantlets obtained by culturing on hormone-free Murashige-Skoog solid medium at 25°C in 16 h/day light were transplanted to pots. At the early cultivation stage, levels of geranyl isovalerate and isobutyl angelate were the same. At the later cultivation stage (20 weeks), isobutyl angelate became the main compound, and the production of geranyl isovalerate decreased gradually.

Introduction

Camomile (Family: Compositae), indigenous to Northern Europe and West Asia, is cultivated throughout the world as a medicinal plant[1]. Two kinds of camomile; German or Hungarian one (*Matricaria chamomilla* L.) and Roman or English camomile (*Anthemis nobilis* L.) are known in general. *A. nobilis* grows to 30-60 cm in height, has fine dawn-like leaves and is used as medicinal substance or raw material for perfumes. This *A. nobilis* has been long used as a flavor or scent in camomile tea, bath products, candy, beverage, cigarettes, *etc.* Its essential oil is used as a fragrance in shampoos, soap, perfumes, *etc.* Furthemore, azulene, contained in the essential oil, has excellent medicinal value as an antiphlogistic.

In essential oil of *A. nobilis* obtained by field cultivation, more than 100 substances including 20 derivatives of angelates were identified by Bicchi *et al.*[2], Hasebe *et al.*[3], Klimes and Lamparsky[4]. They reported that angelates were about 60% of the total essential oil (main compound was isobutyl angelate) and angelic acid was assimilated by much ester, resulting in production of angelates. In addition, Hasebe *et al.*[3] reported that the substances having strong floral and fruity Camomile-like flavor were isobutyl angelate and isoamyl angelate. Thus considerable studies have been reported about the essential oil production of *A. nobilis* cultivated in soils, but there are few reports on the productivity of essential oil in shoot cultures. Recently, Fauconnier *et al.*[5] reported the difference in the biosynthetic capability between *A. nobilis* plants cultured *in vitro* and plants cultivated in *A. nobilis* in the field. In addition, Szoke *et al.*[6] reported on the production of essential oil by callus

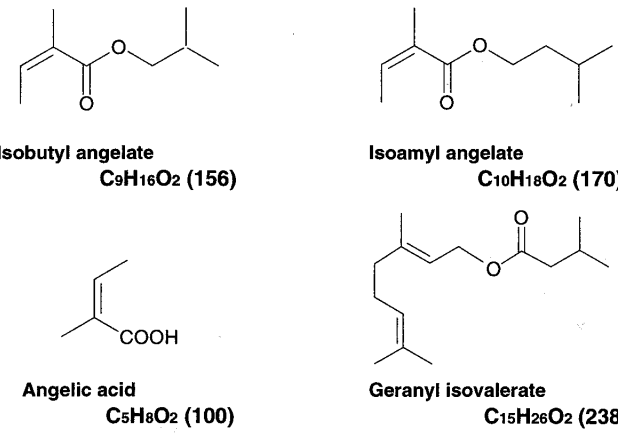

Fig. 1 Structures of essential oil components quantified by GC-MS.

cultures of *M. chamomilla* and comparison of its productivity under the light and the dark conditions. But, there are no reports on the productivity of essential oil in the *A. nobilis* shoots cultured under various light conditions.

In this study, we describe the essential oil production in the shoots and roots of *A. nobilis* cultured in hormone-free Murashige-Skoog liquid medium under different light conditions, because the light was one of the principal factors in the study of essential oil production in *Mentha arvensis* L. cultures described in our previous report[7]. The compounds analyzed by GC-MS are isobutyl angelate, isoamyl angelate, angelic acid and geranyl isovarelate (**Fig. 1**). In addition, we discuss the growth of the plants regenerated from shoots *in vitro* and cultivated in pots, and the change of the essential oil yield in potted plants.

Materials and Methods

1. *Plant materials*

About 2 cm apical buds of *Anthemis nobilis* cultivated in a field were immersed in 75% ethanol for 30 seconds and washed once with sterile distilled water. Then they were disinfected with 2% 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[8] (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. About 1 cm apical buds of plantlets cultured on hormone-free MS medium were used for experiments.

2. *Preparation of media*

MS medium was employed to prepare liquid and solid media for experiments. The liquid medium (30 m*l*) was prepared by dispensing MS medium (containing 3% saccharose) to 100 m*l* Erlenmeyer flasks after adjusting to pH 5.8, and autoclaved at 120°C for 15 min. The solid medium was prepared by adding 0.2% w/v Gelrite to MS medium and 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.

3. *Culture conditions*

Shoots cultured either on solid or in liquid medium were incubated at 25°C under different light conditions: 16 h/day light (4,000 lux) or in the dark.

4. *Transplantation to soil and cultivation*

Plants obtained *in vitro* were transplanted to pots containing the mixture of soil, sand and leaf mold (5 : 1 : 1), and cultivated at 25°C (16 h/day light, 4,000 lux).

5. *Essential oil analysis*

After fresh weight measurement of cultured shoots (leaves and stems) and their roots and cultivated plants (aerial parts) and their roots, these samples were immediately frozen by liquid nitrogen and stored in a deep freezer (−20°C). Essential oil in *n*-hexane extracts of these samples were determind by GC-MS. Ethyl heptanoate was used as an internal standard. The retention times of isobutyl angelate, isoamyl angelate, angelic acid and geranyl isovarelate were 15.64, 20.21, 36.53, and 41.91 min, respectively. The analytical conditions are as follows:

Gas chromatograph: HEWLETT-PACKARD 5890A
Column: J & W DB-WAX (0.25 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: HEWLETT-PACKARD 5970 (MSD)
Ionization: Electron bombardment (70 eV)

Results and Discussion

1. *Shoot growth and essential oil production in liquid medium*

The shoots (*ca.* 1.0 cm length) grew on hormone-free MS solid and liquid medium, showing lateral branching and rooting. However the shoots cultured in MS liquid medium grew more rapidly than

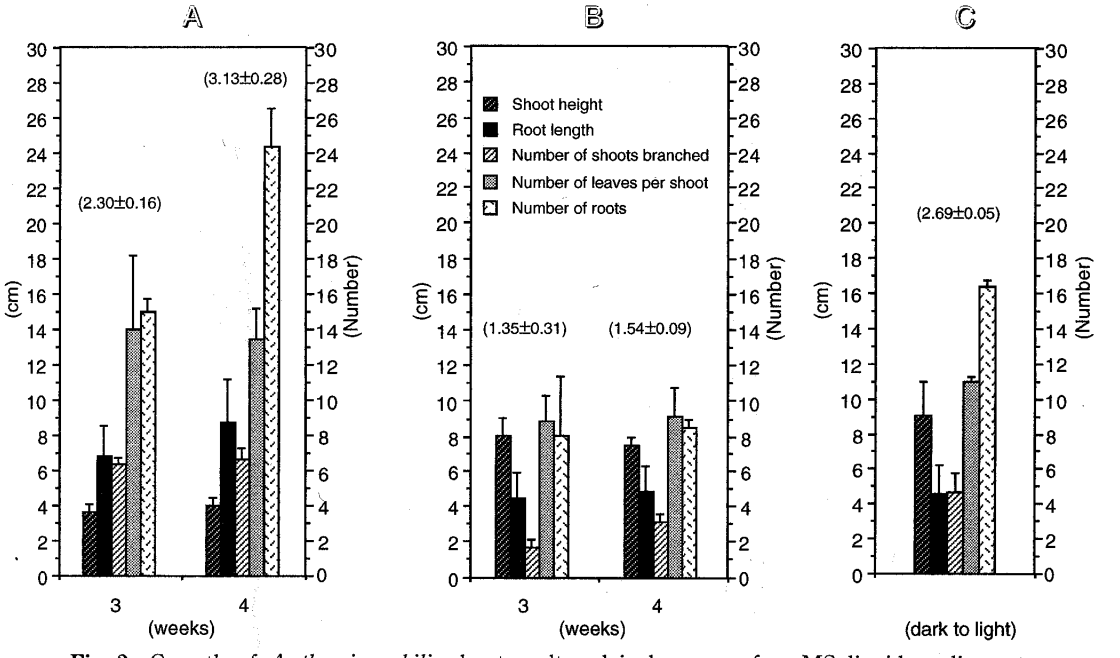

Fig. 2 Growth of *Anthemis nobilis* shoots cultured in hormone-free MS liquid medium at 25°C.

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.

* One shoot (*ca.* 1 cm) inoculated in 100 m*l* Erlenmeyer flask was cultured on a rotary shaker at 100 rpm.

Numbers in parentheses show fresh weight (g). Vertical bars indicate standerd deviation ($n=5$).

those on the solid medium (data not shown). In order to determine the suitable basal medium for the shoot growth, 4 media (MS, half strength of macroelement MS, Gamborg B5, Woody Plant) were tested. Among them MS medium was superior for shoot growth (data not shown). Therefore hormone-free MS liquid medium was employed for further experiments.

The shoots cultured in hormone-free MS liquid medium under 16 h/day light for 10 days grew into plantlets with more than 5 roots. The fresh weight of the plantlets was about 3 g after 4 weeks of culture, showing lateral branchings and many roots (**Fig. 2-A**). In the dark, tall and etiolated plantlets with about half the number of lateral branchings of those cultured in the light was observed at week 4, also the root growth was poor (**Fig. 2-B**). The shoot buds cultured in the dark for 3 weeks and then placed under 16 h/day light for 1 week exhibited intermediate growth when compared with the growth of the shoots cultured in 16 h/day light and in the dark for 4 weeks except for shoot height (**Fig. 2-C**).

As shown in **Fig. 3**, the production of four compounds analyzed by GC-MS in the shoots were affected by the light condition. In 16 h/day light, these four compounds were detected at almost the same levels at week 3 and 4. The etiolated shoots cultured in the dark produced geranyl isovalerate as the main compound. In addition, isoamyl angelate and angelic acid were not detectable even by GC-MS analysis. When the shoots were cultured in the dark and then placed under 16 h/day light, geranyl isovalerate was detected at 54 μg/g fresh weight as the main compound, and isobutyl angelate and angelic acid were detected at a less amount.

In the roots of plantlets obtained from shoot cultures, angelates were not detected by GC-MS

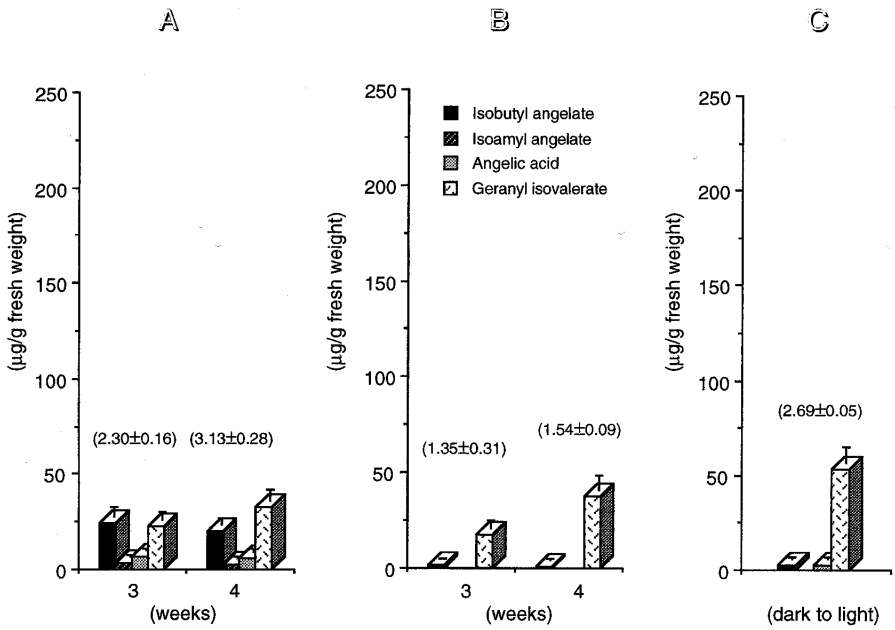

Fig. 3 Essential oil production of *Anthemis nobilis* shoots cultured in hormone-free MS liquid medium at 25°C.

A: Shoots* were cultured in 16 h/day light. B: Shoots* were cultured in the dark. C: After shoots* were cultured in the dark, they were placed under 16 h/day light for 1 weeks.

* One shoot (*ca.* 1 cm) inoculated in 100 m*l* Erlenmeyer flask was cultured on a rotary shaker at 100 rpm.

Numbers in parentheses show fresh weight (g). Vertical bars indicate standard deviation ($n=3$).

analysis. However geranyl isovalerate was produced at about 175 and 225 μg/g fresh weight at week 3 under 16 h/day light and dark conditions, respectively. The content in the roots was about 6-8 fold higher than that in the shoots.

In the shoots cultured on hormone-free MS solid medium, production of essential oil showed a similar tendency to that in the shoots cultured in the liquid medium, but the fresh weight was one fifth under 16 h/day light conditions and one eighth in the dark, and the growth was not satisfactory compared with that in the liquid medium.

These results suggest that geranyl isovalerate was produced in greater amounts than angelates in the shoot cultures. Furthermore, we demonstrated that geranyl isovalerate was specifically found in the roots.

2. *Time course of shoot growth and essential oil production in liquid medium*

Since a relatively large amount of geranyl isovalerate, that was not identified in essential oil analysis in the plants cultivated in the field, were produced in the shoots cultured in the liquid medium, the growth of shoots and the essential oil production in the liquid medium were investigated.

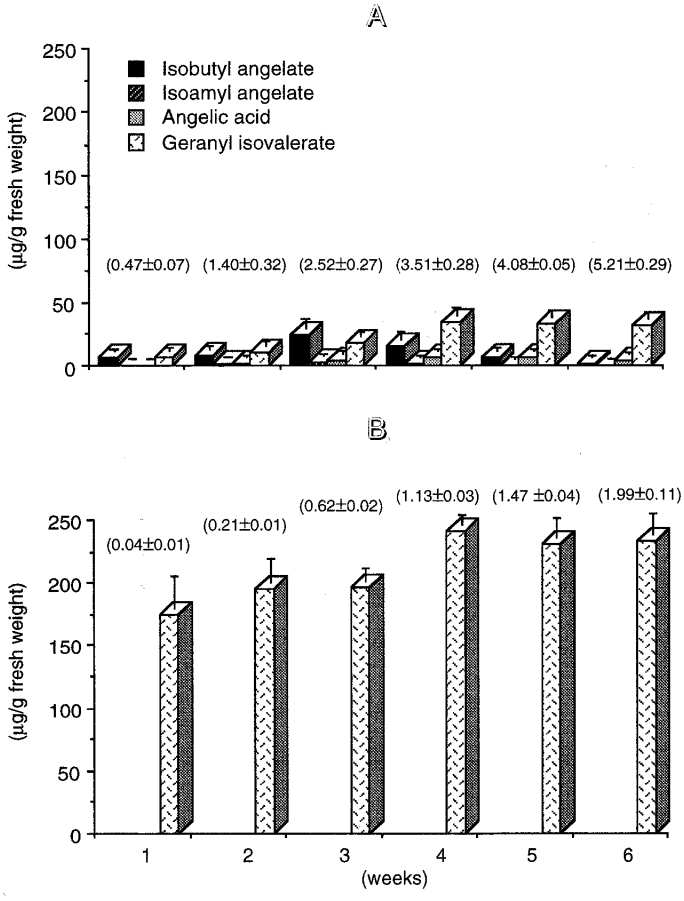

Fig. 4 Essential oil production of *Anthemis nobilis* shoots cultured in hormone-free MS liquid medium at 25°C under 16 h/day light for 6 weeks.
A: Essential oil content of shoots. B: Essential oil content of roots.
One shoot (*ca.* 1 cm) inoculated in 100 m*l* Erlenmeyer flask was cultured on a rotary shaker at 100 rpm.
Numbers in parentheses show fresh weight (g). Vertical bars indicate standard deviation ($n=3$).

The shoots cultured in hormone-free MS liquid medium under 16 h/day light for 6 weeks grew well, showing about 10 lateral branchings and about 25 roots. By GC-MS analysis in the shoots, geranyl isovalerate was detected as the main compound at the later culture stage (**Fig. 4-A**). After 5 weeks of culture, plants had grown to full size in the flask, and no Camomile-like odor was smelt. In the roots, only geranyl isovalerate was produced and that at a high content (**Fig. 4-B**).

In the dark, tall and etiolated plantlets were observed, and their root growth was poor. The etiolated plantlets placed under 16 h/day light for 3 weeks grew into plantlets with many lateral branchings (about 10) and roots (about 20). In the shoots cultured in the dark, only geranyl isovalerate was detected by GC-MS analysis. When the apical buds were cultured in the dark and then placed under 16 h/day light conditions, small amounts of isobutyl angelate was detected, and production of geranyl isovalerate increased rapidly (**Fig. 5-A**). In the roots, even in the dark only geranyl isovalerate was detected by GC-MS analysis (**Fig. 5-B**).

These results suggest that in the plantlets cultured *in vitro* geranyl isovalerate was produced higher than isobutyl angelate known as the main compound and characteristic flavoring substance of *A. nobilis*. It was also found that the light was indispensable for angelate production. Since the production of geranyl isovalerate increased remarkably when the light condition was changed from the dark to 16 h/day light, it is necessary to establish a suitable light condition for geranyl isovaler-

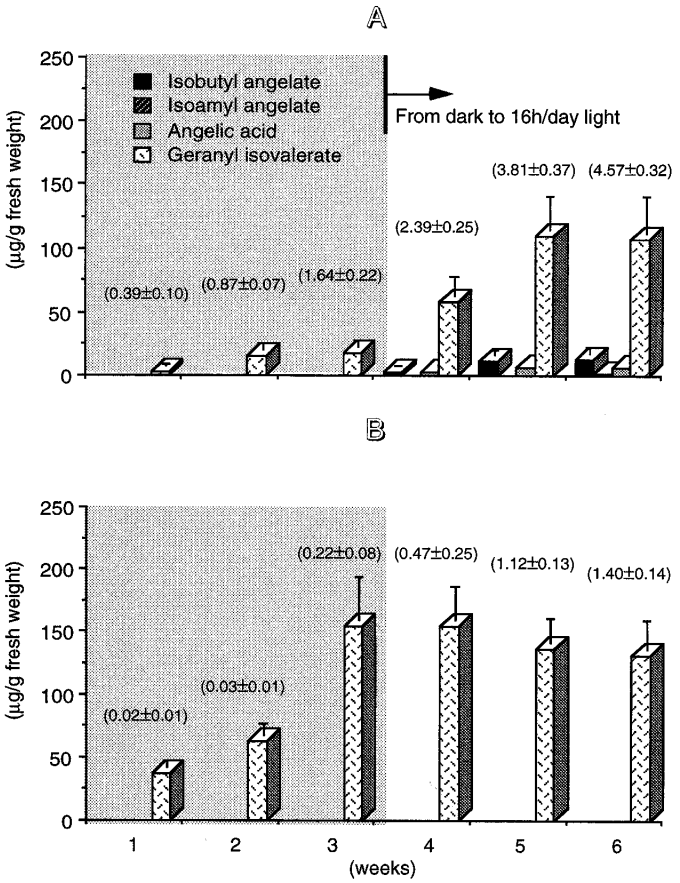

Fig. 5 Essential oil production of *Anthemis nobilis* shoots cultured in hormone-free MS liquid medium at 25°C.
After shoots were cultured in the dark for 3 weeks, they were placed under 16 h/day light for 3 weeks.
The explanation is the same as in **Fig. 4**.

ate production.

3. *Time course of essential oil production in plants cultivated in soil*

Despite the fact that the main compound of *A. nobilis* cultivated in a field is normally isobutyl angelate, geranyl isovalerate was produced in greater amounts than isobutyl angelate in *in vitro* shoots even when they were cultured under 16 h/day light. Since this finding suggested the differences in essential oil production between the cultures *in vitro* and the plants cultivated in soil, *A. nobilis* plantlets obtained by culturing *in vitro* for 3 weeks were transplanted to soil, and the essential oil production was investigated by GC-MS analysis. The fresh weight increased gradually until 20 weeks of cultivation (13 g fresh weight at week 20). In the shoots cultivated in soil, the content of isobutyl angelate increased and became the main compound at week 20 (about 150 μg/g fresh weight). The content of isoamyl angelate also increased gradually, whereas that of geranyl isovalerate decreased (**Fig. 6-A**). In the roots, geranyl isovalerate was produced in a large amount and it decreased gradually until 20 weeks, and isobutyl angelate was only detected in a small amount at week 20, although the other 2 compounds were not detected (**Fig. 6-B**).

These results suggest that, the essential oil production in *in vitro* cultures of *A. nobilis* was

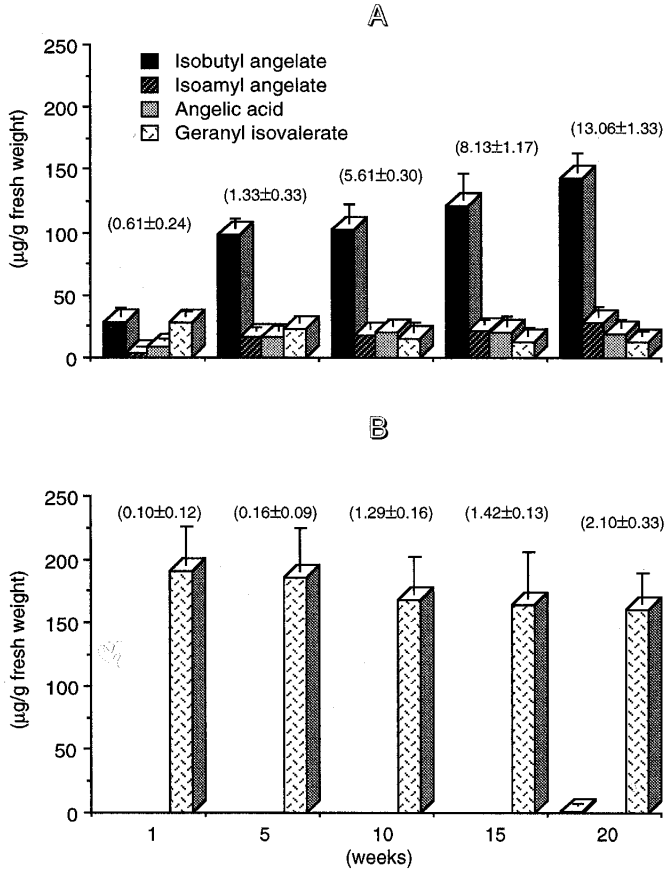

Fig. 6 Essential oil production of regenerates of *Anthemis nobilis* cultivated in pots at 25°C under 16 h/day light.
A: Essential oil content of shoots. B: Essential oil content of roots.
Plantlets cultured on hormone-free MS solid medium at 25°C under 16 h/day light for 3 weeks were transferred to the pots and cultivated.
Numbers in parentheses show fresh weight (g). Vertical bars indicate standard deviation ($n=3$).

Table 1. Essential oil composition of *Anthemis nobilis* shoots culture in MS liquid medium and plantlets cultivated in pots.

	Total reconstructed ion current (%)					
	Shoots cultured in liquid medium				Plantlets cultivated in pots*2	
	dark for 3 wks	light for 1 wk*1	light for 3 wks	light for 6 wks	5 wks	20 wks
Isobutyl angelate	1.35	1.98	9.88	1.22	15.84	21.88
Prenyl isobutyrate	N. D.	1.66	2.51	0.15	1.27	1.08
β-methallyl angelate	N. D.	0.92	3.28	0.14	4.98	8.22
Isoamyl angelate	N. D.	0.64	2.44	0.34	3.02	5.02
2-methyl butyl angelate	N. D.	1.30	4.29	0.63	6.17	8.25
3-methyl pentyl angelate	N. D.	1.70	4.35	0.92	12.17	15.91
Pinocarveol	N. D.	N. D.	1.93	0.89	2.29	4.71
Germacrene D	19.23	27.57	21.19	25.89	15.19	10.40
(E, E)-α-farnesene	49.03	44.80	38.60	57.10	32.16	21.23
Angelic acid	N. D.	0.77	0.78	0.63	4.93	2.26
Geranyl isovalerate	30.39	18.66	10.74	12.08	1.97	1.03

Hexane extract of the leaves and stems of plants 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 Plantlets were cultured on hormone-free MS solid medium at 25°C under 16 h/day light for 3 weeks.

different from that in the field cultivation, and it was confirmed that a large amounts of angelates were produced in the field cultivation, and geranyl isovalerate was produced in greater amount than angelates in the cultures *in vitro*. Therefore the essential oil production was significantly affected by the different environment, such as culture *in vitro* and cultivation in soil. In the regenerates cultivated in 16 h/day light for 20 weeks, tiglates were not detected though they were found in small amouns in the field-cultivated plants[3]. Further experiments will be required.

4. *Simultaneous analysis of essential oil*

Table 1 shows the results of the simultaneous analysis of 11 essetial oil constituents (percent ratios to the total essential oil content) in the shoots of plantlets cultured in liquid medium and regenerated plants cultivated in soil.

In the shoots cultured in the liquid medium, α-farnesene and geranyl isovalerate were detected in large amounts whereas angelates were detected in small amounts by GC-MS analysis. On the other hand, when the plantlets obtained by culturing on the solid medium were transplanted to pots, the content of geranyl isovalerate decreased rapidly and that of isobutyl angelate increased remarkably. At the later cultivation stage (20 weeks), isobutyl angelate became the main compound.

These results suggest that geranyl isovalerate, α-farnesene, isobutyl angelate and other angelates showed a significant change in content by changing the cultivation condition from *in vitro* to soil. It was considered that a large amount of geranyl isovalerate and α-farnesene might be produced in the culture *in vitro*, so that the biosynthetic capability might be strongly affected by cultivation condition and other environmental factors.

Conclusion

A. nobilis shoots cultured in MS liquid medium showed good growth, but the angelates, known as the main compound and chracteristic flavoring substance of *A. nobilis*, were produced in a small

amount. The shoots cultured *in vitro* produced geranyl isovalerate which was not detected in essential oil of plantlets cultivated in a field. In addition, it was found that the light was an indispensable factor for production of angelates but not for the production of geranyl isovalerate, and geranyl isovalerate was detected at the highest content in the roots without the light. The present results demonstrated that there were significant differences in biosynthetic capability between culture *in vitro* and cultivation in soil. There are reports on the essential oil production of *Eupatorium cannabinum*[9] and *Cymbopogon martinii*[10] that accumulate geranyl isovalerate, but there are no reports on the biosynthetic route. Further studies based on the results obtained in this report are expected for clarification of the biosynthesis of these essential oil.

Acknowledgement

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《和文要約》

Anthemis nobilis L. のシュート培養及び再生植物体における生育と香気成分生産

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圃場栽培している *Anthemis nobilis* L. 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液体培地で培養し GC-MS 分析を行うと，*A. nobilis* の主成分であり，特徴的な香気成分として知られている angelate 類の生産量は少なく，栽培系とは異なり高含量で geranyl isovalerate が生産された．また，geranyl isovalerate は，培養で得られた幼植物体の根部において多量に生産されることが分かった．増殖した幼植物体を土壌に移植した結果，geranyl isovalerate の含量は徐々に減少し，angelate 類の含量が急激に増加して 20 週目において isobutyl angelate が主成分となった．よって，培養系と栽培系での環境条件の変化から，香気成分生産に大きな違いが引き起こされることが判明した．