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GERANYL ISOVALERATE ACCUMULATION IN ADVENTITIOUS ROOT CULTURE OF *ANTHEMIS NOBILIS*

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Key Word Index—*Anthemis nobilis*; compositae; angelates; geranyl isovalerate; root culture; shoot culture.

Abstract—Adventitious root cultures of *Anthemis nobilis* were established from roots of *in vitro* plantlets. GC-MS analysis of the n-hexane extract of the root cultures revealed that they accumulated geranyl isovalerate, a compound which is generally not detectable in parental plants, and negligible amounts of other constituents. Geranyl isovalerate was also detected in the aerial parts of *in vitro* plantlets but not in shoot cultures having no roots.

INTRODUCTION

Roman chamomile, *Anthemis nobilis* L. is native to northern Europe and West Asia, and is one of the important medicinal herbs cultivated in the world. Its flowers have been long used for medicinal purposes and its essential oil, usually obtained from the entire aerial parts, is used as a fragrance in shampoos, soap and perfumes. In addition, azulene present in the essential oil has excellent medicinal value because of its anti-inflammatory property [1].

Many phytochemical studies have been reported on the essential oil of *A. nobilis* grown in the field and over 100 substances have so far been identified. In these reports, it has been demonstrated that angelates comprise approximately 65% of the total essential oil with isobutyl angelate predominating (30% of essential oil) [2–4]. However, there are a few reports on the production of essential oil by plant tissue cultures. Recently, Fauconnier *et al.* reported on the difference in the constituents of essential oil from field-cultivated and *in vitro* cultured *A. nobilis* [5, 6].

In a previous study, we established shoot cultures of *A. nobilis* and analyzed their essential oil constituents by GC-MS [7]. We found that the aerial part of the *in vitro* plantlets contained less angelates but contained geranyl isovalerate which could not be detected in field-grown plants. Furthermore, we found that this compound was specifically accumulated in the root part of *in vitro* plantlets.

In this study, we established adventitious root cultures of *A. nobilis*. In addition, we also established shoot cultures having no roots for comparison of their productivity with adventitious root cultures and *in vitro* plantlets.

The constituents of essential oil analyzed were isobutyl angelate (**1**), isoamyl angelate (**2**) (the main constituent in *A. nobilis*), angelic acid (**3**) (which is easily converted into angelates in oil), and geranyl isovalerate (**4**) (found in *in vitro* cultures).

RESULTS AND DISCUSSION

Effect of auxin on growth and essential oil production by adventitious root culture

For establishment of adventitious root cultures, adventitious roots were cultured in MS liquid medium [8] containing IAA (1 mg/l), NAA (0.5 mg/l) or IBA (0.1 mg/l) for 5 weeks. The roots proliferated satisfactorily in all cultural conditions, especially in the medium containing NAA (0.5 mg/l) whether under 16 hr light or in the dark (1.11 g fr.wt under 16 hr light and 1.24 g fr.wt in the dark) (Table 1). In the dark, thin and long white roots were formed, while green roots were formed under 16 hr light. However, two morphologically distinct types of roots were produced under 16 hr light. Thus thin and short roots were produced in the presence of NAA, and thick and long roots in the presence of IAA and IBA.

In the roots, **4** was detected as the sole constituent. Its yield was, on the whole, higher in the roots grown under 16 hr light than those in the dark and the type of auxin had more effect on the yield under light

Table 1. Effect of auxin on growth and essential oil production of *A. nobilis* adventitious roots cultured in MS liquid medium at 25° for 5 weeks.

Conditions	Auxin (mg/l)	Fr. wt (g)	Geranyl isovalerate* (μg/flask)
16 hr light	IAA (1.0)	0.72 ± 0.09	140.21 ± 7.18
16 hr light	NAA (0.5)	1.11 ± 0.05	49.05 ± 1.14
16 hr light	IBA (0.1)	0.52 ± 0.06	92.39 ± 1.43
Dark	IAA (1.0)	0.35 ± 0.15	43.59 ± 1.41
Dark	NAA (0.5)	1.24 ± 0.14	40.39 ± 15.98
Dark	IBA (0.1)	0.50 ± 0.01	35.41 ± 6.78

*Isobutyl angelate, isoamyl angelate and angelic acid were undetectable by GC-MS

conditions (Table 1). Contrary to the growth characteristics, the highest yield was obtained in the roots cultured with IAA under 16 hr light. It is noteworthy that not only the light condition affected the yield of **4**. Thus the thick and long roots generated in the presence of IAA or IBA yielded more of **4** than thin and short roots generated in the presence of NAA. Thus the accumulation and/or biosynthesis of **4** seems to be associated with root morphology.

Growth and essential oil production by adventitious root culture in various liquid media

Since the highest yield of **4** was obtained in the roots cultured in the IAA (1 mg/l)-containing medium, the effect of basal medium (MS, 1/2 MS, B5 [9] and WP [10]) was investigated by using IAA (1 mg/l) as auxin (Table 2).

The roots grew better under 16 hr light than in the dark except when grown on B5 medium. Best root growth (0.94 g fresh weight) was obtained in 1/2 MS liquid medium after 5 weeks of culture.

Table 2. Growth and essential oil production of *A. nobilis* adventitious roots cultured in various liquid media containing 1 mg/l IAA at 25° for 5 weeks.

Conditions	Medium	Fr. wt (g)	Geranyl isovalerate* (μg/flask)
16 hr light	MS	0.75 ± 0.09	147.58 ± 10.97
16 hr light	1/2MS	0.94 ± 0.12	188.62 ± 6.54
16 hr light	B5	0.63 ± 0.09	84.41 ± 1.42
16 hr light	WP	0.86 ± 0.22	94.35 ± 28.74
Dark	MS	0.66 ± 0.08	32.96 ± 10.88
Dark	1/2MS	0.52 ± 0.05	16.71 ± 5.66
Dark	B5	0.69 ± 0.09	34.50 ± 2.40
Dark	WP	0.68 ± 0.16	68.97 ± 9.77

*Isobutyl angelate, isoamyl angelate and angelic acid were undetectable by GC-MS

4 was the main constituent in the roots and its yield was much higher in the roots grown under 16 hr light than those in the dark. Accompanying the growth, the highest yield of **4** was obtained in the roots cultured in 1/2 MS liquid medium under 16 hr light. These were deemed to be the optimum culture conditions for the production of **4**.

Time course of growth and essential oil production by adventitious root culture

The root segments were cultured in 1/2 MS liquid medium containing IAA (1 mg/l) and growth and essential oil production were periodically determined (Fig. 1).

Adventitious roots began to proliferate after about 1 week of culture and several elongated roots (5–10 mm) were observed after 2 weeks of culture. After 2 to 3 weeks of culture, the proximal parts of the roots began to turn green and the roots grew more rapidly resulting in a 50-fold fr.wt increment (1.1 g) after 6 weeks of culture compared with after 1 week of culture (0.02 g).

The roots accumulated only **4**, the yield of which increased until 6 weeks of culture resulting in 200 μg/flask (Fig. 1).

Shoot growth and essential oil production in liquid medium

In our previous study, we found that *A. nobilis* shoot cultured in MS liquid medium showed good growth and small amounts of **4** (*ca.* 25 μg/g fr.wt) were produced in the shoot part of the *in vitro* plantlets, and large amounts of **4** (*ca.* 200 μg/g fr.wt) were produced in the root [6]. In addition, in this study, we found that the **4** was specifically detected in adventitious root cultures. Therefore, a shoot culture with no root system was established to confirm the biosynthetic capability of the shoot part for producing **4**.

Shoot cultures having no roots were obtained by culturing apical shoot in MS liquid medium with BAP (0.5 mg/l). However, some shoot cultures were vitrified and their growth was suppressed. Low yields of **1** and **2** were detected in unvitrified shoots by GC-MS analysis, but **4** was not. In vitrified shoots, somewhat surprisingly, no essential oils were detected by GC-MS analysis (Table 3).

In this study, we found that shoot cultures having no roots do not produce **4**. Thus the root is the organ which specifically biosynthesizes **4**, since rooted shoots contained it, whereas shoots without roots did not. The **4** biosynthesized in the root part of rooted shoots is presumably transported into the aerial parts.

EXPERIMENTAL

Plant materials

Shoots of *Anthemis nobilis* (*ca.* 2 cm length) grown in a field were immersed in 75% EtOH for 30 sec and

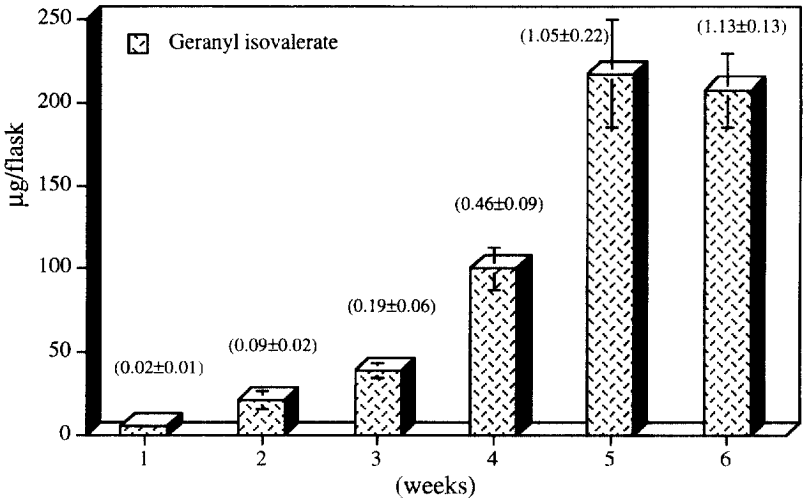


Fig. 1. Growth and essential oil production of *A. nobilis* adventitious root cultured in 1/2 MS liquid medium with 1 mg/l IAA. Vertical bars indicate standard deviation ($n = 3$). Numbers in parentheses show fresh weight (g).

Table 3. Growth and essential oil production of *A. nobilis* shoots cultured in MS liquid medium at 25° for 3 weeks

Plant conditions	Cytokinin (mg/l)	Fr. wt (g)	Isobutyl angelate (μg/flask)	Isoamyl angelate (μg/flask)	Angelic acid (μg/flask)	Geranyl isovalerate (μg/flask)
Unvitrified	BAP (0.5)	3.62±0.35	31.72±9.15	5.02±2.29	N.D.*	N.D.
Vitrification	BAP (0.5)	2.35±0.55	N.D.	N.D.	N.D.	N.D.
Shoots of plantlet	Hormone-free	2.25±0.27	62.04±18.19	7.18±3.35	11.23±3.81	46.19±3.81

*N.D. shows not detectable by GC-MS.

rinsed once with sterile distilled water. Then they were surface-sterilized in 2% NaClO containing Tween 20 (1 drop/40 ml) for 10 min and washed with sterile distilled water three times. Apical buds (*ca.* 5 mm length) excised from the disinfected shoots were cultured on hormone-free MS solid medium to give the axenic plantlets. Apical and lateral buds (*ca.* 2 cm) of the axenic plantlets were subcultured at 8-week intervals. The roots from *in vitro* plantlets were cultured in MS liquid medium [8] containing IAA (1 mg/l) to give adventitious roots. The three distal roots (*ca.* 2 cm) of the adventitious roots were subcultured at 8-week intervals. For the experiment with adventitious root culture, three distal adventitious roots (*ca.* 2 cm) were inoculated into liquid medium. For the experiment with shoot cultures, one apical shoot (*ca.* 1 cm) was inoculated into liquid medium.

Preparation of media

Liquid medium (30 ml, 3% sucrose) was dispensed into 100 ml Erlenmeyer flask after adjusting to pH 5.8 with dil KOH, and autoclaved at 120° for 15 min.

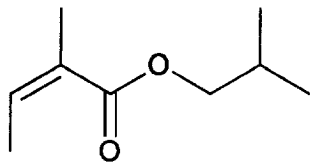
For induction and culture of adventitious roots, auxin (1.0 mg/l IAA, 0.1 mg/l IBA or 0.5 mg/l NAA) was added to the liquid medium. To maintain shoot cultures having no roots, 0.5 mg/l BAP was added to the liquid medium.

Culture conditions

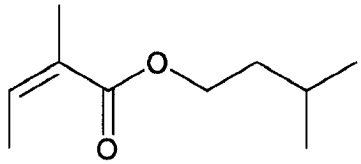
Adventitious roots and shoots were cultured on a rotary shaker (100 rpm) at 25° either under 16 hr light (4000 lux) or in the dark.

Essential oil analysis

After fr. wt measurement of cultured roots and shoots (leaves and stems), they were immediately frozen in liquid N_2 and stored in the deep freezer. Constituents of essential oils in *n*-hexane extracts of these samples were determined by GC-MS by using ethyl heptanoate as an int. standard. The analytical conditions are as follows: GC (HEWLETT-PACKARD 5890A), Column: J&W DB-WAX(0.25 mm i.d. × 60 m), Column temp.: 60° (5 min. hold) to 220°, 3°/min. Injection port temp.: 250°, Detection temp.: 250° (hydrogen ionization-detector), Injection vol: 1 μl (split ratio 70:1), Mass analyzer: HEWLETT-PACKARD 5970 (MSD), Ionization: Electron bombardment (70 eV).



1
$C_9H_{16}O_2$ (156)*



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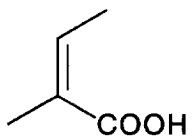

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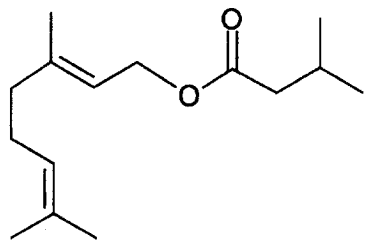
$C_5H_8O_2$ (100)



4
$C_{15}H_{26}O_2$ (238)

*Numbers in parentheses show Mr.

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