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MONOTERPENE BIOSYNTHESIS BY CALLUS TISSUES AND SUSPENSION CELLS FROM *PERILLA* SPECIES

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Key Word Index—*Perilla frutescens* var. *crispa*; Labiatae; callus tissue; biosynthesis; monoterpenes; perillaketone and isoegomaketone.

Abstract—The volatile oils from both the callus tissues and the suspension cells of *Perilla frutescens* Britton var. *crispa* Deane (Taiwan Aoshiso) were analysed by GC and GC/MS. Major components found in the original plant leaves were also produced by *de novo* biosynthesis in the callus tissues and the suspension cells. The most abundant monoterpenes were limonene, linalol, perillaketone and isoegomaketone.

INTRODUCTION

The callus tissues of many medicinal plants biosynthesize biologically active substances [1]. Monoterpenes, which are responsible for the important odours in the food and purfume industries, are produced by several cultured tissues and suspension cells [2–5]. In previous work it was found that the callus tissues or the suspension cells of *Perilla frutescens* (Taiwan Aoshiso) biosynthesized monoterpenes, such as linalol and limonene. [5]. However, several compounds remained unidentified. Thus, in this work, monoterpenes from the callus tissues and the suspension cells were reinvestigated. It was found that the callus tissues and the suspension cells used here possessed the potency to biosynthesize monoterpenes which were found as the prominent components in the volatile oil from the original plant.

Table 1. Volatile components from the leaf of *Perilla frutescens*

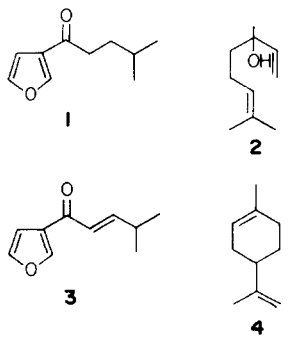
Peak No.	Identity	Peak area (%)	Retention time (min)
1	β-Pinene	0.07	2.86
2	Limonene	0.11	3.35
3	3-Hexen-1-ol	0.88	3.43
4	*p*-Cymene	0.01	4.14
5	*p*-Cymenene	0.04	5.05
6	3-Hexen-1-ol	0.16	5.42
7	3-Octanol	0.11	5.58
8	1-Octen-3-ol	0.51	6.55
9	—*	0.19	7.57
10	Benzaldehyde	0.04	8.05
11	Linalol	5.60	8.52
12	Copaene	0.06	9.10
13	β-Elemene	6.18	9.56
14	—*	1.42	11.41
15	—*	2.32	11.85
16	Caryophyllene	2.68	12.33
17	—*	0.58	12.91
18	Perillaketone	69.17	14.09
19	Isoegomaketone	1.21	16.21
20	α-Ionone	0.26	16.69
21	—*	0.36	17.75
22	—*	0.17	20.73
23	Eugenol	0.10	22.45
24	Myristicin	0.15	24.77
Total		92.83	—

*Unidentified sesquiterpene.

RESULTS AND DISCUSSION

Volatile components from the original plant leaves

The volatile components were separated from the original plant leaves [6] and investigated by GC and GC/MS. The components were identified as shown in Table 1. The most prominent compound in the oil was perillaketone (**1**). Other major components were β-elemene (6.18%), linalol (**2**) (5.60%), caryophyllene (2.68%) and unidentified sesquiterpenes [M^+ at m/z 204 and 204 (1.42 and 2.32%), respectively]. The other monoterpenes identified were isoegomaketone (**3**) (1.12%) and limonene, (**4**) (0.11%). Eugenol (0.11%) and myristicin (0.15%) were found as the phenylpropanoids. From the chemosystematic point of view, plants belonging to *Perilla* sp. are conveniently divided into three categories, namely, those which produce (a) perillaldehyde, (b) isoegomaketone and perillaketone and (c) perillaketone, naginataketone and ersholtzione [7, 8]. It is clear that *P. frutescens* (Taiwan Aoshiso) belongs to category (b).



Growth of the callus tissues

The callus tissues were induced from the young leaves on the surface of Murashige and Skoog agar medium with the addition of (2,4-dichlorophenoxy)acetic acid (2,4-D) and kinetin as growth regulators. The typical growth of the callus tissues is illustrated in Fig. 1. After 4 weeks with limited illumination at 25°, an inoculum of the callus tissues (1.0 g, wet wt) on the agar surface with 2,4-D (1.0 ppm) and kinetin (5.0 ppm) yielded 11.1 g (wet wt) of the callus tissues. The growth of the suspension cells was the same as described in the previous report [5].

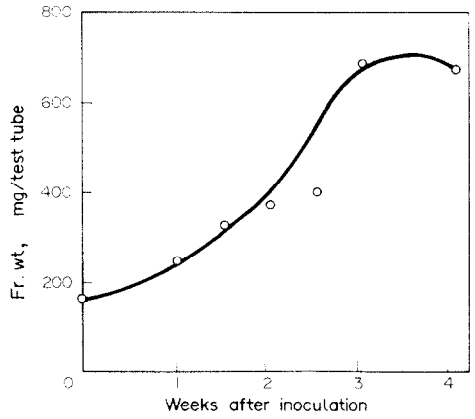


Fig. 1. Growth of the callus tissues, on Murashige and Skoog agar medium (10 ml) with 2,4-D (1.0 ppm) and kinetin (5.0 ppm).

Monoterpenes from the callus tissues

Monoterpenes from the callus tissues, which were subcultured three times from the explant, and from the suspension cells were obtained by steam distillation. The yield of the volatile oil was 23 mg from 12.0 g (wet wt) of the callus tissues. The volatile components were analysed by GC (Table 2) and by GC/MS. The volatile constituents from both the callus tissues and the suspension cells were essentially the same as those from the original plant leaves. Compounds identified were limonene (**4**), linalol (**2**), perillaketone (**1**) and isoegomaketone.

The callus tissues and the suspension cells used in this work possessed the ability to produce the monoterpenes found in the original plant leaves. The callus tissue (10–50 g) was obtained from one explant (2.6 mg) by three subcultures. Monoterpene biosynthesis was also observed in the callus tissues which were subcultured at least five times. The volatile oil in 12 g of the callus tissue at the third generation, which would have been 'carried over' from the explant was calculated to be in the order of 0.5 to 3.1 μg. This compares with 23 mg of the total volatile components actually obtained. The suspension cells were obtained from the eighth generation of the callus tissue. These facts indicate that monoterpenes were biosynthesized in the callus or the suspension cells. The callus tissues used in this study might offer a good experimental system for the study of monoterpene biosynthesis or catabolism.

EXPERIMENTAL

The *Perilla* sp. used in this work, *Perilla frutescens* Britton var. *crispa* Deane, was a *Perilla* cv adapted for cultivation in a warm climate. Similar varieties are seen in various part of Japan and Taiwan [9]. The seeds of this plant were a gift from Dr. Koresawa, Ehime Agricultural Station, Matsuyama, Japan. The seeds were germinated and grown in pots for 3 months.

Callus induction and growth. The leaf callus was derived from the young leaves of Taiwan Aoshiso. The callus tissues were induced from 5 mm^2 of explant on the agar surface (0.9% Difco Bactoagar) of Murashige and Skoog basal medium containing sucrose (30 g/l.). Various combinations of the growth regulators were added to the medium, namely, 2,4-D, 0, 0.1, 1.0, 2.0, 4.0, 6.0 and 8.0 ppm and kinetin 0, 0.1, 1.0 and 5.0 ppm. No induction was observed without any growth regulators. Good results were obtained, when (a) 2,4-D (1.0 ppm) and kinetin (5.0 ppm), (b) 2,4-D (2.0 ppm) and kinetin (1.0 ppm) or (c) 2,4-D (1.0 ppm) and kinetin (1.0 ppm) were added. After the induction of the callus, the callus tissues were subcultured every 4 weeks onto Murashige and Skoog agar medium with 2,4-D (1.0 ppm) and kinetin (5.0 ppm) at 25° under subdued illumination (fluorescence lamp, 1500 lx). Growth was measured on 10 ml of the agar medium in 18 × 1.8 cm (i.d.) test tubes by using the callus of the third generation. Weights of inocula used were *ca* 0.17 g.

Wet wts of the callus tissues were measured every 84 hr after inoculation for 4 weeks. The suspension culture was prepared using the eighth generation of the callus in a similar medium but without agar [5].

Extraction of the volatile oils. The callus tissues (12.0 g) were immersed in 100 ml CH_2Cl_2 at 5° for 24 hr. The extract was concd to 20 ml and steam-distilled at atm pres. for 10 min to yield the volatile oil (23 mg). The volatile oil (1 mg) was separated from the suspension cells (10.2 g, wet wt) in the same manner.

The volatile oil from the original plant leaves was separated by a simultaneous distillation and extraction method [6]. The leaves (50.0 g) were homogenized in H_2O (600 ml) at *ca* 0° with a blender for 5 min. The resulting soln was transferred into the

Table 2. Volatile components from suspension cells of *Perilla frutescens*

Peak No.	Identity	Peak area (%)	RR_t* (A)†	RR_t* (B)‡
2	Limonene	21	0.67 (0.66)	1.93 (1.85)
11	Linalol	trace	1.24 (1.20)	2.09 (1.96)
18	Perillaketone	48	1.63 (1.56)	2.85 (2.91)
19	Isoegomaketone	6.5	1.86 (1.76)	3.11 (3.09)

*Relative to cyclohexanol; values in parentheses are the relative retention times of authentic samples.

†(A) 10% Carbowax 20 M.

‡(B) 10% Apiezon L (5% Igepal).

apparatus for simultaneous distillation and extraction. The volatile oil was obtained by distillation and extraction with 30 ml Et_2O for 90 min. After concn, *in vacuo*, 190 mg of the volatile oil was obtained.

The volatile components were analysed by GC. The volatile oils from the callus tissues and the suspension cells were separated on a 5 m × 3 mm (i.d.) stainless steel column packed column of 10% Carbowax 20 M and on a 3 m × 3 mm (i.d.) stainless steel column packed with Apiezon L containing 5% Igepal. The volatile oil from the leaves was separated on a 30 m × 0.3 mm (i.d.) WCOT glass column coated with Carbowax 20 M. GC conditions were as follows: Carbowax 20 M: column temp. 60–200° at 2°/min, injector temp. 220°, detector temp. 220°, N_2 carrier gas at a flow rate of 20 ml/min. Apiezon L: column temp. 100–200° at 3°/min, injector temp. 220°, detector temp. 220°, N_2 carrier gas at a flow rate of 15 ml/min, WCOT coated with Carbowax 20 M: column temp. 70–150° at 5°/min, injector temp. 170°, detector temp. 170°, N_2 carrier gas at a flow rate of 0.78 ml/min, split ratio of 1:85.

Conditions for GC/MS analysis were as follows: 3 m × 3 mm stainless steel column packed with 5% OV-17, column temp. 80–220° at 4°/min, He carrier gas at a flow rate of 15 ml/min. MS were measured at 27 eV. The volatile components from the original plant leaves were separated on a WCOT glass capillary column coated with FFAP (50 m × 0.25 mm, i.d.); column temp. 70–220° at 3°/min. The ionizing voltage was 20 eV.

Components in the volatile oil from the leaves. MS of the volatile components from the leaves were as follows: β-pinene (*m/z*: 136 $[M]^+$, 121, 93, 69); limonene (*m/z*: 136 $[M]^+$, 121, 107, 93, 79, 68); 3-hexen-1-al (*m/z*: 98 $[M]^+$, 83, 69, 57, 41); *p*-cymene (*m/z*: 134 $[M]^+$, 119, 91), *p*-cymenene (*m/z*: 132 $[M]^+$, 91], 3-hexenol (*m/z*: 82 $[M-H_2O]^+$, 67, 55, 43); 3-octanol (*m/z*: 112 $[M-H_2O]^+$, 101, 83, 59, 45); 1-octen-3-ol (*m/z*: 99, 85, 72, 57); benzaldehyde (*m/z*: 106 $[M]^+$, 105, 77); linalol (*m/z*: 136 $[M-H_2O]^+$, 121, 93, 80, 71); β-elemene (*m/z*: 204 $[M]^+$, 189, 175, 161, 148, 133, 121, 107, 93, 81, 68, 55); caryophyllene (*m/z*: 204 $[M]^+$, 189, 161, 148, 133, 119, 107, 93, 81, 69, 55); perillaketone (*m/z*: 166 $[M]^+$, 123, 110, 95, 67); isoegomaketone (*m/z*: 164 $[M]^+$, 149, 135, 121, 109, 93, 77, 43); α-ionone (*m/z*: 192 $[M]^+$, 177, 159, 147, 136, 121, 109, 93, 77, 43); eugenol (*m/z*: 164 $[M]^+$, 149, 133, 94) and myristicin (*m/z*: 192 $[M]^+$), 161, 133].

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