Essential Oils from Hairy Root Cultures and Field Cultivated Roots of Valerian (*Valeriana sisymbriifolium*)

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Abstract

Background: Plant cell cultivations are considered as an alternative to agricultural processes for producing valuable phytochemicals (secondary metabolites). The use of plant cell cultures has overcome several inconveniences for the production of secondary metabolites.

Objective: The essential oils isolated from roots of 24-month-old field grown valerian (*Valeriana sisymbriifolium*) and hairy root cultures were analysed by GC and GC-mass spectrometry.

Methods: Transformed root cultures of valerian were established by inoculation of sterile plantlets grown with *Agrobacterium rhizogenes* strain R1601. Qualitative and quantitative differences were found between the essential oils from the non-transformed roots and those from the hairy roots.

Results: There are some major differences in the hairy root culture and field grown valerian essential oils especially in valeranone, valerenal, valerenyl acetate, valerenic acid and valerenol compounds. The essential oils from the plant roots were obtained in a yield of 13.2% bornyl acetate, valerenal 12.7%, ∝-Pinene 7.50 compared with that from transformed root culture identified as kessyi alcohol (10.10%) and kessyl acetate (9.90%), as the main constituents.

Conclusion: Inoculation with *Agrobacterium rhizogenes* strain R1601 was found to be an effective means of inducing hairy root formation on *Valeriana sisymbriifolium.*

Keywords: *Valeriana sisymbriifolium*, *Agrobacterium rhizogenes*, Hairy root culture, Essential oil

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Introduction

Valerian (*Valeriana sisymbriifolium*), a member of the Valerianaceae family, is a perennial herb that is widely used as a sleep aid. The dried rhizome and roots of *V. sisymbriifolium* comprise the herbal drug valerian, which has been used for at least 1000 years [9, 18]. The dried rhizome and roots of valerian is widely used as a mild sedative and sleep aid for insomnia, excitability, and exhaustion. Valerian has depressant activities on nervous system, with antispasmodic, and equalising effects-acting as a sedative inagitated states and a stimulant in fatigue [2, 4].

The composition of the essential oil isolated from root parts of *V. sisymbriifolium* has been a subject of extensive studies [20, 25, 26], mainly due to its medicinal properties [8] and taxonomic value [28]. Most of the investigations have focused on the two major groups of constituents, the valepotriates and the sesquiterpenes. The clinical use of valerian as a sedative mainly because of the valepotriates present and the volatile oil constituents, notably valerenic acid [3].

Fast growing adventitious transgenic roots obtained by infection of dicotyledonous plants with *Agrobacterium rhizogenes* (AgR) can be viewed as a way of establishing `immortal' cell lines which can be useful in plant tissue culture [5, 21, 24]. In this process, bacterial genetic material is integrated in the plant chromosome causing the proliferation of tumorous roots. Hairy root cultures are potentially applicable to the production of root-derived metabolites. An important part of the research concerning hairy root is related to the production of medicinally secondary metabolites [7, 17, 19, 23].

The aim of this study is a comparison between the essential oils from the roots of field plant grown and those from hairy root cultures of *Valeriana sisymbriifolium*.

Materials & Methods

Plant material

Valerian 24-month-old roots were collected from plants growing in the Medicinal Plants Garden of Tehran in the Shahed University in June 2009. Field treatments for field culture consisted of nitrogen fertilizer (Urea) at 0, 30, 60, 90, 120 and 150 kg ha^{-1} at the seedling and flowering stages.

Valerian seedlings used in the transformation procedure were grown under aseptic conditions from seeds collected from pure plant population in 2008. The seeds were soaked overnight in H_2O prior to washing in a 10% detergent solution for 10 min, followed by surface disinfection with 70% EtOH for 30 s and 6% NaOCl for 5 min [10, 12]. After rinsing four times with sterile H_2O, the seeds were germinated on Solid Schulz Medium, without growth regulators, at 25°C in a 16 h light/8 h dark photoperiod (42 $\mu Em^{-2}s^{-1}$).

A. rhizogenes Strain (R1601) was provided by the Microbiology Department of the Glasgow University, Scotland U.K. AgR were maintained on solid YMB medium [7, 8]. AgR Strain was inoculated in liquid YMB medium, 48 h prior to transformation assays.

Two-week-old valerian seedlings were randomly wounded with the tip of a sterile hypodermic needle carrying one drop of the bacterial suspension. The seedlings were co-cultivated with the bacteria on MS/2 solid medium (half strength Murashige and Skoog medium [15], with 30 g/l of sucrose, in a 16 h light/8 h dark photoperiod at 24°C, for 48 h. They were then transferred to solid MS/2 medium supplemented with antibiotics (500 mg/l ampicillin or 250 mg/l carbenicillin plus

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250 mg/l cefotaxime). Roots that developed were transferred to liquid Schenk and Hildebrandt (SH) medium without antibiotics, maintained in the dark at 24°C on an orbital shaker (80 rpm) and sub-cultured every 15 days.

Root samples from the 24 months non-transformed roots and hairy roots culture, were freeze-dried for 2 days, at 10^{-1} mbar and -42 C. The essential oils, from both hairy root culture and non-transformed roots, were performed by hydrodistillation for 3 h, using a Clevenger-type apparatus [13]. The oil samples isolated by hydrodistillation were used to estimate the oil yields, and to determine the percentage composition of the oils, since the chance of artifact formation must be considered smaller when the latter method is used.

Gas chromatography

GC analyses were performed using a twin FID instrument, a data handling system and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (30 m * 0.25 mm i.d., film thickness 0.25 μm) and a DB-Wax fused-silica column (30 m * 0.25 mm i.d., film thickness 0.25 μm). The oven temp. was programmed, 45± 1758ºC, at 3ºC min^{-1}, subsequently at 15ºC min^{-1} up to 240ºC and then held isothermal for 10 min; injector and detector temps were 220ºC and 240ºC, respectively; carrier gas, H_2 at 30 cms^{-1}. Samples were injected using the split-sampling technique with a ratio of 1:50. Percentage composition of oils was computed using the normalization method from the GC peak areas without correction factors. Results have obtained for every plants materials after two injections; for the approximately 10 oil samples isolated from the hairy root material harvested periodically, mean values of all of these samples were calculated after each sample was injected twice.

Gas chromatography-mass spectrometry

The GC-MS unit was equipped with a DB-1 fused-silica column (30 m * 0.25 mm i.d., film thickness 0.25 μm) and interfaced with an ion trap detector (ITD; software version 4.1). Oven temps were as described above; transfer line temp., 280ºC; ion trap temp., 220ºC; carrier gas, He at 30 cms^{-1}; split ratio, 1:40; ionization energy, 70 eV (electron Volt); ionization current, 60 μA (Microampere); scan range, 40-300 u (atomic mass unit); scan time, 1 s. The identity of the components was assigned by comparison of their RIs, relative to C_9-C_{17} *n*-alkanes and MS with corresponding data of components of reference oils or of synthetic compounds.

The field and laboratory experiments were arranged as complete randomize block design (CRBD) and complete randomize design (CRD), respectively with three replications. Analysis of variance (ANOVA) using SAS software. The differences between treatments were determined by Duncan's multiple range test (DMRT).

Results

The significant difference ($p<0.05$) have observed in valerian root yield (kg ha^{-1}) at 30, 60, 90, 120 and 150 kg ha^{-1} nitrogen fertilizer (urea) application (Table 1). The highest and lowest root yield (kg ha^{-1}) was 5300 and 3320 kg ha^{-1} respectively, at 150 kg ha^{-1} nitrogen fertilizer and control treatments.

There were reduction about 14.30, 11.43, 17.15, 17.15 and 25.72% in valerian essential oil (%) at 30, 60, 90, 120 and 150 kg ha^{-1} nitrogen fertilizer application respectively, compared with control (Table 1). Valerian

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essential oil (%) in all fertilizer treated area never exceeded that observed in the control (Table 1).

Compared with the untreated control, nitrogen fertilizer at 60, 90, 120 and 150 kg ha^{-1} significantly ($p<0.05$) (27.58, 51.72, 52 and 65.50%) increased the root length (cm). Significant increased in the root diameter per plant (mm) at 90, 120 and 150 kg ha^{-1} nitrogen fertilizer were 16.94, 27.11 and 28.81% respectively (Table 1). Results showed significant increase in valeranone, valerenal, valerenyl acetate, valerenic acid and valerenol compounds grown under field condition (non-transformed roots) at 30, 60, 90, 120 and 150 kg ha^{-1} nitrogen fertilizer compared to hairy roots (Table 2). Instead, compared with the non-transformed roots at different nitrogen levels, hairy roots significantly increased the δ-Elemene, ∝-Humulene, Isovaleric acid, δ-Cadinene, Myrtenol, δ-Cadinol, Kessyl acetate and Kessyl alcohol compounds.

Hairy roots of *V. sisymbriifolium* were established successfully in the dark in liquid SH culture media. The hairy roots grown in SH medium showed a higher biomass increase, expressed either in fresh weight and/or dry weight (Fig. 1). Having started with the same root inoculum size (10 g^{-1}), results showed that *V. sisymbriifolium* hairy root cultures demonstrated higher fresh and dry weight values when grown in the dark in SH, attaining values of approximately 16 and 400 mg fresh and dry weight respectively, at the end of 40 days (Fig. 1). Valerian hairy roots grown in SH media, showed a 40-fold increase in fresh weight at the end of 40 days (Fig. 1). The qualitative and quantitative identified components of the essential oil samples from the valerian hairy roots and plant roots grown under different nitrogen treatments are listed in Table 2. Results showed that the oil of the transformed roots is in its composition from that of the field grown plants. The essential oils were obtained in about 0.45% (v/w) and 0.28% yield from the non-transformed roots and hairy roots, respectively.

Fifty-seven components identified from oil of the normal roots by gas chromatography mass spectrometry (GC-MS), identify 51 components from oil of roots that consist of 89% the essential oil (Table 2). The main component is made up of monoterpene hydrocarbons (27%) with ∝-pinene, ∝-fenchene and camphene as major constituents of this class of compounds, monoterpene esters (18%), sesquiterpene hydrocarbons (14%), oxygenated sesquiterpenes (7%) and such typical valerian cyelopentanoid sesquiterpenes (16%) as valerenal, valerenyl acetate, valerenic acid, valerenol and valerenyi isovalerate. The major components of the normal oil were bornyl acetate (15.3 %) and valerenal (11.5%). These two compounds being considered as characteristic constituents of *V. sisymbriifolium*.

Generally, the oils from the hairy roots revealed some qualitative similarities, at least with regard to their main components. The analysis of the volatile constituents in the essential oil of the hairy roots by GC and GC-MS indicated the presence of at least 57 compounds and 37 of these, amounting to 65% (w/w) of the essential oil, were identified. The main component is mainly composed of sesquiterpene hydrocarbons and oxygenated sesquiterpenes which constitute altogether over 40% (w/w) of the compounds detected. Comparison with the normal oil, there is a significant reduction (7-fold) in the accumulation of monoterpene hydrocarbons (4.5%), whilst the spectrum of oxygenated monoterpenes is very similar in both normal and transformed oils.

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The oxygenated sesquiterpenes such as kessane, valeranone, valerenyl acetate, valerenic acid, and valerenyl isovalerate were absenet in the transformed oil. Also, some mono- and sesquiterpene hydrocarbons derivatives identified as well as 3 phenylpropanoid from the volatile oil of the field-grown plants. Isoeugenol, isoeugenyi acetate and benzyi benzoate, have not been identified in the transformed roots. In addition, four components such as ar-curcumene, 1-hexanol, isocaryophyllene and drimenol were identified in the transformed root oil and could not be found in the roots of the field-grown plants.

Table 1 - Effects of nitrogen fertilizer on the Valerian parameters

Nitrogen fertilizer (kg ha^{-1})	Root yield (kg ha^{-1})	Essential oil (%)	Root length (cm)	Root diameter (mm)
0	3320^{a}*	1.75^{a}	14.50^{a}	2.95^{a}
30	3745^{b}	1.50^{b}	16^{a}	3.15^{a}
60	3920^{b}	1.55^{b}	18.50^{b}	3.15^{a}
90	4550^{c}	1.45^{b}	22b^{c}	3.45^{b}
120	4955^{d}	1.45^{b}	23.50^{c}	3.75^{c}
150	5300^{e}	1.30^{c}	24^{c}	3.80^{c}

*Means within each column followed by the same letters are not significantly different at the 5% level according to Duncan's multiple range test.

Table 2- Composition of Essential oil from hairy roots (HR) and field grown plants (non-transformed roots) of *Valeriana sisymbriifolium*

Peak	Compound	HR (%)	NTR 30 kg N ha^{-1} (%)	NTR 30 kg N ha^{-1} (%)	NTR 30 kg N ha^{-1} (%)	NTR 30 kg N ha^{-1} (%)	NTR 30 kg N ha^{-1} (%)
1	Tricyclene	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
2	∝-Pinene	0.80	7.80	7.50	7.09	7.10	7.60
3	∝-Fenchene	1.10	8.50	8.20	8.90	9.10	8.80
4	Camphene	1	8.50	8.90	8.10	8.70	8.60
5	Hexanal	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
6	β-Pinene	0.30	1.50	1.90	1.80	1.20	1.70
7	Sabinene	<0.1	0.30	0.20	0.25	0.19	0.24
8	β-Myrcene	-	<0.1	<0.1	<0.1	<0.1	<0.1
9	∝-Phellandrene	-	<0.1	<0.1	<0.1	<0.1	<0.1
10	∝-Terpinene	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
11	Limonene	<0.1	1.70	1.30	1.60	1.90	1.60
12	β-Phellandrene	-	0.70	0.80	0.60	0.60	0.70
13	γ-Terpinene	<0.1	0.70	0.60	0.60	0.50	0.80
14	P-Cymene	<0.1	0.30	0.25	0.25	0.35	0.30
15	∝-Terpinolene	-	<0.1	<0.1	<0.1	<0.1	<0.1
16	1-Hexanol	<0.1	-	-	-	-	<0.1
17	δ-Elemene	8.50	5.50	5.40	4.90	4.80	5.70
18	Bicycloelemene	-	<0.1	<0.1	-	<0.1	-
19	β-Selinene	<0.1	0.70	0.50	0.60	0.60	0.60
20	Carvacrol Methyl ether	-	0.50	0.50	0.50	0.50	0.50
21	Bornyl acetate	6.90	12.50	13.30	13	13.10	12.90
22	β-Elemene	1.25	0.90	0.85	0.80	1	0.95
23	β-Caryophyllene	3.80	1.60	1.80	1.80	1.70	1.80
24	∝-Gurjunene	2.70	2.70	2.55	2.60	2.80	2.50
25	γ-Elemene	0.50	<0.1	<0.1	-	<0.1	<0.1
26	5-Camphenyl acetate	0.22	<0.1	<0.1	<0.1	-	<0.1
27	Isocaryophyllene	0.50	-	-	-	-	-
28	Aromadendrene	-	1.70	1.90	1.60	1.80	1.90
29	∝-Elemene	-	0.20	0.18	0.17	0.21	0.22

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Continue Table 2 - Composition of Essential oil from hairy roots (HR) and field grown plants (non-transformed roots) of *Valeriana sisymbriifolium*

Peak	Compound	HR (%)	NTR 30 kg N ha^{-1} (%)	NTR 30 kg N ha^{-1} (%)	NTR 30 kg N ha^{-1} (%)	NTR 30 kg N ha^{-1} (%)	NTR 30 kg N ha^{-1} (%)
30	∝-Humulene	3.50	<0.1	-	<0.1	-	<0.1
31	Myrtenyl acetate	2.30	2.30	2.20	2.50	2.10	2
32	Ledene	-	0.50	0.40	0.55	0.50	0.35
33	Isovaleric acid	0.70	0.40	0.45	0.35	0.40	0.40
34	β-Cadinene	0.50	0.20	0.21	0.21	0.19	0.19
35	β-Cubebene	-	1.40	1.35	1.40	1.40	1.35
36	Bicyclogermacrene	0.90	1.50	1.40	1.45	1.50	1.48
37	δ-Cadinene	0.55	<0.1	-	-	<0.1	<0.1
38	Ar-curcumene	1.18	-	-	-	-	-
39	Myrtenol	3.26	-	<0.1	<0.1	<0.1	-
40	Kessane	-	0.50	0.60	0.55	0.50	0.55
41	β-Ionone	1.55	1.35	1.40	1.52	1.55	1.50
42	Ledol	-	0.50	0.55	0.52	0.51	0.54
43	$C_{15}H_{26}O\Psi$	1.20	0.30	0.30	0.35	0.32	0.30
44	Valeranone	-	0.90	0.85	0.95	0.90	0.90
45	δ-Cadinol	0.50	<0.1	-	-	<0.1	<0.1
46	Bisabolol	-	0.40	0.35	0.40	0.30	0.35
47	Valerenal	4.20	12.40	12.50	11.50	12.70	12.90
48	Valerenyl acetate	-	1	0.95	1	1	1
49	Valerenic acid	-	1	0.95	0.85	1	0.90
50	Isoeugenol	-	<0.1	<0.1	<0.1	<0.1	<0.1
51	Kessyl acetate	9.90	0.50	0.40	0.55	0.50	0.45
52	Valerenol	0.35	0.60	0.55	0.58	0.57	0.60
53	Valerenyl isovalerate	-	-	-	<0.1	<0.1	<0.1
54	Kessyl alcohol	10.10	1	0.95	0.80	0.90	1
55	Drimenol	<0.1	-	-	-	-	-
56	Isoeugenyl acetate	-	1.50	1. 70	1.40	1.60	1.55
57	Benzyl benzoate	-	<0.1	-	-	-	<0.1

Discussion

The composition of hairy root oil was different from the field grown roots [6, 7, 8, 16]. It is not surprising that essential oil composition under different nature and culture conditions (hairy roots and field condition grown) was completely different [17, 27]. The hairy root culture is constituted by isolated roots and may deprive the system of precursors and other compounds with an origin elsewhere in the parent plant. Also, compounds in an in vivo system would be translocated into other plant organs, may remain in the place of synthesis or may be excreted to the culture medium, when an in vitro system is considered [14].

According to Kennedy and et al. [11] the essential oil composition of field grown plant roots and that of hairy roots were completely different. Results of this study and those of Granicher and et al. [7, 8] show that there is some differences in essential oil composition of hairy root and that grown under field condition of *V. officinalis*. Yu et al. [29] stated that the essential oil from hairy root clones established from SH culture media can show different growth rates along with differences in metabolite production. The maturity of the root system may be important for the essential oil synthesis. The continuous growth characteristic of transgenic roots may not be compatible with high degrees of maturation and the metabolite production [14].

Similar to this experiment, Santos and et al. [21, 22] reported for the lovage (*Levisticum officinale*) hairy roots grown in SH media, 39-

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fold increase in fresh weight at the end of 35 days. This value are higher than those reported for dill (*Anethum graveolens*) hairy roots grown in MS/2 medium, in which a 13-fold increase in fresh weight was obtained at the end of 30 days [22]. Hairy root cultures of Large Bullwort (*Ammi majus*), culture in MS medium, showed a 150-fold biomass increase at the end of 30 days [12]. A 77-fold fresh weight on wild carrot (*Daucus carota*) hairy roots, increase at the end of 30 days has been reported [1].

Several factors such as the growth phase and/or rate, competition with growth and differentiation and the metabolic breakdown and/or conversion of components may determine the variations in the oil components of hairy root and the field grown roots throughout the growth cycle [14]. Also, the percentage composition of the essential oils is a relative value and it is not possible to ascertain whether the amount of a component increases due to the real rise in its absolute value or to the decrease of the other oil components. Some more experiments, with different methodologies are necessary to find the relationship between oil content and growth cycle [21, 22].

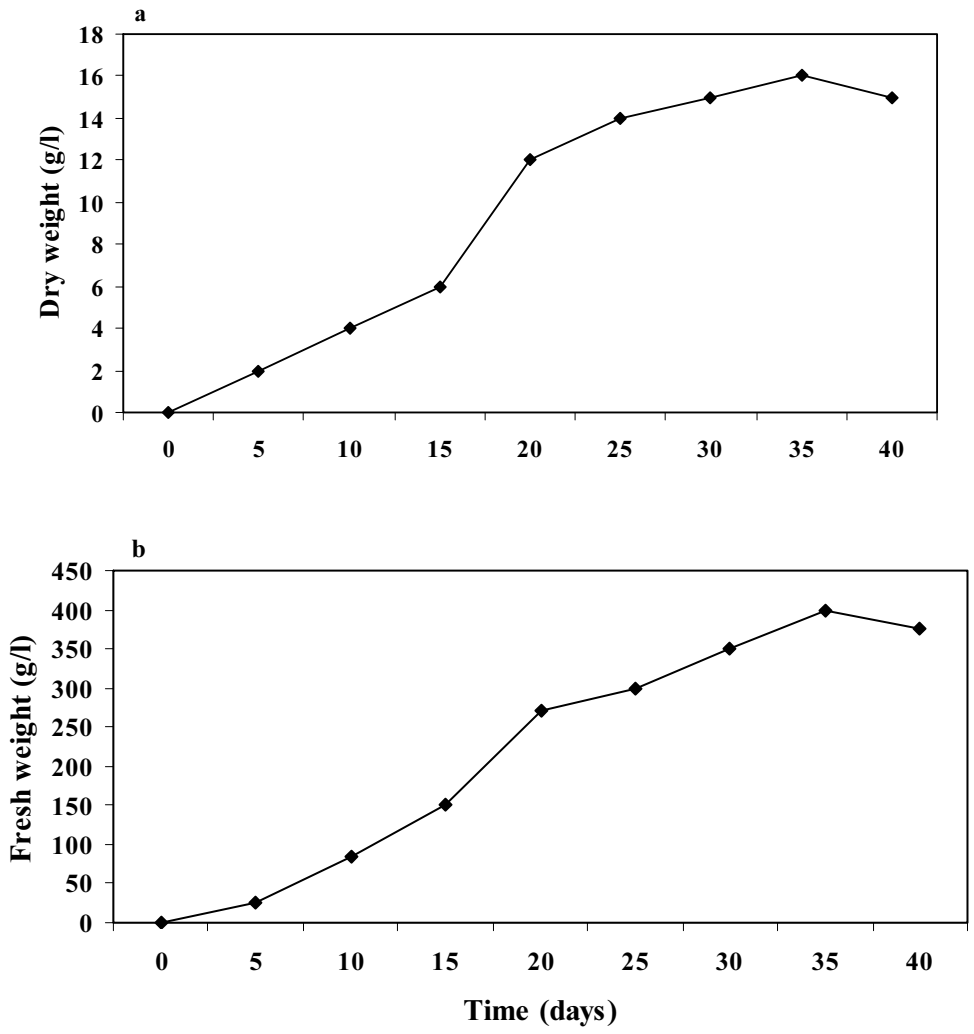

Fig. 1- Dry weight (a) and fresh weight (b) of valerian hairy roots grown in SH liquid media

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In this study, the essential oils from hairy roots showed some qualitative differences, compared with the non-transformed roots; however, these were considerable, depending on the medium and growth conditions applied.

With a stable biosynthetic capacity and an essential oil profile, which is different from that of the plant roots, valerian hairy root cultures show a good potential for a study on the in vitro production of useful secondary metabolites.

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