Composition of the volatile oil, tissue culture and micro-regeneration optimization of wild Yarrow (*Achillea biebersteinii*)

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Abstract

Achillea biebersteinii is a perennial herbaceous medicinal plant that belongs to Compositae family with useful properties, such as: anti-diaphoretic, antihemorrhagic, antiinflammation, antibiotic, antifungal and antioxidant effects. The objectives were; to determine the composition of essential oil components with the use of GC and GC/MS. Forty–two components were found and 98% of the identified oil constitunts categorized with above medicinal properties were; 1, 8 cineole (30.9%), cis-ascaridole (12.8%), alpha-terpinene (9.6%), alpha-terpineole(6.3%) and camphor (4.3%), with 37 minor extra components. Therefore, efforts have been directed to micro-propagation through *in vitro* culture with a view to producing secondary metabolites. So in this research, tissue culture has been optimized in four stages. At first seeds were sterilized then placed in different proportions of MS media. After 10 days the young seedlings emerged and after cutting their roots, were placed on MS media supplemented with different concentrations of BAP (2, 5, 10 mg/lit) in order to study its effects on Shoot induction. At 5 and 10 mg/lit both shoot and callus formed (with higher proportion at 5 mg/lit). At this stage, three characters of young seedlings have been analyzed: The number of leave/ the length of leave and the length of the tallest leave. In the third stage, young seedlings have been placed on MS media with no growth regulator. Then, the young seedlings were transferred to MS media supplemented with 2 mg/lit IBA. The best media for incubator use were MS media supplemented with 5 and 10 mg/lit of BAP.

Keywords: Achillea biebersteinii; Compositae; volatile oil; 1, 8 cineole; secondary metabolites; plant growth regulator

Ghasempour, H., Sh. Dabiri and **M. Cheraghi.** 2012. 'Composition of the volatile oil, tissue culture and micro-regeneration optimization of wild Yarrow (*Achillea biebersteinii*)'. *Iranian Journal of Plant Physiology* 2 (4), 539-546.

Introduction

The genus *Achillea* (Compositae) comprises of 115 species, which are mainly distributed in Europe, Asia, and North Africa (Gabbrielli et al, 1988). Nineteen species of the genus are described in the Flora Iranica (Adam et al., 1998) of which *Achillea biebersteinii* is found in some Western slopes of Zagross mountain habitats in Kermanshah state, west of Iran. *Achillea biebersteinii* (yarrow) is a medicinal plant that grows widely in some regions of Iran and Europe (Masomi S. M., 2001). This medicinal plant has been used as anti-inflammatory, anti-spasmodic, diaphoretic, diuretic, emmenagogues

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Received: July, 2012
Accepted: August, 2012

agent and for treatment of hemorrhage, pneumonia, rheumatic pain and wounds since antiquity and its essential oil is used to cure the nervous and rheumatic pains (Rustaiyan et al., 1999). A lot of efforts have been forwarded to its micro-propagation that have successfully ended with callus induction from leaf and stem explants of this plant (Shirazi et al., 2006; Weyerstahl et al., 1997; Ghasempour et al., 2007; Zebarjadi et al., 2010; Zebarjadi et al., 2011). Also, the effects of the extract of this plant on gram positive and gram negative bacteria, as on *Helicobacter Pylori*, have been studied (Fathiazad and Lotfipour, 2003; Ruataiyan et al., 1998; Taran et al., 2011).

Medicinal plants are the sources for curing and treatment of pains. Due to increasing the side effects of synthetic drugs, efforts have been directed to use medicinal plants (Afsharpour et al., 1996; Bader et al., 2003; Cosentino et al., 1999; Ghasempour et al., 2007).

Plant tissue culture refers to growing plant materials on sterilized conditions. Plant tissue culture techniques and biotechnology variations increases the quality and the rate of product. Cultivation of medicinal plants for the purpose of extraction of active constituents may face certain limitations such as climate, season, water availability, diseases and pests (Arikat et al., 2004; Ghasempour et al., 2007). Such limitations have led to the use of tissue culture techniques for production of the active constituents (Kahrizi et al., 2007; Ghasempour et al., 2007). Tissue culture technique provides a means of rapid propagation of a large number of uniform plants while maintain their genotype. Beneficial use of tissue culture for the purpose of extraction of secondary metabolites include avoidance of collection of endangered wild species, production of secondary metabolites irrespective of seasonal and climatic conditions, and rapid production of secondary metabolites due to rapid growth of cultures *in vitro* (Arikat et al., 2004; Ghasempour et al., 2007; Zebarjadi et al., 2010; Zebarjadi et al., 2011).

The aims of this study were: (a) to determine the physical properties and the chemical constituents of the oils of *Achillea biebersteinii,* particularly in relation to use of the oil as antibacterial, antifungal and antioxidant effects; (b) to set up the plant tissue culture and micro-propagation optimization, in order to find the best culture media for its mass production to be used in incubators for extraction of secondary metabolites. Therefore, the second part of this study was initiated to develop a protocol for *in vitro* micro-propagation of this medicinal plant in order to multiply and regenerate the whole plant. Soil properties and parameters of plant water relations were examined at the collection sites of two habitats in Kermanshah state, Zagross Mountain, western Iran.

Materials and Methods

Plant material

Aerial parts and seeds of *A. biebersteinii* were collected in June and July 2006. The collection sites of two different habitats were as follows: one habitat was located on northern slopes of Bidsorkh heights near the city of Kangavar and the other habitat on southern slope of Bidsorkh heights close to the city of Sahaneh both in Kermanshah state, Zagross Mountain, West of Iran. The oils were extracted and analyzed by GC and GC/Mass.

Isolation procedure

Leaves (100 g) and seeds (100 g) of *A. biebersteinii* were powdered, mixed with 1200 ml and 1000 ml, respectively of distilled water and the essential oils were steam distilled in a Clevenger apparatus for 3 h. The essential oils were dried over anhydrous Na_2SO_4 and stored at 4 ˚C in the dark.

Gas chromatography (GC)

GC analysis of the oil was conducted using a Thermoquest-Finnigan Trace GC instrument equipped with a DB-1 fused silica column (60 m × 0.25 mm i.d., film thickness 0.25 µm). The carrier gas was nitrogen, at a constant flow of 1.1 ml/min. The oven temperature was held at 60 ^{o}C for 1 min, then programmed to 250 ^{o}C at a rate of 4 ^{o}C /min, and then held for 10 min. The injector and detector (FID) temperatures were kept at 250 ^{o}C and 280 ^{o}C, respectively.

Gas chromatography-mass spectrometry

GC-MS analysis was carried out on a Thermoquest-Finnigan Trace GC-MS instrument equipped with a DB-1 fused silica column (60 m × 0.25 mm i.d., film thickness 0.25 µm). The oven temperature was raised from 60 °C to 250 °C at a rate of 5 °C/min, and then held at 250 °C for 10 minutes; transfer line temperature was 250 °C. The quadruple mass spectrometer was scanned over the 45-465 amu (atomic mass unit) with an ionizing voltage of 70 eV and an ionization current of 150 µA.

Identification of components

The constituents of the oil were identified from calculation of their retention indices under the temperature-programmed conditions for *n-alkanes* (C_6–C_{24}) on a DB-1 and DB-Wax columns. Individual compounds were identified by comparison of their mass spectra with those of the internal reference mass spectra library (Wiley 7.0) or with authentic compounds. Identifications were confirmed by comparison of their retention indices with authentic compounds or with those reported in the literature (Shibamoto, 1987). Quantitative data was obtained from FID area percentages (without the use of correction factors).

Water content (WC)

A sample of ten leaves in the field was taken randomly from each ecotype and the sample's fresh weight (FW) was measured. Leaf samples were oven dried and weighed (dried weight = DW) in 70 ˚C for 72h. WC was then calculated using the following formula:

$$WC = [(FW-DW)/FW] \times 100$$

Relative water loss (RWL)

Ten leaves from each region were collected and weighed. The leaves were then wilted at 30 ˚C and reweighed, transferred to the oven (70 °C) for 24h and weight again. RWL was calculated using the following:

$$RWL = (W_1-W_2)/W_3\ (t_1-t_2)$$

Where W_1, W_2 and W_3 are the initial wilted and dried weights; t_1 and t_2 are the time of measurement for initial and wilted weights.

Water potential (ψ_p) was determined by HR 33T Dew Point Microvoltmeter equipped with L – 51 A sensor. The water potential (bars) was calculated by dividing the reading (in µ volts) by -0.75 µ volts bar^{-1} (HR-33T Dew Point Microvoltmeter Instruction, 2001).

Soil analysis

The soil samples were collected in June 2006 at northern slopes of Bidsorkh heights near the city of Kangavar and the other habitat on southern slope of Bidsorkh heights close to the city of Sahaneh both in Kermanshah state, Zagross Mountain, West of Iran northern slopes of Bidsorkh heights near the city of Kangavar both in Kermanshah state, Zagross Mountain, west of Iran. The soils' contents of organic carbon were measured by Walkeli-Black method. Soil samples were treated with H_2SO_4 (96٪) and adjusted with $K_2Cr_2O_7$; after the end of oxidation and reduction reactions the remained $K_2Cr_2O_7$ were titrated with $FeSO_4\ (NH_4)_2\ SO_4\ 6H_2O$ (0.5 N).

The available phosphorus content (P) was measured by Olsen's method (Horta and Torrent, 2007). The potassium contents (K) were measured with a flame photometer, using Jen Way PFP 7. The contents of the microelements Zn, Mn, Fe and Cu were measured by DTPA method.

Essential oil physical properties

Light polarity of the distilled oil was measured by electronic polarimeter (P 3001 KRUSS). Light refraction index was measured by refractometer.

Plant material and seed germination

The seeds were washed thoroughly with tap water, surface sterilized in 70% ethanol for 1 min and then were transferred to 1.5% sodium hypochlorite for 8 minutes on a shaker and finally rinsed with distilled water 3 times. The seeds were then germinated on MS medium solidified with 0.7% agar which contained 2% sucrose and

pH was adjusted to 5.8. The cultures were transferred to a growth chamber (Conviron) at 23 ±2 °C and 16 h / 8h, light/ dark period. The cultures were used as a stock of disinfected plant material for initiation of experiments.

In vitro establishment

Cotyledons were excised from *in vitro* germinated seeds and cultured on MS (Murashige and Skoog) media. Each medium was supplemented with 0, 2, 5, 10 mg/l BAP. Cultures were maintained under the growth chamber room conditions described in the previous section. After 10 days, data on number of leaves and micro-shoot heights were recorded.

Shoot elongation

Micro-shoots formed on MS media were sub-cultured on fresh MS media with 10, 20 and 30 g/l sucrose and 6% agar. Then after, micro-shoots were grown on a growth regulator–free medium for 4 weeks of culture; data were recorded on number of leaves and micro-shoot heights.

Rooting of micro-shoots

Micro-shoots were transferred to MS media supplemented with 2 mg/l IBA and different concentrations of sucrose.

Statistical Analysis

Analyses of variance were carried out in order to determine the effect of treatments. The Duncan's multiple range tests was used for comparing mean performance of treatments for proliferation ability. All data were normalized by transformation using the Arc $\sin\sqrt{x}$ function before statistical analysis.

Results

Hydro-distillation of the air-dried aerial parts of *A. biebersteinii* yielded 0.2% (v/w) of the oil based on the dry weight of sample. The oil was dark blue in color. Analysis of the oil by GC and GC-MS resulted in forty–two components, representing 98% of the total oil (Table 1). The

Table 1
Chemical composition of essential oils of *A. biebersteinii* collected from Bidsorkh Mountains. Contents are given as the percent of total oil and in RI_0= retention index.

component	%	RI*
Alpha- thujene	0.5	922
Alpha-pinene	3.2	930
Camphene	1.2	943
Sabinene	4.00	964
beta-pinene	2.5	971
Myrcene	0.3	977
alpha-terpinene	9.6	1009
P-cymene	5.00	1015
1,8-cineole	30.9	1027
gama-terpinene	1.5	1049
transe-sabinene hydrate	0.3	1054
artemisia alchol	0.1	1067
alpha terpinolene	0.3	1078
linalool	0.6	1082
alpha-campholene	0.2	1103
cis-sabinene hydrate	1.3	1106
Camphore	4.3	1122
Cis-p-menth-2,8-dienole	0.1	1136
Linalyl propionate	1.3	1146
Borneol	0.7	1149
alpha- thujanal	0.1	1157
4-terpineol	0.3	1162
alpha-terpineol	6.3	1172
myrtenal	0.3	1179
Cis-piperitol	0.2	1188
Cis-ascaridole	12.8	1216
1,4-p-menthadien-7-ol	0.2	1256
P-cymene-7-ol	0.2	1259
thymol	0.2	1261
Bornyl acetate	1.0	1267
carvacrol	0.9	1271
Iso ascaridole	1.8	1276
myrtenole	0.1	1298
Cis-jasmonene	0.1	1363
beta-caryophyllene	0.6	1419
Germacrene D	0.3	1489
Geranyl isovalerate	0.4	1494
delta-cadinene	1.0	1513
Caryophyllene oxide	0.3	1573
cubenole	0.5	1616
beta-guaiene	0.1	1619
beta-eudesmol	0.1	1637

major constituents of the essential oil were found to be monoterpene hydrocarbons (Table 1).

***In vitro* establishment**

The statistical analysis of the effects of BAP concentrations on *in vitro* regeneration of A. biebersteinii are presented in Table 4. The results showed that effect of BAP concentrations were significant ($p<0.01$) for *in vitro* micropropagation.

The number of leaves formed on the cotyledons was varied by different concentration of BAP. 5 and 10 mg/l concentrations of BAP were sufficient to promote adventitious shoots, and 0 and 10 mg/l concentration of BAP slightly decreased this parameter. There were no significant difference between 5 mg/l and 10 mg/l BAP for leaves formation. Table 5 shows the effects of different BAP concentrations on number of leaves after 10 days.

Shoot elongation

The statistical analysis revealed that the number of leaves and length of plantlet had no significant difference in SEM medium (shoot elongation medium) with the change in sucrose concentration.

Rooting of micro-shoots

The statistical analysis showed that the highest rooting percentage was obtained in the absence of IBA and in the MS medium (a growth regulator- free medium).

A. biebersteinii showed a high induction potential of callus. On MS media supplemented with 5 and 10 mg/lit BAP both shoot and callus were formed, but the proportion of callus was much higher at 5 mg/lit than the inducted shoot.

Discussion

The results of oil extracts showed that the percentage yield of oil for both habitats were almost the same and the major constituents of the oil were 1,8-cineole, cis- ascaridole, alpha-terpinene, alpha- terpineole, and camphore. Among these components the main constituents were 1,8-cineole (30.9%), cis-ascaridole (12.8%), alpha-terpinene (9.6%), alphaterpineole (6.3%), camphor (4.3%), and 1,8-cineole. Also, 1,8-cineole and camphor have been reported by different researchers as the most common monoterpenes in the oil of the species of

Table 2
A comparison of the leaf water content (WC percent fresh weight), the rate of water loss (RWL) and the leaf water potential ($\psi_{p)}$ in two habitats of *A. biebersteinii*

	Bidsorkh Northern slope	Bidsorkh Southern slope
WC	68.7	76.4
RWL	3.1	3.7
ψ_p bars	-98.6	-97.5

Achillea taxon and Salvia officinalis, *Melissa officinalis* and *Lavandula angustifolia* (Hohmann et al., 1999). In fact, these two compounds are found to be the major oil constituents of *A. auchery* (40.7%), *A. talagonica* (27%), *A. eriophora* (34%), *A. tayget* (26.63%), *A. santolina* (17.6%) *Achillea flacata* 1,8-cineole (24%) and camphor (12%). Also, the minor constituents were reported to be beta-eudesmole, alpha-tujanale and beta-guaiene (Afsharypour, 1996; Rustaiyan, 1998; Rustain et al., 1999; Weyrstahl, 1997).

A number of compounds that were detected in our mentioned analyses are reported to have antibacterial, antifungal activities and are widely used as preservatives. Antibacterial effects of α-terpineol and 4-terpineol have been reported (Taran et al., 2011). Also, antifungal activity has been reported for α-*pinene*, β-*pinene,* ρ- cymene and *linalool* (Taran et al.2011). β-pinene may have antioxidant activity (Hohmann et al., 1999; Lopez-Arnaldos et al., 1995).

The two antifungal components cis-sabinene and α-pinene together constituted 4.5% of the oil (Table 1). The antibacterial 4-terpineol was found in leaf oil only at a low concentration (0.3%). The combined effect of these 3 components was probably sufficient to account for those antibacterial, antifungal and antioxidant effects of the oil of *A biebersteinii*.

There were no differences in contents between oils of the leaves at the two sites and genetic similarity may well be responsible for the leaf-oil resemblance. Genetic similarity between the two habitats is confounded with differences

due to the stable edaphic factors at the two sites and due to transient factors such as the plant water balance (Table 2).

Table 3
Structure and chemical composition of soil in the trial areas at Bidsorkh Northern slope and Bidsorkh Southern slope of mountains (S. P. = saturation percent, O. C. = organic carbon).

Properties	Bidsorkh Southern Slope	Bidsorkh Northern Slope
S. P.	80	70
Soluble mineral Eco×103	0.56	0.53
pH of paste	7.30	7.70
O.C %	>3.70	3.10
P in soil av. ppm	36.6	43.7
K in soil ppm	980	930
Total N %	0.45	0.39
Cu in soil ppm	0.92	0.97
Zn in soil ppm	1.15	1.07
Fe in soil ppm	21.72	25.86
Mn in soil ppm	18	17
Sand %	27	24
Silt %	7	9
Clay %	68	63
Soil texture	Sandy Loam	Sandy Loam

Bidsorkh southern slope had a much higher vegetation density than the Bidsorkh northern slope. It is possible that greater contents of humus, nitrogen, iron and manganese in the soil at Bidsorkh southern slope might have contributed to the high density there (Table 3).

In the second part of this research, that aimed at finding the best tissue culture media for *in vitro* micro-propagation of *A. biebersteinii* in incubator to extract these secondary metabolites the results were as follow: at first stage the best growth of young seedlings was on the 1/4 MS medium. This may be related to detrimental effects of high concentrations of micro and macro elements on this plant, which can reduce germination. The rate of MS and 1/2 MS medium salts may prevent activation of some enzymes that are related to germination (Ghasempour et al., 2007; Zebarjadi et al., 2010; Zebarjadi et al., 2011). In the second stage (SIM) in the experiment concentrations of BAP, three characteristics of young seedlings were analyzed. These included number of leaves, the length of leaves and the length of the tallest leaf. Statistical analysis at the experiment concentrations did not show a significant difference. This may be related to the level of endogenous effects of cytokinins. If the level of endogenous cytokinin in plant is high, the rate of shoot induction will not be affected, and implementing this plant growth regulator in culture media may have reverse effects on shoot induction (Ghasempour et al., 2007; Zebarjadi et al., 2010; Zebarjadi et al., 2011).

In the current study, MS medium was used as a sufficient basic medium for tissue culture of *A. biebersteinii*. Furtheremore, the medium that contained 2 and 5 mg/l BAP, was the most effective medium for micropropagation. Then the 2 mg/l BAP was more optimal, as it was more economic. The optimal concentration of BAP, i.e., 2 mg/l (or 8.8 µM) in the current study was much lower than those reported in wild sage (Salvia multicaulis) (Ghasempour et al., 2007). Arikat (2004) in wild sage has reported that the nodal explants remained green and fresh in the absence of growth regulators in the medium.

In elongation medium, results showed that there was no significant different among sucrose levels for plantlet elongation. This

Table 4
Analysis of variance for leaf formation from cotyledons in different concentrations of BAP in A. biebersteinii.

Source of Variation	Degrees of Freedom	Mean of Squares	Prob.
BAP levels	3	9.511**	0.0067
Error	8	1.094	
Total	11		
CV = 13.72			

**Significant at P≤0.01

Table 5
Mean performance of different concentrations of BAP on regeneration of A. biebersteinii after 10 days.

BAP conc. (mg/l)	Number of leave explants
0.0	2.33 b
2.0	3.57 b
5.0	6.18 a
10	6.56 a

Values in the same column followed by the same letter do not differ statistically at p≤ 0.05

indicates that plantlets can supply the nutrition exigency via photosynthesis phenomena. Therefore, using 10 g/l sucrose is suggested for costs and osmosis pressure problems.

Previous studies have suggested that IBA is a suitable auxin for *in vitro* rooting (Zebarjadi et al., 2011). Arikat (2004) has concluded that auxin is necessary for formation of root in *S. fruticosa*. But our study showed that micro-shoots were able to form roots in the absence of auxin in the medium. This may be due to the influence of endogenous hormone content on rooting ability of micro-shoots.

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