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In vitro callus induction and isolation of volatile compounds in callus culture of *Lallemantia iberica* (M. Bieb.) Fisch. & C. A. Mey.

Seyed Mehdi Razavi*, Homa Arshneshin and Alireza Ghasemian

Department of Biology, Faculty of Sciences, University of Mohaghegh Ardabili, 56199 Ardabil, Iran.
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Abstract:

A modern biotechnological technique to obtain useful natural products from plants is to isolate them from their callus cultures. *Lallemantia iberica* is an annual herb of the *Lamiaceae* family known for its stimulant, diuretic, and expectorant effects in Iranian folk medicine. The present study investigated the induction of plant callus tissue and identification of its volatile compounds. For this purpose, plant seeds of *Lallemantia iberica* were sterilized and cultured in petri dishes lined with an MS medium. After the emergence of seedlings, cotyledon segments were transferred to another MS medium supplemented with different combinations of the plant hormones BAP and 2,4-D. The petri dishes were incubated in a growth chamber at 25 °C for a given photoperiod. The fresh weights of the calli thus produced in the hormonal treatments were measured. In a second stage of the study, the essential oil of the fresh calli was obtained using a Clevenger type apparatus and subjected to analysis by gas chromatography-mass spectrometry (GC-MS). Results showed that callus induction from the cotyledon segments of the seedlings was better accomplished in the MS medium containing phytohormones and in a dose-dependent manner. Maximum callus production was induced in the MS medium supplemented with 2, 4-D (4 mg/L) and BAP (1.5 mg/L) as 3.5g. GC/MS analysis showed that the dominant compounds in the essential oil were Thymol (53.03%), Octane (19.90%), Decane (5.73%), Carvacrol (5.63%), and Octadecane (3.73%).

Keywords: ***Lallemantia iberica*, Callus induction, Volatile compounds, Thymol.**

Introduction:

Higher plants are especially gifted with the capacity to produce a large number of the so-called secondary metabolites which are organic chemicals of high structural diversity, or, indicating the ecophysiological role of the plants. These secondary metabolites offer various biological and pharmaceutical properties. Over the past few decades, much effort has been put into the biotechnological production of secondary metabolites using plant cell or tissue cultures for medicinal or pharmaceutical applications (Vasil and Thrope, 1994). Plant tissue culture is regarded as a new technique used for plant propagation, plant breeding, and regeneration of transgenic plants. In other words, plant tissue culture is now immediately related to commercial applications of basic research into cell biology, genetics, biochemistry, and pharmaceutical sciences (Hall, 1999).

Callus formation is the first event in plant tissue culture. The term callus in the early days of plant biology referred to the massive growth of cells and accumulation of callus was associated with wounding. Today, the same term is used in a broader sense to mean a mass of unorganized parenchyma derived from an explant of mature plant tissue on a gel medium enriched with agar, macronutrients, micronutrients, vitamins, and phytohormones (Ikeuchi *et al.*, 2013). Callus can be produced from a single differentiated cell and is often considered to be representative enough for standard scientific analysis. It has been well documented that calli may be exploited as the source of plant secondary metabolites (Rao and Ravishankar, 2002).

Lallemantia iberica (M. Bieb.) Fisch. & C. A. Mey (*Lamiaceae)* is an annual herb growing to an average height of around 40 cm. It originated from the Caucasian region and expanded to west Asia (Syria, Iran, and Iraq). However, it also grows currently in central and southern Europe. The genus *Lallemantia* has 5 different species distributed in different regions of Iran. *Lallemantia iberica*, also recognized in the literature by such other scientific names as *Lallemantia sulphuraea* and *Dracocephalum ibericum* (Bieb), is locally known as Balango Shahri in Iran. The seeds and leaves of the plant have had wide applications in folk medicine. The leaves are used as a potherb in Iran. People also use its leaves, oil, and seeds as a stimulant, diuretic, expectorant, general tonic, and aphrodisiac in traditional Iranian medicine.

Lallemantia iberica has been cultivated for its seed oil. The seed contains up to 30% a drying oil that is used in the production of furniture polish, paint, and soap (Amanzade, 2011). Published literature indicates that the essential oil from its aerial parts contains a high

*****Corresponding Author, Email: razavi694@gmail.com

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variety of terpenoids (Morteza-Semnani, 2006). It has been also shown that TDZ is used in the production of phenols from the calli and shoots of *L. iberica* (Pourebad *et al*., 2015). The regeneration and micro-propagation of *L. iberica* has been extensively investigated (Ozdem *et al*., 2014). However, to the best of the present authors' knowledge, no research has yet been devoted to the use of optimal hormonal mixtures that could be used for plant callus induction and isolation of callus essential oil which is different in profile from that extracted from the plant's aerial parts. The present work focuses on the induction of callus tissue and extraction of its volatile secondary metabolites.

Materials and Methods:

Plant culture and callus induction: The seeds of *L. iberica* were purchased from Pakanbazr Esfahan Co. They were surface sterilized with sodium hypochloride (10%) and ethanol (70%) and washed three times with distillated water before the sterilized seeds were cultured in the MS medium for germination. After seed germination, the cotyledons of the seedlings were isolated and transferred to solid MS media supplemented with vitamins, sucrose, and different concentrations of phytohormones. A number of hormonal mixtures of 2,4-D:BAP (0.5:0.2, 1.5:0.6, 3:1, and 4:1.5 mg/l) were separately used in the different cultures. Three replications of each culture were used. The pH of each medium was adjusted to 5.8 using NaOH or HCl before autoclaving at 120 °C for 20 min. Isolated stem segments were then incubated under a photoperiod of 8/16 h dark/light using a 2400 lux light regime at 25±1 °C. The callus tissue obtained from each treatment was sub-cultured onto a fresh medium after four weeks. The fresh weight of the callus produced in each treatment was measured after 45 days.

Extraction of essential oil: The essential oil of *L. iberica* obtained from the cultured cotyledon callus (3g) containing 2,4-D and BAP (4:1.5 mg/L) was hydrodistillated in a Clevenger type apparatus for 3 h before it was dried over anhydrous sodium sulfate and stored at 4 ℃ in the dark until use.

Essential oil analysi: The oil was analyzed by GC-MS. The analysis was carried out on a Thermoquest-Finnigan Trace GC/MS instrument equipped with a HP-5MS column (30 m × 0.25 mm i.d., film thickness=0.25 µm). The oven temperature was programmed to increase from 50 ºC to 320 ºC at a rate of 4 ºC/min at which it was finally held for 7 min; the transfer line temperature was 250 ºC. Helium was used as the carrier gas at a flow rate of 0.8 ml/min with a split ratio equal to 1/60. The quadruple mass spectrometer was scanned over the 35-465 amu with an ionizing voltage of 70 eV and an ionization current of 150 µA.

Identification of essential oil components: The ingredients were identified by comparing their retention indices (RI) against those reported in the literature (Adams, 2007) and their mass spectrum against the Wiley library (Wiley 7.0).

Statistical analysis: This experiment was conducted in a randomized complete block design with three replications. SPSS 16 software was used for the statistical analysis. Analysis of variance (ANOVA) followed by Duncan's test was used to evaluate the differences amongst the various groups. The significance level was set at $p<0.05$.

Results and Discussion:

Callus formation and hormonal treatments: Small amounts of the *L. iberica* calli were observed to form in the MS medium lacking phytohormones. However, the phytohormones 2,4-D and BAP were found to induce the generation of considerable amounts of callus in a dose-dependent manner. Maximum callus growth was observed with 2,4-D and BAP(4 :1.5 mg/L) that yielded 3 gr of callus during 45 days (Figs. 1 & 2).

The two hormones 2, 4-D and BAP are reportedly the most commonly used for the formation of callus tissues in different plant species (Amanzadeh et al., 2011). Our results indicated that *Lallemantia iberica* callus formation increased with increasing amounts of both hormones in the culture media. Also, callus was observed to form in an MS medium containing only 2, 4-D while no callus formed in an MS medium containing only BAP. Our literature review indicated that the hormones TDZ, BAP, IBA, 2,4-D, and NAA are also capable of inducing callus formation and regeneration in *L. iberica* (Ozdem *et al*., 2014; Pourebad *et al*., 2015). Comparison of the results obtained fom the present study and those reported elsewhere shows that whereas a combination of 2,4-D/BAP induces callus growth in *L. iberica*, regeneration to form shoots and roots is promoted by a combination of either NAA/BAP or NAA/TDZ.

Callus essential oil distillation and analysis: The hydrodistillation of *L. iberica* callus provided a colorless oil with a yield of 0.1% (v/w). The GC-MS analysis of the essential oil revealed nine components representing 97.52% of the oil. The oil mainly consisted of Thymol (53.03%), Octane (19.90 %), Decane (5.73%), and Carvacrol (5.63 %). The oil was characterized by oxigenated monoterpenes (58.68%) and hydrocarbones (35.13%). Table 1 presents the compounds of the essential oil of *Lallemantia iberica* callus arranged in a descending order of retention time.

Previous reports indicated that *Lallemantia iberica* produces a number of secondary metabolites such as flavonoids, triterpen, and phenolic acid (Khosravi, 2012). Moreover, there have been reports indicating that TDZ enhances the total phenol and flavonoids in the callus and shoot cultures of the plant (Pourebad *et al*., 2015). On the other hand, the aerial parts of the plant produce essential oils rich in monoterpenes and sesquiterpens with antioxidant effects (Amanzade, 2011). The oil is composed of β-cubeben, Linalool, spathulenol, geraniol, bicyclogermacrene, caryophylene oxide, Germacrene-D, β-bourbonene, Trans-geraniol,

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Figure 1. Callus tissue of *Lallemantia iberica*

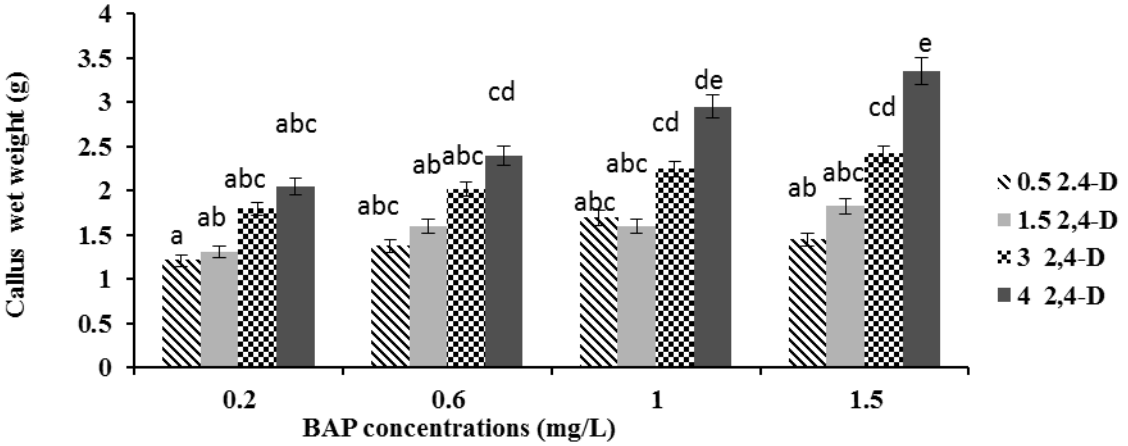


Figure 2. Effects of different concentrations of 2,4-D and BAP on the production of callus tissue of *Lallemantia iberica*. Mean values with the same latters are not significantly different at the 0.05 probability level according to the Duncan's test.

Table1. Composition of the essential oil of *Lallemantia iberica* callus

No	Compounds	%	RI	Rt
1	Heptane	0.94	700	2.43
2	Octane	19.90	800	2.64
3	Valeric acid	0.53	947	5.07
4	Decane	5.73	1000	5.57
5	Dodecane	4.83	1200	9.06
6	Thymol	53.03	1290	11.41
7	Phenol	3.20	1191	15.51
8	Carvacrol	5.63	1299	21.95
9	Octadecane	3.73	1800	30.91
	Total identified	97.52		

RI= retention indices on DB-5 capillary column, RT= standard retention time

pcymene, isophytol, T-cadinol, and terpinen-4-ol (Morteza-semnani, 2006). Our results indicated that thymol and carvacrol are the main components of the callus essential oil obtained from the cotyledon of *Lallemantia iberica*. Thus, it may be concluded that the chemical composition of the oil obtained from the callus is entirely different from those obtained from the other parts of the plant. This finding is confirmed by the fact that metabolic pathways in the callus tissue may be different from those of the other parts.

Thymol with various industrial and pharmaceutical applications is the characteristic monoterpenic compound in the genus *Thymus* of the *Lamiaceae* family. Tyhymol is an isomer of carvacrol with similar bioactivities (Buckingham, 2005). It is part of a naturally occurring class of compounds known as

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biocides with strong antimicrobial attributes when used either alone or with other biocides such as carvacrol (Palaniappan et al., 2010). Numerous studies have demonstrated the antimicrobial effects of thymol, ranging from inducing antibiotic susceptibility in drug-resistant pathogens to powerful antioxidant properties (Ündeğer et al., 2009). Research has also shown that naturally occurring biocides, such as thymol and carvacrol, reduce bacterial resistance to antibiotics through a synergistic effect, and that thymol is an effective fungicide, particularly against fluconazole-resistant strains (Zarrini *et al*., 2010). Because of these properties, thymol has found industrial applications; it is used as a preservative in halothane, an anesthetic, and an antiseptic in mouthwash (Filoche *et al*., 2005).

Given the biological, pharmaceutical, and industrial applications of thymol and carvacrol, further research is required to develop a biotechnological method for their production.

Conclusion:

Based on the results of this study, a hormonal combination of 2, 4-D/BAP was found capable of inducing the formation of *Lallemantia iberica* calli. It was found that the callus of the plant produces various volatile constituents that differ from the essential oil constituents extracted from plant leaves. Finally, Thymol (53.03%) was observed to be the main constituent obtained from *L. iberica* calli with a wide variety of industrial and pharmaceutical applications.

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