



## RESEARCH PAPER

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## Assessment of fennel (*Foeniculum vulgare* Mill.) populations under hormonal treatments in terms of callus and its essential oil profile

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**Key words:** Essential oil, trans-anethole, fennel (*Foeniculum vulgare* Mill.), callus.

<http://dx.doi.org/10.12692/ijb/5.1.190-194>

Article published on July 11, 2014

### Abstract

In this research callogenesis and essential oil content changes of hypocotyl explants callus in five fennel populations (German, Antep, Isfahan, Karaj and Torbatjam) on MS medium under two combined hormonal NAA (1-Naphthaleneacetic acid) + BA (6-Benzylaminopurine) and Kinetin + 2,4-D (2,4-dichlorophenoxy) treatments were studied. The percent of callogenesis, diameter of callus, fresh and dry weight of callus characteristics were evaluated, and also the callus essential oil compounds of populations determined using GC-MS. The analysis of variance showed no significant differences between hormonal treatments for studied characteristics in populations. However, there were significant differences among populations for all traits. The interaction of hormonal treatments and populations was significant only for dry weight trait. Considerable variation was seen in the amount of essential oil components. Highest Trans-anethole (the main metabolite of fennel) content in fennel callus oil, were observed in Antep population under NAA+BA hormonal treatment.

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## Introduction

Fennel (*Foeniculum vulgare* Mill.) with a variety of secondary metabolites as a valuable herb in the treatment of flatulence, coughs, and has the effect of increasing breast-milk production. According to widespread use of fennel essential oil in pharmaceuticals, food, cosmetics, tissue culture and assay of the callus, can be very important. Due to the low cost and simplicity of drug manufacturing plants compared with chemical methods and also due to the many side effects of drugs, the use of medicinal plants has encountered with the substantial growth (Anzidei *et al.*, 1996).

In the recent years, advances in biotechnology research, commercially cultivating plants and production of in vitro secondary metabolites has been discussed. The purpose of this technology is to control plant metabolite biosynthetic pattern in order to make an optimal production or even production of a new metabolite in the plant. There are many reports in connection with the use of tissue culture techniques in medicinal plants (Bhojwani and Razdan, 1996). In vitro culture of cells, tissues or organs provides the accessibility to important secondary metabolites. Production of these metabolites by plant cell and tissue culture has many advantages such as Standardization and improvement of products quality (Zobayed *et al.*, 2004). In addition, Plant cell culture is considered as an effective system for the study of the biological importance of bioactive metabolites in vitro (Yanpaisan *et al.*, 1996). This study is aimed to investigate the effect of growth regulators on five populations of fennel callogenesis and compare them based on the amount of active substance in callus.

## Materials and methods

### *Plant materials and experiment design*

The studied plant materials were consist of five fennel populations: Antep (Turkey), Isfahan (Iran), Karaj (Iran), Torbatjam (Iran), and German which have been compared in two different hormonal composition in terms of the amount of active substance based on the two-factor factorial

experiment in a completely randomized design with three replications.

### *Tissue culture*

A required amount of fennel seed from each population, placed in sterile distilled water for one hour to get drench. Then the seeds, under a laminar hood, sterilized for one min in 70% ethyl alcohol then for 20 min in 2% sodium hypochlorite, and after that rinsed thoroughly for three times with sterile distilled water. These seeds were kept in base MS medium with pH 5.6–5.8, and 8g/lit agar. Root explants, hypocotyls, epicotyl, cotyledon and leaves were used to make callus.

### *Hormonal treatments*

The explants of different populations which were placed in base-MS medium containing agar and two hormonal compositions, 2,4-D and 1mg/lit kinetin and 1mg/lit NAA and BA, were transferred to germinator for callogenesis. Callogenesis percent, weight and size of the callus is measured, then by the use of organic solvents, the callus extract is derived and its ingredients were identified based on comparison of their mass spectra in the apex of each peak with those of analytical standards from Mass Spectral Library.

## Results and discussion

### *Callogenesis*

Among the mentioned explants, root, hypocotyls, epicotyls, cotyledon and leaves were eliminated in callogenesis phase because their callogenic response was poor. The swollen hypocotyls explants of each population placed in callogenesis medium. The hypocotyl explants of each population that were grown in MS medium under two hormonal compositions, revealed 100% callogenesis and the studied populations had no difference in percent of callogenesis. Before data analysis, assumptions of analysis of variance for each studied traits were investigated and were corroborated for all traits except the dry weight of callus. Logarithmic transformation was used to normalize distribution of deviations for dry weight of callus.

### Statistical Analysis

The result of analysis of variance revealed significant differences among populations for callus diameter, fresh and dry weight characteristics at 1% probability level (Table1). Therefore, there were genetic variations between populations in terms of studied traits. No significant differences were observed between two hormonal composition for the diameter, fresh and dry weight characteristics. The interaction of population and hormonal treatment was not significant. The mean comparison for the callus diameter, showed that German population explants

produced greater callus diameter than other populations (Fig. 1). The interaction of population and growth regulator was significant only in callus dry weight. Therefore studied populations had a different response to different levels of hormonal treatments. The highest callus fresh weight is belonged to Antep population at the average of hormonal treatments (Fig. 2), and highest callus dry weight between populations and hormonal treatments belonged to Torbatjam population into medium containing 2,4-D+Kin (Fig. 3).

**Table 1.** Analysis of variance for studied characteristics in callus of fennel populations under different hormonal treatments.

S.O.V	DF	MS		
		Callus dry weight	Callus fresh weight	Callus diameter
Hormone	1	0.079 <sup>ns</sup>	0.001 <sup>ns</sup>	93.982 <sup>ns</sup>
Population	4	1.385 <sup>**</sup>	0.016 <sup>**</sup>	1356.418 <sup>**</sup>
Hormone×Population	4	0.324 <sup>**</sup>	0.001 <sup>ns</sup>	155.553 <sup>ns</sup>
Error	40	0.056	0.001	60.454
CV(%)		10.75	25.93	16.18

ns: non-significant and \*\* significant at the 1% level.

By considering of fresh weight and diameter of callus characteristics, it can be concluded that, because of the differences between populations in callogenesis and their calluses density, a bigger callus was not the heavier one, necessarily. In spite of that, upper consistency between these two characteristics was observed in populations, As the German, Torbatjam, and Antep Populations had very high value for both characteristics compared with Isfahan and Karaj

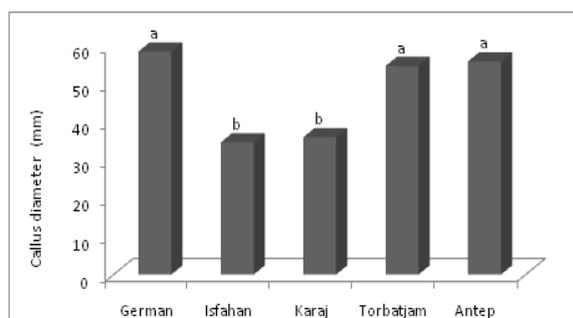
populations. By comparing of dry and fresh weight of produced callus in each population, although fresh weight of Antep population was more than other populations, dry weight of this population was less than Torbatjam population, that it can be caused by the high humidity of calluses of Antep population compared with Torbatjam, also the difference of their dry weights was because of the loss of the humidity.

**Table 2.** Constituents of the essential oil obtained from the five fennel populations callus at under two different hormonal treatments.

Percentage of each chemical compound														Treatments Population	
camphene	$\alpha$ -phellandrene	Cadinene	Verapamil	fenchone	$\gamma$ -terpinene	cisocymene	estragole	myrsene	sabinene	$\alpha$ -pinene	apiol	Limonene	trans anethole		
-	-	-	-	-	-	-	-	-	-	-	5.53	6.835	-	NAA+BA	German
-	-	-	-	-	-	-	-	-	-	-	-	3.5	-	Kin+2,4-D	
-	-	-	2.177	4.06	2.129	3.515	3.256	2.115	2.282	4.13	-	9.69	67.229	NAA+BA	Antep
-	3.68	-	-	2.911	-	-	1.06	-	-	-	-	2.05	6.34	Kin+2,4-D	
2.251	-	-	3.64	-	-	-	-	-	-	-	-	-	-	NAA+BA	Karaj
-	-	3.243	2.115	-	-	-	-	-	-	-	-	-	-	Kin+2,4-D	
-	2.157	-	-	-	-	-	-	-	-	-	-	2.68	-	NAA+BA	Torbat jam
-	-	-	5.5	-	-	-	-	-	-	-	-	-	-	Kin+2,4-D	
-	-	-	-	-	3.47	-	-	-	-	-	1.07	3.72	-	NAA+BA	Isfahan
-	-	-	-	-	-	-	-	-	-	2.553	-	-	-	Kin+2,4-D	

### Essential oil composition

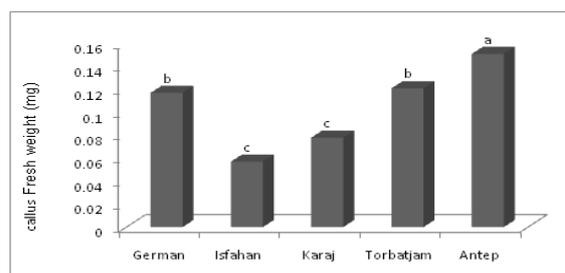
Analysis of callus essential oil compositions at two different hormonal treatments by using gas chromatography (GC) coupled to a mass spectrometer (MS), 14 compositions were identified in essential oil at total (Table 2). Based on GC-MS results, high variations were observed among populations subjected to amounts and kind of the essential oil composition. The highest level of Trans-anethole and estragole as more important substances were measured in extracted essential oil from Antep population that grown under mediums with NAA+BA. The trans-anethole which is of the valuable secondary metabolites in this fennel, significantly (67.229 %) is produced in calluses obtained from Antep population under NAA+BA hormonal treatment. The content of trans-anethole in antep population calluse under 2,4-D+Kin treatment was about 6.34%. In calluses of other populations this effective substance haven't been observed. In many studies on fennel tissue culture and its essential oil components, trans-anethole hasn't been observed (Kirici *et al.*, 2010). Nevertheless Afify *et al.*, (2011) were observed 15.28% of trans-anethole in extracted essence from fennel calluses produced in a medium containing of equal compositions of two growth regulators 2,4-D and Kin (0.5 mg/lit). In the presented survey, the grown callus in a medium containing 2,4-D+Kin with equal proportion of 1:1, 6.34 % of this substance was observed and a remarkable amount of the substance was obtained in the essential oil of the grown callus in Antep population in MS medium containing NAA+BA with proportion of 1:1.



**Fig. 1.** Mean comparison of fennel populations callus diameter using Duncan's test at 5% probability level.

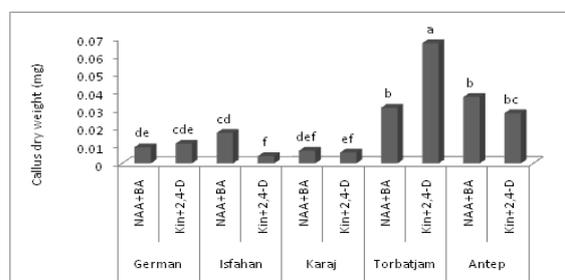
According to the results the most amount of limonene

(9.69 %) was achieved in Antep population with the hormonal composition of NAA+BA.  $\alpha$ -pinene and  $\gamma$ -terpinene are among the other important identified effective substances that were only observed in Antep and Isfahan populations. In Antep population callus with NAA+BA hormonal treatment, 4.13%  $\alpha$ -pinene and 2.13 %  $\gamma$ -terpinene was produced. In the grown callus of Isfahan population,  $\alpha$ -pinene was synthesized with applying 2.553% 2,4-D+Kin treatment and 3.47%  $\gamma$ -terpinene in a medium containing NAA+BA.



**Fig. 2.** Mean comparison of fennel populations callus fresh weight using Duncan test at 5% probability level.

Sabinene, myrcene and cis-ocymene were the secondary metabolites that observed only in Antep population. These aroma compounds in the population's callus were obtained under NAA+BA treatment. Estragol was also produced in Antep population callus under both hormonal treatments. Fenchone is another fennel volatile oil compound which is used in perfumery and it was identified only in Antep population. This substance was obtained by applying in both of 2,4-D+Kin and NAA+BA hormonal treatment at 2.91% and 4.06% amounts respectively. Other populations' callus didn't have this fenchone.



**Fig. 3.** The mean comparison of callus dry weight in fennel populations under different hormonal treatments using Duncan test at 5% probability level.

Two other compounds identified in essential oils compound were the cadinene and camphene that were observed in Karaj population only. The cadinene content in 2,4-D+Kin hormonal treatment was 2.25% and the camphene content under NAA+BA treatment was 3.24%. Apiol is an organic chemical compound that is found in German and Isfahan populations under NAA+BA hormonal treatment at 5.53% and 1.07% amounts respectively. This substance is used to regulate menstrual disorders and it is the main chemical compound in parsley (Kong *et al.*, 2003). The essential oils of Antep population callus under 2,4-D+Kin treatment and in Torbatjam under NAA+BA treatment contained the  $\alpha$ -phellandrene, 3.68% and 2.16% respectively. Verepamil was found in Antep (2.17%) and Karaj (3.64%) populations under NAA+BA hormonal treatment, and also in Karaj (2.12%) and Torbatjam (5.5%) populations in 2,4-D+Kin treatment.

Concerning the mentioned results, it can be concluded that the existence of NAA+BA growth regulators was much more effective on synthesis of valuable secondary metabolites. Among the observations, the type and amount of Auxin or cytokinin, or proportion of Auxin to cytokinin, can change the formation and accumulation of secondary metabolites in the cultured plant cells, and also it is reported that the 2,4-D growth regulator, prevents the production of secondary metabolites in many cases (Bohm and Rink, 1988). The NAA, also increases their secretory and stimulates their activity, and finally increases the amount of produced volatile oil compounds rate (Ramachandra Rao and Ravishankar, 2002).

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