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Effect of Plant Regulations on Callus Essential Oil Content of Fennel (Foeniculum Vulgare Miller) Populations

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Abstract

Foeniculum vulgare Mill., a herbaceous species belonging to the Apiaceae (Umbelliferae) family, is considered as a valuable plant for its pharmaceutical, aromatic and culinary properties. In this research, callus induction and variations in the amount of callus essence in hypocotyl explants of five populations including those of Germany, Turkey, Karaj, Isfahan, and Torbatjam were studied in two hormonal treatments. Two treatments were performed: 1st, the effect of explant sources on MS medium supplemented with 1 mg/liter kinetin and 0.5 mg/liter 2, 4-D; and the 2nd, the effects of 1 mg/liter BA and 0.5 mg/liter NAA, respectively. This study aimed at elucidating the chemical composition of essential oil extracted from callus, subjected in detail by using gas chromatography-mass spectrometry (GC–MS) analysis. Callus essential oil compounds of the populations were analyzed by GC-MS and a considerable variation was observed in the quantity of the essential oil components. Essential oil profiling in callus showed that the main characteristic of the oil was a high content of the limonene, fenchone, and estragole. The results of this study showed the highest level of trans-anethole, the most important secondary metabolite in fennel callus oil in the populations of Antep under BA+NAA hormonal treatment.

Key words: Fennel (Foeniculum vulgar Mill.), Essential oil, GC-MS, Callus, Trans-anethole, Limonene, Fenchone.

Introduction

Medicinal plants have gained increasing interest as important sources of life-saving medicines for the world's population and natural alternatives to chemical drugs. (McGaw et al., 2005). They are widely used to produce highly diverse secondary metabolites, which are limited to taxonomically related species (Gandhi et al., 2014). Essential oil compounds are generally volatile mixtures of various liposoluble organic materials (Carrubba and Catalano, 2009) that are divided into 2 main groups: the first group contains hydrocarbons that are exclusively made up of terpenes, and the second group contains oxygenated compounds, predominantly ketones, aldehydes, esters, alcohol, oxides, and phenols (Olle and Bender, 2010). *Foeniculum vulgare* Mill. commonly called as fennel is a popular medicinal plant species from the Apiaceae (Umbelliferae) family and native to the Mediterranean basin (Hunault et al. 1989). Often referred to as the "star among medicinal species", nowadays fennel is a rich source of secondary metabolites and essential oils, in which details on chemical constituents of essential oil and plant parts and also their pharmacological properties are included. (Olle and Bender, 2010). One of these chemical constituents is trans-anethole, which is much used as a flavoring agent and has great economic importance in the food and cosmetic industries. Moreover, it is used in various illnesses related to endocrine, digestive, respiratory, reproductive, and systems. It is also used as a galactagogue agent for nursing mothers (Anzidei et al., 1996).

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Biotechnological tools are principal for genetic enhancement and proliferation of the medicinal plants by using techniques including genetic transformation and in-vitro regeneration. In addition, the use of plants as bioreactors can be harnessed for the production of secondary metabolites. Many studies have been conducted regarding the use of tissue culture techniques in medicinal plants (Bhojawani and Razdan, 1996). Plant tissue and cell culture technologies can be routinely utilized in the artificially prepared medium under sterile conditions and controlled environment from explants, including meristem cells, stems, leaves, and roots for both the proliferation and extraction ways of secondary metabolites. (Rehman et al, 2003).

This study aimed at the induction of callus production in leaves, cotyledon pieces, shoot apices, and hypocotyl segments of fennel (*Foeniculum vulgare* Mill.), and comparing them with the amounts of 5 populations of fennel, studied in two hormonal treatments.

Materials and Methods

This study was conducted at the Plant Tissue Culture Laboratory of the Jaberebne Hayyan, the Tabriz University of Iran from January 2012 until July 2012.

Plant materials

The plant materials consisted of 5 fennel populations: N6 (German), Antep (Turkey), Karaj (Iran), Isfahan (Iran), and Torbatjam (Iran), which were compared in 2 different hormonal compositions in an absolutely randomized design with three replications regarding the active substance amounts on the basis of the two-factor factorial experiment.

Sterilization Methods and Conditions

Glasses were first washed with detergent and water and dried in an oven at 95°C. Then they were wrapped in a brown cover and autoclaved at 121°C - 15 psi for 15 minutes. Further sterilization was conducted in a laminar flow cabinet, rinsed 2-3 times with distilled water. Instruments used for inoculation e.g. forceps, scalpels, spatula, needles, and blades were sterilized with 70% ethanol. The instruments were placed in a beaker containing ethanol and flamed over spirit lamp before every use. The laminar flow cabinet was irradiated with UV light for 30-40 minutes before inoculation, and then sprayed and scrubbed thoroughly with ethanol. Ultrafilter air was used in the laminar flow cabinet during inoculation (Rehman et al, 2003). Hands were washed first with soap and then rubs 70% isopropanol plus 0.5% chlorhexidine solution.

Inoculation and Incubation.

Before inoculation, the laminar flow was irradiated with UV light for 30 minutes. The ultrafiltered air was turned on. The spirit lamp was placed in the center of the laminar flow. The required amount of fennel seed from each population was rinsed 15 times by the ultra-filtered (0.2-µm pore size) deionized water and a drop of detergent, followed by a 1-minute rinse in 70% ethanol under a laminar hood a 20-minute rinse in 2% sodium hypochlorite solution. Before germinating the seeds, 3 additional rinses in autoclaved sterilized double distilled water were done to remove the residual sodium hypochlorite.

Seed germination

Seeds were placed in sterile vessels, which contained Murashige and Skoog medium (MS medium; Murashige and Skoog, 1962) supplemented with 0.8% agar and 2% sucrose. The pH of the medium was set at 5.6-5.8 before sterilization at 15 psi for 15 minutes. After autoclaving, seeds were placed on germination medium (50 per glass) under Laminar Air Flow and maintained in a growth chamber. They were incubated in darkness at 23 ± 2 °C for 2 weeks until germination.

In vitro plant growth

After 5-7 days, hypocotyls were excised from seedling and root explants, and then epicotyl, cotyledon, and leaves were utilized to make callus. (Fig. 1)

Fig. 1. In vitro plant growth

Explants isolate and culture hormonal treatments

The explants of different populations, cultured on different media, were supplemented with some phytohormones. The explant of different populations including Isfahan (Iran), Antep (Turkey), German, Torbatjam (Iran), and Karaj (Iran) was used to induce the callus. The hypocotyl were cut into 1 cm × 1 cm sizes and placed onto Murashige Skoog MS+1 mg/liter kinetin plus 0.5 mg/liter 2, 4-D, and MS+1 mg/liter BA plus 0.5 mg/liter NAA (1-Naphthaleneacetic acid). The explants were left in the growing room where the temperature was about 25±2 °C and a cool white fluorescent light was used. Each of the media plates that contained ten induced callus was considered as one biological replicate. For subculturing, the same process was repeated for inoculation as previously explained. Calli started growing after two weeks and data were collected for five weeks. The dry and fresh weights of callous mass and the callus induction percentage were measured. To measure the dry weight, we wrapped calli in aluminum foil and kept it at 75° C for 48 hours. We analyzed the statistical data in the form of factorial on a complete randomized design in 5 replications. Then we compared the means with LSD test at a 5% level of significance ($P < 0.05$) by using SAS software. We also plotted the graphs by Excel software. This study aimed to identify the best explant type obtained from in vitro grown seedling of sweet basil and also the most efficient growth regulator concentration and combinations for shoot formation and regeneration. (Fig. 2)

Fig. 2. The explants of different populations cultured on two phytohormones.

Preparation of callus extract and Gas chromatography/mass spectrometry analysis

The callus extract was transferred into spectrometry and extracted three times by 20 ml n-hexane. After collecting the bottom layer of n-hexane extract from the funnel, the essence was collected and dried over Na_2SO4 (anhydrous sodium sulfate) stored in brown bottles in the refrigerator for GC-MS analysis. 1 µl of the extract was injected into a GC-MS (MS model 5975C, GC model 7890, Agilent Technologies, Santa Clara, CA) after filtration with a 0.22-µm syringe filter. GC separation was conducted on an HP-5MS capillary column (Agilent Technologies, Santa Clara, CA) operating at electron impact mode at 70eV. Pure helium gas with a built-in purifier was utilized at a constant flow rate of 1ml/min employed in a splitless mode with an ion source of 280°C and an injector temperature of 250°C. The stepped temperature program was as follows: the initial temperature of the oven was 220°C, hold for 5 min and then a ramp to 300°C at 5°C/min hold for 15 minutes. For the next sample injection, a post-run of 5 min at 300°C was enough. A mass analyzer was utilized in full scan mode scanning from 40-550 m/z and mass spectra were taken at 70 eV. For identification of the compound, manual spectral matching was ascertained by the mass spectral library of National Institute Standard and Technology (NIST) 2.0 and with the help of Automated Mass Spectral Deconvolution and Identification Software (AMDIS) 2.70 by deconvolution of the chromatography peak at the corresponding retention time.

Results and Discussion

Analysis of callus essential oil compositions at two different hormonal treatments was done on 10 essential elements among the studied populations by using gas chromatography (GC) coupled to a mass spectrometer (MS). Based on GC-MS results, the compounds obtained from the two treatments in the whole experiment were trans-Anatole, limonene, fenchone, α-pinene, estragole, sabinene, myrcene, apiol, and verapamil.

Antep population under MS+1 mg/liter BA plus 0.5 mg/liter NAA phytohormones treatment had the highest amount of trans-anethole as an important substance, measured 9.11% and the other level of trans-anethole substance was measured in extracted essential oil from the same population under MS+1 mg/liter kinetin plus 0.5 mg/liter 2, 4-D, phytohormones treatment about 6.34%. German population (N6) grown under both treatments had the trans-anethole, too. In the calli of other populations, this effective substance was not observed. Trans-anethole has not been observed in many investigations on fennel tissue culture and its essential oil components (Kirici et al., 2010). However, Afify et al., (2011) observed 98.73% trans-anethole in the extracted essence of fennel calluses, which was produced in a medium, contained equal compositions of 2 growth regulators Kin (0.5 mg/lit) and 2,4-D. Hasanzadeh et al., (2014) observed 67.229% of trans-anethole in the extracted essence of fennel from the Antep population under 1 mg/liter BA plus 0.5 mg/liter NAA.

Concerning the mentioned results, it can be concluded that the existence of NAA+BA growth regulators was much more effective in the synthesis of valuable secondary metabolites. Among the observations, the type and amount of auxin or cytokinin, or the ratio of auxin to cytokinin, can change the formation and accumulation of secondary metabolites in the cultured plant cells. Moreover. 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 the secretion of these hormones, stimulates their activity, and finally increases the amount of produced volatile oil compounds rate (Ramachandra Rao and Ravishankar, 2002).

According to the results, the most amount of limonene i.e. 4.83% was achieved in the German population with the hormonal composition of 1 mg/liter BA and 0.5 mg/liter NAA.

Fenchone is another fennel volatile oil compound, used in the perfumery, which was identified in German and Antep populations. This substance was obtained by applying in both of hormonal treatment and Isfahan populations under 1 mg/liter BA plus 0.5 mg/liter NAA hormonal treatment, but other callus populations did not have this substance. The highest level of fenchone (5.8%) was measured in the German population, grown under mediums with 1 mg/liter BA plus 0.5 mg/liter NAA hormonal treatment with an equal proportion of 1:0.5.

Estragol is an effective substance that was found in all populations under both treatments. According to the results, the most amounts of estragol, i.e. 2.74%, was achieved in the Isfahan population with the hormonal composition of 1 mg/liter BA plus 0.5 mg/liter NAA. In Antep and German callus populations with 1 mg/liter BA plus 0.5 mg/liter NAA hormonal treatments, myrcene was produced 0.38% and 0.57% respectively. Other populations' calli did not have this substance.

Terpinene was the secondary metabolite observed in the Karaj population under both Antep and German hormonal treatment populations under 1 mg/liter BA plus 1 mg/liter NAA.

α-pinene is among the other important identified effective substances, observed only in Antep and German populations under 1 mg/liter BA plus 0.5 mg/liter NAA, 1.63%, and 1.22%, respectively.

Sabinene is the secondary metabolite that was observed only in the Antep population. This aroma compound in the callus populations was obtained under 1 mg/liter BA plus 0.5 mg/liter NAA treatments.

Apiol is an organic chemical compound that was found in German and Karaj populations under 1 mg/liter BA plus 0.5 mg/liter NAA hormonal treatments at 1.32% and 0.95% amounts respectively. This substance is used to regulate menstrual disorders and it is the main chemical compound in parsley.

Verepamil was found in all populations under 1 mg/liter BA plus 0.5 mg/liter NAA hormonal treatments, and also in German (2.27%), Karaj (1.14%) and Torbatejam (0.93%) populations in 1 mg/liter kinetin and 0.5 mg/liter 2, 4-D treatment.

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