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Micropropagation of wild fennel (*Foeniculum vulgare var. vulgare)* via organogenesis and somatic embryogenesis

Rehab H. Abd-Allah, Ehsan M. Abo Zeid, Mahmoud M. Zakaria* and Samih I. Eldahmy

Department of Pharmacognosy, Faculty of Pharmacy, University of Zagazig, Zagazig 44519, Egypt.

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Wild fennel (*Foeniculum vulgare var. Vulgare*) is a perennial aromatic herb. It is native to the Mediterranean region and currently it iscultivated as an annual or perennial herb worldwide. Dried ripe fruits are commonly used plants part for obtaining essential oil. Fruits oil of wild fennel contains many volatile oils, such as α-pinene, phellandrene, p-cymene, fenchone, estragole, anethole and anisaldehyde. Important biological activities of the volatile oil of fennel fruits are hepatoprotective, hypotensive, anticancer, antioxidant, antibacterial, antifungal, antiviral, hypoglycemic, spasmolytic, analgesic, antipyretic, anti-inflammatory and C.N.S activities. Wild fennel (*Foeniculum vulgare var. vulgare*) is categorized as rare and endangered in the Egyptian flora due to urban sprawl, especially along northern coastal area and it has turn into a retreat in Egypt. Therefore attempts were made to find *in vitro* germination of its seeds as well as to explore the ability of organogenesis and somatic embryogenesis in the produced callus. In order to continue the micropropagation process, transplantation of developed plantlets to the soil was also investigated. This study also looks into the production of different medicinally valuable volatile oils in the formed calluses and micro-propagated plants.

Key words: Wild fennel, *Foeniculum vulgare*, seed germination, callus production, somatic embryogenesis.

INTRODUCTION

*Foeniculum vulgare var. vulgare*family Apiaceae,is common or bitter fennel, which is known as "shamar" in Egypt and also this is the official fennel according to the Egyptian pharmacopeia (Egyptian Pharmacopoeia, 1984). It is native to Mediterranean region but now it is cultivated as an annual or a perennial herb in Argentina, Hungary, Bulgaria, Germany, France, Italy, Greece, china and India (Leung and Foster, 1996; Chevallier, 1996). Important compounds identified in all samples of fennel volatile oils were *trans*-anethole, estragole, fenchone, limonene, *alpha*-pinene and *gamma*-terpinene (Aprotosoaie et al., 2008). The plant has shown various pharmacological properties involving antibacterial (Gulfraz et al*.,* 2008), antispasmodic (Alexandrovich et al*.,* 2003), analgesic (Guang-shou et al*.,* 2011), anti-inflammatory (Ozbek, 2005), antipyretic (Tanira et al*.,* 1996), anxiolytic (Kishore et al*.,* 2012), antioxidant (Singh et al*.,* 2008), (Moon et al., 1985), diuretic (Tanira et al*.,*1996), antihypertensive (Haze et al., 2002), mucolytic (Mills and Bone,2000) and hepatoprotective (Mansour et

*Corresponding author. E-mail: elsameeh@gmail.com, mahmoudelshenawy188@yahoo.com.

al., 2011) activities.

In recent years *in vitro* techniques have received increasing importance in the conservation of threatened plants and this trend is likely to be continued as more species are expected to face the risk of extinction (Kapai et al., 2010). *In vitro* propagation offers an easy, rapid and space-efficient way for mass scale multiplication of plant species.

Available reports on micropropagation of *Foeniculum vulgare var*. *vulgare*do not provide exclusive information about chemical composition of induced calluses or micropropagated plants. This study is aimed to investigate the *in vitro* germination behavior of the seeds of wild fennel (*Foeniculum vulgare var. vulgare*). The ability of explants excised from growing seedlings to form a stable callus was determined representing first step for micropropagation. The ability of produced plant calluses to form somatic embryos to continue the micropropagation process and transplantation to the soil were also investigated. The study also looks into the production of different medicinally valuable volatile oils components in the formed calluses and micropropagated plantlets besides fruits of cultivated and wild fennel as well as the aerial parts of the wild fennel.

MATERIALS AND METHODS

Collection of plants materials

Whole plant and seeds of *Foeniculum vulgare* var.*vulgare*(wild), were collected on April 2013 from SidiBarrani in the north coast of Egypt. Identity of collected material was verified by Assistant Prof. Dr. Eman Shams, Assistant Professor of plant taxonomy, Faculty of science, Cairo University. Voucher specimens were deposited to the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Egypt. Matured seeds were collected from the wild plant on April.

Sterilization

Seeds were sterilized through submerging them 70% ethyl alcohol for different periods 1, 2, 3 and 4 min and then shaking with 5% commercial hypochlorite solution (Clorox®) for different peroids 10, 15, 20, 25 and 30 min. Under the hood, sodium hypochlorite was poured away from seeds then seeds were rinsed thrice with sterile distilled water before applying to media for germination.

Germination of seeds

Some sterilized seeds were transferred to jars (5 seeds/jar) containing solid hormonal free (HF) media of the composition (4.4 g/l M.S.(Murashige and skoog) media, 30 g/l sucrose and 8 gm/l agar) or transferred to the same media composition in addition to 50 mg/l gibberillic acid solution as growth enhancer. The lid of each jar was wrapped with para film and incubated in a growth room at 25°C and a photoperiod of 16 h fluorescent light and 8 h dark. Germination of seeds was investigated using seed germination percentage using the following formula:

$$\text{Seed germination percentage} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds cultured}} \times 100$$

Production of callus

Seedlings of 30-35 days old obtained from seeds germinated on HF solid media, were cut aseptically into pieces of 4-6 mm length of leaf, stem and root which were used as a source of explants. Explants were cultured on sterile solid M.S. medium supplemented with different growth regulators including naphthalene acetic acid (NAA), 6-benzylaminopurine (BAP), 2,4- dichlorophenoxyacetic acid (2,4-D), kinetin (K) and thidiazuron (TDZ) in different combinations and concentrations such as (2,4-D 0.5 mg/l +K 0.1 mg/l), (2,4-D 0.5 mg/l +K 0.5 mg/l), (2,4-D 1 mg/l +K 0.5 mg/l), (2,4-D 1 mg/l + K 1 mg/l), (2,4-D 2 mg/l +K 1 mg/l), (2,4-D 2 mg/l +BAP 0.25 mg/l), (NAA 1 mg/l +K 1 mg/l), (NAA 1 mg/l +BAP 0.1 mg/l) and (TDZ 0.5 mg/l +2,4-D 1 mg/l +BAP 0.1 mg/l) for initiation of callus. Each treatment consisted of five explants per jar with four replicates. All cultures were incubated in a growth room at temperature 25°C ±1 and photo period of 16 h light and 8 h dark. Callus induction and maintenance was investigated using three parameters: callus induction time, callus induction percentage and callus growth rate. Callus induction time is the number of days passed until the callus is formed. Callus induction percentage is calculated using the following formulae:

$$\text{Callus induction percentage} = \frac{\text{Number of callus produced}}{\text{Total number of explants cultured}} \times 100$$

Callus growth rate is represented by the total fresh weight (mg) in different time intervals (days) which was calculated by adjusting the weight of all calluses obtained by all previously mentioned phytohormonal combinations to 1000 mg then increase in callus fresh weight was monitored and calculated at different time intervals (10, 20, 30 and 40 days).

In vitro regeneration

Micropropagation involves direct and indirect techniques which allow the *in vitro* clonal propagation of parts or even cells of the required plant to a whole plant. Direct techniques aim to produce clones of the plant directly from meristematic tissue and buds through direct organogenesis and somatic embryogenesis. Indirect techniques involve the production of callus through which a whole plant could be produced using the above two methods.

Direct micropropagation

Direct micropropagation is rare; it involve direct shooting or rooting from buds or other meristematic tissue. This should be under the influence of different phytohormones. *Foeniculum vulgare var. vulgare* produced seedlings were cut in to different explants of root, stem and leaf, and subjected to different types of phytohormones and their combinations as the previously mentioned hormones and cultured on M.S. solid media at 25±1°C and 16 h photo period either with sucrose containing media or sucrose free media and examined periodically macroscopically and microscopically to detect any signs of organogenesis or somatic embryogenesis.

Indirect micropropagation

After callus was successfully produced, representing stage I of indirect micropropagation, stage II began and involved trials to make shoots, roots or somatic embryos from callus cells. Different conditions were applied on different callus cells produced in order to induce organogenesis or embryogenesis. Phytohormones were used solitary and in combinations with different concentrations and

hormonal free media were also used with high light intensity to induce photosynthesis in the callus cells and thus encourage the cells to differentiate into shooting cells. Media with auxins only were used to induce rooting, media with cytokinins only were used to induce shooting and also some callus were transferred to hormonal free media. In each trial, cells from callus were tested under microscope to detect the production of somatic embryos.

In vivo culturing of the micropropagated plantlets

Plantlets were placed in perforated pots containing autoclaved asbestos pertile soil and all pots were covered with transparent plastic bags to maintain high humidity and allow easy illumination. Pots were placed in a growth room at temperature 25°C ±1 and photo period of 16 h light and 8 h dark. The transparent plastic bags were perforated after two days. Each pot was irrigated every two days with a little amount of water. The transparent plastic bags were removed away after seven days.

Trial for hardening the root system

Some plantlets were put in test tubes containing hormonal liquid media of the auxin (NAA 1 mg/L) and the bottom of the tube was covered with cellophane as roots favor darkness, and tubes were incubated for 30 days in the growth room at 25±1°C and 16 h photoperiod.

Analysis of volatile oils produced by callus, micropropagated plants and fruits of cultivated, wild fennel as well as the aerial parts of the wild fennel

All extracts ofcallus and micropropagated plants were prepared by the following procedures: Callus or micropropagated plants were crushed in a mortar with double distilled n-hexane coupled with ultra-sonic waves at 40°C for 15 min, maintained in well closed jars in a shaker at low speed overnight, then filterated and concentrated to 1 ml and passed to GC-MS analysis.

Fruits of cultivated, wild fennel as well as the aerial parts of the wild fennel were hydro-distilled in the Clevenger apparatus. volatile oils of each were collected after 3 h and water traces was removed using anhydrous sodium sulphate. The oils were kept in refrigerator then passed to GC-MS analysis. GC-MS analysis was conducted in Agilent 6890 gas chromatograph equipped with an Agilent mass spectrometry detector, with a direct capillary interface and fused silica capillary column PAS -5 ms (30 m x 0.32 nun x 0.25 um film thickness). Samples were injected under the following conditions: helium was used as carrier gas at approximately 1 ml/min, pulsed split less mode. The solvent delay was 3 min and the injection size was 1.0 ul. The mass spectrophotometry detector was operated in electron impact ionization mode ioning energy of 70 e.v. scanning from m/z 50 to 500. The ion source temperature was 230 °C and the quadrupole temperature was 150°C .The electron multiplier voltage (EM voltage) was maintained 1250 v above auto tune. The instrument was manually tuned using perfluorotributyl amine (PFTBA). The GC temperature program was started at 40 or 60°C as mentioned in the tables then elevated to 280°C at rate of 8°C / min. and 10 min. hold at 280°C the detector and injector temperature were set at 280 and 250°C, respectively. Wiley and Nist 05 mass spectral data base was used in the identification of the separated peaks.

RESULTS AND DISCUSSION

The wild fennel, *Foeniculum vulgare var*. *vulgare* (Fam. Apiaceae) has become a rare plant in Egyptian flora now due to many human activities such as overgrazing and urban sprawl. This situation inspired the authors to carry out this study which focused on different techniques of micropropagation to succeed in making *in vitro* plant regeneration and this was also accompanied with comparing the volatile oil content of fruits of cultivated and wild fennel as well as the aerial parts of the wild fennel to detect differences between them.

Seed germination percentage

*Foeniculum vulgare*seeds sterilized by different time slots of 70% ethyl alcohol and commercial hypochlorite (5%) were cultured on free hormonal media or with 50 mg/l gibberillic acid solution either on solid or semisolid media as well as in Petri dishes containing sterile Whatman grade number 1 filter papers moisted with either sterile distilled water or sterile liquid hormonal free media or with 50 mg/l gibberillic acid solution and incubated in growth room at 25°C and a photoperiod of 16 h light and 8 h dark. Seed germination percentage is shown in Table 1. The results shows that immersing in ethyl alcohol (70%) for 1 and 2 min along with immersing in sodium hypochlorite for 10, 15 and 20 min were done but some seeds showed bacterial or fungal contaminations with these procedures but immersing in ethyl alcohol (70%) for 3 and 4 min along with immersing in sodium hypochlorite for 25 and 30 min showed no contamination but different germination percentages. Only, immersing in ethyl alcohol (70%) for 3 min along with immersing in sodium hypochlorite for 25 min showed complete germination with no contamination. Also, it was figured that increasing time of sterilization more than that inhibits the surface of some seeds decreasing the rate of germination. The same observations were obtained upon using 50 mg/L gibberillic acid solution without any changes in the time of seed germination or germination percentage.

Callus induction

The produced seedlings were used as explants for production of callus. Induction of callus was studied using different parameters such as the percentage of callus induction (Callus capacity), days for callus induction and callus growth rate measured through callus weight and callus diameter. Different phytohormones and phyto-hormone-combinations were used in this study and the successful attempts in callus production are listed in Table 2 and Figure 1. Generally, Stem, root and cotyledonary leaves can induce formation of callus butit was obviously that stem explants were the best to initiate callus over other root and leaf explants. Also hormonal combinations (2,4-D 1 mg/l +k 1 mg/l), (NAA 1 mg/l +BAP 0.1 mg/l) and (TDZ 0.5 mg/l +2,4-D 1 mg/l +BAP 0.1

Table 1. Results of germination trials with seed germination percentage through 30 days observations.

70%	Clorox® 5%				
Ethanol	**10 min**	**15 min**	**20 min**	**25 min**	**30 min**
1 min	Contamination	contamination	contamination	Slight contamination	Slight contamination after 8 days
2 min	Contamination	contamination	Slight contamination	Slight contamination after 7 days	Slight contamination after 13 days
3 min	Contamination	contamination	Slight contamination after 14 days	Sterile and 100% germination	Sterile and 60% germination
4 min	contamination	Slight contamination after 5 days	Sterile and 70% germination	Sterile and 50% germination	Sterile and 0% germination

Table 2. Callus capacity of different explants of seedlings on different hormonal combinations.

Media	Root (%)	Stem	Leaf	Mean
(2,4-D 0.5+k 0.1)	100	90	100	96.66667
(2,4-D 0.5+K 0.5)	100	100	100	100
(2,4-D 1+K 0.5)	50	100	50	66.66667
(2,4-D 1+K 1)	100	100	100	100
(2,4-D 2+K 1)	100	100	90	96.6667
(2,4-D 2+BAP 0.25)	100	100	90	96.66667
(NAA 1+K 1)	80	80	80	80
(NAA 1+BAP 0.1)	100	100	100	100
(TDZ 0.5+2,4-D 1 + BAP 0.1)	100	100	100	100
Mean	92.2222	**93.3333**	90.0000	

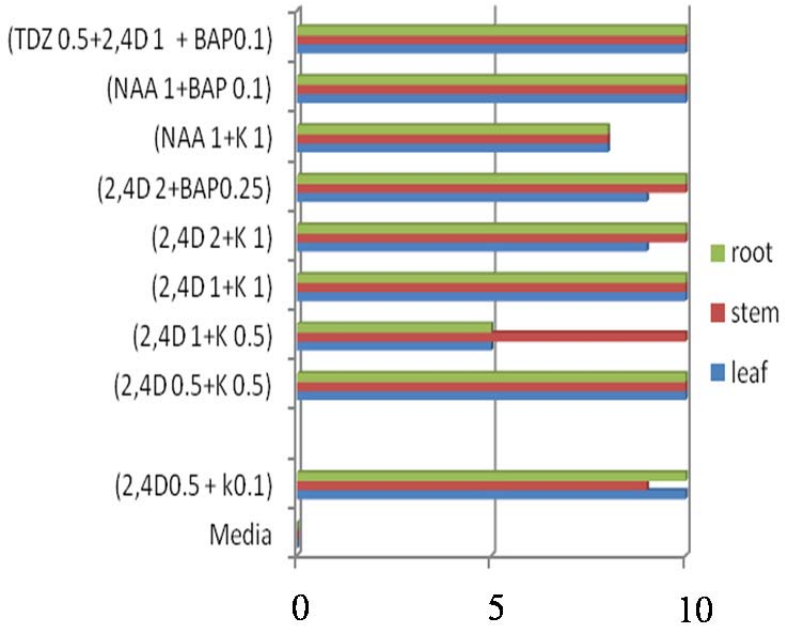

Figure 1. Callus capacity of different explants of seedling on different hormonal combinations.

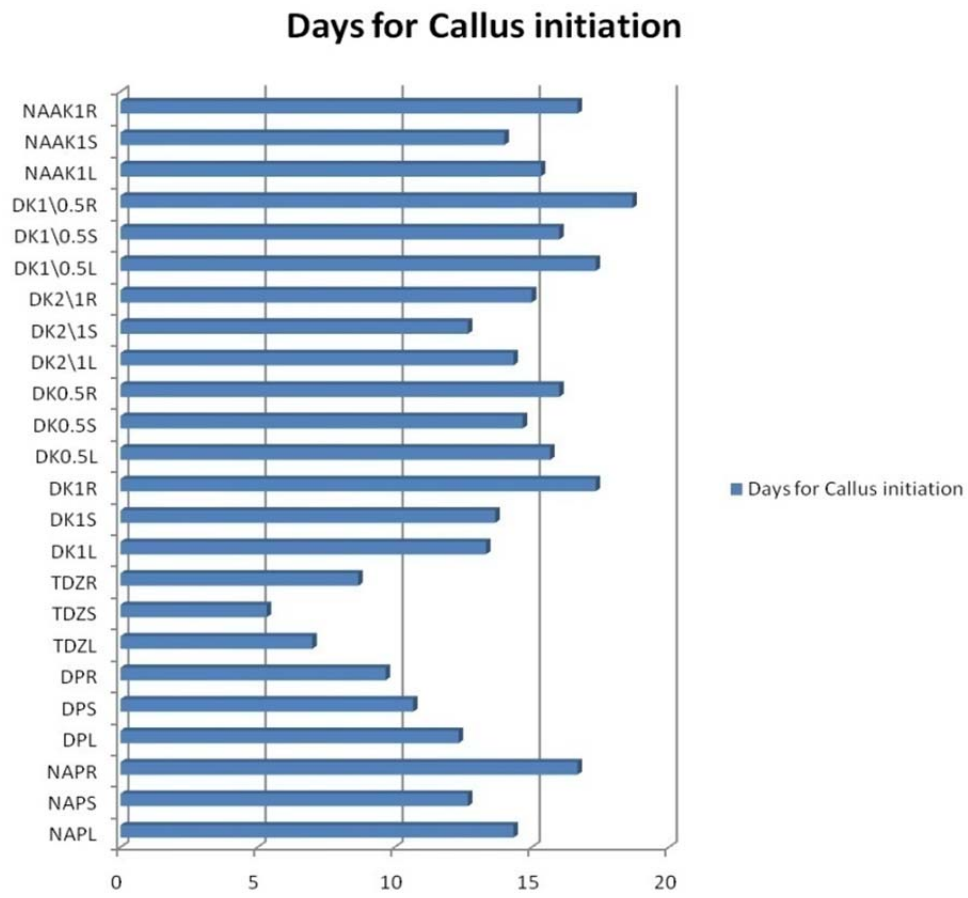

Figure 2. Callus induction days of *Foeniculum vulgare* var. *vulgare* (Fam.Apiaceae) different explants of seedlings on different hormonal combinations (2,4-D 0.5+K 0.1), (2,4-D 0.5+K 0.5), (2,4-D 1+K 0.5), (2,4-D 1+K 1), (2,4-D 2+K 1), (2,4-D 2+BAP 0.25), (NAA 1+K 1), (NAA 1+BAP 0.1) and (TDZ 0.5+2,4-D 1+BAP 0.1).

mg/l) were the best to initiate callus over other hormonal combinations. Callus induction time is illustrated in Figure 2 and Table 3. All previously mentioned hormonal combinations were able to induce callus but the hormonal combination TDZ 0.5 mg/l +2,4-D 1 mg/l mg/l +BAP 0.1 mg/l was the fastest to induce callus formation, followed by the hormonal combination 2,4-D 2 mg/l +BAP 0.25 mg/l.

Callus growth rate

Callus growth rate was monitored and calculated at different time intervals (10, 20, 30 and 40 days) on different hormonal combinations as illustrated in Table 3 and Figure 3.

From the illustrated growth curve ,the best hormonal combinations used were TDZ 0.5 mg/l +2,4-D 1 mg/l + BAP 0.1 mg/l and 2,4-D 2 mg/l +BAP 0.25 mg/l that showed significantly marked growth rate more than other hormonal combinations.

The interesting point is that the hormonal combination (TDZ 0.5 mg/l +2,4-D 1 mg/l + BAP 0.1 mg/l) has never been used or mentioned before in any literature for fennel or any umbelliferous plant and it showed highly significant results in callus formation, callus growth and also micro-propagation as we will illustrate later.

All calluses produced from the previous culture were subcultured on the same media with the same hormonal combinations initiated them and incubated in for 30 days at the same conditions. Not all hormonal combinations were able to maintain the growth of callus. Only the hormonal combinations (2,4-D 0.5 mg/l +K 0.1 mg/l), (2,4-D 0.5 mg/l +K 0.5 mg/l), (2,4-D 1 mg/l +K 0.5 mg/l), (2,4-D 1 mg/l +K 1 mg/l), (2,4-D 2 mg/l +K 1 mg/l), (2,4-D 2 mg/l +BAP 0.25 mg/l) and (TDZ 0.5 mg/l +2,4-D 1 mg/l +BAP 0.1 mg/l) were able to maintain callus growth but (NAA 1 mg/l +BAP 0.1 mg/l) and (NAA1 mg/l +K 1 mg/l) failed to maintain the growth of callus and the best growth of callus of root, leaf and stem was also noticed in the first subculture from (2,4D 2 mg/l +BAP 0.25 mg/l) and (TDZ 0.5 mg/l + 2,4-D 1 mg/l + BAP 0.1 mg/l) hormonal

Table 3. Callus induction time and callus growth rate of *Foeniculum vulgare var. vulgare* (Fam. Apiaceae). Explants were taken from plant seedling growing under sterile conditions. The experiments were repeated 3 times using 12 explants. The results show the mean and standard error of mean was not put for simplification and did not exceed 10 % of mean.

Code	Treatment					Days for callus initiation	Callus weight mg/days			
	NAA	BAP	2,4D	Kin	TDZ		mg/10 days	mg/20 days	mg/30 days	mg/40 days
NAPL	1	0.1				14.33	50.20	451.00	1103.00	1734.00
NAPS	1	0.1				12.67	62.00	367.00	1423.00	1874.00
NAPR	1	0.1				16.67	36.00	319.00	1056.00	1698.00
DPL		0.25	2			12.33	99.00	399.00	1745.00	2322.00
DPS		0.25	2			10.67	108.00	415.00	1987.00	2455.00
DPR		0.25	2			9.67	104.00	387.00	1604.00	2302.00
TDZL		0.1	1		0.5	7.00	112.00	1440.00	1450.00	2640.00
TDZS		0.1	1		0.5	5.33	130.00	514.00	2223.00	2780.00
TDZR		0.1	1		0.5	8.67	125.00	453.00	2114.00	2498.00
DK1L			1	1		13.33	59.00	206.00	949.00	1499.00
DK1S			1	1		13.67	82.00	274.00	1145.00	1546.00
DK1R			1	1		17.33	73.00	244.00	922.00	1402.00
DK0.5L			0.5	0.5		15.67	44.00	123.00	377.00	533.00
DK0.5S			0.5	0.5		14.67	79.00	156.00	412.00	587.00
DK0.5R			0.5	0.5		16.00	65.00	142.00	344.00	421.00
DK2\1L			2	1		14.33				
DK2\1S			2	1		12.67				
DK2\1R			2	1		15.00				
DK1\0.5L			1	0.5		17.33				
DK1\0.5S			1	0.5		16.00				
DK1\0.5R			1	0.5		18.67				
NAAK1L	1			1		15.33				
NAAK1S	1			1		14.00				
NAAK1R	1			1		16.67				

Explant code: L = leaf, S = stem, R = root.

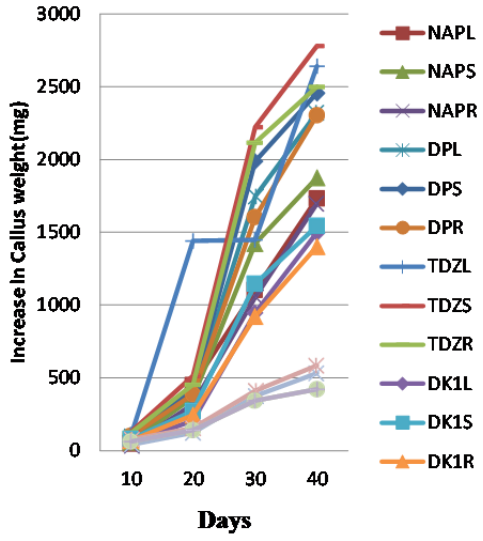

Figure 3. Callus growth rate of *Foeniculum vulgare var. vulgare* (Fam.Apiaceae) measured in the terms of increase in callus weight (mg) per days.

combinations. Subsequent subcultures were done for each 30 days old callus obtained from the first subculture from the hormonal combinations (2,4-D 2 mg/l +BAP 0.25 mg/l) and (TDZ 0.5 mg/l +2,4-D 1 mg/l + BAP 0.1 mg/l) as they showed the best growth and for the hormonal combinations (2,4-D 0.5 mg/l + K 0.5 mg/l) and (2,4-D 1 mg/l + K 1 mg/l) as they showed the best growth for the most common used hormonal combination 2,4-D and kinetin.

The growth of callus on these hormonal combinations was maintained, increased and also the best growth of callus of root, leaf and stem was noticed in the second subculture from (2,4-D 2 mg/l +BAP 0.25 mg/l) and (TDZ 0.5 mg/l +2,4-D 1 mg/l + BAP 0.1 mg/l) hormonal combinations.

In vitro regeneration

Trials to make clones of the plant either directly from the explants or indirectly after callus formation were done ultimately to achieve this point using different hormones

either solely or in combinations or even hormonal free media to get clones of the whole plant.

Direct micropropagation

Foeniculum vulgare var. *vulgare* produced seedlings were cut in to different explants of root, stem and leaf, and subjected to different types of phytohormones and their combinations as the previously mentioned hormones and cultured on M.S. solid media (sucrose containing or sucrose free) at 25±1°C and 16 h photo period, however there were no signs of propagation (neither organogenesis nor somatic embryogenesis).

Indirect micropropagation

From the differently used hormones and hormonal combinations, none of them showed somatic embryogenesis but only NAA 1 mg/l +BAP 0.1 mg/l and NAA 1 mg/l +K 1 mg/l were able to produce organo-genesis from their calluses and NAA 1 mg/l +BAP 0.1 mg/l hormonal combination was the only one able to continue micropropagation. Culture on the hormonal combination NAA 1 mg/l + BAP 0.1 mg/l was repeated thrice with the same conditions to ensure the occurrence of indirect organogenesis and micro-propagation each time from leaf, root and stem calluses.

All calluses of 30, 60, 90, 120 and 150 days old produced from the previously mentioned hormonal combinations from root, leaf and stem explants, were subsequently transferred to HF, K 0.5 mg/l and (K 0.5 mg/l + BAP 0.5 mg/l) solid and semi-solid MS media either sucrose containing media or sucrose free media beside their monitoring on their hormonal combinations inducing callus and incubated at 25˚c ± 1 and photoperiod 16 h light and 8 h dark. In each trial, cells from callus were examined under microscope to detect the production of somatic embryos.

There were no signs of somatic embryogenesis on HF, K 0.5 mg/l and (K 0.5 mg/l +BAP 0.5 mg/l) solid and semi-solid MS media sucrose free MS media. Somatic embryogenesis has been occurred only in 90, 120 and 150 days old callus from the hormonal combination (TDZ 0.5 mg/l +2,4-D 1 mg/l +BAP 0.1 mg/l) of stem and leaf explants as well as callus from the hormonal combination (2,4-D 1 mg/l +K 1 mg/l) of stem explants, only on HF solid and semi-solid sucrose containing MS media that was detected by the formation of globular, heart, torpedo and cotyledonary forms of embryos as shown in Figure 4.

Callus that were able to give these forms of somatic embryos, started to give nodules, then dwarf shoots and roots till forming complete micropropagated plants of roots, shoots and leaflets. These plants were transferred to fresh media at every 30 days time intervals to maintain their vitality. Only plants of formed by stem explants callus callus on the hormonal combination (TDZ 0.5 mg/l +2,4-D 1 mg/l +BAP 0.1 mg/l), were able to continue the indirect micropropagation process.

***In vivo* culturing of the micropropagated plantlets**

After placing the micropropagated plantlets in the perforated pots and incubation, plantlets remain vital for only 10 days maximum without showing any sign of growth and start to wilt after that. This was due to weakness of the root system.

After hardening the root system by placing plantlets with their roots in contact with liquid auxin (NAA 1 mg/l) for 30 days and roots started to get thicker and formed brownish nodules as showed in Figure 5, plantlets showed a marked growth and increased in length from 9 cm to 13 cm in 15 days and maintained their vitality for 20 days but started to wilt after that.

GC-MS analysis

All prepared extracts of callus or micropropagated plants either by organogenesis or somatic embryogenesis were analysed using GC-MS technique as previously illustrated to detect the valuable produced volatile oils in each of them. The results are illustrated in Tables (4, 5, 6 and 7).

Analysis of calluses extracts showed that only calluses extracts of hormonal combinations (TDZ 0.5 mg/l +2,4-D 1 mg/l +BAP 0.1 mg/l) and (2,4-D 2 mg/l +BAP 0.25 mg/l) were able to form different volatile oils components such as *m*-menthane, *p*-menthane, *p*-menth-2-ene, cumene, *dl*-limonene, *m*-cymene, *trans*-anethole, *cis*-methyl-isoeugenol, *beta*-pinene, *gamma*-terpinene, *alpha*-terpinolene and propiovanillone as shown in Tables 4 and 5. Although, the notable point was that these volatile oils components were formed in very low concentrations in different analysed calluses. This has given the green light for proceeding towards micropropagation to optimize formation of different valuable volatile oils components.

Analysis of extracts of micropropagated plants obtained either by organogenesis or somatic embryogenesis also showed different valuable volatile oils componenets such as *trans*-*p*-menthane, *cis*-*p*-menthane, *m*-menthane, 6,6-dimethyl-menth-2-ene, *cis*-asarone and apiole. The most interesting point was that apiole which is a valuable antimicrobial, insecticidal and colon anti-proliferative volatile oil which is a major constituent of parsely (23%) and Dill (16.8%), was formed in different extracts in highly different concentrations which may be in some extracts higher than parsely or Dill.

Apiole percentage in extracts of micropropagated plants obtained by organogenesis varied between those obtained from leaf, root and stem callus on the hormonal combination NAA 1 mg/l + BAP 0.1v as shown in Table 6.

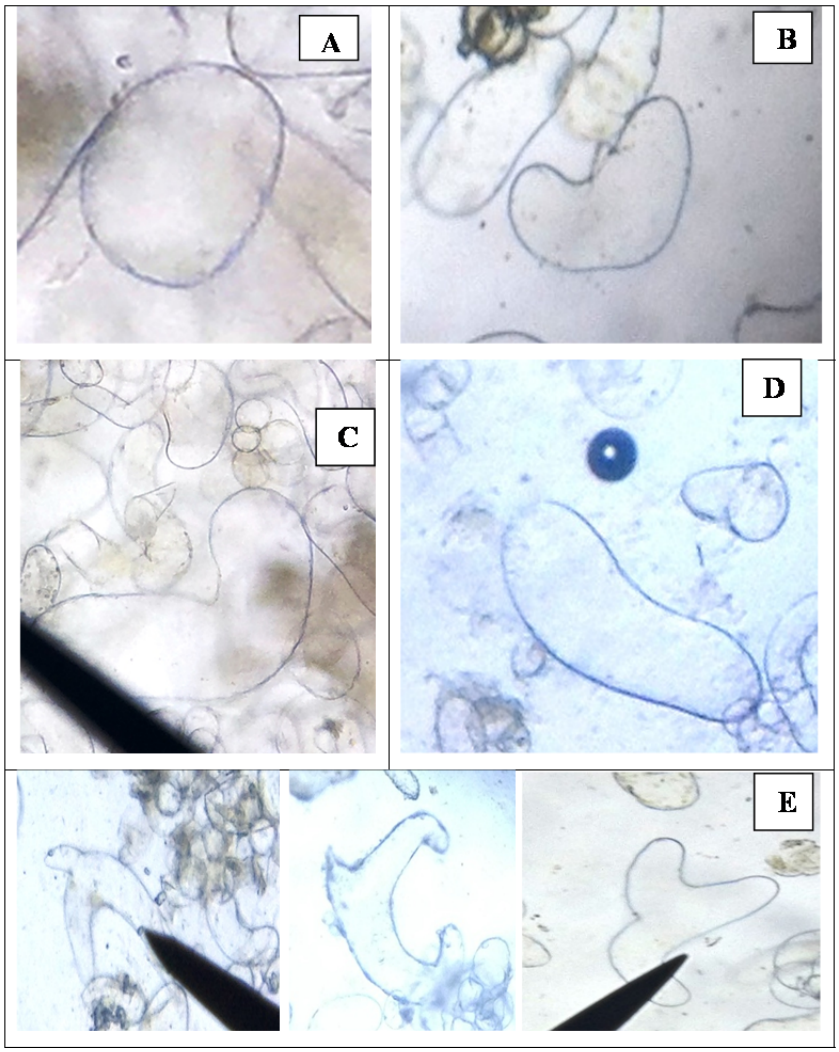

Figure 4. Different forms of somatic embryos of callus cells; A:globular form of embryo; B:early heart form of embryo; C:late heart form of embryo; D:torpedo form of embryo; E: dicotyledonary forms of embryo.

Figure 5. Hardened root system of *in vitro*micropropagated plants of wild fennel after placement in liquid media containing the auxin NAA (1 mg/l) for 30 days.

While apiole obtained by the solely hormonal combination (TDZ 0.5 mg/l +2,4-D 1 mg/l +BAP 0.1 mg/l) of stem callus that showed somatic embryogenesis, reached about 53% .

The analysis of hydrodistilled volatile oils of fruits of both cultivated and wild fennel as well as the hydrodistilled volatile oil of aerial parts of wild fennel showed different medicinally valuable volatile oils components such as *trans*-anethole, *l*-limonene, *beta*-myrcene, *alpha*- phellandrene, fenchone, *cis* and *trans*thujone. The most interesting point was that estragole which is a constituent responsible for most of the genotoxicity and carcinogenicity of the oil was present in lower percentages in the fruits of the wild plant (12.36%) as well as the aerial parts (38.61%) in comparison with the fruits of the cultivated plant (57.12%). Also, it was obviously clear that there is an inverse relationship between the percentage of estragole

Table 4. Different volatile oils identified by GC-MS analysis of stem explants callus of (TDZ 0.5 +2, 4-D 1+ BAP 0.1) hormonal combination.

No. at 60˚c	M^+	BP.	Fragments	R.T	Identification	% in TDZ stem Callus
1	140	97	123,112, 81,71,55	6.66	m-menthane	0.01
2	120	105	91,77,65,51	7.71	cumene	0.12
3	136	68	121,105,93,79, 53,39	7.84	dl-limonene	0.05
4	134	93	121,105,77,67,55	8.83	m-cymene	0.02
5	148	148	133,117,105,93,77,63,51	12.87	trans-anethole	0.01
6	178	178	163,147,135,115,107,91,77, 65,55	16.33	Cis-methyl- isoeugenol	0.42

Table 5. Different volatile oils identified by GC-MS analysis of different explants callus of (TDZ 0.5+2,4-D 1+BAP 0.1) and (2,4-D 2+BAP 0.25) hormonal combinations.

N0. At 40˚c	M^+	BP.	Fragments	R.T	Identification	% in TDZ ROOT	% in TDZ LEAF	% in DBAP ROOT	%in DBAP LEAF	% in DBAP STEM
1	140	97	123,112, 81, 71,55	9.19	*p*-Menthane	0.25	0.32	-	-	-
2	136	93	121, 69,53	9.35	*beta*-Pinene	-	-	-	0.07	-
3	138	97	125,111,83,69,55,41	9.56	*p*-Menth-2-ene	-	-	0.14	-	-
4	140	97	121,111,81,69,55	9.74	*m*-Menthane	0.17	0.2	0.2	-	-
5	136	68	119,93,51	10.55	*dl*-Limonene	-	-	-	0.34	-
6	136	93	121,105,77,55	11.23	*gamma*-Terpinene	-	-	-	0.07	-
7	136	121	105,93,79,67	11.88	*alpha*-Terpinolene	-	-	-	0.05	-
8	180	151	123,91,57	12.11	Propiovanillone	-	-	-	-	0.37

Table 6. Showing different volatile oils identified by GC-MS analysis of plantlets derived from indirect organogenesisof NAA1+BAP0.1 hormonal combination from leaf, stem and root explants callus.

No. at 40°C	M^+	BP.	Fragments	R.T	Identification	% in ROOT	% in STEM	% in LEAF
1	140	97	123,112,81,71,55	9.19	*Trans*-*p*-Menthane	0.02	-	-
2	140	97	123,112,81,71,55	9.29	*Cis*-*p*-Menthane	-	-	0.15
3	140	97	121,111,81,69,55	9.74	*m*-Menthane	0.43	-	0.16
4	168	97	151,137,125,111,83,69,55	13.9	6,6-dimethyl-menth-2-ene	0.31	-	-
5	222	222	195,177,147,119,91,65	21.79	**Apiole**	**13.72**	**39.1**	**41.55**

Table 7. Showing different volatile oils identified by GC-MS analysis of plantlets of somatic embryogenesis derived from (TDZ 0.5+2,4-D 1+BAP 0.1) of stem explants callus.

No. at 40°C	M^+	BP.	Fragments	R.T	Identification	% in TDZ stemplantlets
1	208	208	193,177, 165,148,133,121,105,91,77,57	17.22	*cis*-Asarone	2.93
2	222	222	195,177, 147,119,91,65	21.79	**Apiole**	**53.02**

and *trans*-anethole in the same part of the plant.

Conclusion

The wild fennel *Foeniculum vulgare var. vulgare*contains many valuable volatile oils. In this study, conditions for seeds sterilization, plant germination, callus production and plant micropropagation were investigated. The best condition for seed sterilization was immersing in ethyl alcohol (70%) for 3 min along with immersing in sodium hypochlorite (5%) for 25 min showed complete germination with no contamination.

The best hormonal combinations for callus induction were

(TDZ 0.5+2,4-D 1+BAP 0.1) and (2,4-D 2+BAP 0.25) which also showed the best growth rate particularly from stem explants. Also, NAA 1+ BAP 0.1 hormonal combination was the best for organogenesis while TDZ 0.5 +2,4-D 1 +BAP 0.1 hormonal combination was the only one that showed somatic embryogenesis. Transplantation trials were ultimately conducted but never completed as wilting was occurring each time due to weakness of root system. Even after hardening of root system, plants remained vital for longer time but ended with wilting. GC-MS analysis of callus and micropropagated plants showed valuable volatile oils particularly apiole which showed significantly higher percentages in the micropropagated plants. GC-MS analysis of the volatile oils of fruits of cultivated, wild fennel as well as the aerial parts of the wild fennel showed different medicinally valuable volatile oils but the most notable point was the reduction of estragole percentage in the fruits of the wild fennel in comparison of the fruits of the cultivated fennel.

Conflict of interests

Theauthors didnot declare any conflict of interest.

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