Phytochemical Evaluation and Plant Regeneration Through Somatic Embryogenesis of *Ferulago angulata* subsp. *Carduchorum* (Boiss. and Hausskn), an Endangered Medicinal Plant

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Abstract

Background: ***Ferulago angulata*** **is an important medicinal plant and endemic to Iran which is an endangered Plant.**

Objective: **The aim of this study was to achieve an efficient method for propagation of this plant and evaluation of essential oil content and composition in habitat.**

Metthods: **Essential oils were extracted by hydrodistillation using Clevenger-type apparatus and its composition was analyzed by the GC and GC-MS. For callus induction, explants were cultured on MS medium supplemented with NAA, 2,4-D and BA. For somatic embryogenesis, the calli were cultured on MS medium supplemented with full, 1/4 and 1/10 fold of growth regulators and MS medium without growth regulator. For maturation, embryogenic clumps were transferred on MS medium supplemented with BA, 2,4-D and 30 or 60 g l^{-1} sucrose. The small embryogenic clumps were transferred to medium with GA3.**

Results: **The main components of essential oils were Ocimene (29.84%), followed by α-pinene (27.01%), Isobornyl formate (7.7%) and gamma-Terpinene (4.85%). The highest percentage of callus induction observed in the root and hypocotyl explants. The maximum somatic embryogenesis was obtained in MS medium with 0.5 mg l^{-1} 2, 4-D via hypocotyls derived explants. The highest frequency of maturation was obtained in MS medium containing 30 g l^{-1} sucrose without growth regulators.**

Conclution: **This plant had a noticeable amount of essential oil with valuable ingredients and tissue culture techniques could be applied for its mass propagation, conservation of germplasm and *in vitro* regeneration.**

Keywords: ***Ferulago angulata*****, Callus induction, Embryogenesis, Essential oil**

Introduction

Ferulago angulate species belongs to the family *Appiaceae* is an important medicinal plant that growth in Iran [1]. The oil has long been used as an additive to edible oil (eg. Rughan Kermanshahi) and food preservative [2]. *Ferulago* species are used in folk medicine for their sedative, tonic, digestive and anti-parasitic effects [3]. Antibacterial and antifungal activities have previously been investigated for *F. angulata* subsp. *carduchorum,* and inhibitory effects for microorganisms have been observed [1].

The genetic diversity of *F. angulata* in the wild populations is getting endangered at an alarming rate because of ruinous harvesting practices for production of medicines and for food preservative. This plant is conventionally propagated through seed but is hampered by the seed dormancy. Low seed germination in Apiaceae family is known [4]. Consequently, *F. angulata* subsp. *carduchorum* is recorded as a vulnerable species in the Red Data Book of Iran [5]. Propagation via seed is insufficient due to low viability. Large-scale propagation is a prerequisite to meet pharmaceutical needs without the extirpation of this highly valuable medicinal plant [6]. Hence, there is an urgent need to conserve this valuable germplasm.

Somatic embryogenesis enables large numbers of plantlets to be produced within a short span of time. Embryogenesis can be widely applied to shorten the long sexual cycle and other problems like limited seed availability. Further, genetic improvement is another approach to augment the drug yielding capacity of the plant [7, 8]. To date, there are no reports on an efficient culture system for regeneration in this species. In the present study, for the first time, we report the effect of different plant growth regulators on the indirect somatic embryogenesis in *Ferulago angulata* subsp. *Carduchorum.*

Materials and Methods

Chemical analysis

Oil extraction

The aerial part of plant, which was gathered from mountain (2365 m above sea level, Lat: 35′12′′N; Lon: 46′24′′E), was carefully dried one week at room temperature (25±2°C) in darkness. The extraction of the essential oil was performed using Clevenger-type apparatus according to standard technique (20). 50g of the fine powdered-aerial parts of the *F. angulata* were subjected to hydro-distillation for until full recovery essential oil. Resulted oils were dried over anhydrous sodium sulfate (0.5 gr) and stored in tightly closed vials at 4 °C before chemical analyses.

Analysis of essential oil

Gas chromatography combined with mass spectrometry was used for the identification of the components. The analysis was performed on an Agilent 5973C mass selective detector coupled with a Hewlett-Packard 6890 gas chromatograph equipped with a capillary column (30 m × 0.25 mm ID; film thickness 0.25 μm). To identify the constituents of the essential oil, the essential oil samples was diluted by n- hexane and then these were injected to GC / MS. Helium was used as the carrier gas at a flow rate of 0.5 ml/min. The injector and detector temperatures were kept at

290 °C. Oven temperature program was kept at 50 °C for 5 min, then raised to 240 °C at the rate of 3 °C/min then raised to 300 ºC with the ramp of 15 ºC/min and held isothermally for 3 min. The response time was 75 minutes. Helium was used as the carrier gas at a flow rate of 0.5 ml/min in split ratio of 1:50. Ionization voltage was 70 eV. Ion source and interface temperatures were 220 °C and 290 °C, respectively. The oven program temperature was the same as GC analysis. Copper scan range was adjusted 40 to 500. CHEMSTATION software was used for analysis. Identification of the major constituents of the essential oil was accompanied based on comparison between their retention indices (RIs), retention indices of published data, Standard Mass Spectral fragmentation pattern (Wiley/NBS) and computer matching with NIST and Wiley 275 libraries. The percentage of each essential oil compositions was calculated from GC peak areas [9, 10, 11].

Plant material

The immature seeds and aerial parts (flowering time) of *F. angulata* subsp. *Carduchorum* collected in September 2012 from Shahoo Mountains of Kermanshah province, west of Iran (2365 m above sea level, Lat: 35′12″N; Lon: 46′24″E). Then, the seeds were washed thoroughly under running tap water for 48 h, The soaked seeds were treated with 70% alcohol for 90sec, and then rinsed with sterile water for three times. The seeds were then surface sterilized in 2.5% sodium hypochlorite solution (NaOCl) for 20 min and followed by three washes in succession with sterile water. The mature embryos subsequently were shifted out from seed coats via hand pressure and were cultured on 1/2MS medium [12].

Callus induction

For callus induction, root, hypocotyl and cotyledon explants from 20-days-old seedlings were cultured on sterile MS medium supplemented with 0, 0.5, 1 and 2 mg l^{-1} α-naphthaleneacetic acid (NAA) or 0, 0.5, 1 and 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with 0, 0.5, 1 and 2 mg l^{-1} Benzyladenine (BA). The cultures were incubated at darkness. The calli induced from various explants were subcultured on the same medium in every 4 weeks. Each callus was divided into 2– 4 mm diameter pieces during transfer and subcultured up to three times.

Embryogenesis

For somatic embryogenesis, calli were cultured on MS medium supplemented with different concentrations of 2,4-D or NAA in combination with BA, four types of medium were used for embryogenesis, MS medium supplemented with 0, 0.5, 1 and 2 mgl $^{-1}$ NAA or 0, 0.5, 1 and 2 mg l^{-1} 2,4-D in combination with 0, 0.5, 1 and 2 mg l^{-1} BA, MS medium supplemented with 1/4 of growth regulators, MS medium supplemented with 1/10 of growth regulators and MS medium without any growth regulator. The explants were subcultured on the same medium after 8 weeks. The percentage of indirect embryogenesis was calculated after 12 week.

Maturation and development of somatic embryos

To find the best medium for development

of somatic embryos and embryo maturation, small embryogenic clumps (~100 mg fresh weight) comprising roughly 30–40 globular and heart-shaped embryos were used as the explant. The explants were transferred on different media supplemented with plant growth regulators (0, 0.1, 0.5, 1 and 2mg $^{-1}$ BA in combination with 0, 0.1 and 0.5 mg l^{-1} 2, 4-D) and different amount of carbohydrates (30 g l^{-1} or 60 g l^{-1} sucrose). The number of mature somatic embryos was calculated after 4 weeks.

Germination of somatic embryo

To find the best medium for germination and plantlet formation, small embryogenic clumps comprising roughly 10 globular and heart-shaped embryos were used as the explants, and transferred to different media. Different strengths of full, one-half and one-quarter MS were evaluated for somatic embryo germination. In addition to germination and plantlet formation media were supplemented with 0, 0.5, 1 and 2 mg l^{-1} Gibberellic acid (GA_3). The frequency of secondary somatic embryo formation was examined during the germination stage. The percentage of germinated mature embryo was calculated after 20 days.

Acclimatization

Well-developed plantlets were thoroughly washed in water to remove the agar and transferred to garden soil mixed with perlit, cocopit and vermicoolit (2:1:1) in plastic cups and placed in a glass house under humidity (90%). The plantlets were watered on alternate days. The relative humidity was reduced gradually and after 2 months the plants were shifted out of the glasshouse and placed in shade (approximately 60% shade) under natural conditions. The plants were transferred to pots after 2 months and eventually to the field.

Culture condition and Statistical analysis

The media were supplemented with 3% sucrose (w/v) and solidified with 7 g l^{-1} agar. The pH of each medium was adjusted to 5.7–5.8 before autoclaving for 15 min. Four explant segments were cultured in each Petri dish. The cultures were incubated at 23 ± 2°C under 16 h photoperiod of 80 µmol m^{-2} s^{-1}irradiance provided by cool fluorescent lamps. The treatments were arranged in a completely randomized design (CRD) with three replication and data were analyzed using MSTATC software. Also the mean of treatments were compared by using Duncan's multiple range tests at α=5% level.

Results

Chemical analysis

The oil yield was 2.24 (ml/100 gDM) and according to GC-MS analysis, 34 constituents were identified, representing 96.40% of the total oil that belonged to monoterpenes (81.7% hydrocarbon and 1.92% oxygenated monoterpenes) and sesquiterpenes (5.08% hydrocarbon and 7.7% oxygenated sesquiterpenes) groups of constituents. The main components were (z-β) Ocimene (29.84%), followed by α-pinene (27.01%), Isobornyl formate (7.7%), gamma-Terpinene (4.85%), Myrcene (3.56%), allo-Ocimene (3.22%), Germacrene D (2.92%), Camphene (2.8%), Limonene (2.64%), p-Cymene (2.46%), (E-β) Ocimene (1.51%) and β-Pinene

(1.22%) (Table 1).

Table 1- Composition of the essential oil of aerial part of *F. angulata* (flowering time)

NO	RT	Area%	Compound	sample KI	Adams KI	material
1	11.18	0.07	Tricyclene	924	927	MH
2	11.33	0.19	α-Thujene	927	930	MH
3	**11.75**	**27.01**	**α-Pinene**	**935**	**939**	**MH**
4	**12.61**	**2.8**	**Camphene**	**952**	**954**	**MH**
5	12.84	0.21	Thuja-2,4(10)-diene	957	960	MH
6	13.79	0.6	Sabinene	975	975	MH
7	**14.06**	**1.22**	**β-Pinene**	**981**	**979**	**MH**
8	**14.63**	**3.56**	**Myrcene**	**992**	**991**	**MH**
9	15.62	0.67	α-Phellandrene	1011	1003	MH
10	16.11	0.1	α-Terpinene	1021	1017	MH
11	**16.61**	**2.46**	**p-Cymene**	**1030**	**1025**	MH
12	**16.78**	**2.64**	**Limonene**	**1033**	**1029**	**MH**
13	16.9	0.18	β-Phellandrene	1036	1030	MH
14	**17.15**	**29.84**	**(z-β)Ocimene**	**1041**	**1037**	**MH**
15	**17.65**	**1.51**	**(E-β)Ocimene**	**1050**	**1050**	**MH**
16	**18.31**	**4.85**	**gamma-Terpinene**	**1063**	**1060**	MH
17	19.69	0.57	Terpinolene	1089	1089	MH
18	**21.88**	**3.22**	**allo-Ocimene**	**1133**	**1132**	**MH**
19	22.77	0.39	cis-Verbenol	1151	1141	MO
20	22.97	1.2	trans-Verbenol	1155	1145	MO
21	24.7	0.18	Terpinene-4-ol	1189	1177	MO
22	26.16	0.08	Verbenone	1220	1205	MO
23	26.64	0.07	trans-Carvacrol	1230	1217	MO
24	**29.52**	**7.7**	**Isobornyl formate**	**1291**	**1239**	**Others**
25	33.5	0.18	α-Copaene	1381	1377	SH
26	33.87	0.08	β-Bourbonene	1389	1388	SH
27	35.8	0.29	gamma-Elemene	1434	1437	SH
28	36.69	0.09	(z,β) Farnesene	1456	1443	SH
29	37.47	0.05	β-Acroadiene	1474	1471	SH
30	37.81	0.88	gamma-Curcumene	1483	1483	SH
31	**38.09**	**2.92**	**Germacrene D**	**1489**	**1485**	SH
32	38.7	0.2	Bicyclogermacrene	1504	1500	SH
33	39.56	0.25	delta-Cadinene	1526	1523	SH
34	41.31	0.14	Germacrene B	1570	1561	SH
	Total	**96.40**				
		81.7	Monoterpene Hydrocarbons	MH		
		1.92	Oxygenated Monoterpenes	MO		
		5.08	Sesquiterpene Hydrocarbons	SH		
		7.7	Oxygenated Sesquiterpenes	SO		

Callus Induction and Embryo Germination

The embryo of seeds cultured on MS medium and Germination were occurred normally between all of them. Calli were obtained from root, hypocotyls and cotyledon explants. The data on callus initiation is given in Table 1. The calli derived from root were white and yellow pigmented and watery with smooth surface and the calli derived from hypocotyls segments were watery (Figure 1a), friable, light yellow and fast growing. Cotyledon segments showed a mixture of green and yellowish callus with compact texture. The calli had green appearance in media supplemented with BA. Also Callus formation was affected by kind of the explants. Root explants were most effective for callus induction, followed by hypocotyls and cotyledon. The higher percentage of callus induction (100%) observed in the root and hypocotyls explants (Table 2). The efficiency of callus formation enhanced by increase the concentration of BA. There is no callus induction (0%) in MS medium supplemented with different concentrations of BA (0, 0.5, 1 and 2 mg l^{-1}) in contrast the explants have a positive response when BA was applied in combination with auxins.

Embryogenesis from the subcultured callus

After 8 weeks, massive somatic embryogenesis initiated on calli derived depending on the auxin type and its concentration (Figure 1 b, c). Induced embryos were observed in masses and could be separated easily. The average of embryogenesis percentage ranged from 0.0%

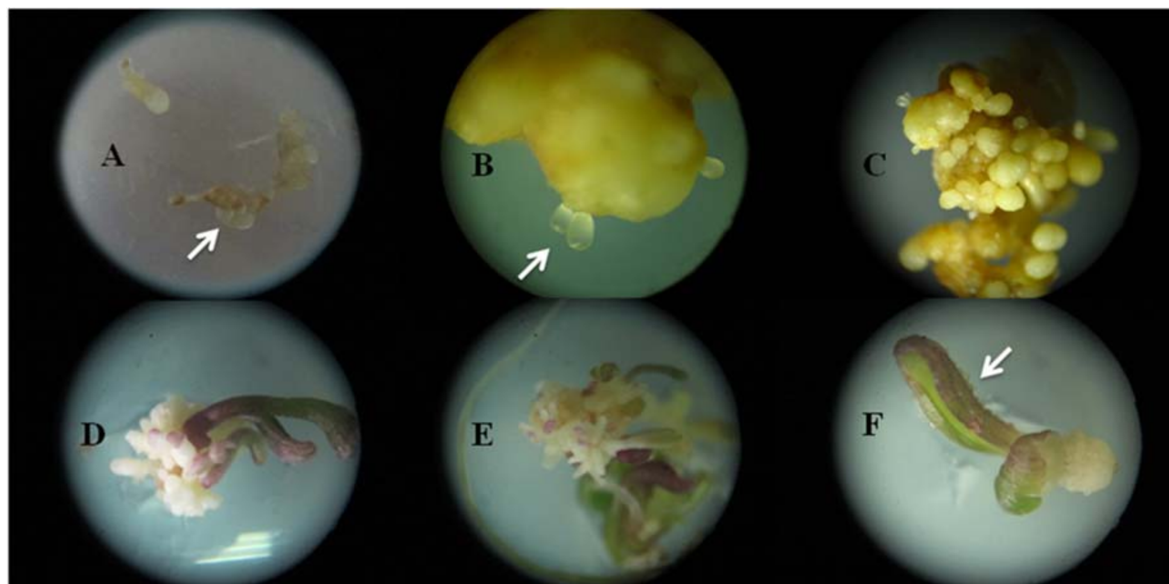

Figure 1- Callus induction and plant regeneration of *F. angulata*. A: Watery Callus induction in hypocotyls explants B: Embryogenic callus induction, C: Embryogenesis, D: Somatic embryo germination, E: Initiation stage of plantlets, F: Secondary embryogenesis

Table 2- Combination of different concentrations of 2, 4-D, NAA and BA for callus induction from root, hypocotyl and cotyledon explants *of F. angulata* ssp. *charduchorum*

Callus induction(%)			Growth regulators		
Cotyledon (%)	Hypocotyls (%)	Root (%)	NAA ($mg.l^{-1}$)	BA ($mg.l^{-1}$)	2,4-D ($mg.l^{-1}$)
0^{f}	0^{f}	0^{f}	0	0	0
0^{f}	50^{b-e}	100^{a}	0	0	0.5
33.33^{c-f}	83.33^{ab}	100^{a}	0	0.5	0.5
41.67^{b-e}	50^{b-e}	100^{a}	0	1	0.5
41.67^{b-e}	66.67^{a-c}	83.33^{ab}	0	2	0.5
0^{f}	83.33^{ab}	100^{a}	0	0	1
0^{f}	66.67^{a-c}	100^{a}	0	0.5	1
41.67^{b-e}	66.67^{a-c}	100^{a}	0	1	1
41.67^{b-e}	58.33^{a-d}	100^{a}	0	2	1
0^{f}	75^{ab}	83.33^{ab}	0	0	2
75^{ab}	100^{a}	100^{a}	0	0.5	2
25^{d-f}	75^{ab}	100^{a}	0	1	2
75^{ab}	97.67^{a}	100^{a}	0	2	2
0^{f}	75^{ab}	100^{a}	0.5	0	0
16.67^{ef}	75^{ab}	83.33^{ab}	0.5	0.5	0
33.33^{c-f}	50^{b-e}	100^{a}	0.5	1	0
16.67^{ef}	25^{d-f}	83.33^{ab}	0.5	2	0
0^{f}	100^{a}	66.67^{a-c}	1	0	0
16.67^{ef}	100^{a}	100^{a}	1	0.5	0
50^{b-e}	66.67^{a-c}	100^{a}	1	1	0
16.67^{ef}	50^{b-e}	100^{a}	1	2	0
0^{f}	66.67^{a-c}	100^{a}	2	0	0
50^{b-e}	25^{d-f}	100^{a}	2	0.5	0
0^{f}	50^{b-e}	100^{a}	2	1	0
83.33^{ab}	83.33^{ab}	100^{a}	2	2	0

to 100% based on the type of auxin, its concentration and type of explants. Also calli derived from hypocotyls segments showed significantly higher frequency of somatic embryogenesis than the calli derived from root and cotyledon segments (Table 3). MS medium supplemented with 0.5 mg l^{-1} 2,4-D produced the highest frequency of somatic embryogenesis (100%) in hypocotyls derived callus (Table 3). Somatic embryogenesis was enhanced by increase of auxin and decrease of BA. 2,4-D was more efficient in embryogenesis than NAA. The aggravate of NAA to medium had the minimum response to somatic embryogenesis on derived callus from hypocotyls and cotyledon but NAA had more than efficient on embryogenesis in root explants. Adding of BA to both NAA and 2,4-D slightly increased the formation of compact callus.

Maturation and development of somatic embryos

MS medium supplemented with various sucrose concentrations (30 g l^{-1} l and 60 g l^{-1} l) evaluated for somatic embryo development

Table 3- Effects of different concentrations of 2,4-D and NAA in combination with BA on somatic embryogenesis

Medium before transferring to embryogenesis medium	New culture media for embryogenesis	Explants		
		Cotyledon (%)	Hypocotyl (%)	Root (%)
0.5D	0.5D	0[k]	100[a]	66.67[c]
0.5D1B	0.5D1B	0[k]	8.443[i]	0[k]
0.5D1B	0.05D0.1B	0[k]	8.333[i]	0[k]
0.5D1B	0	16.33[h]	3[j]	0[k]
0.5D2B	0.5D2B	0[k]	8.333[i]	0[k]
0.5D2B	0.05D0.2B	0[k]	41.33[f]	16.67[h]
0.5D2B	0	0[k]	8.333[i]	0[k]
1D	1D	33.33[g]	8.333[i]	0[k]
1D1B	1D1B	0[k]	16.67[h]	8.333[i]
1D1B	0.1D0.1B	0[k]	16.67[h]	0[k]
1D1B	0	0[k]	33.33[g]	0[k]
2D	2D	8.333[i]	33.33[g]	0[k]
2D0.5B	2D0.5B	8.333[i]	8.333[i]	0[k]
2D0.5B	0	0[k]	83.33[b]	8.333[i]
2D1B	2D1B	16.33[h]	66.67[c]	8.333[i]
2D1B	0.2D0.1B	57.67[d]	8.333[i]	0[k]
2D1B	0	0[k]	8.333[i]	0[k]
1N	1N	0[k]	66.67[c]	42.33[f]
1N0.5B	1N0.5B	0[k]	50[e]	8.333[i]
1N1B	1N1B	0[k]	8.33[h]	0[k]
1N1B	0.1N0.1B	0[k]	33.33[i]	0[k]
2N	2N	0[k]	83.67[g]	33.67[g]
2N0.5B	0.2N0.05B	0[k]	8.333[i]	0[k]
2N0.5B	0	33.67[g]	8.333[i]	0[k]
2N2B	2N2B	0[k]	8.333[i]	0[k]
2N2B	0.2N0.2B	0[k]	16.67[h]	0[k]
2N2B	0	0[k]	8.667[i]	0[k]

For somatic embryos induction, calli were subcultured on previous MS medium (callus induction media), MS medium supplemented with 1/10 of growth regulators and MS medium without any growth regulator. N: NAA B: BA D: 2,4-D

and maturation. The highest frequency of somatic embryo development (637.3) was observed on developmental medium supplemented with 60 g l^{-1} sucrose and 0.1 mg l^{-1}BA. The highest frequency of fully developed mature embryos (200.1) was obtained on MS medium containing 30 g l^{-1} sucrose without any growth regulator (Table 4). Sucrose concentration above 30 g l^{-1} in the maturation medium showed a reduction in the number of mature somatic embryos and an increase of the total somatic embryo number. On the other hand, In this medium, few mature embryos was observed with two cotyledons, but there was various category of somaclonal variation in some of them including only one cotyledon or cotyledons differing in size and shape, lack of cotyledon and cotyledon without root.

Germination of somatic embryo

Development of Somatic embryo started within 4th weeks of culture on germination medium. Somatic embryos became light green (Figure 1d). 1/2MS medium supplemented with 2 mg l^{-1} GA3 showed highest percentage of somatic embryo germination (86%). The highest frequency of secondary somatic embryo (Figure 1f) development (53.33) was observed on MS medium without any growth regulator (Table 5). In this study, the adding of GA3 enhanced the germination percentage of

somatic embryos and decrease of secondary embryogenesis frequency (Figure 1e).

Acclimatization

Finally, the plants were successfully hardened, and transferred to glasshouse. The total of 200 plantlets were produced and 70 plantlets were transplanted into soils. Regenerated plantlets through somatic embryos showed normal growth (Figure 2) in 82% of cultures.

Table 4- Effects of different concentrations of 2,4-D and BA in combination with carbohydrate source on embryo maturation

Growth regulator			Maturation	Total number
2,4-D (mg.l^{1})	BA(mg.l^{-1})	Sucros (g.l^{-1})		
0	0	30	200.1^{a}	430.7^{ab}
0	0	60	41^{db}	398.3^{ab}
0.1	0	30	74.67^{ab}	329.7^{ab}
0.1	0	60	117^{ab}	635.3^{a}
0.5	0	30	118.3 ^{ab}	344.3^{ab}
0.5	0	60	129.3^{ab}	290.7^{ab}
0	0.1	30	22^{b}	309.7^{ab}
0	0.1	60	52^{b}	637.3^{a}
0.1	0.1	30	54.33^{b}	463.7^{ab}
0.1	0.1	60	53.33^{b}	306.3^{ab}
0.5	0.1	30	38.67^{be}	220.7^{b}
0.5	0.1	60	37.67^{b}	192.3^{b}
0	0.5	30	13.33^{b}	284^{ab}
0	0.5	60	19.67^{b}	435.7^{ab}
0.1	0.5	30	21.33f^{b}	387.3^{ab}
0.1	0.5	60	10.67^{b}	269.3^{ab}
0.5	0.5	30	9f^{b}	178.3^{b}
0.5	0.5	60	11.33^{b}	173^{b}
0	1	30	12.33^{b}	173.7^{b}
0	1	60	12.67^{b}	225.7^{b}
0.1	1	30	6.333^{b}	195.3^{b}
0.1	1	60	8^{b}	195.7^{b}
0.5	1	30	14.33^{fb}	171^{b}
0.5	1	60	7^{gb}	128.7^{b}
0	2	30	7.667^{b}	148.3^{b}
0	2	60	10^{fb}	209^{b}
0.1	2	30	4.333^{b}	131.7^{b}
0.1	2	60	5^{b}	226.7^{b}
0.5	2	30	5.667 ^{b}	129.7^{b}
0.5	2	60	8.667^{b}	129.7^{b}

Table 5- Effects of different concentrations of GA_3 on somatic embryo germination

MEDIA	GA3 ($mg.l^{-1}$)	Germination (%)	Secondary somatic embryo number
Full MS	0	30^{e}	53.33^{a}
1/2 MS	0	48.3^{e}	22.67^{c-e}
1/4 MS	0	31.6^{de}	21.33^{c-e}
Full MS	0.5	50^{c-e}	37.33^{b}
1/2 MS	0.5	70^{ab}	11.33^{de}
1/4 MS	0.5	68.3^{a-e}	14^{de}
Full MS	1	85^{a}	24.67^{cd}
1/2 MS	1	80^{ab}	14.67^{de}
1/4 MS	1	63.3^{b-d}	10.33^{e}
Full MS	2	45.3^{de}	17.33^{de}
1/2 MS	2	86^{a}	32^{bc}
1/4 MS	2	76^{a}	14.33^{de}

Figure 2- Acclimatization A: Plantlets B: Initiation stage of acclimatization C: Acclimatized plants after 8 weeks of transferred to plastic cups

Discussion

Ferulago angulata subsp. *Carduchorum* as the medicinal and valuable Iranian endemic plant is currently an endangered species [5] that its domestication is necessary. The highest frequency of callus induction was achieved from root explants at the end of fourth weeks. In plant cells most of the basic physiological

processes such as cell division, cell elongation, polarity, and differentiation are controlled that the auxins and cytokinins have most effective roles [13] also similar result for callus induction has been reported in *Dorema ammoniacum* [14]. According to our observation various color and feature of calli were monitored in media with different growth regulator and type of explants. Similar result in *Dorema ammoniacum* and *Corylus avellana* indicated that texture and type of callus depended on the type of growth regulators and concentrations [14, 15]. In *F. angulata*, similar to *Cuminum cyminum* [16] no differentiated embryo was observed on the callus induction or maintenance media. However, some Apiaceace such as *Daucus carota* L. (carrot) and *Foeniculum vulgare* Mill (fennel) showed somatic embryo formation during the induction phase [17].

All evaluated explants were able to undergo somatic embryogenesis and plant regeneration under the culture conditions tested for embryogenesis; however, the degree of morphogenic response was dependent on kind of the explants. Hypocotyls were most quick to react in all treatments, while the response of root was slow. The highest frequency of embryogenesis was achieved from hypocotyls explants in media supplemented with 0.5 mg l-1 2,4-D. Plant regeneration depends on various factors such as age of the explants, basal medium, growth regulators, genotype, culture conditions and etc… [18]. It is well documented that 2,4,-D is an effective supplement for the induction of potentially somatic embryo form calli in Apiaceae [17]. In our study, somatic embryogenesis was induced by decrease of auxin and cytokinins concentration. It is generally accepted that complete removal of auxin or a decrease in its level in the culture medium following the callus induction phase can serve as a trigger for somatic embryogenesis [19]. Also similar results were reported in *F. assa-foetida* [20], a close relative of *F. angulata* in family Appiaceae in contrast in a few systems, cytokinins alone were found to be effective in somatic embryo induction and maturation [21]. In derived-callus from hypocotyls and cotyledon explants the least somatic embryogenesis were observed in presence of NAA, but in root explants NAA had most efficient on embryogenesis. The two different auxins activate different pathways for the control of cell division and cell elongation. 2,4-D stimulates cell division and inhibits cell elongation, whereas NAA leads to an increase of cell elongation at a reduced frequency of cell divisions [22]. When auxin controls cell division and cell elongation through different receptors, there must be different pathways. When different receptors are involved, they are expected to differ in their affinity for different ligands [23]. Therefore, auxin can activate different signal transduction pathways [24]. The existence of differential signal transduction pathways lead to the different responses [25]. The auxin receptor driving cell division shows a different ligand pattern than the receptor controlling cell elongation. The response of cell division to auxin has been shown recently to require the activity of a putative heterotrimeric G-protein, whereas the response of cell elongation was not dependent on this G-protein [26]. G-proteins conduct the

auxin-triggered signal from an unknown receptor with high affinity for 2,4-D toward the mechanism responsible for the stimulation of cell division. This unidentified receptor (Rx) displays high affinity for 2,4-D and NAA preferentially binds ABP1, leading to an induction of cell elongation. ABP1 binds NAA with high affinity [27]. ABP1 preferentially binds NAA and leads to cell elongation. A second unknown receptor, Rx, preferentially binds 2,4-D and activates cell division through G-protein-mediated signal transduction. Both pathways show a negative crosstalk. The differential effect of 2,4-D and NAA on explants embryogenesis might be explained, in theory, as a consequence of the different transport properties of the two synthetic auxins through the cells. It seems that the gene encoding the ABP1 (NAA receptor) is more active than the gene encoding the Rx in root cells, so embryogenesis in root cells occurred higher than another cells through NAA. It means that the gene encoding the ABP1 is expressed in root cells. But perhaps, in the cotyledon and leave cells, the gene encoding the RX protein (2,4-D receptor) is more active than the gene encoding ABP1. Further studies are require to better understanding the expression profile of genes encoding these two proteins under control of an inducible promoter. Fully developed mature embryos were obtained on MS medium containing 30 mg l^{-1} sucrose while, it showed an inhibitory effect in 60 mg l^{-1}. Sucrose concentration at 30 mg l^{-1} was found to be critical for maturation of somatic embryos in the present study. The medium supplemented with 0.1 mg l^{-1} BA with 60 mg l^{-1} sucrose was the best condition for development of somatic embryos. Sucrose is one of the most important carbon sources, and it has been used frequently in plant tissue culture [28]. Carbon and energy source, and at high concentrations it enhanced somatic embryo induction frequency caused by osmotic stress [29]. Addition of cytokinin in the maturation medium promoted the somatic embryo maturation and regeneration of plantlets. Abnormal somatic embryos in this maturation medium, are normally associated with the use of 2,4-D or other strong auxins in the induction medium. The high concentration and long periods are required to start a sequence of events leading to embryogenesis and abnormalities by auxins. Also long-term cultures can increase the occurrence of somaclonal variation [30]. Morphological abnormalities have been observed in other plants such as carrot [31] and cacao [32].

Addition of GA_3 to MS, 1/2 MS or 1/4 MS increased the average of shoots recovery in all mediums. Although shoot recovery was achieved from all of three media types, inclusion of GA_3 was found to be significantly beneficial, also shoots regeneration in 1/2 MS was more than 1/4MS. Addition of plant growth regulators are essential for efficient regeneration in *F. angulata*. Embryo germination was defined as embryos showing hypocotyls elongation and root development, whereas plantlet conversion was considered as embryos showing normally developed cotyledons and epicotyls after germination [33]. In addition, about 60% of germinating embryos formed secondary somatic embryos at the lower portion of the hypocotyls or root ends. These secondary somatic embryos were

slightly more abundant in medium without any growth regulator. MS medium without growth regulators is a typical medium for inducing somatic embryogenesis in many plants [34]. These secondary somatic embryos, which are capable of successive embryogenesis under optimized culture, are being studied to establish repetitive somatic embryogenesis in *F. angulata*. Also the maximum average number of germinated embryos (200) that later converted into plantlets.

Further experiments are needed to achieve a higher conversion and survival frequency of plantlets after germination or hardening off in potting mixtures, respectively. Improvement of Apiaceae family plants following the classical breeding procedure is generally slow, laborious and time consuming [16, 17]. Regenerated plantlets through somatic embryos showed normal growth in 82% of cultures (Figure 2). The method described here is useful for producing large scale propagation and conservation of this important herb.

The composition of essential oil generally was according to other investigations [35, 36, 37]. The evaluation of chemical composition of aerial parts of *F. angulata* provided from Shahoo Mountain by Taran *et al* (2010) showed that the cis-ocimene (27.9%), *α*-pinene (25.7%), bornyl acetate (3.9%) and *α*-terpinene (0.1%) were the major components of essential oil. The variability and diversity of the reports regarding the chemical composition content of *F. angulata* essential oil can be attributed to different method used for extraction of the essential oil, geographical conditions, climate and seasonal variations and the stage of the plant growth [38]. Antibacterial and antioxidant activities of essential oil of *F. angulata* have been observed previously, these properties could be attributed to the presence of monoterpene hydrocarbon (*α*-pinene, myrcene, *α*-terpinene, *β*-pinene and ocimene) and sesquiterpene (Germacrene D) compounds. Several *in vitro* studies have demonstrated that these natural compounds possess potential activities as antibacterial, anthelmintic, antifungal and antiviral agents [35, 39]. Gamma-Terpinene, volatile essential oil derived from *Melaleuca alternifolia*, was showed antimicrobial properties against various human pathogens [40]. As well as the gamma- terpinene that drived from essential oil of *Lippia multiflora* was showed antioxidant, anti-inflammatory and anti-proliferative activities [41]. Our results indicated that *F. angulata* essential oil might be a potential rich source of compounds with antibacterial and antioxidant properties. Further work is required to study of secondary metabolite.

Conclusion

Plant tissue culture techniques could be applied for mass propagation, conservation of germplasm, study and production of bioactive compounds, and for genetic improvement of this plant. In this present paper, we report influence of media additives, type of explants, and plant growth regulators on indirect somatic embryogenesis, somatic embryo maturation, germination and plantlet formation in *F. angulata* ssp *carduchorum.* Somatic embryo maturation and high frequency germination of plantlets achieved in the present study can be useful pathway for

propagation of *F. angulata.* Further work is required to produce secondary metabolite and study of somaclonal variation of this important medicinal plant.

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