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Original Contribution

ESTABLISHMENT OF AN EFFICIENT CALLUS INDUCTION AND PLANT REGENERATION SYSTEM IN *DUCROSIA ANETHIFOLIA* (DC.) AN IMPORTANT MEDICINAL PLANT

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ABSTRACT

Ducrosia. Anethifolia (DC.) is an important medicinal herb belonging to the family Apiaceae. The present study was undertaken to study the effect of different concentrations of plant growth regulators and various parts of *D. anethifolia* on callus induction and regeneration. Various explants: leaf, node, stem, root, and petiole taken from the actively growing plants. Explants were cultured on Murashig and Skoog (MS) medium supplemented with 2-4- Dichlorophenoxyacetic acid (2, 4-D; 1–4 mg.l^{-1}) or α- Naphatalenacetic acid (NAA; 1-4 mg.l^{-1}) alone or in combination with N_6-Benzil adenine (BA; 0-1 mg.l^{-1}) or Kinetin; (KIN 0-1 mg.l^{-1}) for callus induction. The best response (100%) was observed in stems on MS medium with (2 mg.l^{-1}NAA and 1 mg.l^{-1}BA) and on MS medium containing (3 mg.l^{-1} NAA and 1 mg.l^{-1} BA) with stem, leaf and node. These calli were transferred to MS medium supplemented with various concentrations of BA (1–2 mg.l^{-1}) or KIN (1–2 mg.l^{-1}) alone or in combination with indole 3-aceticacid (IAA: 0.1–0.5 mg.l^{-1}) or NAA (0.1–0.5 mg.l^{-1}) or indole butyric acid (IBA: 0.1–0.5 mg.l^{-1}) for shoot regeneration. Calli derived from Stem segment Showed significantly highest frequency of shoot regeneration. Rooting of in vitro raised shoots was mostly induced on Quarter strength MS (100%). Regenerated plants established in original habitat. This model procedure can be used for as a source for the isolation of secondary metabolites from *D. anethifolia*.

Key words: *Ducrosia anethifolia*, Medicinal plant, Plant Regeneration, Callus Induction

INTRODUCTION

Ducrosia anethifolia (DC.) (Apiaceae) is a perennial medicinal herb, native in Iran, Afghanistan and Pakistan. In pharmacological and biological tests, extracts and fractions of *D. anethifolia* and some other species of *Ducrosia* are reported to have antimicrobial, antimycobacterial and antifungal effect (1). Alpha-pinene which constitutes 12.4% of essential oil of *Ducrosia anethifolia* is likely responsible for antianxiety effect without having sedative effect in mice (1).

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Medicinal plants are the most exclusive source of life saving drugs for majority of the world's population. The commercial production of secondary metabolites using plant culture is normally limited by their low yield. In the search for alternatives to production of desirable medicinal compounds from plants, biotechnological approaches, specifically, plant tissue cultures, are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites (2). The utilization of plant cells for the production of natural or recombinant compounds of commercial interest has gained increasing attention over past decades (3).

N-decanal (70.1%), α- pinene (12.4%) and dodecanal (5.4%) are vastly utilized in industrial companies as major components of essential oils. N-Decanal is used in fragrances and flavoring (4). In chemical industry, selective oxidation of alpha-pinene with some catalysts gives many compounds for perfumery, such as artificial odorants. An important oxidation product is verbenone, along with pinene oxide, verbenol and verbenyl hydroperoxide (5). Dodecanal is one of common ingredient in perfumery and have been used in soap, detergent, beauty care and household products. The substance is partially non-toxic, and is a permitted food additive (GRAS) in both U.S. and the UE (6).

Based on the vast usage of constituents of this important medicinal plant extract, there is a must for providing a protocol for the in vitro regeneration as a stepping stone for releasing secondary metabolite. Further, genetic improvement is another approach to augment the drug-yielding capacity of the plant (7).

Although Al-Yemeni (1999) reported breaking of seed dormancy in *D. Anethifolia* with sulphuric acid. In vitro culture of this species as a practical method toward other finding has not been that much noticeable (8).

The objective in the present study was to evaluate influence of varied concentrations of hormones and explants on callus induction and regeneration *D. anethifolia*.

MATERIALS AND METHODS

Plants of *Ducrosia anethifolia* were collected in October 2007 from wild stands in Karaj and grown in controlled hygienic conditions in Institute of Medicinal plants, ACECR, Karaj, Iran (lat:35° 58' N, Lon: 50° 41'E). Then, plants were washed thoroughly in running tap water. After that, they were removed under laminar airflow cabinet followed by treatment with ethanol for 30 second. Thereafter, explants were exposed with solution of 3% Tween 20 (v/v) together for followed by immersion in a 2/5% sodium hypochlorite solution for 3min. Finally, they were rinsed three times with sterile distilled water for 1 min. Parts of approximately 1 cm segments were excised from leaf, shoot, root, petiole and node.

1. Callus induction

The surface sterilized explants were cultured on MS (Murashige and Skoogs, 1962) medium (9) including 6-benzyladenine (BA; 0 and 1 $mg.l^{-1}$) and Kinetin (KIN; 0 and 1 $mg.l^{-1}$) alone or combination with 2,4-Dichloro phenoxyacetic acid (2,4-D; 1–4 $mg.l^{-1}$) and α-naphthaleneacetic acid (NAA; 1-4 $mg.l^{-1}$) for callus induction. All experiments were incubated in dark condition. After 6 weeks of culture, callus formation was recorded. All callus subcultured on to medium containing the same or lower concentrations growth regulators.

2. Plant regeneration

After callus production, calli (100-200 mlg) were transferred to MS medium supplemented with various concentrations of BA (1–2 $mg.l^{-1}$) or) KIN (1–2 $mg.l^{-1}$) alone or in combination with indole 3-aceticacid (IAA: 0.1–0.5 $mg.l^{-1}$), NAA (0.1–0.5 $mg.l^{-1}$) and indole butyric acid (IBA: 0.1–0.5 $mg.l^{-1}$) for shoot regeneration.

3. Elongation and Establishment of shoots

After 3 weeks shoots were transferred to new treatments including: Full MS, 1/2 MS, 1/4 MS, 1/2 concentrations of (NH_4NO_3, KN_3), (MS+0.001BA+0.0003 NAA), (MS+0.002 BA+0.0004 NAA) which last two were 1/100 concentration of our best regeneration media**.** After 6 weeks of first regeneration culture, data were measured for regeneration percentage and number of shoot per callus.

4. Plant rooting and hardening

Elongated shoots (more than 10 cm) were excised from each culture transferred to quarter strength MS medium containing 3٪ (w/v) sucrose and 0.7% (w/v) agar. The medium was further supplemented with 0, 0.1, 0.2, 0.5 and 1 $mg.l^{-1}$ IBA and NAA individually. Measured traits included response percentage; number of root and mean root length were recorded after 4 weeks.

Plantlets with well-developed roots were removed from the culture medium. Next, roots were washed gently under running tap water. After a while, plantlets were transferred to plastic cups containing autoclaved chopped perlit and farmyard manure, garden soil (1:1:1) and placed in a glass house under high humidity. The relative humidity was reduced gradually and after 60 days the plantlets were transplanted to pots in greenhouse and finally planted out.

5. Culture condition

The pH of the medium was aligned to 5.7 before adding agar for entire of treatments. They were autoclaved at 105 kPa and 121 ˚C for 20 min. All the cultures were incubated at 23±2˚C under 16 h

photoperiods of 35-40 µmol m^{-2} s^{-1} irradiance provided by cool white fluorescent lamps.

6. Statistical analysis

Experiments were carried out in a randomized design (CRD) and each experiment had 3 replicates and 10 explants in each replicate. Data were analyzed statistically using MSTAT software. The treatment means were compared using Duncan's multiple range tests at 5% probability level.

RESULTS

Explants of *D. anethifolia* grown on MS medium containing different plant growth regulators swelled and formed calli in the course of 2 weeks of culture. The data on callus initiation is given in **Table 1**. The remarkable callus induction (100%) was obtained from leaf, stem and nodal segments in medium supplemented with (3 $mg.l^{-1}$NAA +1 $mg.l^{-1}$ BA). The calli from root explants were white to creamy with compact texture (Fig.1C), while calli derived from stem leaf **(Figure 1A, B)** and node appeared green to greenish creamy, friable and nodular. Whitish calli derived root in media with KIN became green gradually, also calli derived stem in media supplemented with KIN were green but calli were found cream in absence of KIN.

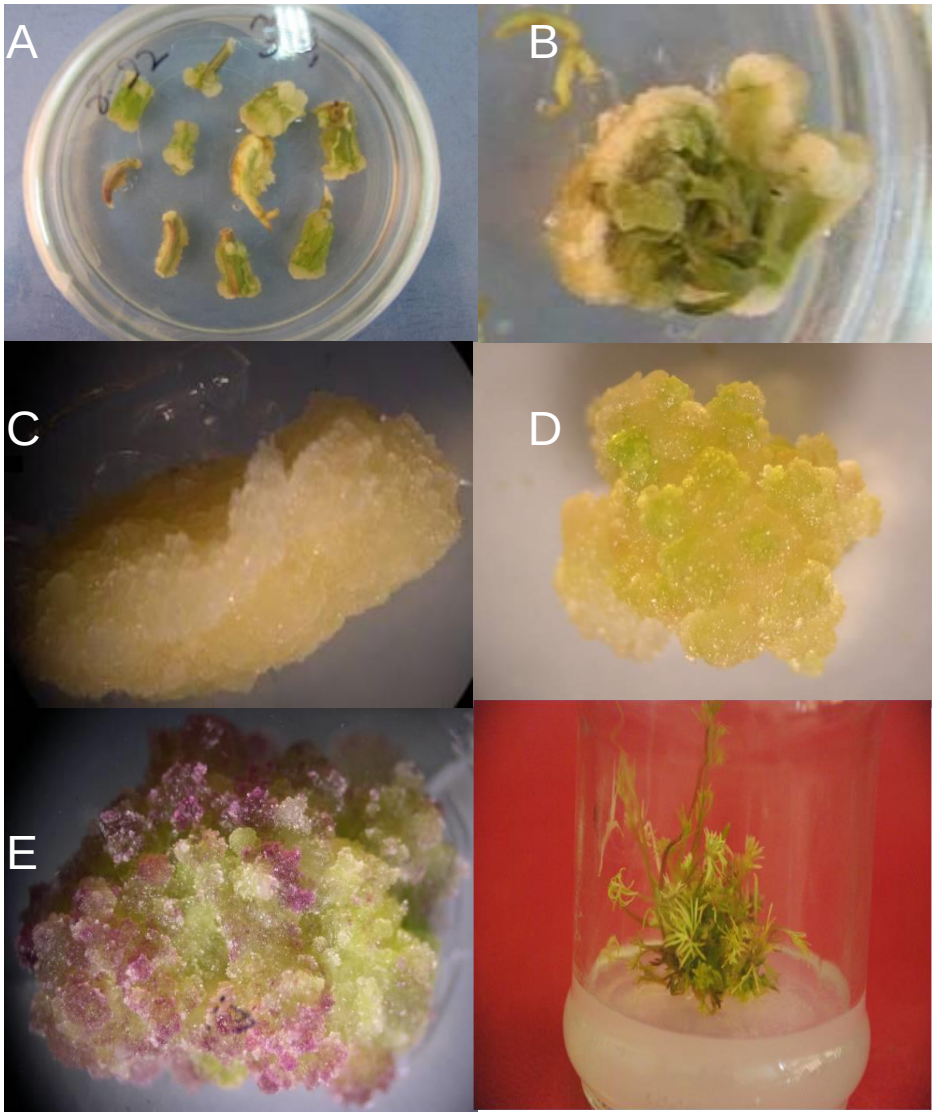

Figure 1. Callus induction of *Ducrosia anethifolia*. **A.** induction of green Callus in Stem explants on MS medium supplement with 3 mgr^{-1} 2, 4-D and 1 mgr^{-1} BA. **B**. Appearance of callus in brims of leaf explants on MS medium with 2 mgr^{-1} NAA and 1 mgr-1 BA. **C**. White and compact Callus in Root explants on MS medium supplement with 1mgr^{-1} KIN and 1 mgr^{-1} 2, 4-D. **D**. appearance of green spots on the surface of the callus after 1- 2 Week. **E**. Emersion of pigment cells and anthocyanin synthesis in 1 mgr^{-1} 2, 4-D in calli of D. Anethifolia. **F**. Withered shoots in D. Anethifolia.

Table 1. *Effect of different concentrations of 2, 4-D and NAA alone and in combination with BA or KIN for callus induction from Root, Stem, Leaf, Nodal of D.Anethifolia*

Plant Regulators (mgl^{-1})				Callus Induction (%)			
Auxines		Cytokinines		Explants			
Stem			Leaf	Nodal	Root	KIN	BA NAA 2,4-D
4	0		40 F-P	35 G-P	35 G-P		40 F-P
4	1		26.66 I-P	33.33 H-P	51.46 C-N		46.66 E-O
3	0		26.66 I-P	50 D-N	16.66 L-P		26.66 I-P
3	1		40 F-P	70 A-H	40 F-P		53.33 B-M
2	0		23.33 J-P	53.33 B-M	70 A-H		26.66 I-P
2	1		16.66 L-P	91.6 A-C	92.06 A-C		60 A-K
1	0		40 F-P	46.2 E-O	36.66 G-P		26.66 I-P
1	1		33.33 H-P	86.66 A-E	23.33 J-P		60 A-K
4		1	26.66 I-P	20 K-P	20 K-P		6.66 OP
3		1	33.33 H--P	63.33 A-IJ	16.66 L-P		46.66 E-O
2		1	46.66 E-O	93.33 AB	73.33 A-H		46.66 EFG-O
1		1	80 A-F	23.33 J-P	40 F-P		40 F-P
4	0		13.33 M-P	20 K-P	3.33 P		20 K-OP
4	1		40 F-P	66.66 A-I	79.16 A-F		46.66 E-O
3	0		0 P	10 N-P	13.33 M-P		20 K-P
3	1		60 A -K	100 A	100 A		100 A
2	0		10 N-P	23.33 J-P	13.33 M-P		40 F-P
2	1		53.33 B-M	100 A	66.66 A-I		90.46 A-D
1	0		20 K-P	10 N-P	13.33 M-P		20 K-P
1	1		53.33 B-M	76.66 A-G	80 A-F		93.33 AB
4		1	33.33 H-P	36.66 G-P	46.66 E-O		40 F-P
3		1	53.33 B-M	56.66 B-L	50 D-N		53.33 B-M
2		1	66.66 A-I	86.66 A-E	63.33 A-J		80 A-F
1		1	60 A-K	66.66 A -I	53.33 B-M		100 A
	1		13.33 M-P	3.33 P	9.76 N-P		33.33 H-P
		1	20 K-P	13.33 M-P	23.33 J-P		33.33 H-P

Means within a column followed by the same letter are not significantly different by Duncan's multiple range test (P>0.05)

Calli of *D. Anethifolia* in all of explants and hormones always had a good odor that is persumably due to the activation of secondary metabolite synthesis like n- decanal, α-pinene and dodecanal. Pigment cells and anthocyanin synthesis were also observed in calli of *D. Anethifolia* which became purple to red in medium containing 2, 4-D (**Figure 1E**: MS+ 1 $mg.l^{-1}$ 2, 4-D).

The calli derived from various explants of *D. anethifolia* were cultured on MS media supplemented with different concentrations of BA (1 and 2 $mg.l^{-1}$), KIN (1-2 $mg.l^{-1}$) or IAA, IBA or NAA (0.1-0.5 $mg.l^{-1}$) individually for shoot regeneration **(Table 2).** One week after transfer, to regeneration medium, calli became bigger and greenish spots appeared on the surface **(Figure 1D)** and buds emerged on calli on the 4^{th}-6^{th} weeks. The regeneration percentage and number of shoots per callus differed in calli derived from various explants. Calli derived from leaf segments showed significantly higher frequency of regeneration and number of shoot than calli derived from stems and nodal explants **(Table 2 and 3).**

Therefore, medium contained with 1 $mg.l^{-1}$ BA plus 0.3 $mg.l^{-1}$ NAA (72.67 %) and 2 $mg.l^{-1}$ BA in combination with 0.4 $mg.l^{-1}$ NAA were the perfect for shoot regeneration (77.67 %) in stem-derived callus. Also stem-derived callus in medium with 2 $mg.l^{-1}$ BA in combination with 0.4 $mg.l^{-1}$ NAA had the most number of shoot per callus (7.66) **(Table 3 and Figure 2.).** No shooting was initiated at medium with various concentrations of IBA and IAA in combination with different concentration of KIN and BA. Among three types of auxins NAA was found to be comparatively more effective than other two auxins IBA and IAA at different concentrations tested for producing shoots.

Table 2. *Effect of different concentrations of NAA alone and in combination with BA or KIN for percentage of regeneration from Stem, Leaf and Nodal of D.Anethifolia*

Plant Regulators (mgl^{-1})			**Regeneration (%)**		
Auxines	Cytokinines		Explants		
	Stem	Leaf	Nodal	KIN	BA NAA
	1		42.66 $^{B-F}$	41.33 $^{B-H}$	30.00 $^{F-L}$
0.1	1		53.33 ^{BC}	40 $^{C-I}$	41.66 $^{B-G}$
0.2	1		55 ^{B}	36.66 $^{D-J}$	45 $^{B-E}$
0.3	1		72.66 ^{A}	43.33 $^{B-F}$	16.66 $^{L-R}$
0.4	1		15 $^{M-S}$	13.33 $^{N-S}$	15 $^{M-S}$
	2		46.66 $^{B-D}$	45.33 $^{B-E}$	17 $^{L-R}$
0.1	2		51.66 ^{BC}	36.67 $^{D-IJ}$	10.00 $^{O-S}$
0.2	2		32.33 $^{E-K}$	26.67 $^{I-N}$	28.33 $^{G-M}$
0.3	2		41.67 $^{B-G}$	48.33 $^{B-D}$	23.33 $^{J-O}$
0.4	2		77.67 ^{A}	23.33 $^{J-O}$	18.33 $^{K-Q}$
		1	13.33 $^{N-S}$	12.67 $^{N-S}$	11.67 $^{O-S}$
0.1		1	21.67 $^{K-P}$	20.00 $^{K-P}$	12.33 $^{N-S}$
0.2		1	23.33 $^{J-O}$	15.00 $^{M-S}$	5.00 $^{Q-S}$
0.3		1	13.00 $^{N-S}$	11.67 $^{O-S}$	18.33 $^{K-Q}$
0.4		1	23.33 $^{J-O}$	8.33 $^{P-S}$	13.33 $^{N-S}$
		2	10.00 $^{O-S}$	5.00 $^{Q-S}$	10.00 $^{O-S}$
0.1		2	18.33 $^{K-Q}$	11.67 $^{O-S}$	3.33 ^{RS}
0.2		2	23.33 $^{J-O}$	15.00 $^{M-S}$	10.00 $^{O-S}$
0.3	0	2	28.00 $^{H-M}$	11.67 $^{O-S}$	23.00 $^{J-O}$
0.4	1	2	28.00 $^{H-M}$	13.67 $^{N-S}$	17.00 $^{L-R}$
0	0	0	5.00 $^{Q-S}$	3.33 ^{RS}	1.66 ^{S}

Means within a column followed by the same letter are not significantly different by Duncan's' multiple range test (P>0.05)

Table 3. *Effect of different concentrations of NAA alone and in combination with BA or KIN for number of shoot per callus from Stem, Leaf and Nodal of D.Anethifolia*

Plant Regulators (mgl^{-1})			**Number of shoot per callus**		
Auxine	Cytokinines		Explants		
	Stem	Leaf	Nodal	KIN	BA NAA
	1		5.00 $^{B-E}$	2.83 $^{E-M}$	5.16 $^{B-D}$
0.1	1		3.26 $^{D-K}$	1.46 $^{K-N}$	2.06 $^{G-N}$
0.2	1		4.90 $^{B-E}$	1.36 $^{K-N}$	2.36 $^{F-MN}$
0.3	1		6.66 ^{AB}	1.36 $^{K-N}$	1.66 $^{J-N}$
0.4	1		2.66 $^{F-M}$	2.66 $^{F-M}$	4.16 $^{C-G}$
	2		2.33 $^{F-N}$	2.66 $^{F-M}$	2.83 $^{E-M}$
0.1	2		4.00 $^{C-I}$	3.26 $^{D-K}$	2.46 $^{F-N}$
0.2	2		4.10 $^{C-H}$	1.76 $^{I-N}$	2.00 $^{G-N}$
0.3	2		4.10 $^{C-H}$	1.93 $^{G-N}$	3.23 $^{D-K}$
0.4	2		7.66 ^{A}	5.10 $^{B-D}$	3.80 $^{D-IJ}$
		1	1.10 $^{K-N}$	1.80 $^{I-N}$	1.33 $^{K-N}$
0.1		1	1.60 $^{J-N}$	1.16 $^{K-N}$	1.30 $^{K-N}$
0.2		1	2.33 $^{F-N}$	6.00 $^{A-C}$	1.00 $^{K-N}$
0.3		1	2.36 $^{F-N}$	1.03 $^{K-N}$	2.50 $^{F-N}$
0.4		1	2.16 $^{G-N}$	1.10 $^{K-N}$	2.66 $^{F-LM}$
		2	1.86 $^{H-N}$	2.50 $^{F-N}$	2.33 $^{F-N}$
0.1		2	3.13 $^{D-L}$	1.66 $^{J-N}$	0.83 $^{L-N}$
0.2		2	2.13 $^{G-N}$	4.500 $^{C-F}$	2.33 $^{F-N}$
0.3	0	2	2.66 $^{F-M}$	1.800 $^{I-N}$	1.90 $^{G-N}$
0.4	1	2	3.23 $^{D-K}$	1.56 $^{J-N}$	1.66 $^{J-N}$
0	0	0	1.00 $^{K-N}$	0.66 ^{MN}	0.33 ^{N}

Means within a column followed by the same letter are not significantly different by Duncan's multiple range test (P>0.05)

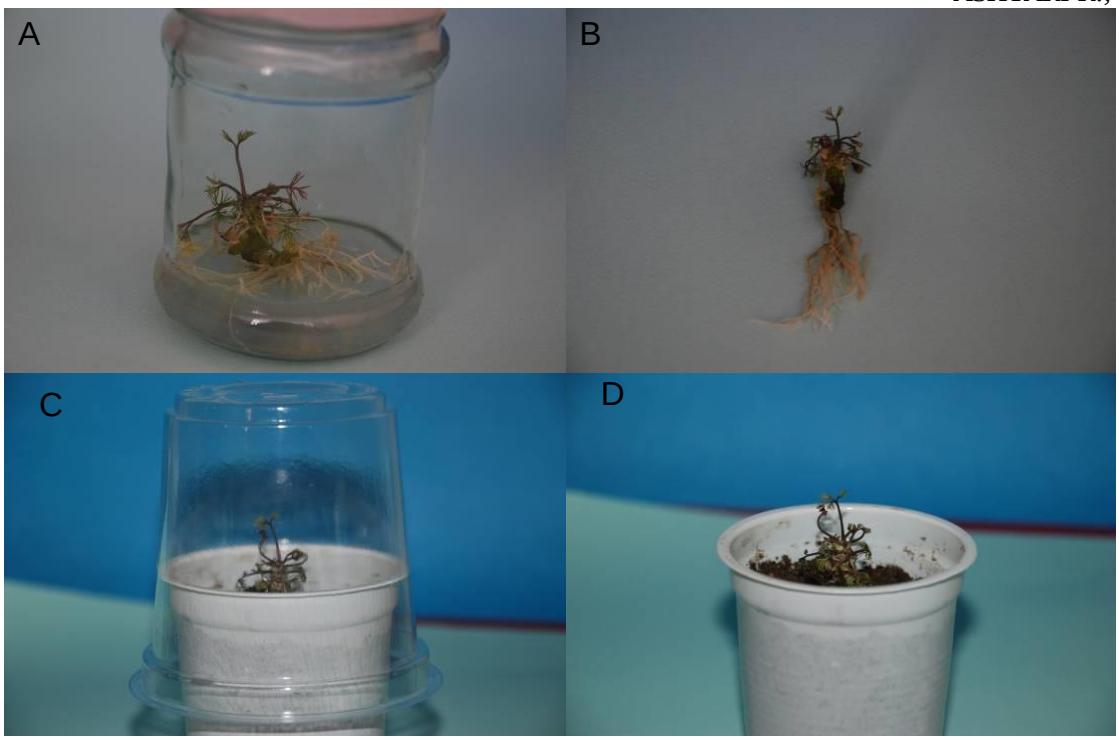

Figure 2. Regeneration and acclimatization of *Ducrosia anethifolia* .**A**. Shooting and Rooting in Vitro. B. Intact plant after exiting of the In vitro condition. **C.** Perfect plant under protected conditions. **D.** Perfect and acclimatized plant in vase

But after 3 weeks regenerated shoots faded and turned yellow. The shoot proliferation stopped and finally wilted shoots turned to brown and died **(Fig 1F).** In order to revive the withered shoots were transferred to diverse media without hormone or in 1/100 concentrations of the best regeneration media. In this case, 1/2MS medium free hormone that was most beneficial for trigging both renewing and proliferation. Also this medium spontaneously caused root generation in most of the shoots **(Table 4).**

Table 4. *Shoot's features in elongation media*

Elongation and Establishment of shoots Media	Shoots quality
1. Full MS	Most of the shoots revitalized; Leaves turned to fresh and green, shoots steadily proliferated, Mostly rooted
2. 1/2 MS*	All Shoots revived; Leaves turned to fresh and green, shoots increasingly propagated, Mostly rooted
3. 1/4 MS	%50 of the shoots thrived ; Leaves were fresh and green, shoots slightly proliferated, Thoroughly Rooted
4.1/2Concentration of (NH_4NO_3,KN_3)	Partially shoots renewed, frequently kept dying
5. MS+0.001BA+ 0.0003NAA	Shoots still wilted and turned to brown and entirely died
6. MS+0.002BA+0.0004 NAA	Shoots kept withering and turned to brown and completely died

The shoots were transferred to 1/4 strength MS medium free hormone that supplemented with various concentration of IBA and NAA (0, 0.1, 0.2, 0.5, 1 $mg.l^{-1}$) for rooting. Roots were evaluated after four weeks of culture. The rooting response to different treatments is shown in **Table 5**. The best result for rooting was achieved at 1/4th strength of MS auxin free medium. Among two types of auxins NAA was found to be comparatively more effective than IBA at different concentrations tested for producing roots. Regenerated plantlets were morphologically uniform having normal leaf shape and growth.

Table 5. *In Vitro rooting of shoots on quarter-strength MS medium fortified with different concentrations of IBA and NAA*

1/4 MS Auxin concenteration (mgl^{-1})		Response	Number of	Mean root
NAA	IBA	%	root	length
0	0	75 ^{A}	3.836 ^{A}	4.333 ^{A}
	0.1	5 ^{E}	0.6658 ^{E}	0.8333 ^{E}
	0.2	8.333 ^{E}	0.9342 ^{DE}	1.667 ^{DE}
	0.5	11.67 ^{DE}	1.256 ^{CDE}	1.867 ^{CDE}
	1	13.33 ^{DE}	1.52 ^{CD}	2.000 ^{BCD}
0.1		20 ^{CD}	1.282 ^{CDE}	1.533 ^{DE}
0.2		26.66 ^{C}	1.788 ^{BC}	2.400 ^{BCD}
0.5		42 ^{B}	1.788 ^{BC}	3.000 ^{B}
1		46.67 ^{B}	1.989 ^{BC}	2.900 ^{BC}

Means within a column followed by the same letter are not significantly different by Duncan's' multiple range test (P>0.05)

DISCUSSION

In our research, the best composition for stimulating Callus proved to be in medium with NAA/BA combination from leaf, stem and nodal explants. Similar result in *F. assa foetida* indicated that presence of both NAA and BA is necessary for optimum callus formation from various explants (2). On the contrary, combination of NAA with BAP did not promote calogenesis in *C. urucurana* (10).

In general, addition of cytokinins to medium increased the frequency of callus formation in *D. Anethifolia* **(Table 1).** HE et al (2006) reported the identical consequence in *Arctium lappa* L (11).

According to our observation various color and feature of calli were monitored in media with different hormones. In other experiment Martin (2003) confirmed that texture and type of callus depended on the type of growth regulators and concentrations (12). In this case special example occurred in media supplemented with KIN which calli appeared green in presence of KIN. Overall, Cytokinines promote the conversion of etioplasts (which are chloroplasts that have not been exposed to light) into chloroplasts via stimulation of chlorophyll synthesis (13).

George (1996) reported that Growth regulator effaces on the RNA metabolism by inducing the transcription of messenger RNA capable of coding proteins required for the growth and hence, promoting a chaotic cell proliferation callus formation (14). In plant cells most of the basic physiological processes such as cell division, cell elongation, polarity, and differentiation are controlled, amongst other factors, by phytohormones, above all by auxins and cytokinins (15).

Results of this study indicated that pigment cells and anthocyanin synthesis occurred in calli of *D. Anethifolia* in medium containing 2, 4-D. MS medium along with isolated 2, 4-D or in combination with cytokinins simplifies callus formation and anthocyanin development in *D.Anethifolia*. In *Cleome rosea* the highest biomass accumulation of anthocyanin was obtained in the callus cultures initiated on medium supplemented with 0.90 μM 2, 4-D

(16). MS-medium with 1 mg.l^{-1} 2, 4-D and 3 mg.l^{-1} BAP was the best medium for both callus and anthocyanin production in some ornamental Plants (17).

Calli of *D. Anethifolia* under any circumstances had a pleasant scent which is undoubtedly because of the secondary metabolite synthesis like n- decanal, α-pinene and dodecanal in calli cells which are common ingredients in perfumery.

Cytokinin alone or in combination of auxin was indispensable for the regeneration of *D.Anethifolia*. Our outcome is comparable with Irvani et al (2010) in regeneration of *Dorem ammoniacum* (18). The use of BAP and NAA for the plant regeneration has been reported in several plants such as *Centella Asiatica* (19).

Although in *D. anethifolia* various explants were found to be totipotent to regenerate, the calli derived stem has been considered to be the appropriate explants for inducing the multiple shoots in the present investigation likewise, *D. carota* (20).

The specific differences in the regeneration potential of different organs and explants have various explanations. Shoot regeneration also was strongly influenced by the explants type. The significant factors include differences in the stage of the cells in the cell cycle, the availability of or ability to transport endogenous growth regulators and the metabolic capabilities of the cells (21).

We observed that BA is more effective than KIN in regeneration of *D. Anethifolia*. In agreement with our outcome Sharma and Wakhlu (2003) reported the same in *Heracleum candicans* (22). BA proved more useful compared to KIN in shoot induction for callus produced from various explants (2). BA induces production of natural hormones such as zeatin within the tissue and thus works through natural hormone systems that were metabolized easier than artificial growth regulators (23).

NAA was practically used as proper auxin for regeneration of *D. Anethifolia* against two other auxins (IBA and IAA). Higher concentration of NAA has favored the plant differentiation more rapidly in *D. carota* (20). Inhibitory effect of IAA on shoot regeneration of *Capsicum annuum* is reported (24).

After 3 weeks in vitro regenerated shoots withered, turned yellow and shriveled. Length of time intervals between subcultures may cause this problem. Dehydrated shoots were recovered in 1/2MS medium devoid growth regulators that were useful for growth of shoots and propagation **(Table 4).**

The hyperhydric plantlets reversed to normal plantlets when plant growth regulators were removed from culture medium (25). The incidence of hyperhydricity increased with an increase in the concentration of cytokinins. Hyperhydricity was most severe with BA treatment although all treatments caused hyperhydricity at higher concentrations (26).

1/4 strength of MS free hormone has the highest rooting also NAA was found to be better than IBA in terms of producing roots for *D. Anethifolia*. The equal result was procured in an endemic Umbelifera named *Hydrocotyl Confera* which NAA identified utmost for inducing roots (27). The rooting of the shoots in (*Benincasa. hispida* L.) occurred on 1/4th strength of MS medium supplemented with IBA or NAA (28). IBA was introduced as best rooting auxin for Apiaceae members like, *Thapsia garganica* (29). Plant tissue culture techniques have been increasingly applied to many medicinal plants in particular for mass propagation, conservation of germplasm, study and production of bioactive compounds, and for genetic improvement (30).

CONCLUSION

In conclusion, we report a protocol for the successful regeneration of *D. Anethifolia* through callus cultures derived from varied explants. Maximum percentage of callus induction was observed on MS medium supplemented with 3 mg.l^{-1} NAA and 1 mg.l^{-1} BA. MS medium contained with 1 mg.l^{-1} BA plus 0.3 mg.l^{-1} NAA (72.67 %) and 2 mg.l^{-1} BA in combination with 0.4 mg.l^{-1} NAA were the best media for shoot regeneration (77.67 %) in stem-derived callus. Further work is required to produce secondary metabolite of this important medicinal plant.

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Abbreviations

2, 4-D: 2, 4-Dichlorophenoxyacetic acid
BA: N6-benzyladenin
IAA: Indole-3-acetic acid
IBA: Indole butyric acid
KIN: Kinetin (6-furfurylamonopurine)
NAA: α-naphthaleneacetic acid
MS: Murashige and Skoog (1962) medium

REFERENCES

1. Hajhashemi, V., Rabbani, M., Ghanadi, A., Davari, E., Evaluation of antianxiety and sedative effects of essential oil of Ducrosia anethifolia in mice, *CLINICS*, 65(10); 1037-1042, 2010.
2. Zare, A.R., Solouki, M., Omidi, M., Irvani, N., Mahdi Nezad, N., Rezazadeh, Sh., Callus Induction and Plant Regeneration in Ferula Assafoetida L. (Asafetida), an Endangered Medicinal Plant, *TJS*, 8 (1); 11-18, 2010.
3. Canter, P.H., Thomas, H., Ernst, E., Bringing medicinal plants into cultivation: opportunities and challenges for biotechnology, *Trends Biotechnol*, 23; 180-185. 2005.
4. Rychlik, M., Schieberle, P., Grosch, W., Compilation of Odor Thresholds, Odor Qualities and Retention Indices of Key Food Odorants, Institut für Lebensmittelchemie der Technischen Universität München und Deutsche Forschungsanstalt für Lebensmittelchemie, 1998.
5. Neuenschwander, U., Mechanism of the Aerobic Oxidation of α-Pinene, In: *ChemSusChem*. 3(1); 75–84, 2010.
6. Amiri, H., Meshkat, A.l., Sadat, M.H, Lari Yazdi, H., Chemical composition of the essential oil of Eremostachys Laevigata bung, *DARU J Pharm Sci*, 15(1); 41- 44, 2007.
7. Karuppusamy, S., Kiranmai, C., Aruna, V., Pullaiah, T., Micropropagation of *Vanasushava pedeta* An Endangered Medicinal Plant of India, *Plant Tissue Cult & Biotech*, 16(2); 85-94, 2006.
8. Al-Yemeni MN, Basahy AY., Breaking of seed dormancy in Ducrosia. Anethifolia (DC.) Boiff. Pak, *J. Bot*, 31(2); 247-252, 1999.
9. Murashige, I., Skoog, F., A revised medium for rapid growth and bioassays with tobacco tissue cultures, *Physiologia Plantarum*, 15(3); 473-497, 1962.
10. Ednabel, C.L, Renato, P., Rairys, C.N., Callus Induction in Leaf Segments of *Croton urucurana* BAILL. Ciênc Agrotec Lavras, 32; 1 17-22, 2008.
11. HE, W.T., HOU, S.W., WANG, C.Y., Callus Induction and High-Frequency Plant Regeneration from Hypocotyls and Cotyledon Explants of *Arctium. Lappa* L., In Vitro Cell Dev, *Biol.—Plant*, 42; 411–414, 2006.
12. Martin, K.P., Efficacy of different growth regulators at different stages of somatic embryogenesis in *Eryngium foetidum* L. a rare medicinal plant., In Vitro Cell. Dev. *Biol—Plant*, 40; 459–463, 2003.
13. Pospišilová, J., Synková, H., Rulcová, J., Cytokinins and water stress, *Biologia Plantarum*, 43 (3); 321-328, 2000.
14. George, E.F., Plant propagation by tissue culture: part 1: the technology. Edington: Exegetics, 574p, 1996.
15. Zažímalová, E., Petrášek, J., Morris, D.A., The Dynamics of Auxin Transport in Tobacco Cells, Plant physiol, 207–224, 2003.
16. Simões, C., Bizarri, C.H.B., Cordeiro ,L.S., Castro, T.C., Coutada, L.C.M., Silva, A.J.R Albarello, N., Mansur, E., Anthocyanin production in callus cultures of *Cleome rosea*: modulation by culture conditions and characterization of pigments by means of HPLC-DAD/ESIMS. *Plant Physol Bioch*, 47(10): 895-903, 2009.
17. Taha, H.S., Abd E-Rahman, R.A., Fathalla M.A., Aly, U.E., Successful Application for Enhancement and Production of Anthocyantn Pigment from Calli Cultures of Some Ornamental Plants. *AJBAS*, 2(4): 1148-1156, 2008.
18. Irvani, N., Solouki, M., Omidi, M., Zare, A.R., Shahnazi, S., Callus induction and plant regeneration in *Dorem ammoniacum* D. an endangered medicinal plant., *Plant Cell Tiss Organ Cult*, 100(3); 241-360, 2010.
19. Deshpande, H.A., Chalse, M.N., Bhalsing, S.R., Centella Asiatica LINN: Plant Regeneration through Leaf Derived Callus,

Journal of Herbal Medicine and Toxicology, 4 (2); 119-122, 2010.
20. Pant, B., Manandha, S., In vitro propagation of Carrot (*Daucus Carota*) L., *Scientific World*, 5; 51-53, 2007.
21. Akin-Idowu, P.E., Ibitoye, D.O., Ademoyegun, O.T., Tissue culture as a plant production technique for horticultural crops, *AJB*, 8 (16); 3782-3788, 2009.
22. Sharma, R.K., Wakhlu, A.K., Regeneration of Heracleum candicans Wall Plants from Callus Cultures through Organogenesis., *J. Plant Biochem. Biotech*, 12; 71-72, 2003.
23. Zaerr J.B., Mapes, M.O., Tissue culture in forestry (Bonga J M, Durzen DJ, Editors)., Martinus Nijhiff and Dr W Janik Publishers, London, 231-255, 1982.
24. Ashrafuzzaman, M., Hossain, M.M., Razi Ismail, M., Shahidul Haque, M., Shahidullah Shahin-uz-zaman, S.M., Regeneration potential of seedling explants of chilli (Capsicum annuum), *AJB*, 8 (4); 591-596, 2009.
25. Yang, C., Qin, y., Sun, X., Yuan, Sh., Lin, H., Propagation of Sedum spectabile Boreau in Leaf Culture in Vitro., *Not Bot Horti Agrobo*, 40(1);107-112, 2012.
26. Michael, W., Wendy, B., Stirk, A., Dolezal, K., Van Staden, J., Optimizing the micropropagation protocol for the endangered Aloe polyphylla: can meta-topolin and its derivatives serve as replacement for benzyladenine and zeatin? *Plant Cell Tiss Organ Cult*, 90; 15–23, 2007.
27. Karuppusamy, S., Aruna, V., Kiranmai, C., Pullaiah, T., In Vitro Propagation of an Endemic Umbelifer, *Hydrocotyl Confera*, IJBT, 6; 541-544, 2007.
28. Thomas, T.D., Sreejesh, K.R., Callus induction and plant regeneration from cotyledonary explants of ash gourd (*Benincasa hispida* L.), *Scientia Horticulturae*, 100; 359–367, 2004.
29. Makunga, N.P., Jager, A.K., Staden, J.V., Improved in vitro rooting and hyperhydricity in regenerating tissues of *Thapsia garganica* L., *Plant Cell Tiss Org Cult*, 86; 77-86, 2006.
30. Nalawde, S.M., Tsay, H.S., In vitro propagation of some important Chinese medicinal plants and their sustainable usage, In Vitro Cell, Dev, *Biol.—Plant*, 40; 143–154, 2004.