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Original Contribution

CALLUS INDUCTION AND PLANT REGENERATION IN *FERULA ASSA FOETIDA* L. (ASAFETIDA), AN ENDANGERED MEDICINAL PLANT

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ABSTRACT

Plant regeneration in *Ferula assa-foetida* L. (Asafetida), a medicinally important vulnerable species, was achieved through the mediation of callus derived from root, hypocotyl and cotyledon. Murashige and Skoog (MS) medium with 1 mg l^{-1} α-naphthaleneacetic acid (NAA) and 2 mg l^{-1} N6-benzyladenine (BA) was most effective (100%) for the proliferation of callus for root explants. MS medium containing 1-3 mg l^{-1} BA or KIN alone or in combination with 0.2 or 0.5 mg l^{-1} NAA facilitated indirect organogenesis. BA was superior to KIN in shoot morphogenesis. Hypocotyl-derived callus subcultured on medium with 1 mg l^{-1} BA and 0.2 mg l^{-1} NAA showed maximum percentage of cultures regenerating shoots (81.1%), with a mean of 7.4 shoots per callus. Root-derived callus was inferior, which yielded a mean of 2 shoots on medium with 2 mg l^{-1} BA. Rooting of in vitro raised shoots was best the on half-strength MS medium containing 2.5 mg l^{-1} indole-3-butyric acid (IBA) with a mean of 7.23 roots per shoot measuring a mean length of 5.34 cm. The plantlets acclimatized and transferred to soil exhibited 90% survival.

Key words: *Ferula assa foetida* L., seedling explants, shoot regeneration, Murashige and Skoog medium, vulnerable species.

INTRODUCTION

Ferula assa-foetida (Apiaceae), commonly known as Asafetida is an herbaceous, perennial medicinal plant indigenous to Iran (1) Asafetida is a very effective medicinal herb that acts mainly on the digestive system, cleansing and strengthening the gastro-intestinal tract. The pungently flavoured gum-resin that is obtained from the root by incisions is alterative, anthelmintic, antiperiodic, antispasmodic, carminative, deobstruent, deodorant, expectorant, laxative, sedative and stomachic (9).

The commercial and pharmacological need of Asafetida is achieved usually by exploiting the wild populations. Indiscriminate collection of this plant from the wild has posed a serious threat to its existence in the wild populations, especially when the plants are harvested well before seed set. The plant is conventionally propagated through seed but is hampered by the seed dormancy. Further, the methods of extraction employed are almost invariably crude and unsystematic (16). Consequently, Asafetida is recorded as a vulnerable species in the Red Data Book of Iran (8). On the other hand, improvement of Apiaceae plants following the classical breeding procedure is generally slow, laborious and time consuming (18). Hence, there is a strong need for proactive understanding in the conservation, cultivation and sustainable usage of this species for future use.

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Recent years witnessed an increased interest in tissue culture techniques which offer a viable tool for mass multiplication and germplasm conservation of rare, endangered and threatened medicinal plants (6, 11, 17, 20). Further, indirect organogenesis offers the induction of variability and thereby the improvement of the crops. In vitro propagation has been accomplished in many Apiacean species, including *Centella asiatica* (19, 13), *Eringium foetidum* (7, 12), *Cuminum cyminum* L (3).

Despite being a valuable medicinal plant, very little work has been done on tissue culture of *Ferula assa foetida*. There has been only one report on somatic embryogenesis via hypocotyl explants of this plant (5). Here we describe the first methods for plant regeneration through the mediation of callus derived from seedling explants of *F. assa-foetida*, an endangered medicinal plant.

MATERIAL AND METHODS

1. Preparation of plant materials

The mature seeds of *F. Assa-foetida* collected in July 2007 from a biological reserve (Nasr Abad) in the nearby county Yazd, Iran (1900 and 2050 m above sea level) were washed thoroughly under running tap water for 3 days. The soaked seeds were treated with 70% ethyl alcohol for 2 min, then rinsed with sterile water for three times. The seeds were then surface sterilized in 2.5% sodium hypochlorite solution for 20 min and was followed by three washes in succession with sterile water. The surface sterilized seeds were cultured on 1/4MS (Murashige and Skoog, 1962) medium supplemented with 30 g l^{-1} sucrose and 7 g l^{-1} agar. To overcome dormancy, the plates were kept in a refrigerator (4 ˚C) for 30 days. After cold stratification pre-treatment, the cultures were incubated at 23 ± 2 ˚C under 16 h photoperiod of 80 µ mol m^{-2} s^{-1} irradiance provided by cool fluorescent lamps. The cultures were used as a source of plant material for establishment of explants before initiating the experiments.

2. Callus induction

Aseptically excised root, hypocotyl and cotyledon explants (root and hypocotyl of 10 mm, cotyledon of 5 mm^2) from 20-days-old seedlings were cultured on sterile MS medium fortified with 0-4 mg l^{-1} α-naphthaleneacetic acid (NAA) or 0-4 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) alone and or in combination with 0-2 mg l^{-1} benzyladenine (BA) or 0-2 mg l^{-1} kinetin (KIN) for callus induction. After 6 weeks of culture, callus formation was evaluated. All the calluses obtained were subcultured onto medium containing the same or lower concentrations of same growth regulators after every 6 weeks for callus proliferation.

3. Regeneration

The shoot regeneration was attempted by transferring 6-week-old calluses on MS medium with 1-3 mg l^{-1}BA or 1-3 mg l^{-1} KIN and in combination with lower levels of NAA (0.2 and 0.5 mg l^{-1}). After 6 weeks of culture, data were recorded for percent of shoot formation and number of shoots.

4. Root formation

Healthy shoots (2 cm height) excised at the end of 6th week were transferred onto rooting medium consisting of half strength MS medium with 1-5 mg l^{-1} NAA, IAA or IBA. The data were recorded on percent root formation, number and length of roots after 4 weeks.

5. Culture conditions

All the media were supplemented with 3% (w/v) sucrose and medium was gelled with 0.8% (w/v) agar. The pH of all media was adjusted to 5.8 before the addition of agar. The media were sterilized under 1.5 kg cm^{-2} and 121 ˚C for 20 min. All the cultures were grown at 23 ± 2 ˚C under 16 h photoperiod supplied by two Philips TL 40W fluorescent tubes.

6. Acclimatization

Plantlets gently washed in distilled water to remove agar from their roots were planted in plastic cups (upper dia 6 cm × height 8 cm) containing sand, fertile soil and vermiculite (1:1:1) and placed in a glasshouse with 90% humidity. After 2 months, the plants were transferred to glasshouse and placed in shade (approximately 60% shade) under natural conditions. The plants transplanted to pots (upper dia 20 cm × height 30 cm) after 2 months were transferred subsequently to the field.

7. Experimental design and data analysis

The treatments were arranged in factorial experimental based on Completely Randomized Design (CRD) with three replicates per treatment and ten explants per replicate. Data given in percentages were subjected to arcsine transformation before statistical analysis. Data were analyzed statistically using MSTATC software. The mean values of different treatments were compared using Duncan's multiple range tests (at 5% level).

Table.1 *Callus induction from different explants of F. assa-foetida on MS medium with different growth regulators.*

Growth Regulators (mg l^{-1})				Callus induction (%)		
2,4-D	NAA	BAP	KIN	root	hypocotyl	cotyledon
MS basal media				15^{wx}	0^{y}	0^{y}
1				37.1^{f-u}	28.6^{m-x}	24^{q-x}
1		1		50^{d-n}	50.4^{d-m}	39.6^{f-t}
1		2		60.5^{c-g}	57.5^{d-h}	43.8^{e-q}
2				45.2^{e-q}	26.3^{o-x}	18.6^{u-x}
2		1		63.3^{b-f}	29.2^{l-x}	25.4^{p-x}
2		2		59.6^{c-g}	30.4^{k-w}	19.2^{u-x}
4				27.1^{o-x}	0y	0^{y}
4		1		28.3^{n-x}	0^{y}	0^{y}
4		2		23.8^{t-x}	16^{v-x}	0^{y}
1			1	46^{e-s}	42.3^{e-s}	34.6^{k-w}
1			2	46.8^{d-p}	46^{e-q}	33.4^{h-v}
2			1	53.3^{d-k}	42.3^{e-s}	0^{y}
2			2	60^{c-g}	37.4^{f-u}	31.7^{j-w}
4			1	22.4^{s-x}	19.1^{u-x}	0^{y}
4			2	23.6^{q-x}	0^{y}	0^{y}
	1			63.6^{b-f}	47.5^{d-o}	36.9^{f-u}
	1	1		90.2^{a-c}	62.4^{b-f}	56^{d-j}
	1	2		100^{a}	64^{b-f}	52.5^{d-l}
	2			69.6^{b-e}	57.7^{d-h}	33.1^{h-w}
	2	1		92.8^{ab}	63.6^{b-f}	43.1^{e-r}
	2	2		89.5^{a-c}	74.9^{a-d}	62.4^{b-f}
	4			57.5^{d-i}	27.1^{o-x}	24.3^{q-x}
	4	1		57.7^{d-i}	24.9^{q-x}	0^{y}
	4	2		64.6^{c-g}	34.8^{h-v}	26.5^{o-x}
	1		1	50.8^{d-o}	42.4^{f-s}	33.8^{h-v}
	1		2	54^{d-k}	46^{e-q}	40^{f-s}
	2		1	40^{f-s}	30^{k-w}	34^{h-v}
	2		2	43.1^{e-r}	28.2^{m-x}	20.9^{t-x}
	4		1	33.1^{i-w}	22.9^{r-x}	12.9^{x}
	4		2	38^{g-u}	29.8^{l-x}	18^{u-x}

Means followed by the same letters within columns are not significantly different at the 5% level.

RESULT

The seeds cultured on basal medium devoid of any plant growth regulator germinated normally, developing into seedlings with roots and shoots without any callus formation.

Root, hypocotyls and cotyledon derived from 20-days-old in vitro seedlings cultured on MS medium with different levels of NAA and 2,4-D either alone or in combination with different levels of BA or KIN **(Table. 1).** The earliest visible sign of callus growth from root explants was noticeable between 3 and 5 days of incubation, while callus formation on cotyledon explants was observed after 3 weeks culture. In hypocotyl and cotyledon cuttings, calluses were formed from the basal cut end, whereas in root cultures the calluses were produced all over the surface of the root. Callus morphology also differed. The calluses from root explants were white pigmented and watery with smooth surface and then turned brown, while calluses derived from hypocotyl segments became soft or friable, sticky, yellow-white to cream colored and nodular type with proembryogenic structures **(Fig. 1B)**. Cotyledon explants developed nodular, green, organogenetic and compact callus **(Fig. 1A)**. The callus became brownish after 6 weeks of culture on medium with more than 2 mg l^{-1} NAA or 2,4-D alone or in combinations with KIN, particularly the callus from root explants.

NAA alone or in combination with BA or KIN induced shoots and roots as well as callus at varying frequencies depending on the concentration and type of explants (data not shown). Callus formation was significantly influenced by the explants type, type and concentration of growth regulators **(Table. 1)**. The frequency of callus induction from root segments was significantly higher than that from hypocotyls and cotyledon segments. NAA in combination with BA was most effective for consistent callus induction. Hence, the medium supplemented with 1mg l^{-1} NAA and 2 mg l^{-1} BA was the most effective for the proliferation of callus from root explants. Individual treatment of 2,4-D and NAA showed few response for callus induction, particularly the calli from hypocotyl and cotyledon explants **(Table. 1)**.

The effect of 2,4-D and NAA were quite reducible when used in higher concentration. Hence, No callus induction was obtained on medium containing 4 mg l^{-1} 2,4-D in hypocyyl explants. Cotyledon segments also incubated in the present of 4 mg l^{-1} 2,4-D and various concentration of BA or KIN did not develop any callus. The hypocotyl and cotyledon segments inoculated on without growth regulators showed no callus induction **(Table 1)**.

Proliferated calli of the three explants, after two subcultures on the callus induction medium, were transferred to MS medium with various combinations of BA or KIN alone or in combination with NAA to evaluate their potential for shoot formation **(Fig.1D)**. BA proved more useful compared to KIN in shoot induction for callus produced from various explants **(Table 2**). Shoot regeneration also was strongly influenced by the explants type. Shoot regeneration was better in the calluses derived from hypocotyl as compared to cotyledon and root explants **(Fig.1C)**. Therefore, shoot production being and number of shoots highest (81.1 % and 7.4 shoots per callus, respectively) when callus produced from hypocotyls were placed on medium with 1 mg l^{-1} BA plus 0.2 mg l^{-1} NAA **(Table 2)**. Root derived callus induced less regeneration compared to that of other explants. Therefore, callus produced from root incubated in the present of 3 mg l^{-1} BA or kinetin and various concentration of NAA did not develop any shoot **(Table 2)**. Presence of KIN at concentrations higher than 2 mg l^{-1} decreased shoot formation. Hence, callus produced from root and cotyledon explants inoculated on MS medium supplemented with 3 mg l^{-1} KN alone and or in combination with either of 0.2 or 0.5 mg l^{-1} NAA did not produce any shoot buds **(Table 2**).

The mean number of shoots decreased with increase in the concentrations of NAA **(Table 2)**. When callus derived from different explants were cultured on media containing BA or KIN in combination with NAA, it is evident that the mean number of shoots was closely related to concentration of the NAA supply. Increasing NAA decreased the frequency and number of shoots concomitant with an increase in the amount of callus **(Table. 2)**. MS basal medium was unable to evoke shoot regeneration from root and cotyledon derived calli, while it was observed that there was a considerable increase in shoot number on MS hormone free in calli produced from hypocotyl explants.

The shoots were subcultured on the same medium and were allowed to grow till they attained height of 3 cm within 6 weeks. Then, Individual shoots (2–3 cm) with their apical leaves were cultured separately in half-strength MS medium supplemented with IAA, NAA or IBA for root formation. Root induction was observed in all the treatments. Of the three auxins tested, IBA was found to be comparatively more effective than other two auxins NAA and IAA at different concentrations tested for producing roots **(Table. 3).** Among the different concentrations used, maximum number of roots (7.23) per shoot and highest rooting (88.8%) were obtained with 2.5 mg l^{-1} IBA within 4 weeks in the culture medium **(Fig. 1E)**. A further increase of IBA and NAA concentration above 2.5 mg l^{-1} decreased the rooting percentage, number of roots per shoot and length of the roots. Explants inoculated on the entire media generally induced callus at the base of the shoots **(Table. 3).**

Plantlets having well-developed roots were transferred to small plastic cups containing sand, fertile soil and vermiculite (1:1:1) and the humidity was maintained at approximately 90% by covering with plastic **(Fig. 1F)**. They were watered twice every 4 days. About 35–40% of the plants transferred into soil died after one to two weeks, mostly because of fungal infection. After 2 months the plants were transferred to larger pots and after acclimatization, the 70-day-old plants were transferred to field.

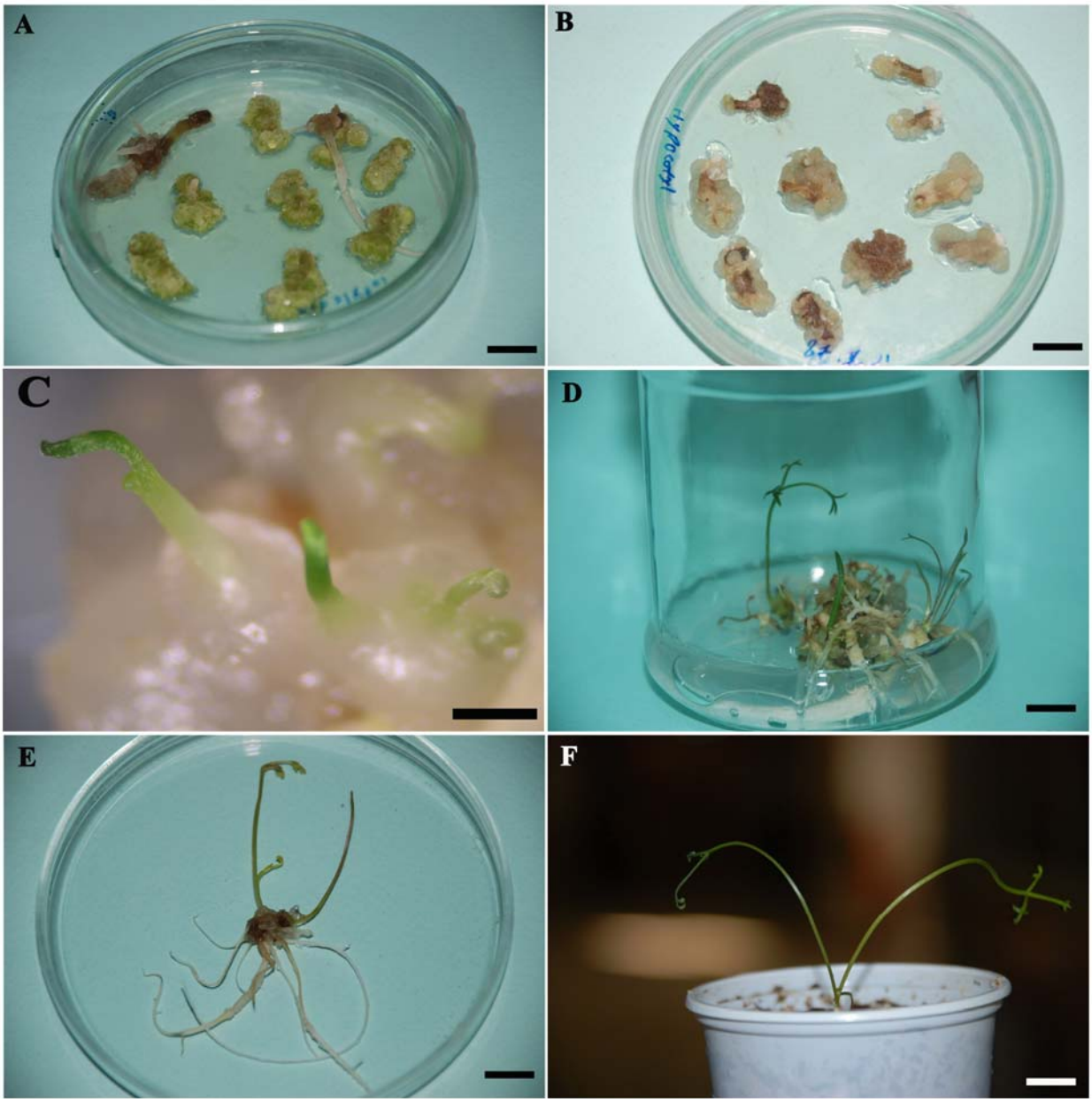

Fig.1. Callus induction and plant regeneration processes of various explants in *Ferula assa foetida*. A. Callus induction and root formation of cotyledon explants on MS medium supplemented with 1 mg l^{-1} and 2 mg l^{-1} BA after a culture period of 6 weeks (bar=1 cm), B. Sticky and creamy callus of hypocotyl explants on MS medium containing 2 mg l^{-1} NAA with 2 mg l^{-1} BA after subculture (bar=1 cm), C. Initiation shoot regeneration of hypocotyl explants on MS medium containing with 2 mg l^{-1} BA and 0.2 mg l^{-1} NAA (bar=0.5 cm), D. Shoot regeneration from the hypocotyl -derived calli on MS medium fortified with BA (1 mg l^{-1}) and NAA (0.2 mg l^{-1}) 5 weeks after culture (bar=1 cm), E. Root induction from multiplied shoots on half-strength MS medium supplemented with IBA (2.5 mg l^{-1}) after 6 week of culture (bar=1 cm), F. A healthy plant in plastic cups containing sand, fertile soil and vermiculite (1:1:1) (bar= 1cm).

DISCUSSION

Although *F.assa foetida* is a very valuable medicinal plant, there is no study about callus induction and in vitro propagation of this plant. We, therefore, aimed to develop an in vitro culture protocol for the high-efficiency regeneration of Asafetida plant by plant regeneration from callus cultures.

The best frequency of germination was observed in seeds placed at 4 ˚C for 30 days. The need for a period of cold stratification for dormancy breaking has been reported for other Apiaceae plants (15). The chilling treatment enhances the maturation of the embryo eliminating seed dormancy (4).

Table.2 *Shoot induction efficacy of root, hypocotyls and cotyledon-derived callus of F. assa-foetida on MS medium with different growth regulators (after 6 weeks).*

Growth regulators (mg l^{-1})			root		hypocotyl		cotyledon	
BAP	**KN**	**NAA**	**Shoots (%)**	**No. of shoot**	**Shoots (%)**	**No. of shoot**	**Shoots (%)**	**No. of shoot**
MS basal media			0^{p}	0^{o}	24.6^{g-m}	2.53^{h-m}	0^{p}	0^{o}
1			0^{p}	0^{o}	50^{b-f}	5^{c-e}	31.3^{e-l}	3.7^{fg}
1		0.2	18^{i-o}	1.56^{l-n}	81.1^{a}	7.4^{a}	40^{b-h}	5^{c}-^{e}
1		0.5	10.6^{o}	1.33^{n}	60^{a-c}	6.2^{b}	30^{e-l}	3.3^{f-j}
2			27.4^{f-m}	2^{k-n}	48^{b-f}	4.2^{d-f}	33^{e-l}	2.4^{h-n}
2		0.2	20.7^{h-n}	1.43^{mn}	64^{ab}	5.2^{cd}	39.1^{b-h}	2.8^{g-k}
2		0.5	12.3^{m-o}	1.4^{n}	52.5^{a-e}	5.2^{cd}	34^{c-k}	3.23^{f-j}
3			0^{p}	0^{o}	21.2^{h-n}	3.5^{f-g}	23.5^{g-m}	2.4^{h-n}
3		0.2	0^{p}	0^{o}	30.7^{e-l}	2.9^{g-k}	29.6e-l	2.53^{h-m}
3		0.5	0^{p}	0^{o}	36.9^{c-i}	3.33^{f-i}	31.3^{e-l}	2.3^{i-n}
	1		0^{p}	0^{o}	33^{d-l}	2.03^{k-n}	22.4^{l-o}	1.5^{mn}
	1	0.2	0^{p}	0^{o}	44^{b-g}	3.4^{f-i}	35.7^{c-j}	2^{k-n}
	1	0.5	0^{p}	0^{o}	37^{c-i}	2.9^{g-k}	27.4^{f-m}	2.2^{j-n}
	2		11.93^{no}	1.4^{n}	51.7^{b-f}	3.7^{fg}	36^{c-j}	2.67^{g-l}
	2	0.2	16.5^{j-o}	1.8^{k-n}	57.6^{a-d}	4.07^{ef}	50^{b-f}	3.5^{f-h}
	2	0.5	0^{p}	0^{o}	65.1^{ab}	5.3^{bc}	34^{c-k}	2.77^{g-k}
	3		0^{p}	0^{o}	24^{k-o}	2.2^{j-n}	0^{p}	0^{o}
	3	0.2	0^{p}	0^{o}	22.4^{g-m}	2.37^{i-n}	0^{p}	0^{o}
	3	0.5	0	0^{o}	28.3^{f-m}	3.23^{f-j}	0^{p}	0^{o}

Means followed by the same letters within a column are not significantly different using Duncan's Multiple Range Test at 5% level.

Table.3 *In vitro rooting of shoots on half-strength MS medium fortified with different auxins.*

IBA	NAA	IAA	Root formation (%)	No. of roots	Mean root length (cm)
1/2MS basal media			22.67^{e}	2^{f}	1.26^{g}
1			71.1^{b}	5.45^{b-d}	3.56^{bc}
2.5			88.8^{a}	7.23^{a}	5.34^{a}
5			63^{b}	4.8^{ab}	4.11^{ab}
	1		50^{cd}	3^{c-e}	2.57^{ef}
	2.5		63^{b}	4.05^{a-c}	3.74^{b-e}
	5		51.1^{bc}	3.36^{cd}	3.16^{b-d}
		1	38^{d}	2.55^{e}	2.3^{f}
		2.5	44^{cd}	3.1^{de}	3.12^{d-f}
		5	51.1^{bc}	3.27^{cd}	2.67^{c-f}

Means within a column followed by the same (a–g) letters are not significantly different by Duncan's multiple range test ($P > 0.05$).

In the present investigation, maximum callus induction was observed from the root explants in NAA/BA combination. Overall, percentage of callus induction was lower for all explants on media with 2,4-D plus KIN when compared to NAA plus BA combinations **(Table 1)**. These results clearly show that NAA played a central role in the callus

induction. Results of this study indicated that the presence of both NAA and BA in the medium is necessary for optimum callus formation from various explants of *F. assa foetida.*

Though the positive effect of NAA in combination with BA or KIN was observed in the present study, in some the combinations tried, there was callus formation accompanied by spontaneous root development **(Fig 1A).** Martin (2004) reported a similar observation in *Eryngium foetidum* over the effect of NAA and KIN on root formation during callus induction (12). Hypocotyl and cotyledon explants of *ferula assa foetida* cultured on MS basal medium did not form callus. These results indicated that plant growth regulators, especially auxins, were absolutely necessary for callus induction. Browning of callus cultures were observed during subculture. Chand *et al*. (1997) eliminated browning of cultures of *Pimpinella anisum L*. by shortening the frequency of subculture to 2 weeks (2). Browning of the callus, which has been reported previously (13, 10), may be due to the activation of secondary metabolite synthesis.

The most *in vitro* studies on Apiaceae member concern somatic embryo induction and development (13, 7 21, 18). Only few reports mention plant regeneration by callus in this family (10, 19). Transition of callus to regeneration pathway was affected by interaction effect between BA and NAA. In fact, this interaction played a key role in regeneration through indirect organogenesis pathway. In our study, the combination of BA and NAA substantially increased the frequency of responding various explants (Table 2). Our observations are in agreement with Tiwari *et al* (2000) who reported micropropagation in *Centella asiatica* in the combination of NAA and BA for nodel explants (19). The callus formation increased with increasing concentration of BA in the regeneration media. Similar results were observed by Ignacimuthu *et al* (1999) in *Eryngium foetidum*, the medium when supplemented with BA along with 2,4-D presented considerable increase in the frequency of callus induction (7).

Different tissues may have different levels of endogenous hormones and, therefore, the type of explant source would have a critical impact on the regeneration success (22). In our study, when root, hypocotyl and cotyledon explants were compared, it was clear that hypocotyl explants were much more productive for shoot formation than root and cotyledon explants **(Table 2)**. Plant regeneration from root segments was unsuccessful. It seems that root derived calli were not totipotent for plant regeneration. Similarly, hypocotyl derived calli was also reported as the most responsive explant in terms of shoot organogenesis with *Cuminum cyminum* L. (18).

In general, shoots grown with IBA, IAA or NAA produced root primordia at the base after four weeks of culture. The tendency of NAA on callus formation during *in vitro* root development was also reported in *Eupatorium triplinerve* (11). A similar problem of callus formation was also encountered by earlier workers (10). The positive effect of IBA on root induction was reported in *Centella asiatica* (19) and *Cuminum cyminum* (18). The roots formed initially were tender, whitish but subsequently changed to dark brown color in the mature shoots might possibly be due to the biosynthetic activity of essential oils.

Acclimatization of *in vitro* developed plantlets was difficult, as some of the plantlets had hyperhydric shoots. Most of these plantlets did not survive. Future research should be directed towards reducing.

The micropropagation protocol for plantlets reported herein provides a starting point for the conservation of *F. assa foetida* and possibly an alternative future source for the bioactive compounds. We are acutely aware of the limitations of this protocol as the low survival percentage of plantlets necessitates further optimization of the acclimatization steps and warrants more comprehensive investigation. The transfer of rooted plantlets or micro cuttings to an optimal acclimatization medium would maximize their survival during the transitional period from culture vessel to an ex vitro environment and ensure minimal losses common to these stages of in vitro propagation.

In conclusion, we report for the first time a protocol for the successful regeneration of *F.assa foetida* plants through callus cultures derived from root, cotyledon and hypocotyl explants. The frequency of regeneration from hypocotyl derived calli described here for asafetida is high enough to encourage us to carry out protoplast culture, somatic hybridization and genetic transformation. Further work is needed to maintain the capacity of plant regeneration from callus cultures in order to employ this callus as experimental material.

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ABBREVIATIONS

MS: Murashige and Skoog; BA: N6-benzyladenine; NAA: α-naphthaleneacetic acid; IBA: indole-3-butyric acid; 2,4-D: 2,4- dichlorophenoxyacetic acid; KIN: kinetin;

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