Somatic Embryogenesis of *Ferula assa-foetida*

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Abstract

Regenerated plantlets were obtained from *Ferula assa-foetida* (Apiaceace) through indirect and direct somatic embryogenesis, for the first time. Callus was induced on hypocotyl explants from seedlings of two ecotypes (Shirkooh and Tabas) on Murashige and Skoog (MS) basal medium supplemented with 0.5-4 mg/L kinetin along with 0.1-1 mg/L α-naphthalene acetic acid (NAA) for 12 weeks. Embryogenic calli developed within 4 weeks after transferring the calli to hormone-free MS medium. Induction/maintenance MS medium supplemented with 1.5 mg/L kinetin and 1 mg/L NAA was most effective and provided a high embryogenesis frequency (31%) associated with a large mean number of mature somatic embryos per explant (8.4) in Tabas ecotype. According to our data, the presence of kinetin in the callus induction medium with NAA enhanced subsequent differentiation of somatic embryos on the hormone-free medium. About 40-50% of regenerated somatic embryos germinated into complete plantlets. Direct somatic embryogenesis without an intervening callus phase was induced from intact seedlings on hormone-free medium within 12 weeks. Embryo induction was observed all over the seedling surface with the highest numbers on hypocotyl segments. By this procedure, the maximum mean number of embryo per seedling was 42 in Shirkooh ecotype and more than 50% of cotyledonary embryos were developed not only into normal plantlets, but rooted simultaneously when cultured on hormone-free MS medium. Also, histological observations revealed different stages of embryogenicity such as globular, heart, torpedo, and cotyledonary stages in *F. assa foetida*.

Keywords: *Ferula assa-foetida,* Callus induction, Somatic embryogenesis, Hypocotyl explant, Seedling.

Introduction

Ferula assa-foetida L. (Apiaceae) is a medicinal plant indigenous to Iran and Afghanistan. This plant is one of the most important among thirty species of *Ferula* distributed in Iran. This species is growing wild or recently cultivated in several areas of the country. Asafoetida or Anghouzeh (in Persian) is an oleo gum resin, obtained by incision from the roots (Sadraei *et al*. 2003). It has been reported in Iranian folk medicine to be antispasmodic, aromatic, carminative, digestive, expectorant, laxative, sedative, nervine, analgesic, anthelmintic, aphrodisiac and antiseptic (Sadraei *et al.* 2003, Khajeh *et al.* 2004).

Apparently, due to lack of materials with sufficient quality for planting, commercial plantations of this important medicinal species have not been widely attempted. Presently, only the wild ecotype is exploited for extraction purposes. Due to overexploitation and lack of organized cultivation, the wild ecotypes are endangered. There are a number of constraints for the propagation and conservation of this species through conventional methods like vegetative and seed propagation. The major ones are variations in edaphic and climatic factors, low percentage of seed set and seasonal dormancy. The propagation of this species in its natural habitat occurs rarely evidenced by close field observations. For these reasons we tried to find an alternate method of rapid micropropagation for this species. Micropropagation through somatic embryogenesis can be widely applied to shorten the long sexual cycle and other problems like limited seed availability (Chandrasekhar *et al*. 2006).

The formation of somatic embryos in Apiaceae based on callus cultures was first reported in *Daucus carota* L. (carrot). Subsequently, somatic embryogenesis was reported in *Foeniculum vulgare* Mill (fennel) and *Apium graveolens* L. (celery) (Hunault *et al*. 1989, Tawfik & Noga 2002). Several investigators have worked extensively on plant regeneration through somatic embryogenesis in other plant species of this family (Ignacimuthu *et al*. 1999, Ul Haq 2005).

Since, there is no report providing evidence for somatic embryogenesis in *F. assa-foetida*, for the first time we established *in vitro* culture systems for induction of indirect and direct somatic embryogenesis in this plant leading to plantlet regeneration.

Materials and Methods

Plant materials and seed germination: Mature seeds of two *F. assa-foetida* ecotypes (Shirkooh and Tabas, Yazd province, Iran) were collected during September 2006 from their natural habitats. They stored in capped plastic bags at 4°C in the laboratory until initiation treatments. The seeds were chemically scarified using H_2SO_4 (75% v/v) for 10 min, surface sterilized by pre-washing with tap water for 1 h. Then, in a laminar flow cabinet, they were soaked in 70% alcohol for three min, disinfected with 20% commercial bleach + 2 drops of Tween 20 per 100 mL for 30 min, and finally rinsed 3 times with sterile distilled water. Thereafter, the surface sterilized seeds were cultured onto agar-solidified (0.4%) hormone-free basal MS medium (Murashige and Skoog, 1962) containing 3% sucrose for germination. The pH of the medium was adjusted to 5.7–5.8 prior to autoclaving at 120˚C and 1.2 $kg.cm^{-2}$ for 15 min. The cultures were incubated in darkness at 23 ± 2 ºC.

Induction of somatic embryos: For callus induction, hypocotyl segments (5-7 mm) were excised from sterile seedlings 4-5 days after radicle emergence and were cultured on MS medium supplemented with different concentrations of *a*-naphthalene acetic acid (NAA, 0.1- 1 mg/L) and combination with kinetin (0.5 - 4 mg/L). The media were supplemented with 3% sucrose (w/v) and solidified with 0.8% agar. The pH of each medium was adjusted to 5.7–5.8 before autoclaving for 20 min. Disposable Petri dishes containing 25 mL agar-gelled medium were used. Four hypocotyl segments were cultured in each Petri dish.

Some intact seedlings were placed on MS medium without growth regulator for induction of direct somatic embryogenesis. The cultures were incubated at 23 ± 2 ºC under a light intensity of approximately 2000 lux provided by growth chamber with 12/12 h photoperiod.

Each experiment was arranged in a randomized complete-block design (RCBD) with 4 replicates. The percentage of explants producing callus in the different media was recorded 12 weeks after explants culture.

Development of somatic embryos and plantlet formation- Explants forming embryogenic callus were scored 4 weeks after transferring the calli to hormone-free media and percentage of response was calculated. The number of indirect mature somatic embryos was determined 4 weeks after the incubation. The percentage of direct embryogenesis and mean number of embryos per seedling was also recorded after 12 weeks. Then the mature indirect and direct somatic embryos were transferred to hormone-free MS medium for germination and plantlet formation. The regenerated plantlets (10 cm in height) were washed in tap water to remove agar from roots and were planted in jiffy pots (6 cm diameter). The plantlets were watered on alternate days and were placed in plastic trays (45×30×15 cm) for maintaining high humidity.

Histological studies: For histological confirmation on the initiation and development of somatic embryos, the tissues with indirect and direct somatic embryos at different stages were fixed in FAA for 24 h. The fixed samples were washed for 30 min with tap water. After washing, the tissues were dehydrated by transferring embryos through an ethanol–xylol series and then embedded in paraffin. Tissues were sectioned to 7 mm with a microtome, mounted on glass slides, and stained with Haematoxylin-Eosin. Photographs were taken under Zeiss light microscope (Laboval, Germany).

Statistical analysis: The data pertaining to percentage of embryogenesis and mean number of embryos per culture were calculated and statistically analyzed by the Duncan's multiple range test (DMRT). To detect significant differences among treatment levels, data from experiments were subjected to ANOVA (analysis of variance) using the SPSS program (version 12). All of data were transformed to the scale $\sqrt{x}$ + 0.5 for data normalization.

Results

Indirect somatic embryogenesis: Different hormonal combination of kinetin and NAA were used in MS basal medium, for callus induction on hypocotyl

segments. Hypocotyl explants formed callus during 12 weeks of the incubation on the induction medium. The calli from hypocotyl explants were hard, greenish white to translucent. We found out that the frequency of callus induction varied from 25-100% in dependence on the ecotype, kinetin and NAA concentrations. The highest percentage (100%) of callus was observed in both ecotypes in MS medium supplemented with 1 mg/L kinetin with 0.5 mg/L NAA. Results of the callus evaluation (given as callus-formation points) after three months of growth in MS medium are presented in Table 1. Callus growth varied widely among ecotypes in the same medium. During the induction phase no somatic embryo formation was observed for the callus in maintenance media (Fig. 1a).

Table 1- Effects of different concentrations of kinetin and NAA on callus formation on *F. assa-foetida* hypocotyl explants from two ecotypes after 8 weeks of culture.

Growth regulators (mg/L)		Callus formation points*	
		Ecotype	
Kinetin	NAA	Shirkooh	Tabas
0	0	0	1
0	0.1	2.2	0.6
0	0.5	2.4	1.4
0	1	1	2
0.5	0	0.2	0.9
0.5	0.1	1.75	1.2
0.5	0.5	0.9	2.75
0.5	1	2.3	1.8
1	0	0.3	0.75
1	0.1	2	1.8
1	0.5	3.2	2.9
1	1	2.75	1.5
1.5	0	0.5	0.9
1.5	0.1	1.1	0.6
1.5	0.5	2.7	2.4
1.5	1	1.9	2.25
2	0	0.4	0.9
2	0.1	2	1.7
2	0.5	2.6	1.4
2	1	2.9	2.1
4	0	0.8	0.3
4	0.1	1.4	0.9
4	0.5	2.9	1.5

When the calli were transferred to the fresh hormone-free media, nodular and greenish yellow embryogenic calli with varying degrees of compactness were developed within 4 weeks (Fig. 1b). The microscopic examination of embryogenic callus indicated the existence of somatic embryos in different stages of development (Fig. 1c). The development of embryogenic calli was asynchronous. Globular, heart-shaped, torpedo and cotyledonary stages were observed throughout the incubation period on media (Fig. 1c). At this stage, development of clear bipolar embryos with organized shoot and root portion was observed. Embryos at the cotyledonary-stage showed the presence of two prominent cotyledons (Fig. 1f). Most of embryos were normal in morphology, but a few abnormal somatic embryos were also observed whereby some had either one, three or more cotyledons (Fig. 1b). Secondary embryos were sporadically formed all over the surface of the primary embryos.

Among the analysed ecotypes; Tabas ecotype gave good response in percent of somatic embryogenesis. However, only some cultures exhibited somatic embryogenesis after 4 weeks in the hormone-free medium. The frequency of embryogenesis in two ecotypes ranged from 6 to 31% on MS medium containing different concentrations of kinetin and NAA (Fig. 2). The best hormonal combination was 1.5 mg/L kinetin with 1 mg/L NAA, where embryo formation in two ecotypes of Shirkooh and Tabas were 25% and 31%, respectively (Fig. 2). The Maximum mean numbers of mature somatic embryos were 8.4 for Tabas ecotype in medium supplemented with 1.5 mg/L kinetin and 1 mg/L NAA and 6.56 for Shirkooh ecotype in media containing 2 mg/L kinetin and 0.5 mg/L NAA (Fig. 3). Non-embryogenic calli were induced on the hypocotyls explants in all of the media containing only kinetin concentrations. Adventitious root and shoot organogenesis were mostly observed on non-embryogenic calli. In both ecotypes, approximately 40-50% of the cotyledonary somatic embryos germinated and grew into normal young plantlets on the hormone-free media within 4 weeks (Fig. 1g). All the plantlets formed from somatic embryos were phenotypically identical to that of the original plant at the end of 4 week culturing period (Fig. 1h). The young plantlets were transferred to pots (Fig. 1i).

Different concentrations of kinetin and NAA had important effects ($P<0.01$) on the somatic embryos production. Significant differences were also observed between ecotypes ($P<0.01$) for the somatic embryo induction.

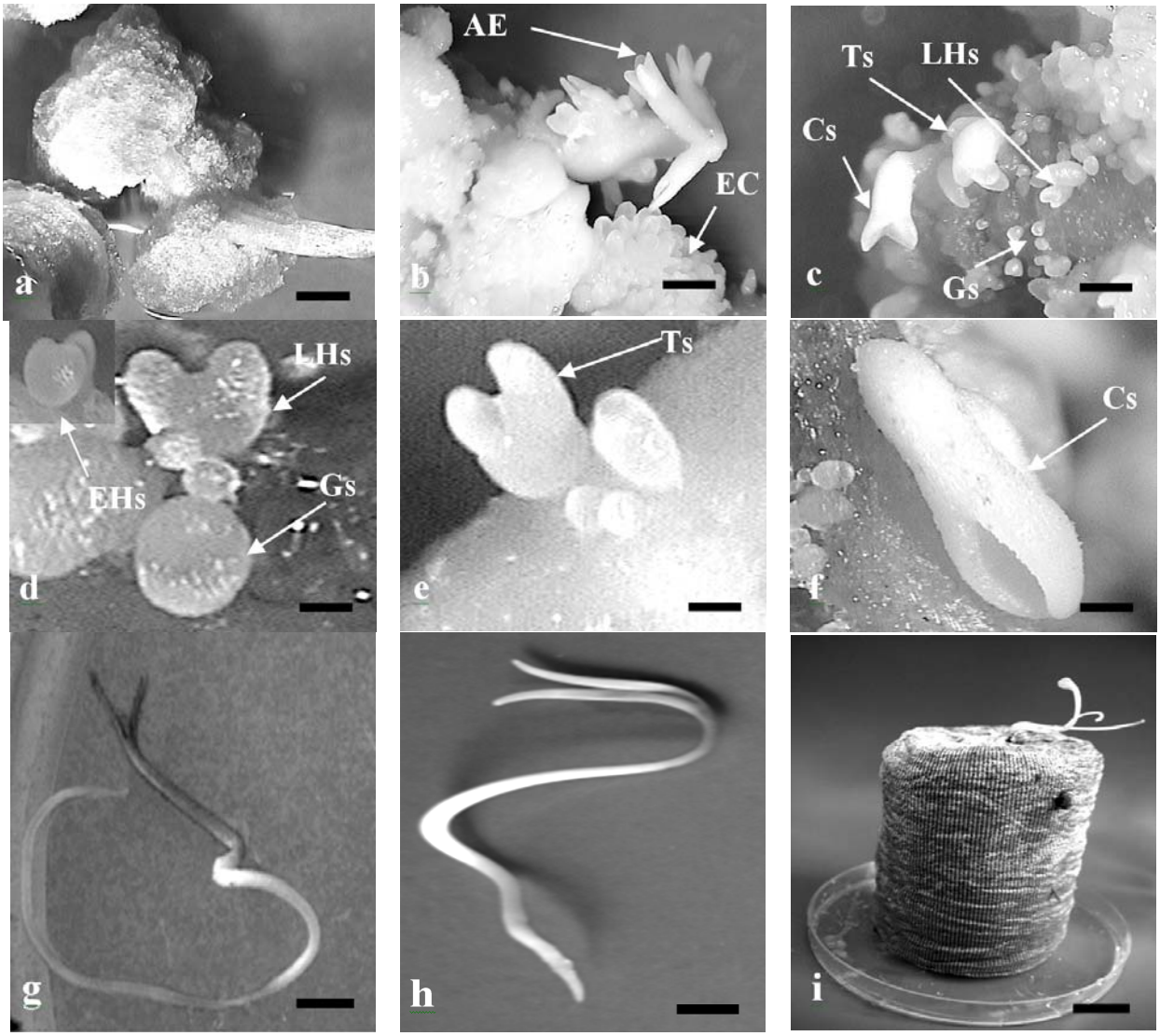

Figure 1- Plantlet regeneration by somatic embryogenesis of *F. assa-foetida*; a, Induction of callus on medium supplemented with kinetin (2 mg/L) and NAA (0.5 mg/L) after 12 weeks culture (bar = 3.6 mm); b, Embryogenic callus induced on hormone-free medium (bar = 3.4 mm); c, Embryogenic callus indicated the existence of somatic embryos in different stages of development (bar = 3.4 mm); d, Intact seedling exhibiting direct somatic embryos at various developmental stages (bar = 1.8 mm) (Inset) somatic embryo at early heart-shaped stage; e, Direct torpedo-shaped somatic embryo (bar = 2 mm); f, Embryo at cotyledonary stage (bar = 3 mm); g, Mature somatic embryo grew into plantlet (bar = 6 mm); h, Plantlet from zygotic embryo (bar = 6 mm); i, Plantlet transferred to jiffy pot (bar = 6.5 mm).
Abbreviations: AE, abnormal embryo (embryos with more than two cotyledons); EC, embryogenic callus; Gs, globular stage; EHs, early heart-shaped stage; LHs, late heart-shaped stage; Ts, torpedo-shaped stage.

The double and triple interactions were significant for kinetin × NAA ($P<0.01$), kinetin × ecotype ($P<0.05$) and NAA× kinetin × ecotype ($P<0.01$), except for NAA × ecotype. ANOVA for mean frequency of somatic embryo induction showed significant at 5% level (Fig. 2). Similarly, ANOVA for mean number of embryos per explant showed combination of kinetin and NAA was significant at 5% level (Fig. 3).

Direct somatic embryogenesis: Intact sterile seedlings were transferred to hormone- free solid

MS medium. After 8 weeks of culture, somatic embryos at different stages of development were formed directly on the surfaces of all parts of responded seedlings (Fig. 1d-f). Induction of somatic embryos was predominantly initiated on hypocotyl sections of seedlings. The frequency of direct somatic embryo formation on intact seedlings was the highest in the Shirkooh ecotype (50%) as compared to Tabas ecotype (20%). Maturation of these embryos was achieved after 12 weeks of culture on hormone-free MS medium. Maximum numbers of mature somatic embryos per seedling were obtained 42 and 19 for Shirkooh and Tabas ecotypes, respectively. Somatic embryos were developed and characterized with two well developed cotyledons and roots (Fig. 1f). More than 50% of the mature cotyledonary embryos from both ecotypes germinated and grew into plantlets with typical morphology on the media within 4 weeks (Fig. 1g).

Histological observation: To confirm the origin of embryo induction histological studies were conducted. The first sign of somatic embryogenesis on embryogenic callus (indirect) or on seedlings (direct) was marked by the appearance of globular structures that were attached to the surface by suspensor-like structures (Fig. 4a). Development of globular embryos appeared to progress through typical heart-, torpedo-shaped and well-differentiated cotyledonary embryos (Fig. 4b-e). These embryos later became detached from the mother tissue, and development of clear bipolar embryos with organized shoot and root portion was observed. The cotyledonary stage embryos showed the presence of two prominent cotyledons (Fig. 4e). In most of the developmental aspects of direct and indirect somatic embryogenesis, the embryos resembled the zygotic embryos of intact seeds (Fig. 4f).

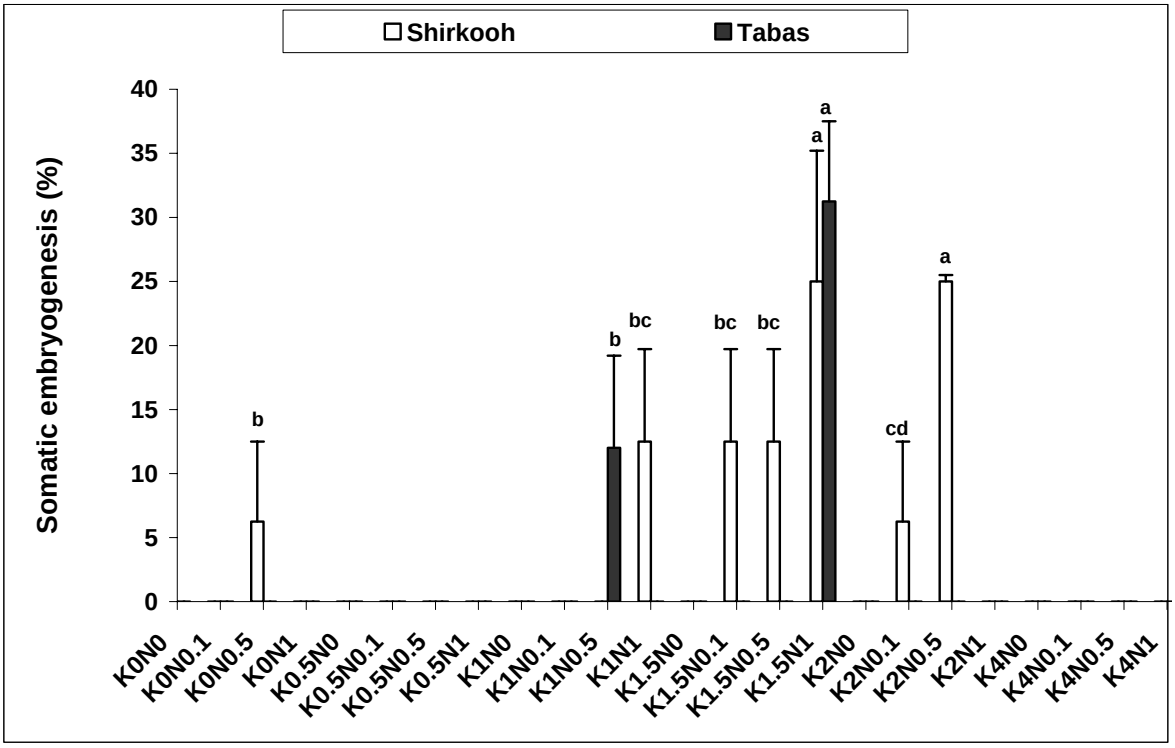

Figure 2- Effect of induction/maintenance medium supplemented with different concentrations (mg/L) of kinetin (K) and NAA (N) on the percent of somatic embryogenesis formation on calli from hypocotyl segments of *F. assa-foetida,* after 4 weeks of subculture in free-hormone medium. The columns are means of somatic embryogenesis percentages, SEs are indicated by bars. Also columns denoted by different letters are significantly different at 0.05 level of probability.

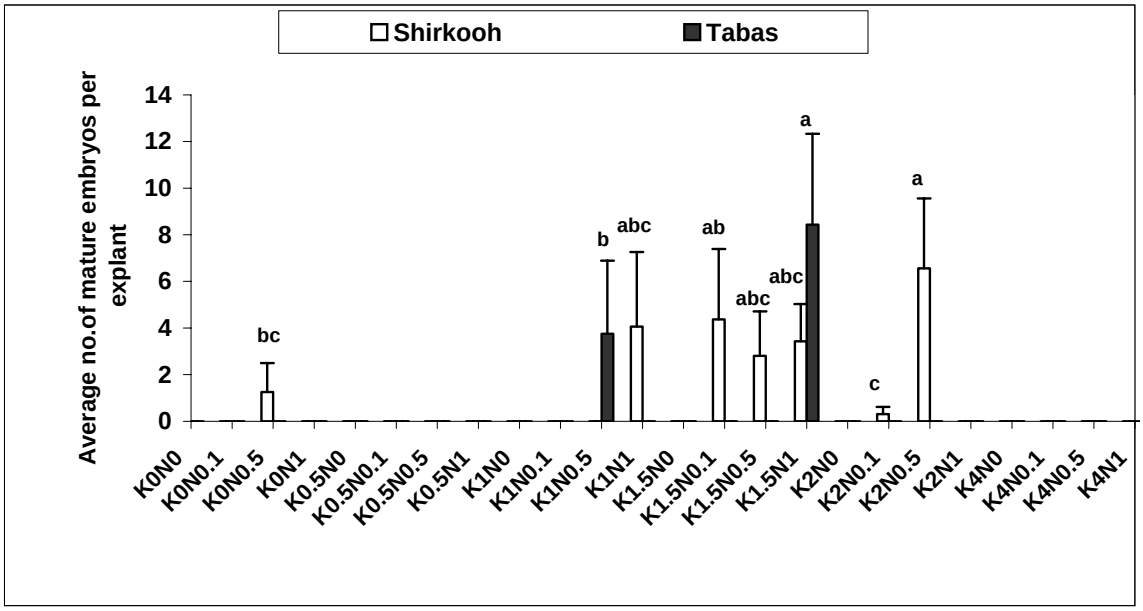

Figure 3- Effects of induction/maintenance medium supplemented with different concentrations (mg/L) of kinetin (K) and NAA (N) on mean numbers of mature embryos per hypocotyls explants of *F. assa-foetida* L. after 8 week of subculture in free-hormone medium. The columns are means of somatic embryos number per explants, SEs are indicated by bars. Also columns denoted by different letters are significantly different at 0.05 level of probability.

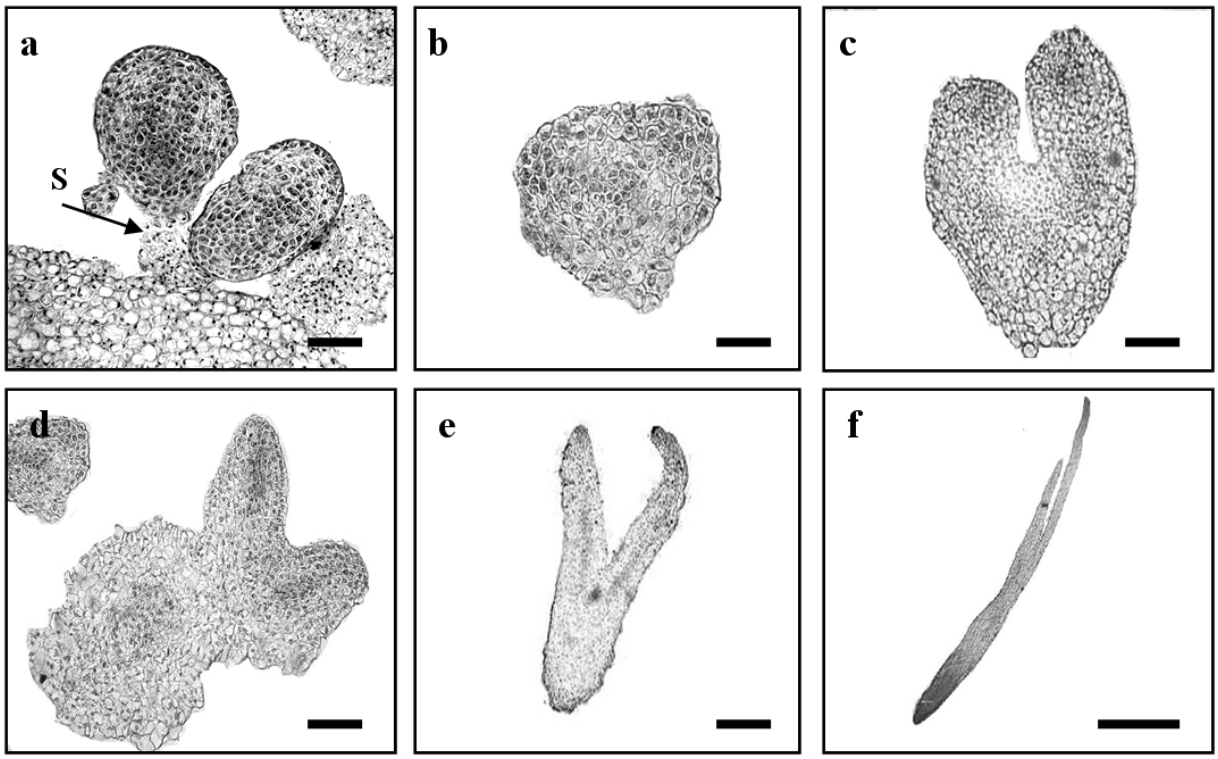

Figure 4. Histology of somatic embryogenesis of *F. assa-foetida*. *a*, Longitudinal sections of globular embryo development on the surface of the explant (bar = 60 µm), S: suspensor-like structure; *b*, Early heart-shaped embryo (bar = 30 µm); *c*, Late heart-shaped embryo (bar = 60 µm); *d*, Torpedo shaped embryo (bar = 60 µm); *e*, Cotyledonary embryo showing two distinct cotyledons with root meristem (bar = 200 µm); *f,* Zygotic embryo (bar = 300 µm).

Discussion

Up to now, in *F. assa-foetida* there is no report on tissue culture for inducing somatic embryogenesis. In this study, we describe tissue culture protocols for the plantlet regeneration through indirect and direct somatic embryogenesis.

The hypocotyl explants were incubated in a series media for callus induction. In the presence of kinetin with NAA in the callus induction media, higher percentage of the explants produce callus. All the treatments with only kinetin concentrations produced fewer callus throughout the duration of the culture period. This result shows that callus formation is affected not only by kinetin concentration in the culture media. As the case for callus induction and maintenance of other Apiaceae such as *Foeniculum vulgare* (fennel), *F. assa-foetida* callus was more proliferative with kinetin than without it in the NAA containing medium (Tawfik & Noga 2002).

In *F. assa-foetida*, similar to cumin (Tawfik & Noga 2002) 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 (Hunault *et al*. 1989).

We used only hormone-free MS media, for embryo initiation and proliferation on calli. Large numbers of the somatic embryos at different stages were found on embryogenic calli. Germination of the somatic embryos was achievable on hormone-free MS medium. The occurrence of the developmental process of the embryogenesis of *F. assa-foetida* in the absence of hormone is a common response with the other Apiaceae species (Tawfik & Noga 2002). In this context, it was interesting that we have observed a low but reproducible frequency of somatic embryogenesis even when calli are incubated in hormone-free medium. Enhanced maturation of somatic embryos was observed on embryogenic calli that have been proliferated on media with higher concentrations of kinetin in comparison to NAA. Exogenous hormones, especially auxins and cytokinins, are needed for the activation of somatic cells and for the entry to the division cycle (Pasternak *et al*. 2002, Pola & Sarada 2006). The initiation and maturation of somatic embryos on the hormone-free medium was affected by the hormone supplements in the preceding callus induction media. We concluded that kinetin is important for somatic embryogenesis in *F. assa-foetida*. Our finding confirmed results that were obtained by Tawfik and Noga (2002) on cumin. Histological analysis showed that characteristics of the somatic embryos were the same as those induced from immature zygotic embryos (Fig. 1h). The somatic embryos which had a clear bipolar root and shoot organization grew into normal young plantlets.

The results obtained in this research using *F. assa-foetida* corroborated a significant influence of the genotype, auxin and cytokinin concentrations on somatic embryogenesis for the Shirkooh and Tabas ecotypes. It has been indicated that the genotypes influenced differently on callus induction, somatic embryogenesis and plant regeneration. Some authors reported that the success of *in vitro* cultures largely depends on the nutrition, growth regulators, variety and the interaction between the variety and medium (Khaleda & Al-Forkan 2006, Klcova *et al*. 2004, Tripathi & Tripathi 2003).

Our obtained results showed that both the percent and the number of somatic embryos formed directly from seedlings in *F. assa-foetida* were higher than indirect somatic embryogenesis. Moreover, induction and development of direct somatic embryos to young plantlets was completed in 4 months whereas through using callus induction and indirect somatic embryogenesis protocol it was accomplished in 5 months. Thus, the response time was reduced by at least 4 week using the direct somatic embryogenesis from intact seedlings in the present work. Furthermore, the direct somatic embryogenesis protocol is simpler and lower cost than that of indirect somatic embryogenesis since it uses only hormone-free medium to induce both direct somatic embryogenesis and the regeneration of plantlets. On the other hand, due to genetic variability (somaclonal variations) within tissue cultures, the propagation by indirect embryogenesis carries the risk of producing plants that may differ genetically from each other and from the parental plant (Sahrawat & Chand 2001, Bach & Pawiowska 2003, Desai *et al*. 2004, Thengane *et al*. 2006, Gatica *et al*. 2007, Santacruz-Ruvalcaba *et al*. 1998).

For the first time, in this report we describe a simple and efficient protocol that uses intact seedlings of *F. assa-foetida* from which direct somatic embryos can be induced at a high frequency. However, further studies need to be conducted to find the cultural conditions to increase the frequencies of somatic embryogenesis induction and rate of plant recovery from the somatic embryos and *ex vitro* acclimatization of regenerated plant.

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