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Short communication

Induction of Callus and Somatic Embryogenesis from Cotyledon Explants of *Fagonia indica* Burm.

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Abstract

To explore the potential for *in vitro* rapid regeneration of *Fagonia indica* Burm., cotyledon explants were obtained from 10-day old aseptically germinated seedlings that cultured on MS medium which supplemented with 1.0-5.0 µM 2,4-D. Callus was initiated from cotyledon explant on MS medium supplemented in all concentrations of 2,4-D investigated. 2,4-D was more effective than IAA and NAA for callus induction, but auxins and cytokines' combination is essential for the green callus induction for somatic embryogenesis. Induced calluses were subcultured on media containing 2.5-5.0 µM BA or Kin combined with different concentrations IAA, NAA and 2,4-D. Elimination of 2,4-D and a slight increase of BA concentration induced of the relating somatic embryogenesis. The maximum globular structure embryos were further enlarged and produced the somatic embryos in MS basal medium supplemented with 5.0 BA µM and also combined with either 2.5 µM NAA. Continued formation of the globular embryo occurred in these mediums for 12 months. The maximum germinations of embryos were observed in MS medium + 1.5% sucrose without growth regulators.

Key words: *Fagonia indica* Burm. f., Somatic embryogenesis, Callus

Abbreviations: Murashing and Skoog (MS), 2,4-Dichlorophenoxyacetic acid (2,4-D), (IAA), α-naphthaleneacetic acid (NAA), 6-Benzylaminopurine (BA), Kinetin (Kin).

Introduction

Fagonia indica Burm. Belonging to Zygophyllaceae is a small spiny shrub widely distributed in the warm and arid areas of all countries specially India, Iran, Egypt and Pakistan [1]. Plants belonging to the genus *Fagonia* are often used in folk medicine, mainly as a popular remedy for the treatment of various skin lesions. An aqueous decoction of the plant is a popular remedy for cancer in the indigenous system of medicine [2]. It was reported that some species of this medicinal plant have anti-microbial, antihypertensive, antitumor, antioxidant and analgesic activities [3]. *F. indica* contains valuable secondary metabolites including terpenoids, saponins, sterols, proteins, trace elements and ß-carboline alkaloids [4]. The antimicrobial activity of its flavonoid compounds has been explored previously, while the nutritive values of it and of other species growing wild in the Rajasthan region of India have also been evaluated [1]. Propagation of *F. indica* through seed is unreliable due to poor germination. On the other hand, harvesting the plant on mass scale from natural habitat leads to depletion of natural population. Therefore, there is need to develop a plantation method either by developing the method for improvement of seed germination or by micropropagation.

Somatic embryogenesis is a valuable technique in medicinal plant improvement programs, such as for propagation and genetic transformation. An

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efficient and reproducible regeneration system is essential for the successful genetic engineering of plants [5]. Development of standard technique for callus culture and somatic embryogenesis in *Fagonia indica*. will help for *in vitro* production of secondary metabolites, utilization and in evolving improved *Fagonia* species by *in vitro* breeding method. Furthermore it was reported that callus culture technique was used to produce biologically active compounds [6]. The aim of the present study is to introduce a simple one medium formulation protocol for callus culture and somatic embryogenesis in this valuable medicinal plant.

Materials and Methods

Plant material and germination

Seeds were obtained from ripened, mature fruits of *F. indica* which were collected from natural population in around Yazd, Iran. The seeds were washed five times with sterilized distilled water then disinfected by immersion in 0.1% $HgCl_2$ for 5 minute. Then seeds were rinsed five times with sterile distilled water. The sterilized explants were aseptically transferred to MS basal media.

Establishment of callus and culture condition

Cotyledon explants were obtained from 10-day old seedlings grown aseptically. The cotyledon explants were then cut into small fragments of about 5×5 mm. These discs were cultured on the MS medium with different concentrations (0.0-15 µM) alone and combination of BA, NAA, IAA and 2,4-D for callus initiation and somatic embryogenesis and complete plant regeneration. Furthermore, for each hormonal treatment, the cotyledon explants were placed in the medium with either the abaxial surface facing up or down. Cultures were maintained in the culture room at 25±2^{o}C with light intensity of 2000-3000 lux provided by cool white fluorescent light for 16 h photoperiod regulated by a timer. Callus induction was visually evaluated and scored to aid a more rapid screening out of the options.

Embryo maturation

After 5 weeks in culture, embryogenic callus at different developmental stages was transferred to MS medium + 1.5% sucrose without growth regulator for embryo maturation. These cultures were incubated on test tubes for a period of four weeks in a room of average temperature of 25±2^{o}C. At two-week intervals; small embryogenic callus were removed aseptically from each culture, carefully separated and examined for a range of embryonic stages.

Statistical analysis and Histological observation

A completely randomized design was used throughout all experiments. The experiments were repeated three times with 10 replicates each. The mean standard error and ANOVA were calculated, and mean separations were carried out using DMRT [7] at 5% level of significance. Calluses for histological observations were fixed in IAA (95% ethyl alcohol:glacial acetic acid:formaldehyde:water, 10:1:2:7), dehydrated with a tert-butyl alcohol series, embedded in paraffin wax, sectioned at 10 µm thickness and stained with 0.5% safranin [8].

Results and Discussion

Induction of callus

The cotyledon explants of *F. indica* were remained green and fresh for two weeks but failed to be morphogenic respond and callus formation on hormone-free MS medium. All cotyledons explant cultured on MS medium fortified with phytohormones grew in size and callus formation began from cut portion in the same explant within 10-15 days of incubation. Cotyledon explants of *F. indica* Brum. were cultured on MS medium with various levels auxins and cytokinin alone and in combination for induction of callus. After 3 weeks of culture incubation indicated that medium with high concentration of 2,4-D alone up to 5.0 µM showed a remarkable callus formation. The calluses obtained from this medium was faster growing, delicate, mostly spongy, and white creamy (Fig. 1.a). While culturing in media amended with 2,4-D and BA combination, data in (Table 1) revealed that, the green calli with high frequency of callus formation were at lower concentration of 2,4-D at 2.5 µM. The frequency of callus varied depending on the hormonal combination used. 2,4-D was more effective than IAA and NAA for callus induction. The outcome of present study, and also earlier results suggest that mostly the auxin NAA or 2,4-D in combination with cytokinin particularly BA; was most effective for initiation and growth of callus in *F. indica*. In the treatment with 2,4-D, response in the form of callus production was observed only at the cut edges of the explants and

on the abaxial surface, even when placed face down. Eman *et al,.* [6] reported that, hypocotyle of *F. indica* Brum. was the most suitable explant to induce calluses (60.833%) on MS medium supplemented with 5mg/l Kinetin + 1 mg/1 NAA, this medium gave both the highest percentage and the highest amount of calli under the same conditions. This result shows that callus formation in a few cases is affected among other factors by orientation of the explants on the culture medium [9]. This report is in agreement with the findings of Oluwaseun and Erhinmeyoma [10] in which they observed that callus formation occurred only on the abaxial surface of *Parkia biglobosa* cotyledon, which had been placed face up. The highest frequency of callus induction was 89% at the end of fourth weeks. It was recorded on medium supplemented with 2.5 µM of 2,4-D with 5.0 µM of BA. Similar combination of auxin with cytokinin for callus induction has been reported by Dode *et al.,* [11]. Our studies also is in accordance with Yasmin *et al.,* [12] and further revealed that 2,4-D and BA combinations is essential for the green callus induction for somatic embryogenesis. This is in contradictory to the observation by Sasse [13] in *Peganum harmala.* Kim *et al.*, [14] were succeeded to develop callus from the immature zygotic embryo on MS medium fortified with 2,4-D in *Catharanthus roseus*. The considerable callus formation was also in the saponin producing species of *Dioscorea sp*. with 2,4-D [4, 15, 16]. Nikam *et al,.* [17] noted that extensive callus induction and proliferation was obtained in leaf explant on MS medium supplemented with 5.0 µM BA and 2.5 µM NAA. Calluses were maintained over a period of two years. The embryogenic and non embryogenic response was consistent during the maintenance of callus. The influence of auxin and cytokinin on callus proliferation was evaluated at different time intervals. Callus obtained from an above-mentioned medium was used to investigate the influence of growth regulators on the induction of somatic embryogenesis (Table 1).

Table 1 Effects of different concentrations of auxin and in combination with cytokinins for callus induction from cotyledon explant of *F. indica*

MS + Growth regulators (µM)					Percentage of explant induced callus	Mean Dry weight of callus induction±SE (g)
BA	Kin	2,4-D	IAA	NAA		
-	-	-	-	-	0.0	-
-	-	1.0	-	-	55 ± 1.1^{gh}	0.56 ± 0.3^{h}
-	-	2.5	-	-	65 ± 1.3^{e}	0.61 ± 0.4^{fg}
-	-	5.0	-	-	92 ± 1.7^{a}	0.80 ± 0.2^{b}
-	-	7.0	-	-	72 ± 1.3^{cd}	0.78 ± 0.4^{c}
2.5	-	2.5	-	-	67 ± 1.7^{e}	0.58 ± 0.6^{fg}
2.5	-	5.0	-	-	72 ± 1.4^{cd}	0.73 ± 0.5^{dc}
2.5	-	7.0	-	-	70 ± 1.8^{d}	0.69 ± 0.3^{e}
5.0	-	2.5	-	-	89 ± 1.3^{ab}	0.87 ± 0.3^{a}
5.0	-	5.0	-	-	80 ± 1.9^{b}	0.81 ± 0.9^{b}
5.0	-	7.0	-	-	65 ± 1.6^{e}	0.77 ± 0.6^{c}
2.5	-	-	2.5	-	59 ± 1.8^{g}	0.67 ± 0.5^{e}
5.0	-	-	5.0	-	65 ± 1.3^{e}	0.70 ± 0.3^{d}
2.5	-	-	-	2.5	67 ± 1.5^{e}	0.71 ± 0.4^{d}
5.0	-	-	-	5.0	75 ± 1.5^{c}	0.78 ± 0.4^{c}
-	2.5	2.5	-	-	73 ± 1.0^{c}	0.53 ± 0.1^{h}
-	5.0	5.0	-	-	70 ± 1.3^{d}	0.68 ± 0.5^{e}
-	2.5	-	2.5	-	51 ± 1.9^{h}	0.58 ± 0.4^{fg}
-	5.0	-	5.0	-	63 ± 0.9^{f}	0.69 ± 0.3^{e}
-	2.5	-	-	2.5	62 ± 1.1^{f}	0.65 ± 0.5^{f}
-	5.0	-	-	5.0	71 ± 1.7^{d}	0.71 ± 0.7^{d}

The values represent the mean ± SEM calculated on three independent experiments, each based on minimum of 15 replicates. Values followed by the same letter were not significantly different at 5% level (DMRT).

Embryogenesis from the subcultured callus

Induction of somatic embryogenesis from cotyledon callus was tried on MS medium with various concentrations of cytokinins (BA and Kin) and auxins (NAA, IAA and 2,4-D) alone and in combination (Table 2). According to Godbole *et al.,* [18], the 2,4-D and BAP combination was essential for initial culture establishment for callusing and subsequently, elimination of 2,4-D and a slight increase of BA concentration induced somatic embryogenesis. The maximum frequency of embryo formation of *Tribulus terrestris* was reported by Nikam *et al,.* [17] on MS medium containing 5.0 µM BA and 2.5 µM NAA together with 75 mg l^{-1} casein hydrolysate. Small protuberances were also observed from the top of these nodular calluses. Obembe *et al.,* [19] and Ehsanpour [20] all reported small protuberances on the top of growing calluses and described these as certain globular or spherical morphogenic manifestations, which can actually develop into embryoids or ordinary shoots. Embryogenic potentialities of the callus showed a difference to some extend which depending on the growth regulators' supplements (Table 2).

In media, all the concentration of BA alone and BA in combination with NAA showed that somatic embryogenesis started from the cotyledon callus after 3 weeks of sub-culturing. Embryo formation the fourth, fifth weeks of culture might create the environmental stress, such as availability of less water and also nutrients [17]. There have been several reports of increased somatic embryogenesis following starvation stress [21]. However, MS medium containing selected concentration of 5.0 µM BA induced further somatic embryoids differentiation (Fig. 1.b). The highest number of somatic embryos per culture was 5.1 embryo per 1 g of callus (Fig. 1.c).

Table 2 Effects of different concentrations and combination of cytokinin and auxin for somatic embryogenesis in *F. indica*

Media Composition	Time course Growth (weeks)	Callus response Parameter	Mean No. of embryos per 1 g of callus
MS + 5.0 µM BA + 2.5 µM 2,4-D	3	NE	-
	4	NE	-
	5	NE	-
MS + 5.0 µM BA + 2.5 µM NAA	3	GP	-
	4	EC	3.4 ± 0.5
	5	EC	3.3 ± 0.2
MS + 5.0 µM BA + 2.5 µM IAA	3	NE	-
	4	GP	-
	5	EC	2.2 ± 0.1
MS + 5.0 µM Kin + 2.5 µM 2,4-D	3	NE	-
	4	NE	-
	5	NE	-
MS + 5.0 µM Kin + 2.5 µM NAA	3	NE	-
	4	NE	-
	5	GP	-
MS + 5.0 µM Kin + 2.5 µM IAA	3	NE	-
	4	GP	-
	5	EC	0.9 ± 0.06
MS + 5.0 µM BA	3	GP	-
	4	EC	1.4 ± 0.08
	5	EC	1.7 ± 0.1
MS + 1.5% sucrose	3	GP	1.6 ± 0.09
	4	NE	2.9 ± 0.2
	5	NE	5.1 ± 0.4

All data calculated on three independent experiments, each based on minimum 10 replicates.
NE: Non-embryogenic Callus, GP: Globular Protuberance, EC: Embryogenic Callus,

The transfer of friable nodular callus into MS medium + 1.5% sucrose without growth regulators facilitated the development of somatic embryos through different stages, from globular to early cotyledonary stages (Fig. 1.b, d).

Zimmerman [22] observed that removal of growth regulators especially auxins from the culture medium is a prerequisite to 'switch off' several genes or to synthesize new gene-products that are necessary for the successful completion of embryo development. Briefly, this study has established the callogenic capacity of cotyledon explants of *F. indica* It has also established the possibility of inducing somatic embryogenesis from the induced calluses.

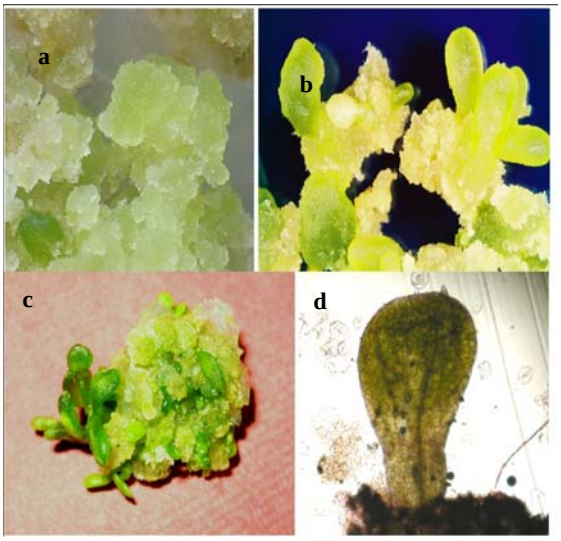

Fig. 1 Somatic embryogenesis in *Fagonia indica*, a. Embryogenic leaf callus. b & d. Globular embryo (longitudinal section). c. Embryo at different developmental stages (globular heart and cotyledonary) after 5 weeks of culture.

Further research in order to advance the embryoids to plantlets and their establishment in the field will be the next step. Meanwhile, this protocol offers itself not only as a highly efficient method for mass clonal propagation of this species but also for its conservation. It also opens up research on genetic transformation to improve this medicinal plant species.

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