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In vitro regeneration in medicinal plant fenugreek (*Trigonella foenum-graecum* L.)

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Abstract
Fenugreek (*Trigonella foenum-graecum* L.) is a medicinal plant used as a traditional medicine. Two of the most important medicinal components are diosgenin and trigonelline that have many medicinal properties including anticancer, decreasing blood sugar and cholesterol. *In vitro* optimization of callus induction and shoot regeneration is the first step in the optimum production of secondary metabolites through plant tissue culture techniques and genetic engineering. To achieve this goal, leaf, hypocotyl and embryo axis explants were cultured on Murashige-Skoog (MS) medium containing various concentrations of naphthalene acetic acid (NAA) (0, 0. 5, 1.5 and 2 mg L^{-1}) in combination with 6-benzyl adenine (BA) (0, 0.5, 1 and 1.5 mg L^{-1}) producing 48 treatments. The data were analyzed by the non-parametric Kruskal-Wallis test and the means were compared by the U test of Mann Whitney at 5% probability level. The result of the analysis indicated a significant difference between treatments. Simultaneous callus induction and shoot regeneration occurred in the MS medium supplemented with 0.5 mg L^{-1} NAA without BA for the embryo axis explant. However, higher frequency of callus induction for leaf and hypocotyl explants was obtained in the higher concentration of NAA (2 mg L^{-1}).

Keywords: Callus induction; Embryo axis; Fenugreek; Naphthalene acetic acid; Plant regeneration

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Introduction

Fenugreek (*Trigonella foenum-graecum* L.), a crop from the Fabaceae family, is an annual forage legume and also an aromatic and spice crop. It is grown in South Asia, West Asia, South-east Asia, North Africa, Middle East, Mediterranean Europe, China, Australia, North America and Argentina. India is the largest producer of fenugreek across the world but does not have much export since the internal consumption is high (Basu and Agoramoorthy 2014; Basu *et al.* 2014). About 100 species of this genus as wild and cultivated plants in the world have been identified (Martin *et al.* 2008), of which 33 species are distributed in many parts of Iran including Isfahan, Damghan, Central, Azarbaijan, Fars and Khorasan (Mozafarian 1995). Fenugreek is an appropriate source of steroidal sapogenins, mainly diosgenin used as a traditional medicine with anti-inflammatory activity (Patel *et al.* 2012). Furthermore, it is used for the medication of leukemia, colon cancer, hypercholesterolemia and climacteric syndrome (Lepage *et al.* 2009). The medicinal properties of this species are due to the presence of some phytochemicals including diosgenin, galactomannan and 4-hydroxy isoleucine (Zandi *et al.* 2015). The seed and leaf extracts of *T. foenum-graecum* demonstrate pronounced antidiabetic (Phadnis *et al.* 2011), anticarcinogenic (Shabbeer *et al.* 2009), antihypertensive (Balaraman *et al.* 2006), hepatoprotective (Kaviarasan *et al.* 2007), immunomodulating (Bin-Hafeez *et al.* 2003) and other therapeutic effects. The anticancer activity of diosgenin has been mentioned as it affects many molecular candidates critical to tumorigenesis (Raju and Rau 2012). Diosgenin is one of the

important sapogenins from that is used to synthesize the steroidal drugs and hormones such as glucocorticoids, progesterone and testosterone in the pharmaceutical industry worldwide (Fazli and Hardman 1968; Acharya *et al.* 2010). Diosgenin has an oestrogenic effect on the mammary gland that is of much interest to the pharmaceutical industry (Nigam 20180. It also control the cholesterol metabolism which is responsible for the changes in the lipoxygenase activity of human erythroleukemia cells as well as morphological and biochemical properties in megakaryocyte cells (Beneytout *et al.* 1995; Nappez *et al.* 1995).

The demand for *T. foenum-graecum* with higher production of diosgenin and trigonelline made the tissue culture efforts necessary (Mehrafarin *et al.* 2010). For successful application of the tissue culture techniques in plant breeding, the potential of each plant for callus induction and plant regeneration must be determined (Wang 2011). Fenugreek cell and tissue culture is used for the production of secondary metabolites of economic interest from plant shoots (Mehrafarin *et al.* 2011) and roots (Antony 1975). Shekhawat and Galston (1983) investigated various treatments and reported that the medium with 0.1 mg L^{-1} of 6-benzylaminopurine (BA), zeatin, glutamine and asparagines was proper for callus induction and differentiation in fenugreek. Based on Azam and Biswas (1989) fenugreek callus induction and growth were better on MS medium containing naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin and coconut water. El-Bahr (1989) showed that fenugreek callus had the best growth on MS medium supplemented with 3% sucrose and 2 mg L^{-1} 2,4-D. The presence of 2,4-D is essential for callus induction and no callus formation is observed in the absence of 2,4-D (Ahmed *et al.* 2000). Shoot regeneration directly through organogenesis was first reported by Khawar *et al.* (2002) in fenugreek. Embryogenesis was observed directly from explants or indirectly from callus (Razdan 2003). Aasim *et al.* (2009) regenerated shoots of fenugreek plants successfully on MS medium with thidiazuron using apical meristem and cotyledon leaf explants. The potential of apical meristem in shoot regeneration was higher than cotyledon leaf explants. Rezaeian (2011) revealed that 1.0 mg L^{-1} 2,4-D was the best concentration for callus induction and proliferation in all explant types of *T. foenum-graecum*. Additionally, the callus formation enhanced by increasing the concentration of 2,4-D up to 1.0 mg L^{-1}. The shoot regeneration using shoot tips explants on MS medium with basal callus formation was shown by Prabakaran and Ravimycin (2012). El-Nour *et al.* (2013) presented a protocol for callus induction in fenugreek (*T. foenum-graecum* L.) on MS and B5 media containing different types and concentrations of hormones to obtain the best callus induction. The maximum callus was observed on MS medium with 1.5 mg L^{-1}, 2,4-D in hypocotyls and cotyledons explants. Increasing the concentration of 2,4-D up to 1.5 mg L^{-1} significantly decreased the fresh and dry weight of callus (El-Nour *et al.* 2013). *In vitro* propagation methods are crucial to managing the plant genetic resources and are important for the conservation of rare and endangered plant species (Sidhu 2010). In the present research, the effect of different concentrations of NAA and BA on callus induction and shoot regeneration for different types of fenugreek explants was investigated.

Materials and Methods

Plant material

The mature seeds of fenugreek, collected from an agricultural research center in Khorasan Razavi province, Iran, were surface sterilized in 1.5% (w/v) sodium hypochlorite solution for 15 min and rinsed three times with sterile distilled water. To prepare sterile plant material, seeds were then cultured on basal MS (Murashige and Skoog 1962) medium and incubated in a growth chamber for one month. Embryo axis, hypocotyl and leaf explants were used in this research. To prepare the embryo axis, the end of some seeds was cut using a scalpel and the embryo was extruded with pressure on the middle of the seed and the axis section was used as explant. Leaf and hypocotyl segments were also isolated from sterile plants and used as explants. Three explants of each type were cultured.

Culture condition

The effect of different concentrations of NAA (0, 0.5, 1.5 and 2 mg L^{-1}) and BA (0, 0.5, 1 and 1.5 mg L^{-1}) in combination with three types of explant, producing 48 treatments, on callus induction and shoot regeneration were investigated. The pH of the media was adjusted to 5.8 before autoclaving at 121 °C for 15 min. The medium was solidified with 8% (w/v) agar (Sigma, USA). The explants were then cultured in sterile Petri dishes (7×12 mm), each containing 25 ml of culture medium, sealed with parafilm and maintained at 25 ± 2 °C under 16 h photoperiod (30 μ moles m^{-2} s^{-1}).

Statistical analysis

After 4-6 weeks, the number of explants producing callus and regeneration from each replication was counted. The data were analyzed by the non-parametric Kruskal-Wallis test and the means were compared by the of Mann Whitney's U test at 5% probability level.

Table1. Different treatments produced by the combination of NAA, BA concentrations (mg L^{-1}) and different types of explants.

Code	Treatment	Code	Treatment
T1	0 NAA+0 BA+L	T25	1.5 NAA+0 BA+L
T2	0 NAA+0 BA+H	T26	1.5 NAA+0 BA+H
T3	0 NAA+0 BA+E	T27	1.5 NAA+0 BA+E
T4	0 NAA+0.5 BA+L	T28	1.5 NAA+0.5 BA+L
T5	0 NAA+0.5 BA+H	T29	1.5 NAA+0.5 BA+H
T6	0 NAA+0.5 BA+E	T30	1.5 NAA+0.5 BA+E
T7	0 NAA+1 BA+L	T31	1.5 NAA+1 BA+L
T8	0 NAA+1 BA+H	T32	1.5 NAA+1 BA+H
T9	0 NAA+1 BA+E	T33	1.5 NAA+1 BA+E
T10	0 NAA+1.5 BA+L	T34	1.5 NAA+1.5 BA+L
T11	0 NAA+1.5 BA+H	T35	1.5 NAA+1.5 BA+H
T12	0 NAA+1.5 BA+E	T36	1.5 NAA+1.5 BA+E
T13	0.5 NAA+0 BA+L	T37	2 NAA+0 BA+L
T14	0.5 NAA+0 BA+H	T38	2 NAA+0 BA+H
T15	0.5 NAA+0 BA+E	T39	2 NAA+0 BA+E
T16	0.5 NAA+0.5 BA+L	T40	2 NAA+0.5 BA+L
T17	0.5 NAA+0.5 BA+H	T41	2 NAA+0.5 BA+H
T18	0.5 NAA+0.5 BA+E	T42	2 NAA+0.5 BA+E
T19	0.5 NAA+1 BA+L	T43	2 NAA+1 BA+L
T20	0.5 NAA+1 BA+H	T44	2 NAA+1 BA+H
T21	0.5 NAA+1 BA+E	T45	2 NAA+1 BA+E
T22	0.5 NAA+1.5 BA+L	T46	2 NAA+1.5 BA+L
T23	0.5 NAA+1.5 BA+H	T47	2 NAA+1.5 BA+H
T24	0.5 NAA+1.5 BA+E	T48	2 NAA+1.5 BA+E

L= leaf; H= hypocotyl; E= embryo axis

Results and Discussion

The initiation of callus induction and shoot regeneration occurred 2 and 4 weeks after culture of explants on MS medium containing various concentrations of NAA and BA, respectively (Figures 1a and 1c). The results of analysis of data by Kruskal-Wallis test indicated the significant effect of treatments on callus induction (data not shown). Callus induction and shoot regeneration were not observed on MS basal medium when both NAA and BA were not present. This result is consistent with several studies reported by previous researchers (Nickavar and Esbati 2012; Amini *et al.* 2013). Callus induction and shoot regeneration did not occure also in the treatments with BA alone indicating NAA is essential for callus induction and shoot regeneration in fenugreek. Aasim *et al*. (2010) reported that callus was produced only on MS medium containing different concentrations of BA with 0.20 mg L^{-1} NAA, whereas, the explants totally failed to give any response to other cytokinins-auxins combinations. Although, cotyledon node explants failed to generate shoots on MS medium containing BA without NAA, supplementing of NAA in the culture medium promoted shoot regeneration.

We observed somatic embryogenesis in the treatment of 0.5 mg L^{-1} NAA and 1.5 mg L^{-1} BA for the leaf explant (Figure 1b). Afshari *et al.* (2011) reported somatic embryogenesis for this plant in the medium with 0.5 mg L^{-1} NAA and 2.5 mg L^{-1} BAP. Our results demonstrated shoot regeneration in the medium supplemented with 0.5 mg L^{-1} NAA without BA for the embryo axis explant (Figure 1c).

The response of cultures depends on the plant hormone combination and the type of explant. The percentage of callus induction was enhanced by increasing the concentration of NAA to 2 mg L^{-1}. El-Nour *et al.* (2015) suggested that callus formation improved by increasing the concentration of NAA and the maximum callus formation was observed in the MS medium containing 2.0 mg L^{-1} NAA using the hypocotyl explant. Additionally, combinations of NAA + Kinetin and 2,4-D + Kinetin were found to be more

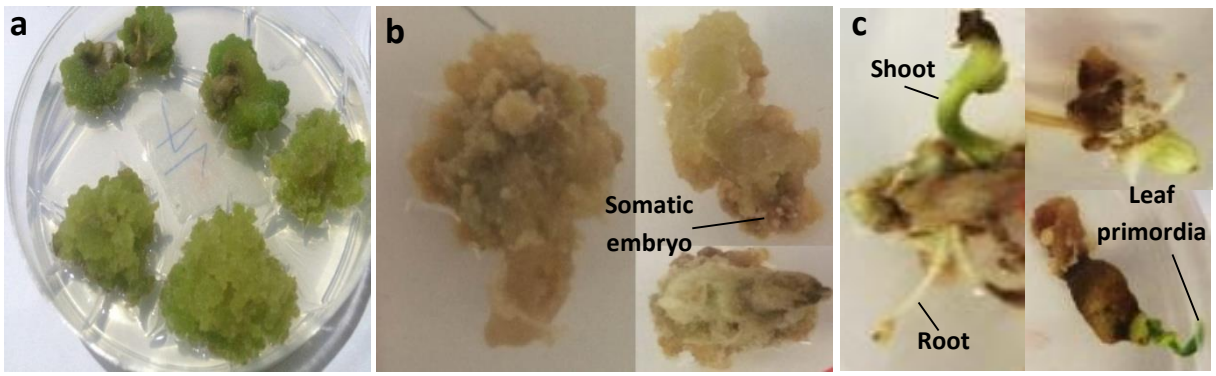

Figure 1. Callus induction on hypocotyl explants (a), somatic embryogenesis in leaf (b) and regeneration in embryo axis and (c) explants in the medicinal plant of fenugreek (*Trigonella foenum-graecum* L.).

effective to induce callus from hypocotyl explants relative to 2,4-D and NAA alone. Saini and Jaiwal (2000) reported that a low concentration of NAA in the medium enhanced the frequency of regeneration and the length of regenerated shoots on the cotyledonary node explants but not for hypocotyl. This can be attributed to the different content of endogenous hormones in various explants. Zatimeh *et al.* (2017) observed the highest number and length of regenerated shoots using a low concentration of NAA (0.1 mg L^{-1}). In our study, increasing the concentration of NAA to more than 0.5 mg L^{-1} had a suppression effect on the regeneration. The shoot regeneration was not observed in the treatments supplemented with higher concentrations of NAA (data not shown) that is concordance with the results of Afshari *et al.* (2011). They reported that the maximum shoot regeneration obtained in the treatment of 0.5 mg L^{-1} NAA and 1.5 mg L^{-1} BA and no regeneration occurred in the treatments without NAA. Also, the callus induction and regeneration were obtained in the treatment with no BA indicating this hormone is not required for the occurrence of measured traits in this plant.

The volume of the calli was higher in the medium supplemented with both auxin and cytokinin. There are many studies confirming the positive role of auxins in combination with cytokinin on callus induction in *Santolina canescens* Lagasca (Casado *et al.* 2002), *Bupleurum fruticosum* (Fraternale *et al.* 2002), *Peganum harmala* (Saini and Jaiwal 2000) and *Acacia tortilis* (Sané *et al.* 2001).

Our data demonstrated that the size of calli was higher in hypocotyl compared to other explants. Zhang *et al.* (2003) reported that hypocotyl explants were most responsive to callus induction and proliferation in fenugreek.

For the first time, we used the embryo axis that revealed better results compared to other explants. Hypocotyl and leaf explants are too old to respond to growth regulators rapidly and efficiently while the embryo axis is younger and more sensitive which responds more quickly to plant hormones (Valizadeh *et al.* 2006). The highest percentage of callus induction for leaf and hypocotyl was observed at the higher concentration of NAA while it was in contrast with the embryo axis. The result of the analysis illustrated that the highest frequency of callus induction was found for the treatments containing 0.5 mg L^{-1} NAA without BA and 0.5 mg L^{-1} NAA and 1.5 mg L^{-1} BA in the embryo axis (Table 2). Therefore, the most efficient treatment for callus induction in fenugreek was 0.5 mg L^{-1} NAA without BA.

Conclusions

We investigated the effect of various explants, NAA and BA on callus induction and shoot regeneration in the medicinal plant of fenugreek. Simultaneous regeneration, callus and root induction in the same medium occurred in the embryo axis explant, shortening the tissue culture time, with no sub-culturing and less infection and chemical consumption. Since regeneration and somatic embryogenesis was found in one of the treatments tested in this research, we suggest to study the effect of other auxins and cytokinins to improve the frequency of these characteristics using different types of explants in the future studies.

Table 2. Mean comparison for the effect of different treatments on the number of explants with callus induction.

Treatment	Number of explants with callus induction	Treatment	Number of explants with callus induction
T1	0.00^{c}	T25	0.00^{c}
T2	0.00^{c}	T26	0.00^{c}
T3	0.00^{c}	T27	2.67^{ab}
T4	0.00^{e}	T28	0.67^{bc}
T5	0.00^{c}	T29	0.00^{c}
T6	0.00^{c}	T30	2.67^{ab}
T7	0.00^{c}	T31	0.00^{c}
T8	0.00^{c}	T32	0.33^{c}
T9	0.00^{c}	T33	0.00^{c}
T10	0.00^{c}	T34	3.00^{a}
T11	0.00^{e}	T35	2.00^{abc}
T12	0.00^{c}	T36	0.00^{c}
T13	0.00^{c}	T37	1.00^{bc}
T14	0.00^{c}	T38	0.67^{bc}
T15	3.00^{a}	T39	0.00^{c}
T16	0.00^{c}	T40	3.00^{a}
T17	0.00^{c}	T41	1.67^{abc}
T18	2.67^{ab}	T42	0.00^{c}
T19	0.00^{c}	T43	1.00^{abc}
T20	0.00^{c}	T44	3.00^{a}
T21	1.67^{abc}	T45	0.00^{c}
T22	0.00^{c}	T46	2.00^{abc}
T23	0.00^{c}	T47	1.00^{abc}
T24	3.00^{a}	T48	0.00^{c}

Different letters in two columns represent significant differences by U test of Mann Whitney at 5% probability level.

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باززایی درون شیشه در گیاه دارویی شنبلیله (*Trigonella foenum-graecum* L.)

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چکیده

شنبلیله (*Trigonella foenum-graecum L.*) یک گیاه دارویی بوده که در طب سنتی مورد استفاده قرار می‌گیرد. دو تا از مهمترین مواد دارویی دیوزجنین و تری گونلین می‌باشد که دارای خواص دارویی بسیار از جمله خاصیت ضد سرطان، پایین آورنده کلسترول و قند خون می‌باشد. بهینه سازی درون شیشه، القای کالوس و باززایی اندام‌های هوایی اولین گام در جهت تولید بهینه متابولیت‌های ثانویه در این گیاه از طریق کشت بافت و مهندسی ژنتیک می‌باشد. به منظور دستیابی به این هدف ریزنمونه‌های برگ، هیپوکوتیل و محور جنینی روی محیط کشت موراشیک و اسکوگ (MS) حاوی غلظت‌های مختلف هورمون‌های نفتالین استیک اسید (NAA) (۰، ۰/۵، ۱/۵ و ۲ میلی‌گرم در لیتر) در ترکیب با ۶-بنزیل‌آدنین (BA)(۰، ۰/۵، ۱ و ۱/۵ میلی‌گرم در لیتر) با تشکیل ۴۸ تیمار کشت شدند. داده‌ها به وسیله آزمون غیرپارامتری کروسکال والیس تجزیه شدند و میانگین‌ها توسط آزمون غیرپارامتری توکی مقیسه شدند. نتایج تجزیه داده‌ها تفاوت معنی‌داری را بین تیمارها نشان داد. القای کالوس و باززایی اندام‌های هوایی به طور هم‌زمان در محیط کشت MS حاوی ۰/۵ میلی‌گرم در لیتر NAA بدون BA و در ریزنمونه محور جنینی رخ داد. بیشترین فراوانی القای کالوس در ریزنمونه برگ و هیپوکوتیل در غلظت بالاتر NAA (۲ میلی گرم در لیتر) به دست آمد.

واژه‌های کلیدی: القای کالوس؛ باززایی گیاه؛ شنبلیله؛ محور جنینی؛ NAA؛ BA