Assessment of Diosgenin Production by *Trigonella foenum-graecum* L. *in vitro* Condition

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**ABSTRACT**

The objective of this study was to analyze and compare the accumulation of diosgenin during different stages of growth in leaf, shoot apical meristem and root apical meristem callus cultures of fenugreek. The levels of this secondary compound were examined by UV Spectrophotometer. The results showed that Callus generation increase was caused by the increased concentration of 2,4-D hormone. The highest callus generation was obtained in shoot apical meristem grown on 1 mg L⁻¹ 2,4-D hormone in 45 days was significant (p<0.05) compared with other treatments. We concluded that the level of diosgenin detected in leaf callus was more than its level in shoot and root callus.

**Key words:** Fenugreek, tissue culture, 2,4-D hormone, diosgenin, UV spectrophotometer

**INTRODUCTION**

Fenugreek (*Trigonella foenum-graecum* L.) is an annual plant from legume family. This native crop is extending from Iran to northern India (Petropoulos, 2002). Fenugreek leaves and seeds have been used extensively for preparing extracts and powders in medicinal performance (Basch *et al.*, 2003; Nithya and Ramachandramurty, 2007). In some region in Asia, the young plants are performed as potherbs and the seeds for herbal medicine usages. The species name "foenum-graecum" means "Greek hay" indicating its use as a forage crop in the past (Petropoulos, 2002). Fenugreek leaves and seeds have been used extensively to prepare extracts and powders for medicinal uses (Basch *et al.*, 2003). Fenugreek is a rich source of steroidal sapogenin. Previous studies reported the anti-diabetic (Broca *et al.*, 2004; Srinivasan and Karundavi, 2005; Khan and Anderson, 2003), Antibacterial (Bonjar, 2004), Anti-oxidant (Semalty *et al.*, 2009) hypocholesterolaemic (Suboh *et al.*, 2004), anti-cancer (Devasena and Menon, 2003), thyroxin-induced hyperglycaemia (Tahiliani and Kar, 2003), anti-inflammatory (Subhashini *et al.*, 2011), antiaflatoxigenic (Mohamed and Metwally, 2009) and protective properties against ethanol toxicity (Thirunavukkarasu *et al.*, 2003) of fenugreek. It also plays an important role in the control of cholesterol metabolism (Bahram *et al.*, 2005).

*Trigonella foenum-graecum* is produced diosgenin as a steroidal sapogenin, belonging to triterpene group and has great significance for pharmaceutical industry due to its oestrogenic effect on the mammary gland (Onchina *et al.*, 2000). It also plays an important role in the control of cholesterol metabolism, variation in the lipoxygenase activity of human erythroleukaemia cells and responsible for morphological and biochemical variations in megakaryocyte cells. Diosgenin is performed mainly as basic material for partial synthesis of the oral contraceptives, sex hormones and the other steroids (Gomez *et al.*, 2004). Methyl protodioscin, as the potent agent by anti-tumor properties, has been synthesized from diosgenin (Cheng *et al.*, 2003). Also the role of ethylene on diosgenin production in *T. foenum-graecum* seedlings reported before (De and De, 2003).
Ozana et al. (2002) concerned about the expression of sapogenin in this plant material contained the roots and hairy roots cultures and its expression in their seeds.

In vitro requesting of this secondary compound is the alternative process for obtaining sapogenin, so that its supply (especially on the industrial scale) is not dependent to the environmental status or on its particular growth stage. The maximum absorbance of UV spectrophotometer in the identifying the callus cultures from Trigonella foenum-graecum is 430 nm. This is used as an identification and quantification procedure of diosgenin production. Therefore, the aim of the present study was to analyze and compare the accumulation of diosgenin during the different stages of growth in the Trigonella foenum-graecum leaf, the shoot apical meristem and the root apical meristem of their callus cultures.

MATERIALS AND METHODS

The experiment was carried out in the Tissue Culture Laboratory of the Department of Biology, Faculty of Science, Islamic Azad University, Mashhad, Iran during October 2009 to October 2010. Fenugreek seeds were prepared by Agricultural Research Laboratory of Khorasan Razavi province, Iran. Seeds were sterilised in 70% ethanol for 25 sec and 2% hypochloride sodium solution for 3 min. The seeds were then rinsed several times in sterile water before being implanted on a 0.7% w/v agar medium. Cultures were maintained at 25°C with continuous light. Three to four weeks after germination, different plant organs (leaves, Shoot Apical Meristem (SAM) and Root Apical Meristem (RAM)) were separated and placed separately in MS culture media. Hormonal treatments involved infiltrating different quantities of 2,4-D (0, 0.5, 1 and 1.5 mg L⁻¹) into the culture media after autoclaving and before the agar gelled. All types of media were sterilized at 121°C for 20 min. The calluses were grown and maintained at 25°C with a 16 h light photoperiod provided by fluorescent tubes (F38W/Gro). Tissues were subcultured at 3-week intervals. Three replicates were maintained for each analysis. All reagents, solvents and standards were of analytical reagent grade.

Three-time subcultured calluses were used to measure growth and the fresh and dry weights were determined at different times after subculturing. At different times, 3 g of the callus was used for the isolation of diosgenin.

Diosgenin was determined by employing the method described by Baccou et al. (1977) and Umetsu et al. (2000) with some modifications. Standard diosgenin and p-anisaldehyde (4-methoxybenzaldehyde) were purchased from Sigma-Aldrich Company. Sulfuric acid and ethyl acetate were both of analytical grade. The diosgenin level was determined by measuring absorbance at 430 nm, based on the color reaction with anisaldehyde, sulfuric acid and ethyl acetate. Briefly, two color developing reagent solutions were prepared: (A) 0.5 mL p-anisaldehyde and 99.5 mL ethyl acetate and (B) 50 mL concentrated sulfuric acid and 50 mL ethyl acetate. The 0.2 mL of the methanol extract was placed in a glass and dissolved in 2 mL of ethyl acetate, 1 mL of each of the reagents A and B were added to the tube and were stirred. The test tube was placed in a water bath maintained at 60°C for 10 min to develop color, then was allowed to cool down for 10 min in 25°C water bath. The absorbance of the color developing solution was measured with a spectrophotometer (V-1100-UV/VIS) at 430 nm. Ethyl acetate was used as a control for the measurement of absorbance. As a reagent blank, 2 mL ethyl acetate was placed in a tube and assayed in similar manner. For calibration curve, 2-40 mg standard diosgenin in 2 mL ethyl acetate was used. Each sample was repeated thrice and the average was taken.
Data analysis: Data were analyzed using the analysis of variance and Duncan's multiple range test at p<0.05 level of significance. Data handling: fresh and dry weights of leaf, shoot apical meristem and root apical meristem and diosgenin content.

RESULTS AND DISCUSSION
Callogenesis: The fresh and dry weights of different explants callus during growth stages were analyzed as described in Table 1.

In the present study three explants of leaf, shoot apical meristem and root apical meristems were used for callogenesis. Significant differences (p<0.05) were detected among different tissues for fresh and dry weights of calluses. Table 1 showed that different 2,4-D hormonal status evaluation in fresh and dry weight of callus in 15, 30, 45 and 60 days after culture presented. During the experiments the highest and lowest of fresh and dry weight of callus attributed to

Table 1: Fresh and dry weights of Root Apical Meristem (RAM), Shoot Apical Meristem (SAM) and leaf (LEAF) callus in different concentrations of 2,4-D during growth

<table>
<thead>
<tr>
<th>Explant 2,4-D (mg L⁻¹)</th>
<th>16 day</th>
<th>30 day</th>
<th>45 day</th>
<th>60 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D</td>
<td>Fresh</td>
<td>Max-Min</td>
<td>Dry</td>
<td>Max-Min</td>
</tr>
<tr>
<td>(g)</td>
<td>(g)</td>
<td>(g)</td>
<td>(g)</td>
<td>(g)</td>
</tr>
<tr>
<td>SAM,0</td>
<td>0.260fg</td>
<td>0.301-0.324</td>
<td>0.623ef</td>
<td>0.030-0.022</td>
</tr>
<tr>
<td>SAM,0.5</td>
<td>0.730de</td>
<td>0.956-0.332</td>
<td>0.703cd</td>
<td>0.084-0.068</td>
</tr>
<tr>
<td>SAM,1</td>
<td>1.052c</td>
<td>1.350-0.794</td>
<td>0.17cal</td>
<td>0.135-0.092</td>
</tr>
<tr>
<td>SAM,1.5</td>
<td>0.541ef</td>
<td>0.520-0.500</td>
<td>0.056de</td>
<td>0.056-0.045</td>
</tr>
<tr>
<td>RAM,0</td>
<td>0.423ef</td>
<td>0.623-0.059</td>
<td>0.022ef</td>
<td>0.041-0.029</td>
</tr>
<tr>
<td>RAM,0.5</td>
<td>1.415b</td>
<td>2.002-1.124</td>
<td>0.087abc</td>
<td>0.128-0.113</td>
</tr>
<tr>
<td>RAM,1</td>
<td>1.74d</td>
<td>2.925-3.013</td>
<td>0.109a</td>
<td>0.172-0.125</td>
</tr>
<tr>
<td>RAM,1.5</td>
<td>1.03ecd</td>
<td>1.966-0.940</td>
<td>0.050cd</td>
<td>0.090-0.067</td>
</tr>
<tr>
<td>LEAF,0</td>
<td>0.335g</td>
<td>0.237-0.183</td>
<td>0.011f</td>
<td>0.025-0.011</td>
</tr>
<tr>
<td>LEAF,0.5</td>
<td>0.340fg</td>
<td>0.511-0.100</td>
<td>0.042def</td>
<td>0.054-0.039</td>
</tr>
<tr>
<td>LEAF,1</td>
<td>0.570ef</td>
<td>1.126-0.726</td>
<td>0.056de</td>
<td>0.099-0.069</td>
</tr>
<tr>
<td>LEAF,1.5</td>
<td>0.298fg</td>
<td>0.301-0.288</td>
<td>0.054d</td>
<td>0.037-0.040</td>
</tr>
</tbody>
</table>

In all columns, means followed by the same letters are not significantly different at p<0.05 level of significance and were analyzed using the analysis of variance and Duncan's multiple range test.
1 mg L\(^{-1}\) of 2,4-D and control plants (0 mg L\(^{-1}\) 2,4-D). The results showed that callus generation increased through increasing the 2,4-D hormone concentration to 1 mg L\(^{-1}\) while its increasing up to 1.5 mg L\(^{-1}\) decreased fresh and dry weight of callus significantly (p<0.05). Among the various concentrations of 2,4-D used for callus induction, the concentration of 2,4-D at 1 mg L\(^{-1}\) proved to be the best for maximum callus induction and proliferation in all kinds of explants (Table 1). The results also showed that 0.5 mg L\(^{-1}\) 2,4-D is a better concentration for callogenesis than 1.5 mg L\(^{-1}\) 2,4-D and control group (Fig. 1, 2).

The results showed that through experimental periods fresh and dry weight of callus increased significantly as their minimum and maximum rates were in 15 days and 45 after culture, respectively. As shown in Table 1, the variations in fresh and dry weights were observed in different times. For all calluses, there was an exponential growth phase between day 15 to day 45 followed by a linear phase (stationary phase) started after day 45. Present investigation demonstrated the effect of auxins on callus induction.

Fig. 1: Callus formed from SAM. Explant after 30 days of inoculation in the 2nd subculture on MS medium containing 1 mg L\(^{-1}\) 2,4-D

Fig. 2: Callus formed from RAM explants after 15 days of inoculation on MS medium containing 1 mg L\(^{-1}\) 2,4-D
Explant from shoot apical meristem growth in 1 mg L\(^{-1}\) 2,4-D had highest potential for callus formation and proliferation while explant from leaf had relatively less potential for callus formation. The callogenesis response was induced after 15 days of in vitro culture in response to 2,4-D added in MS basic media.

According to our results the maximum callus generation rate in 15 days was for RAM explants. But in 45 and 60 days, the maximum callus generation in SAM explant formed in MS culture containing 1 mg L\(^{-1}\) 2,4-D hormone. Different explant segments into different hormonal levels formed different callus levels (Johri and Mitra, 2001). Also, Fitch and Moore (1993) expressed higher callus formation in meristems attributed to easier differentiation of these areas. Slesak et al. (2005) determined the best hormonal status for callus generation of *Brassica napus* is 1 mg L\(^{-1}\) 2,4-D in MS culture. Also the maximum callus generation for *Tanacetum balsamita* L. determined 1 and 0.5 mg L\(^{-1}\) 2,4-D hormone for leaf explant (Shoja et al., 2010).

Callus growth follows a typical logarithmic pattern involving slow initial cell division induction period requiring auxin, a rapid cell division phase involving active synthesis of DNA, RNA and protein followed by a gradual cessation of cell division along with differentiation of callus mass (Hartmann et al., 1999). Among different hormones used for callus induction and proliferation, auxins are reported to be most effective. Among different auxins used, 2,4-D is reported to have better results for callus induction and proliferation. The hormone mediated callus induction and subsequent growth is dependent on certain factors which may trigger the complete chain of events that influence the ability of cultured cells to grow in an organized fashion. Plant tissues, therefore, must have receptors for hormones. Mockeviciute and Anisimoviene (1999) reported before that these hormonal levels are interacted by specific receptors either on the cell membrane or within the cytoplasm.

Affinity and concentration of receptors on the surface of the target tissues determine the type of response. Specific binding site for auxin has been identified (Kim et al., 2001). A class of proteins called expansions mediates the proton ability to cause cell wall loosening. These expansions break the hydrogen bonds between the polysaccharide components of the wall (Cosgrove, 2001). Proton (H\(^{+}\)) pumping and lowering of cytosolic pH result in an elevation of intracellular calcium level (Shishova et al., 1999). Both cytosolic pH and calcium ions act as second messengers in early auxin action (Zhang and Lu, 2003). Calcium ions, either themselves and or along with calcium binding proteins, e.g., calmodulin, activate the protein kinase cascade which in turn activates other proteins including the transcription factors. These factors presumably interact with the auxin-response elements and regulate the expression of auxin-inducible or auxin-responsive genes and exert their effect on cell cycle and stimulate cell division (Johri and Mitra, 2001).

**Diosgenin:** The diosgenin level was determined by measuring absorbance at 430 nm due to the maximum absorbance in this wavelength (Fig. 3). The results of diosgenin concentration of different callus explants during growth stages are given in Fig. 4. A significant variation in diosgenin concentration was found among the different explants (leaf, SAM and RAM). The level of diosgenin accumulated at all the ages analysed (on days 15, 30, 45 and 60) were higher in leaf callus (1.86, 2.0, 2.3 and 1.75 mg, respectively) than in SAM callus (0.35, 0.58, 0.80 and 0.65 mg, respectively) and RAM callus (0.35, 0.41, 0.65 and 0.50 mg, respectively) (Fig. 4). The variation in diosgenin level was observed in different days. In all three callus types, maximum level was attained on day 45 which coincided with the onset of the stationary phase of growth for
Fig. 3: Absorption spectrum of diosgenin

Fig. 4: Diosgenin level of Root Apical Meristem (RAM), Shoot Apical Meristem (SAM) and leaf callus during growth. Vertical bars denote standard deviation

both leaf and SAM cultures. These results suggest that leaf calluses are the most suitable starting material for obtaining diosgenin in vitro. The concentration of diosgenin accumulated in leaf, shoot apical meristem and root apical meristem increased up to day 45 (maximum production) and then began to decrease.

The results of the research showed that all three callus types (leaf, shoot and root) of fenugreek obtained in their culture conditions accumulate diosgenin in the same way as they do in the mother plant (Ortuno et al., 1998). There has been no alteration in the metabolic pathway as a result of in vitro cultivation.

The diosgenin levels accumulated in leaf were 30% of the levels detected in the corresponding organs of the mother plant on day 45.

CONCLUSION

Statistically significant differences were observed between explants, 2,4-D concentrations and days investigated in fresh and dry weight measured. The best explant for callogenesis was Shoot Apical Meristem (SAM). These results provide important knowledge on diosgenin content, emphasizing that fenugreek can be a good source of bioactive compounds. The results also showed
that the maximum levels of this secondary compound in fenugreek were in leaf callus, lower level being detected in Shoot Apical Meristem (SAM) and Root Apical Meristem (RAM) callus. The level of diosgenin accumulated in leaf, shoot apical meristem and root apical meristem increased up to the day 45 (maximum production) and then decreased.

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REFERENCES