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ENHANCEMENT OF SECONDARY METABOLITES IN TISSUE CULTURE OF A MEDICINAL PLANT: *TRIGONELLA FOENUMGRAECUM* L.

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Trigonella foenum- graecum L is an annual forage legume and a traditional spice crop that has been grown for centuries across the Indian subcontinent which is rare and extensively used in traditional systems of medicine. Diabetes mellitus is a heterogeneous group of diseases characterized by chronic elevation of glucose in the blood. It arises because the body is unable to produce enough insulin for its own needs, either because of impaired insulin secretion, impaired insulin action, or both insulin resistance and insulin deficiency. Secondary to pancreatic beta-cell failure the world as the best remedy for the diabetes has been taken in the present investigation for the study of secondary products produced in tissue culture. For callus induction the cotyledonary explants from 8-week old seedlings were excised. These explants were inoculated to MS medium supplemented with various concentrations of (0.5-2.5 mg / L) of auxins such as 2,4–Dichlorophenoxy acetic acid (2,4-D), Indole 3-acetic acid (IAA), Naphthalene acetic acid (NAA). Callus was harvested after 3 weeks, dried and analysed for steroids and glycol alkaloids. All analytical and preparative thin layer Chromatography (TLC) methods were performed with chloroform and methanol (99:1) solvent using silica gel G Plates. Spots corresponding to reference diosgenin and B-Sitosterol were marked by exposing the plates to iodine vapors. The corresponding zones were collected, eluted with chloroform, tested on TLC (Thin Layer Chromatography) and later crystallized. The potentialities of the cell cultures of *Trigonella foenum - graecum* L. producing steroidal compounds of pharmaceutical importance, have been worked out in the laboratory. A relationship of the relative synthesis of steroidal compounds and their kinetics has been established. Incorporation of cholesterol, various growth regulators and nitrogenous sources individually into the medium has been investigated and enumerated establishing the synthesis of the secondary products in callus of *T. foenum-graecum* in *in vitro* cultures.

Several strategies have been adopted for the enhancement of bioactive metabolite production in *in vitro* cultures; one of them is using growth regulators which are often a crucial factor in secondary product accumulation. The type and concentration of auxin or cytokinin or the auxin/cytokinin ratio may alter dramatically both the growth and the product formation in cultured plant cells. Therefore, it is of great interest to evaluate the antidiabetic activity and steroid compounds of *T.foenum graecum* callus

Key words: *In Vitro* culture, callus Induction, Secondary products, *Trigonella foenum - graecum* L. Sapogenin content and Cholesterol

Medicinal plants are the most exclusive source of life saving drugs for majority of the world's population. The utilization of plant cells for the production of natural or recombinant compounds of commercial interest has gained increasing attention over past decades (Canter *et al.* 2005).

Plant cell culture systems represent a potential renewable source of valuable medicinal compounds, flavours, fragrances, and colorants, which cannot be produced by microbial cells or chemical synthesis. The evolving commercial importance of the secondary metabolites has in recent years resulted in a great interest, in secondary metabolism, and particularly in the possibility to alter the production of bioactive metabolites by means of cell culture technology. The principle advantage of this technology is that it may provide continuous, reliable source of plant pharmaceuticals and could be used for the large scale culture of plant cells from which these metabolites can be extracted. Plant cell and tissue cultures hold great promise for controlled production of myriad of useful secondary metabolites on demand. The current yield and productivity cannot fulfill the commercial goal of plant cell-based bioprocess for the production of most secondary metabolites. In order to stretch the boundary, recent advances, new directions and opportunities in plant cell based processes are being critically examined. Recently, there have been growing interests in the application of natural components as antidiabetic agents (Qi *et al*, 2010). A wide range of products claiming

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to lower blood glucose levels or prevent and treat diabetes complications and comorbidities are marketed to the public (Geil *et al,* 2008). One of the most promising amongst them is *Trigonella foenum-graecum* L. (fenugreek, family Leguminasae) seeds. Fenugreek seeds have shown potential as a dietary supplement and cause a marked decrease in the symptoms of DM such as polydipsia, polyuria, urine sugar, renal hypertrophy and glomerular filtration rate (Ulbricht *et al.* 2004). Fenugreek is a spice rich in dietary fibers and has a traditional history of medicinal use in the management of DM in Egypt, southern Europe, India, Asia, and northern Africa (WHO 2007). Fenugreek seeds mainly contain 4-hydroxyisoleucine (4-HI), trigonelline, galactomannan with flavonoids, carotenoids, coumarins, proteins, saponins, and lipids (Basch *et al.* 2003). Fenugreek seeds have previously been shown to have hypoglycemic and hypocholesterolemic effects in type 1 and type 2 DM patients (Gupta *et al.* 2001 and Madar *et al.* 1988) and alloxan induced diabetic animals (Jelodar *et al.* 2005 and Raju *et al.* 2001).

T. foenum - graecum L produce steroidal compounds, such as steroidal saponins, sapogenins and teroidal alkaloids of pharmaceutical importance in tissue cultures (Brain and Lockwood 1976),. The production of secondary metabolites in cell culture systems is largely dependent on environmental and biological factors. Workers have studied the effect of cholesterol on diosgenin in biosynthesis in plants like *Dioscorea deltoidea* (Stohs *et al.* 1969). Similarly the supplementation of various growth regulators in to the medium and their relative effects on callus growth and steroidal metabolism has been studied in *Dioscorea deltoidea* (Hoftman 1967). The nitrogenous substances have also been incorporated into the medium to study their effects on the growth rate and production of metabolites (Kaul and Staba 1968). In the present investigation of research for enhancement of secondary products from callus of cotyledonary explants culture on MS Medium supplemented with various concentrations of auxins in *T. foenum–graecum.*

MATERIAL AND METHODS

Seeds of *Trigonella foenum-graecum* used in the present study were obtained from Agricultural Research Institution, Warangal, Telangana. Seeds were soaked in concentrated Sulphuric acid (H_2SO_4) for 24 hours. These seeds were surface sterilized with 0.1% (W/V) Mercuric chloride ($HgCl_2$) solution for 3-5 minutes, followed by three rinses with sterile distilled water. These seeds were germinated aseptically on MS basal medium (Murashige and Skoog,1962) solidified with 0.8% (W/V) difco bacto agar at pH 5.6 ± 2.0 in 250 ml Ehrlen meyer flasks (50ml medium / flask).

For callus induction and the production of secondary metabolites the cotyledonary (0.6 - 0.8 cm^2), from 6-week-old axenic seedlings were excised. These explants were inoculated to MS medium supplemented with various concentrations of (1.0- 5.0 mg / L) of auxins such as 2,4–Dichlorophenoxy acetic acid (2,4-D), Indole 3-acetic acid (IAA), Naphthalene acetic acid (NAA). All the explant growth regulators were used as auxine alone in culture media on MS medium and maintained by periodic subcultures of 4-6 weeks. (Table-1) (Plate-1). This medium is supplemented with various concentration of cholesterol of 10 to 80 mg/ 100ml Gibberelic acid (GA_3) and nitrogenous sources like calcium nitrate and Urea were added to the medium at 100 and 200mg/L Levels. Growth regulators such as 2,4–Dichlorophenoxy acetic acid (2,4-D), Indole 3-acetic acid (IAA), Naphthalene acetic acid (NAA)(0.5 - 4.0mg/L) (Table-2) were added. Media were adjusted to pH 5.8 before addition of 0.8% agar agar and autoclaved at 121^0 C and 103 K pa for 20 minutes, Cultures were made in 25 x 150 mm cultures tubes. Callus was harvested after 3 weeks dried and analysed for steroids and glycol alkaloids.

The callus were dried in shade and stored in the dark until use. All chemicals like Glucoamylase (Sisco Research Laboratories),Glucose (Sisco Research

Laboratories), Starch (SDF Mumbai), 3,5-Dinitro Salicylic Acid (Spectro Chem), Silica gel for column chromatography 60-120 mesh (SDF chemicals), Silica gel H for TLC (SDF chemicals), Sodium Acetate (SDF chemicals), Glacial Acetic Acid (SDF chemicals) were extra pure and analytical grade while the water was glass distilled.

All analytical and preparative thin layer Chromatography (TLC) methods were performed with chloroform and methanol (99:1) solvent using silica gel G Plates. Spots corresponding to reference Stigmasterol and B-Sitosterol were marked by exposing the plates to iodine vapors. The corresponding zones were collected, eluted with chloroform tested on TLC and later crystallized.

RESULTS

Callus induction ability of cotyledon explants and secondary metabolite production was investigated by using various concentrations of different auxins individually. Callus proliferation was initiated at the cut surfaces of the explants studied and later it covered the entire surface. Both color and texture of the callus also varied with growth regulators supplemented. The results are presented in Table-1 and shown in Plate I. MS medium supplemented with different concentrations (1.0-5.0mg/L) of auxin such as 2,4-D, IAA and NAA, individually exhibited initiation of callus after 15 days of incubation while it took 12-15 days in cotyledonory explants.

For callus induction the explant viz. Cotyledon (0.6-0.8 cm^2) from 6 weeks old axenic seedling were excised and inoculated to MS medium supplemented with various concentrations of 2,4-D, IAA and NAA (1.0-5.0 mg/L). Callus proliferation was initiated at the cut surface of the cotyledon explants.

Effect of 2, 4-D: On 2,4-D supplemented medium early induction was observed in all concentration of 2,4-D. High amount of callus was induced at 2.0 and 3.0 mg/L 2,4-D. Different callusing response was recorded in cotyledon explants at all the concentrations of 2,4-D. Morphology of callus was found to be varied at different levels of 2,4-D. White compact callus was found at all concentration of 2,4-D. Low amount of callus was induced at high concentrations of 2,4-D. Highest percentage (80%) of response was observed at 3.0mg/L 2,4-D.

Effect of IAA:-On IAA supplemented

Table 1: Effect of different concentrations of 2, 4-D, IAA and NAA Production of Secondary metabolites from Cotyledonary explants of *Trigonella foenum-graecum* after 6 weeks of culture**.**

Plant Growth regulators mg/L			% of cultures responding	Morphology	Callusing response
2.4- D	**IAA**	**NAA**			
0.5	-	-	47	White compact	++
1.0	-	-	58	White compact	+++
1.5	-	-	82	Brown Friable	+++
2.0	-	-	76	Brown Friable	++
2.5	-	-	70	White nodulart	+
2.0	0.5	-	58	White compact	+
2.0	1.0	-	60	White friable	++
2.0	1.5	-	68	White friable	+++
2.0	2.0	-	56	Brown compact	+
2.0	2.5		52	Brown compact	+
2.0	-	0.5	52	White compact	++
2.0	-	1.0	82	White compact	++
2.0	-	1.5	86	Brown friable	+++
2.0	-	2.0	78	White friable	+++
2.0	-	2.5	70	White friable	++

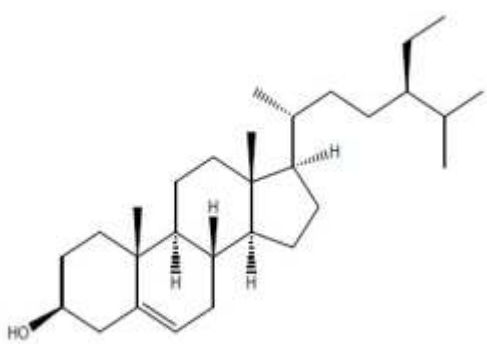
Table 2: Effect of Plant growth regulators and cholesterol 80 mg/100ml, Gibberelic acid (GA_3) Calcium nitrate and Urea 100 mg/100ml on subcultures of Secondary metabolites production from Cotyledonary explants of *Trigonella foenum-graecum* after 6 weeks of culture.

Plant Growth regulators mg/L			% of Diosgenin	% of Gitogenin	% of Tigogenin
2.4- D	**IAA**	**NAA**			
0.5	-	-	0.70	0.30	0.80
1.0	-	-	0.85	0.45	0.93
1.5	-	-	1.00	0.50	1.00
2.0	-	-	1.25	1.00	1.07
2.5	-	-	0.72	0.43	0.82
-	0.5	-	0.85	0.85	0.55
-	1.0	-	0.96	0.80	0.96
-	1.5	-	1.45	1.00	1.00
-	2.0	-	1.96	1.45	1.05
-	2.5		1.00	0.35	0.54
-	-	0.5	0.45	0.30	0.35
-	-	1.0	0.65	0.46	0.53
-	-	1.5	0.68	0.67	0.85
-	-	2.0	1.34	0.70	0.96
-	-	2.5	0.96	0.36	0.32

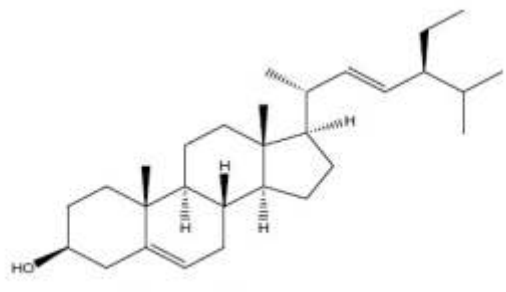
medium moderate amount of callus was observed at 2.0 and 3.0 mg/L IAA. Low amount of callus was observed at 1.0, 4.0 and 5.0 mg/L IAA. Morphology of callus was also found to be varied at different levels of IAA (Table-2). White compact callus was induced at 1.0 white friable callus obtained at 2.0 and 3.0 mg/L IAA. Green nodular callus was observed at 4.0, and 5.0mg/L IAA. 68% callusing response was recorded in cotyledon explants at 3.0 mg/L concentration of IAA.

Effect of NAA: Effect of NAA on callusing ability of cotyledon explants is shown in (Table-1). High percentage (86%) of response was observed at 3.0 mg/L NAA. Responding callus was also varied at different levels of NAA. Whereas moderate amount of callus was observed at 1.0 and 2.0mg/L NAA. High amount of callus was induced at 3.0 and 4.0mg/L NAA. A white compact type of callus was induced at 1.0, 2.0 and 3.0 mg/L NAA. The high concentration (4.0 and 5.0 mg/L NAA induced creamy friable callus (78% and 70%) were cultures responded.

The regulatory effect of cholesterol and growth regulators on steroidal sapogenins have been observed in *Trigonella foenum-graecum.* The maximum increase in diosgenin content over the control have been noticed at 100mg/l of cholesterol and 2.0mg/L IAA while 2.0 mg/L NAA showed the inhibitory effects on the



β-sitosterol



Stigmasterol

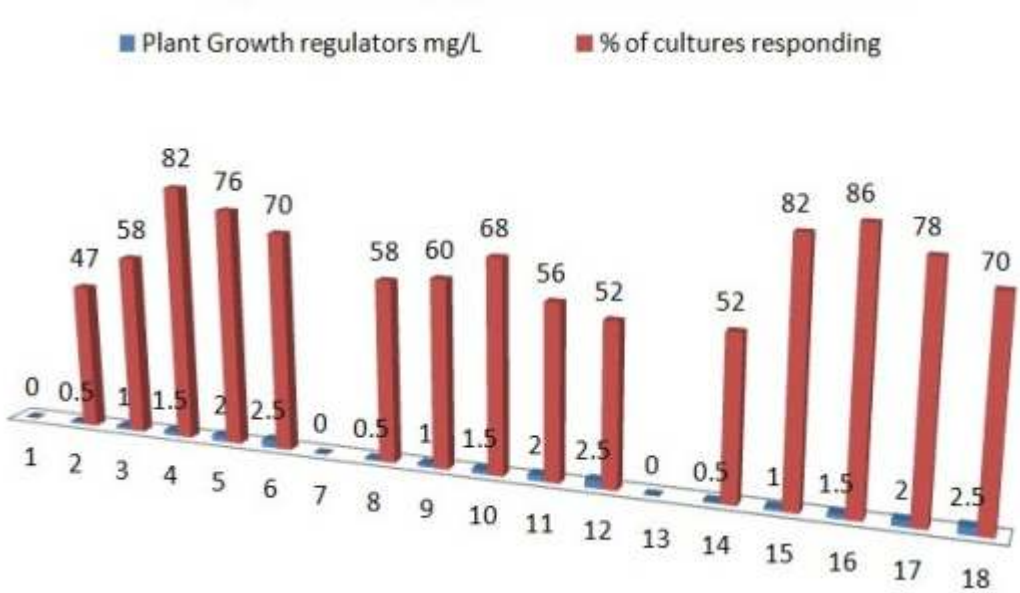


Figure 1: Effect of different concentration of 2, 4-D, IAA and NAA on MS Medium for Producation of Secondary metabolites from Cotyledonary explants of Trigonella foenum-graecum after 6 weels of culture

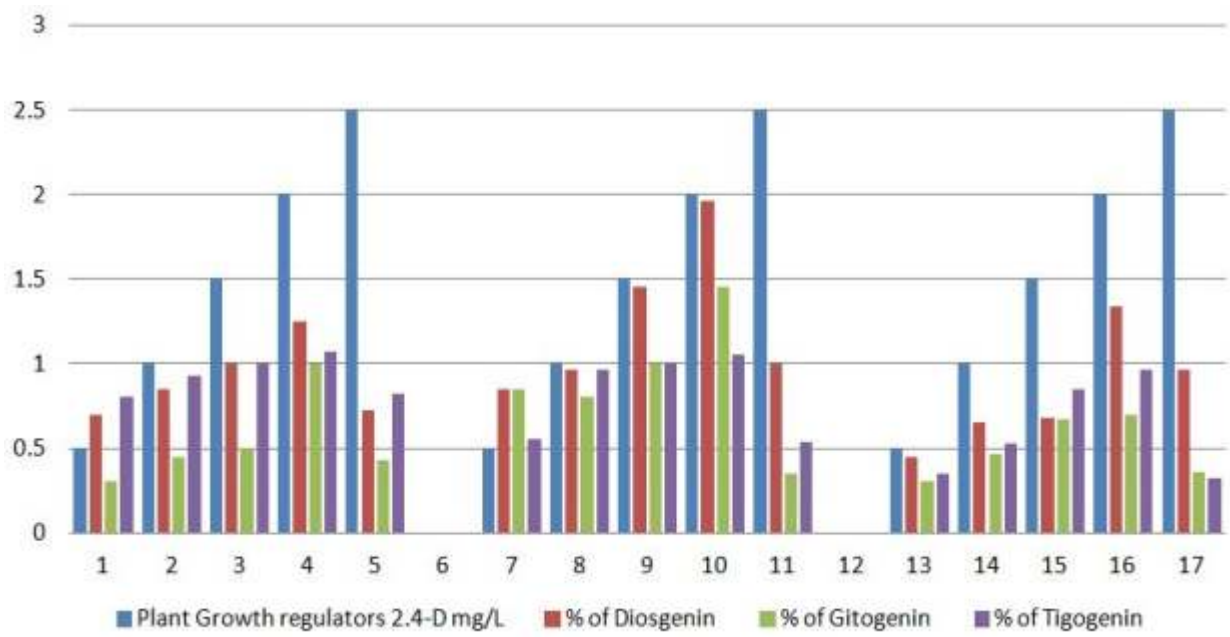


Figure 2: Effect of Plant growth regulators and cholesterol 80 mg/100ml, Glibberelic acid (GA3) Calcium nitrate and Urea 100 mg/100ml in subcultures of Secondary metabolites production from Cotyledonary explants of *Trigonella foenum-graecum* after 6 weeks of culture

production of sapogenins as given in the (Table-2).

Plant growth regulators affected not only growth and differentiation of cultured cells but also have dominant role in the induction and repression of certain biosynthetic path ways leading to accumulation of secondary plant products. Regulatory effect of various growth regulators on steroidal compounds in many plant species has been investigated (Hardmann and Stevens 1978).

The present study shows that cholesterol,

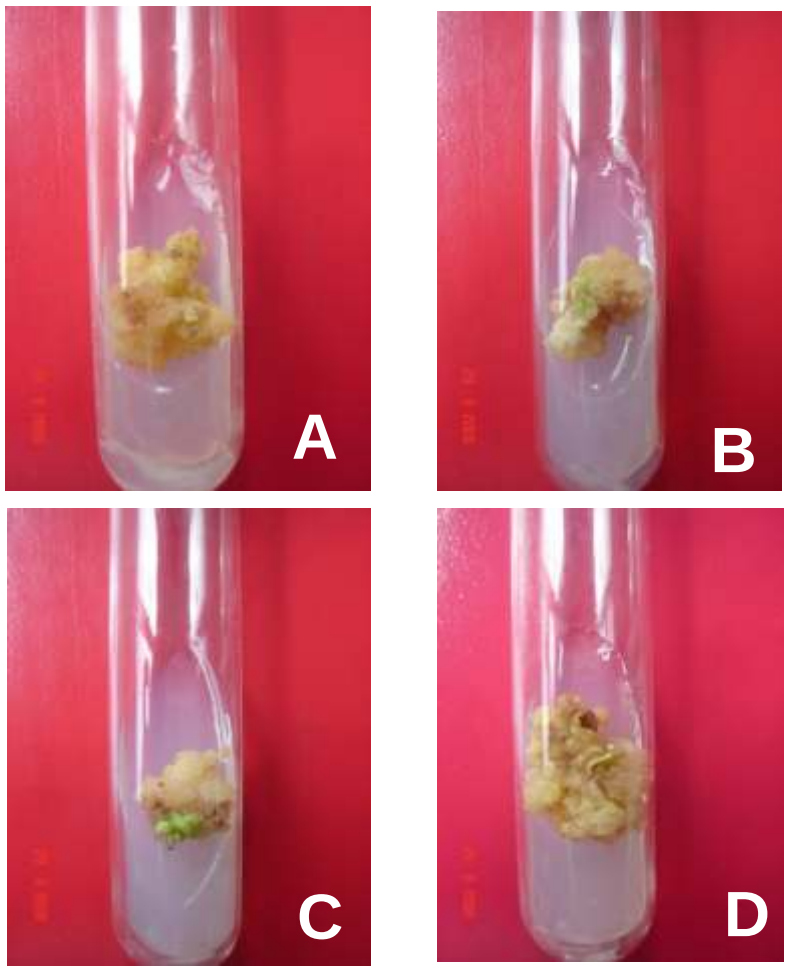


Plate I: Callusing ability of Cotyledonary explants of Trigonella foenum-graecum for production of secondary metabolites on MS Medium supplemented with various concentration of auxins A: Induction of brown friable callus on (1.5 mg/L) 2, 4-D. **B.** Induction of White friable callus on 2.0mg/L 2, 4-D **C.** Induction of brown friable callus on MS + 1.5mg/L IAA. **D.** Induction of brown friable callus on 1.5mg/L NAA.

growth regulators and nitrogenous substances play a prominent role in the synthesis of steroidal compounds in tissue cultures.

CONCLUSION

From methanol extracts of Leaf callus culture in *Trigonella* using Thin Layer Chromatography (TLC) resulted two new chemicals Stigmasterol and β-Sitosterol. As per aim these compounds were characterized by using physico chemical and moderate spectral analysis. These compounds are showing various pharmacological activities. These two steroids are well known phytosterols. The structure of the isolated compounds were identified on the basis of spectroscopic methods and by comparing their physical properties reported in the literature.

B- sitosterol is the most abundant phytosterols and occurs either free or as its glucoside, frequently accompanied by its mono-unsaturated analogue, stigmasterol, which also appears either free or as its glucoside. (Pelletier *et al.* 1995) Anti-inflammatory, analgesic and anti-pyretic activity of β- sitosterol have been reported previously (Gupta *et al.* 1980). β-sitosterol could also modulate antioxidant enzymes *in vitro* (Vivancos *et al.* 2005). A purified mixture of β-sitosterol and stigmasterol isolated from the root extract of *Pluchea indica* successfully established its anti-snake venom activity in experimental animal (Gomes *et al.* 2007).

The mixture of stigmasterol and β-sitosterol

isolated from the cotyledonary explants callus of *Trigonella foenum-graecum* was found to be a moderate inhibitor of glucoamylase, thus may act as an agent to control blood glucose levels in hyperglycemic condition. From the present study, it was apparent that plant exhibits or have the potential to show, a diverse array of biological activities. However, so far only a fraction of these plants have been investigated. There is an urgent need to develop newer, more efficient and reliable bioassays for large scale rapid evaluation of plant extracts.

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