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Current Trends In Fenugreek Bıotechnology And Approaches Towards Its Improvement

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Abstract

Fenugreek (*Trigonella foenum-graecum* L.) plant is cultivated throughout the world as medicinal, food, condiment, dye, and forage. First biotechnological study was carried out in 1945 that reported the effects of diniconazole, a triazole-type fungicide on cell suspension cultures of fenugreek. It was followed by many studies using cell suspension and callus culture emphasising increased production of protein and economically important metabolites like trigonelline, sapogenin, Isoflavonoid pterocarpans, diosgenin, gitogenin and tigogenin from callus, leaves, stems and roots explants. Plant tissue culture studies have emphasied use of callus, cotyloden, hypocotyls and shoot tip epicotyls, apical meristem, cotyledon node and cotyledon leaf explants. Most of the researchers agree difficulty in *in vitro* rooting. Protoplast studies have also been reported using leaf mesophyll and root apices. Leaf mesophyl protoplasts could be converted to leafy shoots whereas, root apices protoplasts gave cell colonies or roots only. Genetic transformation studies using *Agrobacterium rhizogenes* and *A. tumefaciens* are at initial stages and the genes used in the studies only present transformation with either marker or reporter genes. There is a single report on molecular charachterisation of fenugreek from India using 10 RAPD and ISSR primers that revealed interspecific polymorphysm. A review of all this suggests that plant biotechnology of fenugreek is at initial stages of development and there is lot to do for the improvement and breeding of fenugreek.

Keywords: Fenugreek, *Trigonella foenum-graecum* L., secondary metabolites, tissue culture, molecular charachterization

Abbrevıatıons: 2,4-D: 2,4 dicholorophenoxy acetic acid; **ATP**: adenosine triphosphate; **BAP**: *6-Benzylaminopurine*; **EMS**: ethyl methane sulphonate; **GA**: gibberellic acid; **GMGT:** galactomannan galactosyltransferase; **GUS**: β-glucuronidase; **HPLC**: high performance liquid chromatograpghy; **IAA**: indole acetic acid; **IBA**: indole butyric acid; **IPA**: indolepropionic acid; **ISSR**: inter-simple sequence repeat; **MMS**: methyl methane sulphonate; **MS**: Murashige-Skoog; **NAA**: naptahlene acetic acid; **PCR**: polymerase chain reaction; **RAPD**: random amplification of polymorphic DNA; **SPAR**; single primer amplification reaction; **TDZ**: thidiazuran

INTRODUCTION

Fenugreek, *Trigonella Foenum-groecum* L, is indigenous to the Eastern Mediterranean region and largely cultivated in Mediterranean countries of Africa and Europe, Pakistan, Argentina, France, India, and the United States. Both seeds and leaves are used for medicinal purposes, food, condiment, dye, and as forage plant [1, 2]. The leaves are commonly consumed as a vegetable. Fenugreek leaves contain protein (25.0 %), starch (25.9 %), neutral detergent fiber (12.9 %), gum (4.3 %), ash (10.8 %) and lipids (6.5 %). They are a rich source of calcium, iron, β-carotene, and other vitamins. The seeds contain 6-10% lipids, 44–59 % carbohydrates, and 20-30 % protein [3]. The exact number of *Trigonella* species is not known, Taxonomists Linnaeus suggested 260 species of Fenugreek of which only 18 are currently recognized. *T. foenum-graecum, T. balansae, T. corniculata, T. maritima, T. spicata, T. occulta, T. polycerata, T. calliceras, T. cretica, T. caerulea, T. lilacina, T. radiata, T. spinos* are medicinally important [4]. Most species are diploids including *T. foenum-graecum* L., (*2n* = 16 chromosomes). However, 18, 28, 30, 32 or 44 chromosomes are also contain by some *Trigonella* species [5].

The Choline, alkaloid saponins, trigonelline, trigocoumarin, trimethyl coumarin and nicotinic acid, number of steroidal sapgenins including furastanol glycosides, F-ring opened precursors of diosdiosgenin, are found in Fenugreek [6]. It is effective in reducing blood glucose levels, cholesterol, and for gastrointestinal disorders treatments. Studies on Type 1 and 2 diabetics have shown that it aids in insulin secretion to reduce total cholesterol and LDL cholesterol levels; maintaining a blood glucose level and decrease insulin resistance [2,7,8]. Fenugreek is largely self pollinated plant that makes its hybridisation very difficult under field conditions [9, 10]. Therefore, the possible way to breed fenugreek is introduction from among world accessions, induce artificial mutations [10] and use biotechnological approaches. A number of mutants have been produced applying chemical mutagens like ethyl methane sulphonate (EMS), sodium azide (NaN_3) and methyl methane sulphonate (MMS), to seeds [11, 12] including tetraploid plants with economic charachteristics [13, 14]. Very little is known about action of chemical mutagens belonging to different groups [15]. Plant cell and tissue culture in fenugreek has mainly focussed on the productionof secondary metabolites like diosgenin and trigonelline [16, 17]. Fenugreek cells and tissue cultures are used for production of secondary metabolites of economic interest and plant [4] even from roots [18]. The demand of fenugreek metabolites with high diosgenin and trigonelline contents increase the development of tissue culture protocols [19]. This study reviews some biotechnological approaches used for the improvement of fenugreek.

TISSUE CULTURE STUDIES OF *TRIGONELLA FOENUM-GRACEUM* L.

Plant cell and tissue cultures, provides an understanding of physiological, biochemical, and anatomical reactions of cell material under controlled conditions to specified factors in order to gain insight into the intact plant life to its natural environment [20]. Although, Trigonella as medicinal plant is gaining importance in different parts of the world due to derivatives it contains. However, several studies on Trigonella covering fields from secondary metabolites production from cell suspension culture, callus culture, protoplast culture, organogenesis and genetic transformation has been reported.

Cell suspension cultures and Secondary metabolite production

The first tissue culture on fenugreek was carried out by Cerdon et al. [21] and checked the effects of diniconazole (broad spectrum triazole-type fungicide) on cell suspension cultures of fenugreek. They found that 125 µM fungicide added to the fenugreek cell cultures resulted in decreased cell growth by 20 % after 21 days of culture compared to the cell growth in control. They also noted 50 % reduction of total sterol content after treatment with diniconazole treatment leading to sterol biosynthesis inhibition with 14-α- methylΔ8-sterols accumulation. Trisonthi et al. [22] reported promotory effects of mevalonic acid on steroidal sapogenins synthesis in suspension cultures of fenugreek tissue, on MS (Murashige-Skoog) or Miller medium. Suspension cultures of *T. foenum-graecum* on culture medium containing different concentrations of cholesterol induced higher sapogenin contents compared medium without cholesterol by Brain and Williams [23]. Ramesh et al. [16] noted incresed trigonelline concentration (37%) from cultures containing 50 mg/l nicotinic acid compared to 31% trigonelline, obtained from culture with 1 mg/l nicotinic acid only.

Tsiri et al. [24] found that Isoflavonoid pterocarpans, like medicarpin, produced by leguminous plants in response to biotic or abiotic elicitation from either their glycosidic conjugate pools or by *de novo* synthesis. Intact fenugreek seedlings and cell suspension cultures were treated with $CuCl_2$ in order to check the origin of isoflavonoid pterocarpans, like medicarpin, medicarpin in response to copper elicitation. Accumulation of isoflavonoid aglycones and their glycosides were measured by using High Performance Liquid Chromatograpghy (HPLC) and their results showed the clear relationship between copper and *de novo* synthesis of medicarpin.

Callus culture

Joshi and Handler [25] reported nicotinic acid and Sadenosylmethionine conversion into trigonelline in the presence of Adenosine triphosphate (ATP) and $MgCl_2$ in cell-free extracts of root callus cultures. It was also noted that 3 to 4 times more trigonelline in callus cultures of *T. foenum-graecum* compared to seeds and 12-13 times more than roots and shoots. They also reported the stable productivity level upto eight months by successive subculturing of calli and cell suspensions. Eight-week-old callus cultures established from seeds on solid Revised Tobacco medium with 1 mg/l 2,4-D (2,4 Dicholorophenoxy Acetic Acid) was helpful in increasing trigonelline to 4.5 % and increased further to 5.25 and 5.01 % by adding 0.5 and 1.0 mg/l nicotinic acid respectively. Khanna and Jain [26] who induced higher steroidal contents from callus culture compared to seeds and produced diosgenin, gitogenin and tigogenin with other sterols of spirostane derivatives on agar solidified MS medium containing 1 mg/l 2,4-D. They noted that six week old cultures were more prone to the production of diosgenin, gitogenin and tigogenin, other sterols, high growth index and the total steroidal content compared to the seeds. Khanna et al. [27] also noted that suspension cultures of *T. foenum-graecum* with various concentrations of cholesterol increased sapogenin contents compared to medium without cholesterol.

Radwan and Kokate [28] obtained 15.6 mg/g (dry wt) of trigonelline from four-week-old callus cultures of *T. foenum-graecum*. The trigonelline concentration was found 3-4 folds more than seeds and 12-18 times more from roots and shoots of the parent plants. They subjected explants to shocked with high concentrations of 2,4-D, IAA (Indole Acetic Acid), IPA (indolepropionic acid), NAA (Naptahlene Acetic Acid), GA (Gibberellic Acid) and kinetin to determine

the role of these plant growth reulators on induction of callus. It was noted that 1 h shock was enough to stimulate growth of the calli. They also noted that 10 mg/l 2,4-D, IAA, IPA and NAA significantly increased the trigonelline content. Contrarily, treatment with 1 mg/l GA, and 2 mg/l kinetin were not effective. Ahmed et al. [29] studied optimum conditions for callus proliferation from different explants of fenugreek variety Giza-3. All combinations of 2,4-D and kinetin (0, 0.5, 1.0, 1.5 and 2 mg/l; 25 combinations for each explant) induced callus production. No callus formation was observed in the absence of 2,4-D. The best combination for callus induction in the different explants was 2 mg/l 2,4-D and 0.5 mg/l kinetin. Root explants of fenugreek were better in inducing callus compared to other explants. The roots induced maximum callus growth after fourth week of culture. Subcultures were repeated 12 times with 4 weeks interval. Maximum growth rate of tissues derived from leaves, stems and roots were 63.9, 64.6 and 71.9 mg/day after 10, 11 and 12 subcultures, respectively. Inverse relationship between dry weight and trigonellene content was found from subcultures 1-4 parallel thereafter. Trigonelline content of leaves, stems and roots in vivo were 0.45, 0.21 and 0.29 mg/g dry weight respectively, compared with 0.61, 0.30 and 0.40 mg/g dry weight in callus derived from these tissues

Oncina et al. [17] cultured leaf, stem and root calli on MS, White and Gamborg's B5 media along with coconut milk, malt extract and NAA for diosgenin production by callus cultures of *T. foenum-graecum*. Comparison among the three culture media showed that MS medium containing coconut milk (15 % v/v) and NAA (3×10^{-6} M) was found the best medium for callus growth from leaf explant stem or root explant. They achieved maximum diosgenin levels of 2.2 mg/g dry weight after 45 days on leaf calli followed by stem (0.74 mg/g) and root (0.60 mg/g). Prabakaran and Ravimycin [30] assessed chlorophyll pigment content in the cullus with different morphology and the *in vitro* regenerated plants. Total chlorophyll value was estimated as 2.7277 mg/g. The total protein content of *in vitro* regenerated plant was estimated as 0.789 mg/g fresh weight and callus was 0.421 mg/g fresh weight, estimated by Lowry's and acrylamide gel electrophoresis. The seeds of field-grown plant as well as green friable callus obtained using 2,4-D of field-grown plants showed maximum amount of protein content. They noted maximum peroxidase activity in green friable callus obtained from a combination of 2,4-D. Afsharie et al [31] studied different explants including stem segments, embryos, hypocotyls, etc. were cultured on two basal culture media viz. B5 and MS. Moreover, 2, 4-D, Kin, NAA, BAP and IBA were used as plant growth regulators. Their result showed that the medium containing 2 mg/l 2, 4-D in combination with 0.5 mg/l Kinetin found to be the best treatment for callus induction in both MS and B5 media. Combination of 2.5 mg/l BAP+0.5mg/l NAA was the best treatment for somatic embryogenesis in both basal media. Also, combination of 1.5 mg/l BAP+0.5 mg/l NAA was the best hormonal treatment to shoot regeneration in both basal media. According to the results, the treatment containing 1 mg/l IBA was optimum for root induction from regenerated shoots on MS medium.

Protoplast culture

Shekhawat and Galston [32] obtained high yields of mesophyll protoplasts after treatment of treating fenugreek leaves with with purified cellulase with 70 % plating efficiency under appropriate culture conditions. The protoplast-derived colonies developed into rapidly growing green calli and produced leafy shoots on a medium with

0.1 mg/l BAP and zeatin. Addition of glutamine and asparagine to the medium was found essential for rapid cell division, callus growth and differentiation. Christen [33] reported protoplast culture of fenugreek. They reported isolation of protoplasts from the root apices of 48h imbibed seeds. They noted first divisions of root fenugreek protoplasts from 3-4 day culture which resulted in cell colonies subsequently. However, the authors failed to regenrate shoots from these and noted development of a few roots on these colonies. Petropoulos [10], obtained shoot regeneration from fenugreek protoplasts obtained from the root apices of 48h imbibed seeds. The first divisions of protoplasts were observed from 3 day culture followed by cell colonies due to subsequent divisions resulted in roots only. 3-4 folds more trigonelline contents were scored from callus cultures compared to seeds and 12-13 times more than the roots and shoots.

Organogenesis

In vitro plant regeneration through cell suspension culture, callus and protoplast culture had been reported since 1945. But shoot regeneration directly through organogenesis was first reported by Khawar et al [34], who reported shoot regeneration from callus cultures of Fenugreek. But they could not induce roots on the *in vitro* cultured shoots. Later on, *in vitro* shoot regeneration was reported by Aasim et al. [35] by using apical meristem, and cotyledon leaf explants. They cultured explants on different concentrations of thidiazuran (TDZ) with or without Indole Butyric Acid (IBA). Shoot regeneration potential of apical meristem was found more than cotyledon leaf explants. Relatively more shoots were scored on MS medium containing TDZ used singly compared to the medium with TDZ+IBA. However, presence of IBA exerted positive effect on shoot length.

In another study, Aasim et al. [36] cultured 8-10 days old *in vitro* grown cotyledon node explants on MS medium containg 0.05-0.80 mg/l kinetin, 0.25-1.0 mg/l BAP+0, 0.20 mg/l NAA or 0.05-0.80 mg/l TDZ+0, 0.10 mg/l IBA solidified by using 0.22% gelrite. Explant responded well to TDZ with or without IBA and resulted in maximum shoot regeneration and shoots per explant. Maximum number of 22.21 shoots per explant were scored from MS medium with 0.40 mg/l TDZ. However, longer shoots were obtained in the presence of auxins in the culture medium. Thereafter, they transferred the shoot to rooting media containing 0.1 - 1.0 mg/l IBA or NAA. In both studies, they failed to induce rooting and got multiple rooting from single plant in the IBA rooting medium. Prabakaran and Ravimycin [29] micropropagated *T. foenum-graecum* L and biochemical analysis of compounds present in the callus as well as in the *in vitro* plant using cotyloden, hypocotyls and shoot tip epicotyls. They induced shoot regeneration using shoot tips explants on MS medium with basal callus formation:

GENETIC TRANSFORMATION

Agrobacterium tumefaciens are most commoly used for introducing foreign genes into plant cells for transgenic plants production [37]. However, there are very few reports available on the genetic transformation of fenugreek using A*grobacterium*. However, no report is available on the genetic transformation using other techniques like proptoplast or biolistic mediated transformation. First report on genetic transformation was reported by Merkli et al. [38] by using *Agrobacterium rhizogenes* strain A4 for diosgenin. They used callus cultures by using leaves, stems and roots from 30 day old seedlings. Reid et

al. [39] reported fenugreek GMGT (galactomannan galactosyltransferase) that express constitutively in membrane-bound form have endosperm galactomannans with increased average degrees of Gal substitution (Man/Gal about 10 in T_1 generation seeds and about 7.5 in T_2 generation seeds) in seeds of transgenic tobacco lines. Khawar et al. [40] reported genetic transformation of fenugreek using A281 oncogenic strain of *Agrobacterium tumefaciens* to induce tumor on root, cotyledon and hypocotyl explants excised from 1-week-old seedlings, which showed that the plant was highly susceptible to transformation. Tumors (calli) were selected on 50 mg dm^{-3} kanamycin. They were analyzed for β-glucuronidase (GUS) expression. Presence of *uidA* (*gus*) gene, was confirmed by polymerase chain reaction (PCR) amplification.

Rahele et al [41] used different Agrobacterium rhizogenes strains (A4, 9126 and 15834) and infection methods (co-coltivated and injection) to investigate the ability for transformation and production of trigonellin in *T. foenum-graecum* . PCR analysis using rolb gene was used for the identification of transformed hairy roots. The transformation efficiency was 26 % in injection method for whole plants. The higher growth index was obtained in hairy roots induced by two strains, 15834 and 9126, in Zanjan and Borazjan masses, respectively. Detection and identification of trigonellin for a 28 days period was done by high performance liquid chromatography method. The highest amounts of trigonellin in Borazjan and Zanjan hairy roots were 14.89 and 14.03 mM g-1 DW after 28 and 7 days, respectively.

MOLECULAR CHARACTERIZATION

Harish et al. [42] estimated genetic variability among the 10 accessions of fenugreek-*T. foenum-graecum* repository of National Bureau of Plant Genetic Resources at Jodhpur Station, using Random Amplification of Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeat (ISSR) genetic markers. Multilocus genotyping by 10 SPAR (single primer amplification reaction-RAPD and ISSR) primers revealed intraspecific polymorphism in banding patterns. The referenced tree generated after 100 resampling via bootstrap method showed that accession IC-373449, IC-396616 and IC-448830 are distantly placed. Two major clusters were formed from rest of the accessions, one having four accessions viz. IC-448828, IC-448832, IC-448833 and IC-448834 and other having three accessions viz. IC-448835, IC-448837 and IC-396625. Diosgenin and gum content were also estimated. They observed that the study can be useful for intraspecific crosses designing between cultivars of these fenugreek collections or in seed spices breeding programme.

CONCLUSION

Review of literature shows that nothing concurrent has been determined emphasising more and persistanet efforts to improve fenugreeks through biotechnological approaches in addition to traditional plant breeding tools. There is need to use the identified metabolites through computer aided drug designing; quantitative structure activity relationship and structure-based drug designing etc.

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