“IDENTIFICATION OF FACTORS RESPONSIBLE FOR SAPONIN PRODUCTION AND ACCUMULATION IN FENUGREEK SEEDS FOR COMMERCIAL PRODUCTION”

SUBMITTED TO

KADI SARVA VISHWAVIDYALAYA, GANDHINAGAR, GUJARAT, INDIA

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IN

BIOTECHNOLOGY

BY

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UNDER THE SUPERVISION OF

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SYNOPSIS OF Ph.D. THESIS ENTITLED

“IDENTIFICATION OF FACTORS RESPONSIBLE FOR SAPONIN PRODUCTION AND ACCUMULATION IN FENUGREEK SEEDS FOR COMMERCIAL PRODUCTION”

SUBMITTED TO: KADI SARVA VISHWAVIDYALAYA, GANDHINAGAR, GUJARAT, INDIA.

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“IDENTIFICATION OF FACTORS RESPONSIBLE FOR SAPONIN PRODUCTION AND ACCUMULATION IN FENUGREEK SEEDS FOR COMMERCIAL PRODUCTION”

Introduction

*Trigonella foenum-graecum* (Fenugreek, vern. / gujarati : Methi) is dicot plant belonging to family Fabaceae (earlier termed as family Leguminaceae & subfamily Papilionaceae. It is widely used in medicinal applications all over the world. The golden yellow seeds (Acharya, 2008) of Fenugreek have been used extensively in Indian Ayurveda and Chinese medicine for treatment of epilepsy, paralysis, gout, dropsy, chronic cough, diabetes, piles, sinus, and lung congestion, inflammation, infection mitigation, hair treatment, breast enhancement, lactation stimulant, anticancer, anti-fertility, anti-microbial, virucidal, insecticidal, anti-parasitic, hypocholesterolaemic, hyperglycaemia and aphrodisiac effects [ (Mullaicharam, 2013), (Leela, 2008), (Gopu, 2008), (Habori, 2002), (Francis, 2002), (Tiran, 2003), (Broca, 2004), (Devi, 2003)]. Fenugreek is a source of various saponins (Harsha, 2012) that are considered as a part of the plant defense mechanism. Saponins are glycosylated titerpenes and steroids, having properties similar to detergent to disrupt cell walls (Freeman, 2008), (Gonzalez, 2009)]. Fenugreek was reported to contain 81 phytonutrients and one of the most bio active compound Diosgenin [(25R)-5-spirosten-3h-ol] (Aasim, 2010).

Diosgenin is used as precursor for the synthesis of steroidal drugs and hormones such as testosterone, glucocorticoids, and progesterone. It is also found to have anticancer activity and delays skin aging at the time of climacteric [(Agarwal, 2015), (Lee, 2007), (Tada, 2009), (Yan, 2009)]. Russell Marker had developed semisynthetic process of progesterone from Diosgenin in 1940. Diosgenin has estrogenic activity and is used as precursor for the industrial scale synthesis of steroidal drugs and hormones like Progesterone and Norethisterone. Progesteron along with estrogen is used for hormone replacement therapy the treatment of menopausal issues, a $3.7 billion market in only US. Diosgenin is also a medicine for cardiovascular diseases, cancers and contraception [ (Katy, 2007), (Qin, 1997), (Liu, 1993), (Aradhna, 1992), (Rancis, 2014)]. Currently, Mexican yams (Dioscorea spp.) are used for Diosgenin production, though it takes years to grow the yam tuber to a stage that it contains sufficient quantity of diosgenin to be used for pharmaceutical purpose (Rosser, 1985). Fenugreek can be a better option for this because of
shorter growth cycle and low cost of production [(Hardman, 1969), (Petropoulos, 1973)]. It was found that maximum amount of Diosgenin naturally present in Fenugreek in young leaves amounts to 20 mg g–1 dry weight and in mature seeds it ranges from 0.28 to 0.92%. For using Fenugreek as a source of Diosgenin production for pharma industry, it is essential to improve Fenugreek varieties by triggering the cascade of genes responsible for Diosgenin production. The endogenous signals to activate defense mechanism in plants through elicitors have provided the freedom to the researchers to avoid the use of fungal or herbivore stresses. Methyl jasmonate (MeJA), a volatile methyl ester of the chemical jasmonic acid, a plant growth regulator, was first isolated elicitor from a plant pathogenic fungus *Lasiodiplodia* (Botyodiplodia) *theobromae* as plant growth inhibitor and the role of JA in elicitor-induced signal transduction was first described by Gundlach *et al.* in 1992. [(Gundlach, 1992), (Paul, 1992), (Sembdner, 1993)]. Exogenously applied Jasmonic acid, a metabolite of 13-hydroxylinolenic acid, is a plant hormone reported to increase the accumulation of metabolites related to plant defense mechanism [(Acharya, 2008), (Aldridge, 1971), (Gundlach, 1992), (Wasternack, 1997), (Morrisey, 1999), (Chen, 2012)]. MeJA treatment triggers cascade of intracellular signals and also activates de novo transcription of genes like phenylalanine ammonia lyase which are the part of chemical defense mechanism of plants, which ultimately leads to accumulation of secondary metabolites (Heidrun, 1992).

Figure 1: Proposed diosgenin biosynthesis pathway, (Dashed lines indicates multiple steps involved in the pathway)
In plant system, the pathways for the synthesis of secondary metabolites were not studied in detail so it is difficult to identify and trigger correct gene for increasing the content of any particular compound. MeJA treatment elicits the production of HMG-co-A reductase gene which regulates a rate limiting step between two important plant metabolic pathways namely glycolysis and mevalonate pathways and plays a critical role in controlling isoprenoid derivatives related pathways (Chen 2011). In humans, all the pathways are well studied and as human and plants both are the eukaryotes, we have hypothesized the same pathway of human into the plants. Based on our Fenugreek transcriptome study published earlier, we have proposed a pathway for the production of secondary metabolites by combining three pathways, namely glycolytic pathway, mevalonate pathway, and steroid biosynthesis pathway (figure 1). By combining the aforesaid pathways, we have proposed a hypothesis that diosgenin may be formed from squalene 2,3-oxide in two ways, (i) from lanosterol via the formation of cholesterol and (ii) from cycloartenol via the formation of sitosterol. Mehrafarin et al., have tried to explain the biosynthesis of sapogenins from cholesterol but all the steps were not defined completely (Mehrafarin, 2010).

The aim of the present study was to increase the content of Diosgenin produced in the Fenugreek cultivars by triggering defense mechanism through biotic or abiotic stresses. The present study focuses on increasing the naturally occurring diosgenin content in fenugreek plant by various treatments including Gibberellic acid (GA3), ethephon, composts, vermin compost, cakes, protein hydrolysates, nickel and elicitors to increase the overall growth of Fenugreek and finally the yield of seeds and saponins in an attempt to obtain high Diosgenin yields. Attempt has been made to develop a green commercializable procedure for extraction of saponin and to carry out comparative gene expression analysis of saponin synthesis pathway in response to various treatments to establish a marker gene.

**Cultivation of Fenugreek**

Seeds of five fenugreek varieties Gujarat Methi-2 (GM-2), Kasuri, Pusa early branching (PEB), Rajasthan Methi (RMT-1) and Maharashtra methi-5 (MMT-5) were sterilized using sodium hypochlorite solution (4% w/V Merck, UN1791) for 10 minutes. Sterilized seeds were soaked in autoclaved water in petri-dishes for 48 hours in dark for sprouting process. Out of these five varieties, Kasuri variety is different from others, its seeds as well as leaves are comparatively smaller in size. The germinated seeds were transferred to the pots filled with autoclaved soil.
Effect of compost, growth hormones and nickel on growth and yield of fenugreek

Farm soil from Ahmedabad was collected, mashed manually to very fine powder, passed through sieve and autoclaved for about 1 hour to eliminate the microbial contaminants. Approximately, 200g of soil was then transferred to plastic pots of 6 cm dia. at the bottom and 10 cm dia. at the top and 15 cm of depth. 30 sprouted seeds were transferred to each pot and grown for 6 days in sterile room with light:dark cycle of 10:14 hours at 25°C temperature with the relative humidity around 36-40% without any treatment. The seedlings were then treated with two different concentrations of vermicompost, ethephon, GA3 and nickel which were 10^{-4} M and 10^{-5} M GA3, 25ppm & 50ppm ethephon, 10g/kg & 20g/kg Vermicompost and 20mg/kg & 40mg/kg Nickle, selected on the bases of the literature survey and published data.

Table 2: Effects of amendments on the height of one month old fenugreek plants. (Data represent the mean value ± SD (n = 3))

<table>
<thead>
<tr>
<th>Name of varieties</th>
<th>Control</th>
<th>10-4M</th>
<th>10-5M</th>
<th>25ppm</th>
<th>50ppm</th>
<th>10g/kg</th>
<th>20g/kg</th>
<th>20mg/kg</th>
<th>40mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMT-1</td>
<td>18.5±.2</td>
<td>19.5±.3</td>
<td>20.5±.2</td>
<td>20.7±.2</td>
<td>18.5±.2</td>
<td>18.9±.3</td>
<td>20.4±.4</td>
<td>20.7±.2</td>
<td>21.8±.3</td>
</tr>
<tr>
<td>GM-2</td>
<td>19±.3</td>
<td>20.5±.4</td>
<td>21.8±.3</td>
<td>21.5±.4</td>
<td>19.2±.3</td>
<td>20.8±.4</td>
<td>22.5±.3</td>
<td>20.2±.4</td>
<td>22.5±.3</td>
</tr>
<tr>
<td>Kasuri-1</td>
<td>13.5±.2</td>
<td>13.5±.2</td>
<td>14.8±.2</td>
<td>15.2±.2</td>
<td>14.7±.2</td>
<td>14.2±.2</td>
<td>15.0±.2</td>
<td>13.8±.3</td>
<td>14.5±.2</td>
</tr>
<tr>
<td>Kasuri-2</td>
<td>11.2±.5</td>
<td>10.9±.2</td>
<td>11.5±.3</td>
<td>11.9±.2</td>
<td>11.1±.2</td>
<td>11.3±.3</td>
<td>11.8±.2</td>
<td>11.1±.2</td>
<td>11.3±.3</td>
</tr>
<tr>
<td>PEB</td>
<td>18.2±.2</td>
<td>18.7±.3</td>
<td>20.3±.4</td>
<td>20.4±.3</td>
<td>18.6±.4</td>
<td>18.5±.3</td>
<td>20.9±.4</td>
<td>19.8±.4</td>
<td>21.8±.2</td>
</tr>
<tr>
<td>MMT-5</td>
<td>18.9±.3</td>
<td>18.9±.2</td>
<td>20.5±.4</td>
<td>21.1±.2</td>
<td>19.6±.3</td>
<td>18.2±.1</td>
<td>19.0±.2</td>
<td>20.8±.4</td>
<td>21.7±.4</td>
</tr>
</tbody>
</table>

Table 3: Effects of amendments on the fresh weight of one month old fenugreek plants. (Data represent the mean value ± SD (n = 3))

<table>
<thead>
<tr>
<th>Name of varieties</th>
<th>Control</th>
<th>10-4M</th>
<th>10-5M</th>
<th>25ppm</th>
<th>50ppm</th>
<th>10g/kg</th>
<th>20g/kg</th>
<th>20mg/kg</th>
<th>40mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMT-1</td>
<td>650±5</td>
<td>640±5</td>
<td>690±4</td>
<td>680±4</td>
<td>640±5</td>
<td>610±6</td>
<td>670±4</td>
<td>640±4</td>
<td>680±5</td>
</tr>
<tr>
<td>GM-2</td>
<td>720±7</td>
<td>710±4</td>
<td>750±5</td>
<td>790±5</td>
<td>710±6</td>
<td>710±4</td>
<td>750±5</td>
<td>700±5</td>
<td>740±4</td>
</tr>
<tr>
<td>Kasuri-1</td>
<td>380±4</td>
<td>350±4</td>
<td>420±4</td>
<td>410±6</td>
<td>390±3</td>
<td>370±5</td>
<td>390±4</td>
<td>350±3</td>
<td>420±3</td>
</tr>
<tr>
<td>Kasuri-2</td>
<td>375±3</td>
<td>370±5</td>
<td>380±4</td>
<td>379±8</td>
<td>370±4</td>
<td>369±5</td>
<td>382±6</td>
<td>370±4</td>
<td>375±4</td>
</tr>
<tr>
<td>PEB</td>
<td>770±5</td>
<td>750±5</td>
<td>790±7</td>
<td>810±4</td>
<td>760±4</td>
<td>750±4</td>
<td>790±6</td>
<td>740±4</td>
<td>810±5</td>
</tr>
<tr>
<td>MMT-5</td>
<td>690±6</td>
<td>670±6</td>
<td>720±4</td>
<td>710±5</td>
<td>680±5</td>
<td>650±6</td>
<td>710±5</td>
<td>660±5</td>
<td>710±6</td>
</tr>
</tbody>
</table>
Table 4: Effects of amendments on the dry weight of one month old fenugreek plants. (Data represent the mean value ± SD (n = 3))

<table>
<thead>
<tr>
<th>Name of varieties</th>
<th>GA3 Control</th>
<th>GA3 10⁻⁷M</th>
<th>GA3 10⁻⁵M</th>
<th>Etaphon 25ppm</th>
<th>Etaphon 50ppm</th>
<th>Vermicompost 10g/kg</th>
<th>Vermicompost 20g/kg</th>
<th>Nickle 20mg/kg</th>
<th>Nickle 40mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMT-1</td>
<td>380±4</td>
<td>370±4</td>
<td>390±4</td>
<td>395±4</td>
<td>381±4</td>
<td>382±4</td>
<td>397±4</td>
<td>375±4</td>
<td>387±4</td>
</tr>
<tr>
<td>GM-2</td>
<td>400±5</td>
<td>395±5</td>
<td>410±6</td>
<td>415±5</td>
<td>390±5</td>
<td>405±2</td>
<td>410±3</td>
<td>398±3</td>
<td>410±4</td>
</tr>
<tr>
<td>Kasuri-1</td>
<td>60±2</td>
<td>55±3</td>
<td>67±3</td>
<td>68±4</td>
<td>55±2</td>
<td>62±2</td>
<td>68±2</td>
<td>61±4</td>
<td>68±3</td>
</tr>
<tr>
<td>Kasuri-2</td>
<td>58±3</td>
<td>59±4</td>
<td>62±3</td>
<td>63±4</td>
<td>67±3</td>
<td>60±3</td>
<td>63±4</td>
<td>65±4</td>
<td>59±4</td>
</tr>
<tr>
<td>PEB</td>
<td>405±4</td>
<td>398±4</td>
<td>410±4</td>
<td>420±5</td>
<td>410±5</td>
<td>407±4</td>
<td>413±5</td>
<td>400±4</td>
<td>410±4</td>
</tr>
<tr>
<td>MMT-5</td>
<td>390±5</td>
<td>388±6</td>
<td>408±5</td>
<td>398±4</td>
<td>385±4</td>
<td>395±4</td>
<td>405±4</td>
<td>390±3</td>
<td>400±4</td>
</tr>
</tbody>
</table>

Table 5: Effects of amendments on the diosgenin yield of one month old fenugreek plants.

<table>
<thead>
<tr>
<th>Name of varieties</th>
<th>GA3 Control</th>
<th>GA3 10⁻⁷M</th>
<th>GA3 10⁻⁵M</th>
<th>Etaphon 25ppm</th>
<th>Etaphon 50ppm</th>
<th>Vermicompost 10g/kg</th>
<th>Vermicompost 20g/kg</th>
<th>Nickle 20mg/kg</th>
<th>Nickle 40mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMT-1</td>
<td>0.85</td>
<td>0.9</td>
<td>1.54</td>
<td>1.55</td>
<td>1.21</td>
<td>1.12</td>
<td>1.31</td>
<td>0.95</td>
<td>1.27</td>
</tr>
<tr>
<td>GM-2</td>
<td>0.9</td>
<td>0.95</td>
<td>1.65</td>
<td>1.75</td>
<td>1.3</td>
<td>1.32</td>
<td>1.5</td>
<td>1.32</td>
<td>1.45</td>
</tr>
<tr>
<td>Kasuri-1</td>
<td>0.65</td>
<td>0.8</td>
<td>1.24</td>
<td>1.33</td>
<td>1.14</td>
<td>1.33</td>
<td>1.28</td>
<td>0.91</td>
<td>1.22</td>
</tr>
<tr>
<td>Kasuri-2</td>
<td>0.55</td>
<td>0.65</td>
<td>1.15</td>
<td>1.25</td>
<td>1.12</td>
<td>1.23</td>
<td>1.23</td>
<td>0.83</td>
<td>1.15</td>
</tr>
<tr>
<td>PEB</td>
<td>0.75</td>
<td>0.88</td>
<td>1.57</td>
<td>1.62</td>
<td>1.28</td>
<td>1.22</td>
<td>1.33</td>
<td>0.98</td>
<td>1.32</td>
</tr>
<tr>
<td>MMT-5</td>
<td>0.75</td>
<td>0.82</td>
<td>1.46</td>
<td>1.52</td>
<td>1.08</td>
<td>0.94</td>
<td>1.3</td>
<td>0.94</td>
<td>1.23</td>
</tr>
</tbody>
</table>

Effects of amendments on fenugreek plant growth and diosgenin yield

Data on plant height, number of leaves, leaf diameter, leaf length and fresh weight and and dry weight of plant and dry weight of plant was collected after 4 weeks of growth. Data of plant height, fresh weight, dry weight and diosgenin yield is summarized in table 2, 3, 4 and 5.

GA3 was found most effective at 10⁻⁵M concentration in all fenugreek varieties. Diosgenin yield was found highest in GM2 variety was 1.65% which is 183% higher then control and lowest in Kasuri-1 variety was 1.15%. For the rest of the variety, diosgenin yield obtained was 1.57%,
1.54%, 1.46%, 1.24%, 1.15% PEB, RMT-1, MMT-5, Kasuri-1 and Kasuri-2 respectively. In comparison analysis of GA3 treatment among all the varieties of fenugreek, GM2 variety yielded highest amount of diosgenin and growth in terms of fresh and dry weight followed by PEB, RMT-1, MMT-5, Kasuri-1 and Kasuri-2.

In case of treatment with ethephon, optimum results were obtained at 25ppm treatment in all the varieties. Maximum diosgenin yield 1.75 % was obtained in GM2 variety, which was 194% higher than control. In other varieties, diosgenin yield obtained was 1.62%, 1.55%, 1.52%, 1.33%, 1.25% in PEB, RMT-1, MMT-5, Kasuri-1 and Kasuri-2 respectively. After the analysis of growth along with diosgenin yield for ethephon treatment, optimum results were observed in GM2 variety followed by PEB, RMT-1, MMT-5, Kasuri-1 and Kasuri-2.

In reference to treatment of fenugreek plants with vermicompost, optimum results were obtained at 20g/kg concentration in all the varieties. Maximum yield of diosgenin was obtained 1.50% in GM2 variety which was 166% higher than control followed by 1.33%, 1.31%, 1.30%, 1.28% and 1.23% in PEB, RMT-1, MMT-5, Kasuri-1 and Kasuri-2 respectively.

Nickle treatment has given optimum results at 40mg/kg (of soil) concentration in all the fenugreek varieties. Diosgenin yield was found highest in GM2 variety was 1.45% which was 161% higher than control followed by 1.32%, 1.27%, 1.23%, 1.22% and 1.15% in PEB, RMT-1, MMT-5, Kasuri-1 and Kasuri-2 respectively.

**Statistical analysis**
Statistical analysis between control and experimental treatment of the data was analyzed by one way analysis of variance (ANOVA) with post-hoc Tukey HSD Test Calculator. The P-value for the experiment is <0.05 considered significant. ANOVA stasical test performed for 6 independent experiments and one control experiment. The probability of these result (P-value) corresponding to the f-statistics of one-way ANOVA is lower than 0.05, suggesting that the one or more treatments are significantly different. The data presented in table 2, 3, 4 and 5 regarding the effects of the amendments on the fenugreek plant height, fresh weight, dry weight and diosgenin yield in all the experimental plants against the control plants for statistical analysis using the aforementioned test. Data of the plant height, fresh and dry weight presented in tables 2, 3 and 4 respectively was analyzed using statistical analysis with ANOVA test concluded that it is not significant (where P-value was considered 0.05%). It means that amendments described here
were not imparted significant effect on the plant height, fresh and dry weight which is natural as these all were phenotypic data of one month old plants and it may be natural that no much difference can be observed in the phenotypic characteristics of one month old plants. ANOVA results of the table 5, represented significant difference in the data of diosgenin yield in the control and experimental plants where P-value was considered 0.01% which proves that the hypothesis about the effect of amendments on the diosgenin yield is correct.

**Experimental design**

The seedlings of six Fenugreek varieties Gujarat Methi-1 (GM-1), Kasuri-1 and Kasuri-2, Pusa early branching (PEB), Rajasthan Methi (RMT-1) and Maharashtra methi-5 (MMT-5) were treated with various concentrations of MeJA (0.005%, 0.01%, 0.02%, 0.03%, 0.05% and 0.1%) prepared in ethanol on seventh and ninth day. Total of 36 experiments and 6 control pots were grown for 7 days, aforesaid prepared concentration of methyl jasmonate solution was dissolved in 10 ml water and applied in the soil covering the roots of seedlings on 7th day and 9th day. All the seedlings control as well as experimental seedlings were harvested along with roots on 11th day. Harvested seedlings were washed thoroughly with autoclaved water to remove soil from the roots and were stored immediately in the -80°C freezer. From each experiments, 100mg of seedlings were used for RNA extraction and rest were used for diosgenin extraction and analysis.

**Diosgenin extraction: Diosgenin extraction and analysis**

Diosgenin extraction and estimation was done by using a newly developed protocol with few modifications in the method described by Trivedi et al., 2007. Briefly, dried Fenugreek seedlings were ground to fine powder using liquid nitrogen and re-fluxed with 50ml of 2.5 M ethanol sulfuric acid at 80°C for 4 hours. The solution was then filtered using whatman filter paper no.1, diluted with 50ml double distilled water and extracted with 50 ml of n-hexane 3 times which were then pooled and evaporated to dryness at room temperature. Dry residues were dissolved in 25ml mobile phase (acetonitrile:water-90:10) and filtered through 0.22μm filter before HPLC analysis. For HPLC analysis Hypersil ODS C18, 5 μm, 250 x 4.6 mm (Thermo scientific) was used at flow rate of 1ml per minute with mobile phase (acetonitrile: water-90:10) and at 35°C temperature for 30 minutes. 1 mg per ml of Diosgenin (D1634, SIGMA) was used as standard (Trivedi et al., 2007).
Gene Expression study

Primer designing

Two very important genes of metabolic pathways which are 3-hydroxy-3-methylglutaryl-CoA reductase (HMG) and sterol-3-Beta Glucosyl transferase (STRL), were selected as target genes for the current study. HMGR is a key regulatory enzyme of mevalonate pathway it catalyzes the irreversible conversion of 3-hydroxy-3-methylglutaryl co-enzyme A to mevalonate. It has been studied and proven that the HMG is an important rate limiting enzyme for isoprenoid biosynthesis in plants, expression of this enzyme 191 increases the total sterol accumulation by 2 fold and cycloartenol accumulation by 100 fold (Joseph, 1995). In proposed hypothesis, sterol-3-Beta Glucosyl transferase is responsible for the synthesis of sterol 3-β-D194 glucoside (saponin) from sitosterol which is then converted to diosgenin. Primers for the two target genes HMG and STRL were designed from the whole transcriptome data of Gujarat methi variety published by our lab (Kanak, 2013). Proposed diosgenin biosynthesis pathway for fenugreek is given in figure 3. Primer 3 plus software was used for primer designing and designed primers were synthesized in primex facility at Xcelris labs. Primer efficiency of genes and their relative expression was calculated using relative expression software tool (Michael, 2002).

RNA extraction, cDNA preparation and gene expression

Seedlings treated with MeJA were stored in TMS RNA stabilizer (XGtms- 100) solution at -80°C for RNA isolation and gene expression studies. The frozen seedlings collected from all 42 pots including 36 experimental and 6 control pots were subjected to RNA isolation. 50 mg of frozen seedlings were taken for RNA isolation using Xcelgen plant RNA miniprep kit (XG6611-01) following manufacturer’s instructions. The quantity of isolated total RNA was determined by absorbance ratio A260/280, A260/230 on nanodrop spectrophotometer 8000 (Thermo scientific) and quality of total RNA was analysed on 1% denaturing agarose gel and Agilent Bioanalyzer 2100 using the RNA 6000 pico chip (Agilent Technologies, Santa clara, Palo Alto, CA, USA). 500ng of total RNA was then subjected to reverse transcription reaction for first strand cDNA synthesis using M-MLV reverse transcriptase enzyme (XG02032). For preparing first strand cDNA, 500ng of total RNA with 0.5 μl RNase inhibitor was used with oligo dT primers. Reaction mixture used for cDNA synthesis contained 500-1000ng of total RNA, 10mM oligo-dt primer, 0.5 μl RNAses inhibitor and 10mM dNTPs and the reaction was incubated at 65°C for RNA denaturation. After this incubation, 200 units of M-MLV enzyme, reverse transcriptase buffer and 50mM dithiothreitol were added to the reaction and subjected to reverse transcription using three temperatures 25° for 10 minutes, 40° for 60 minutes and 70° for 10 minutes. First
strand cDNA was quantified using nanodrop spectrophotometer and 100 ng was used for gene expression reaction. For standardization, polymerase chain reaction was set up in a gradient thermal cycler (Applied Biosystems Verity 96 well thermal cycler, 0.2 ml) followed by analysis of the amplicons on 2% agarose gel. After standardization of the PCR conditions, same conditions were used for gene expression assay using Light cycler 480II instrument. For gene expression assay, the reaction mixture used containing 1X SYBR green master mix (Light cycler 480 SYBR green Imaster (04707516001), 0.5pM of each forward and reverse primers, 100ng of first strand cDNA, and 3 µl of nuclease free water (total volume 10 µL). All the experiments were performed in triplicate and average CP value was considered for the calculation of gene expression results by basic relative quantification method.

Results

Table 6: Data of the fold change in the expression of HMG and STRL genes and diosgenin yield by HPLC calculation in all the varieties control vs experiment. Data found significant for 0.01% MeJA treatment ( P <0.05) calculated by ANOVA with post-hoc Tukey HSD Test Calculator.
<table>
<thead>
<tr>
<th></th>
<th>HMG</th>
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<td></td>
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<td>1.19</td>
<td>1.01</td>
<td>0.97</td>
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<td>0.38</td>
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</table>

Figure 2: Effect of MeJA treatment on the genes (HMG and STRL) responsible for Diosgenin bio synthesis and diosgenin yield

MeJA is most effective for increasing the diosgenin content in Fenugreek varieties at 100µL/L concentration. It elicits the expression of HMG gene and in turn up-regulates the mevalonate pathway and steroid biosynthesis pathway. In this study our hypothesis for the production of diosgenin from cycloartenol via the formation of sitosterol was proved. This method can increase the temporal diosgenin production in fenugreek from 0.6-0.9% (naturally occurring) to 1-1.8% in
controlled environment in a cost effective manner, it can also be scaled up and hence it can be a viable option for commercial production of diosgenin from fenugreek plants.

Summary
The aim of the present study was to identify the factors responsible for the saponin production in different varieties of fenugreek plant so that it can be increased. Saponins are glycosylated titerpenes and steroids having anti virucidal, anti fungal and anti microbial activity. Saponins are well studied as a part of plant defence mechanism, so for increasing their content we have to activate the plant defence mechanism. Diosgenin is a steroidal saponin and is the most important bio active compound which is often used as raw precursor for the production of steroidal drugs and around 400 hormones. We have developed the method to increase the natural occurrence of diosgenin in fenugreek plant by treating it with specific concentration of methyl jasmonate. Methyl jasmonate treatment triggers cascade of intracellular signals and also activates de-novo transcription of genes like phenylalanine ammonia lyase which are the part of chemical defence mechanism of plants which results in the increase content of diosgenin. Apart from this, we have also developed the method to increase the plant growth so that maximum yield can be obtained form less cultivation. To solve this purpose two plant growth hormones GA3 and ethephon, vemicopmost and a micro nutrient - nickle was selected at two different concentrations. Optimum growth was observed by treatment with GA3 at $10^{-5}$ M and ethephon at 25ppm. To conclude, this study had provided the method for increasing diosgenin content and fenugreek plant growth. Till date, yams were used for commercial production of diosgenin, this method has given a new alternative to the steroid synthesis and diosgenin industry a natural source in cost and time effective manner. It will be a great benefit to steroid industry and to the human health sector also as the steroidal drugs can be produced at comparatively low price.

Application and Future prospective of the study

Results obtained in the present study reveals that, MeJA can be the most effective elicitor at 0.01% concentration for increasing diosgenin content in fenugreek plants for industrial production. If we calculate the same for applying on the field it requires 12.44ml of 0.01% MeJA means 1.24 µl/L MeJA solution per meter square area which costs 0.60 ₹. If we calculate the cost per hectare it requires 12.4 ml of MeJA per hectare means 6000₹ cost. As per the information available on the website of “Spices board India” ideal sowing conditions for fenugreek is 10 cm apart from other plant and 30 cm spacing among rows which requires 10-15 kg seeds per hectare
and yields 1800-2000 kg seeds per hectare (www.indianspices.com). If we calculate economically, fenugreek seed prices is $80 per kg, so it requires $1200 per hectare which would yield $160000 of seeds. Price of around 93% pure diosgenin is $52114 per 100g (Cat no. D1634 SIGMA). As per the present study if 1.8% diosgenin can be obtained from fenugreek, if the yield is 1800-2000 kg seeds per hectare it would give 32.4 to 36 kg diosgenin per hectare. To conclude if fenugreek is grown in 1 hectare area of land, and treated with 0.01% of MeJA it will require $1200 (18.72$) of seeds and $6000 (93.60$) of MeJA which would yield the diosgenin worth $1,50,00,000 (234009 $) (For converting INR to USD 64.10$ per USD was considered as on 13th June, 2015). This can help to bring a break through and can give a new era to the steroid industry.

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