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Optimization of *in vitro* culture conditions for accumulation of diosgenin by fenugreek

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Abstract
Fenugreek as a spice, vegetable and medicinal plant has valuable nutritional properties and is important for diversification of human diet. The most important medicinal properties of this herb are related with the content of diosgenin. Selection of media for optimal and stable production of diosgenin level in plants grown *in vitro* is prerequisite for understanding the biochemical pathway of diosgenin biosynthesis. Changes of concentration of this secondary metabolite in various plant organs during growth are also critical for sampling and isolation procedure. We found that, the concentration of diosgenin depends on the day of harvest, type of medium and plant organ. The highest content of diosgenin in leaves occurs between 21st and 38th day of growth. These results suggest a positive influence of carbohydrates on the efficiency of diosgenin synthesis. The highest content of diosgenin was produced by leaves when compared to stems, roots and callus culture.

Keywords: *Trigonella foenum-graecum,* fenugreek, diosgenin, Murashige and Skoog medium, woody plant medium,

Abbreviations:
MS – Murashige and Skoog medium
WPM – Woody Plant Medium
½ WPM – half-strength Woody Plant Medium
MRM – multiple reaction monitoring
FM – Fresh Mass
DM – Dry Mass
DDM – Diosgenin content in Dry Mass
DFM – Diosgenin content in Fresh Mass
TM – Tissue Moisture

1. Introduction
Fenugreek (*Trigonella foenum-graecum* L.) is widely distributed throughout the world wild or cultivated plant in the Fabaceae family [1]. Fenugreek is commonly used as a traditional food and medicine and is a rich source of steroidal sapogenins, mainly diosgenin that has anti-inflammatory activity and is used for the treatment of leukemia, hypercholesterolemia, climacteric syndrome and colon cancer [2].
Diosgenin is of great interest to the pharmaceutical industry because of its oestrogenic effect on the mammary gland. It also plays an important role in the control of cholesterol metabolism, is responsible for changes in the lipoxygenase activity of human erythroleukemia cells, and for morphological and biochemical changes in megakaryocyte cells [3]. The anticancer activity of diosgenin has been proved, as it affects many molecular candidates critical to tumorigenesis [4]. *In vitro*, this compound contributed to apoptosis of several cancer cell lines (ie. colon HT-29 and HCT-116, breast AU565 and skin M4Beu). Diosgenin is also a substrate for synthesis of oral contraceptives, sex hormones and other steroids [2].
Selection of media for optimal and stable production of diosgenin level in plants grown *in vitro* is prerequisite for effective modulation of the level of this compound and further understanding the biochemical pathway of diosgenin biosynthesis. Changes of concentration of this secondary metabolite in various plant organs during growth are also critical for sampling and isolation procedure. In this report, we describe changes of diosgenin content in fenugreek seedlings grown for up to 40 days on Murashige and Skoog (MS), WPM and half-strength WPM. The level of diosgenin in callus cultures and plant organs maintained *in vitro* was also compared.

2. Materials and Methods

2.1 Plant material

Experiment 1

Seeds of *Trigonella foenum-graecum* (accession no. 19271) were obtained from Botanic Garden in Bonn (Germany). Seeds were sterilized by soaking in 70% ethanol for 2 min and then in a 20% v/v Domestos® solution for 30 min. They were rinsed several times in sterile water before being implanted on solid MS [5] pH 5.65 containing 3% sucrose and 0.8% agar. After germination the 100 plantlets were divided into four parts (25 per medium) and transferred onto solid media 1) WPM with 1% sucrose, 2) half-strength WPM + 1% sucrose ($^1/_2$ WPM) [6], 3) MS supplemented with 1% sucrose, and 4) MS with 3% sucrose. The plants were grown and maintained at 25°C with a 16-h light photoperiod. About 6-8 young leaves from 3 plants per medium were harvested 8 times in 3-6 days intervals to study changes of diosgenin level during plant development. All samples were weighted after harvest and then lyophilized at temperature -42°C and pressure 0.018 mbar. Dried materials were also weighted. Tissue moisture (TM) (%) was determined as TM = 100 x $[(M_f-M_d)/M_d]$, where M_f is the fresh mass, M_d is the dry mass after freeze-drying [7].

Experiment 2

Content of diosgenin was analyzed in callus, leaves, stems and roots of *in vitro* maintained plants. Plants were germinated as described above and grown on MS supplemented with 3% sucrose. For induction of callus 3-4 weeks old plantlets were placed on MS culture media containing 2,4-dichlorophenoxyacetic acid (2,4D, 1 mg/L) or 6-benzylaminopurine (BAP, 4 mg/L). Calli were maintained on the same induction medium. After 5 months of cultivation both calli and plant organs were collected and lyophilized. Fresh and dried masses were weighed. Both callus, leaves, stems and roots were collected from about 10-15 samples. Organs of plants were mixed and crushed with mortar and pestle to prepare uniform sample before analysis.

2.2 Extraction of diosgenin

Diosgenin was extracted with a liquid phase [8] with modifications. Plant material was hydrolyzed with 1 M sulfuric acid in 70% isopropanol (30 ml) for 6 h under reflux. In the next step water (15 ml) was added and obtained solution was extracted three times with hexane (3x20 ml) and finally washed with 2 M NaOH (2x50 ml) and distilled water (2x50ml). Organic phase was evaporated to dry powder in vacuum concentrator.

2.3 Identification and quantification of diosgenin

Diosgenin standard was purchased from Sigma-Aldrich. Unless specified otherwise, all chemicals were of analytical reagent grade and solvents were of HPLC grade. Water was purified using Milli-Q system.

Diosgenin was identified by ultra performance liquid chromatography coupled with mass spectrometry with triple quadrupole. UPLC analyses were performed using a Shimadzu apparatus on a Kinetex C-18 RP column (50 mm x 2.1 mm I.D., 1.7 µm). The isocratic solvent system was methanol-water 8:2 (v/v), the flow rate was set at 0.8 ml/min and the column temperature was maintained at 40°C. The injection volume was 1 µl. All samples were dissolved in 1 mL of methanol.

AB Sciex QTRAP 4500 was equipped with an electrospray ion source operating in positive ion. For targeted metabolites we used very sensitive multiple reaction monitoring (MRM) method. Transition for diosgenin *m/z* 415.3→271.2 and *m/z* 415.3→253.2 was detected. The ESI interface conditions were as follows: ion spray voltage: +5000 V; declustering potential: +156 V; entrance potential: +10 V; temperature: 600 °C. For calculation the concentration of diosgenin, calibration curve was used. The correlation coefficient of calibration curve was $r^2 = 0.997$. Measurements were taken in three replications. ANOVA analysis was performed to test the significance of differences between means in STATISTICA [9].

3. Results and discussion

3.1 Changes of diosgenin content during plant development on different growth media

Analysis of variances showed that diosgenin content in both fresh and dry mass was significantly influenced by type of medium, day of plant growth and interaction of these factors. Mean diosgenin content in fenugreek plant grown on four solid media was significantly different in case of fresh mass (DFM), while differences in diosgenin level in dried material (DDM) were not significant statistically (Tab. 1). The result of analysis showed that the most effective production of diosgenin was on the MS 3% and half-strength WPM media with average DDM 712.7 µg•g-1 and 619.1 µg•g-1, respectively (Fig. 1). The level of diosgenin on the WPM and MS supplemented with 1% sucrose media was significantly lower (507.5, and 449.3 µg•g-1 DW).

Table 1: Significance of variation mean and standard deviations of fresh mass (FM), dry mass (DM), diosgenin content in dry mass (DDM), diosgenin content in fresh mass (DFM), and tissue moisture (TM).

Variable	F(media)	F(organs)	Means and standard deviation				
			Leaves	Stems	Roots	Callus BAP	Callus 2,4-D
FM [g]	6.71**	6.19*	4.1±0.6	4.9±2.9	25.1±5.6	24.2±1.1	24.2±9.3
DM [g]	12.5***	2.70	0.7±0.1	0.8±0.5	1.6±0.1	1.2±0.2	1.3±0.4
DDM [µg·g-1]	2.62	79.46***	529.3±12.2	176.3±69.5	96.5±34.3	77.9±39.3	30.5±9.6
DFM [µg·g-1]	5.29**	152.3***	85.4±2	27.6±10.8	6.1±0.5	3.8±1.6	1.7±0.7
TM [%]	2.40	23.61***	5.2±0	5.4±0	14.6±4.4	18.8±2.2	17.5±1.2

* - significance level <0.05, ** - significance level <0.01, *** - significance level <0.001.

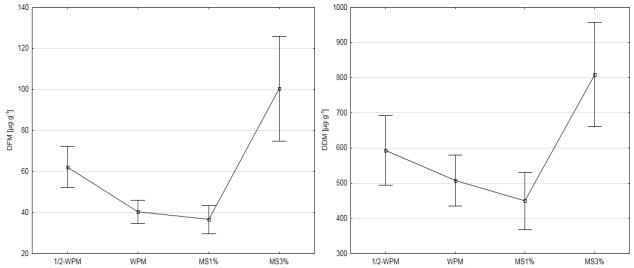

Fig 1: Changes in diosgenin content in fenugreek seedlings grown on different media.

Diosgenin production by fenugreek on various plant media has been investigated. Hairy root cultures were established with *Agrobacterium rhiozgenes* and fourteen different liquid media were investigated [10]. The highest diosgenin content was observed in half-strength WPM medium with 1% sucrose (0.040% dry weight), which represented almost twice the amount detected in the 8-month-old non-transformed roots (0.024%). The hairy roots cultured in WPM (half and full strength) with a low sucrose concentration showed a higher diosgenin content while in MS medium supplemented with 3% sucrose diosgenin was not detectable (<0.4 µg/mL of culture medium). Our results evidence that different media are optimal for the highest content of diosgenin in whole plant and hairy root cultures. However, half-strength WPM medium was the second medium for high diosgenin yield. Moreover, the concentration of sucrose influences diosgenin production. This suggests that the level of carbohydrates may stimulate diosgenin productivity. The quantity of diosgenin obtained in our study ranges from 317.9 µg/g dry weight to 1376 µg/g dry weight, what suggest that aerial parts of fenugreek are better materials to study of saponin biosynthesis.

The changes in diosgenin accumulation in leaves during development of fenugreek was tested in 3-6 days intervals. We observed significant changes of diosgenin content in the time of plant growth on individual media. The highest levels of diosgenin were found in 21st and 27th day on MS medium supplemented with 3% of sucrose. When compared to other media, diosgenin production on $^1/_2$ WPM was the most effective at 38th day with high content of diosgenin – 909.6 µg·g^{-1} dry weight (Fig. 2).

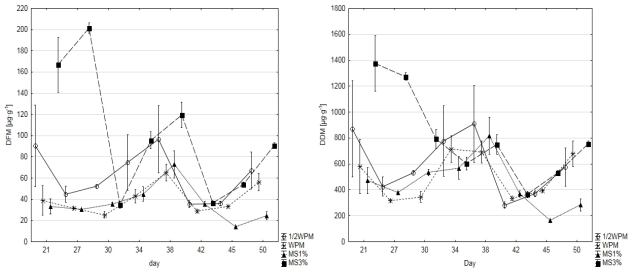

Fig 2: Changes in diosgenin content during development of seedlings.

Our results partly coincided with data reported by Ortuno *et al.* [11], where the highest level of diosgenin was between 15th and 30th day and later decreased. The highest concentration of diosgenin at the beginning of the plant development may be related with environmental hazards and risk of microbial attack. Secondary metabolites represent the adaptations of plants to environmental stress, or they may serve as defensive or protective chemicals against microorganisms, insects, and higher herbivorous predators [12]. Many research articles describe the identification of saponins in plants and their biological activities. They have been reported to have antimicrobial, virucidal, or insecticidal action. In this view, saponins can be considered as a part of the plant defense mechanism and can be classified in a large group of protective molecules [13].

3.2 Content of diosgenin in various organs

Previous *in vitro* cultivation studies revealed that diosgenin occurs in different concentrations in leaves, stem, seeds and root of fenugreek. Leaves were the best source for the isolation of diosgenin [11].

Concentration of diosgenin in dry weight was significantly different ($F=211.9$, $p<0.001$) for leaves, stems, roots and undifferentiated callus tissues. The highest content of diosgenin was found in leaves (525 µg·g^{-1} DDM) (Fig. 3).

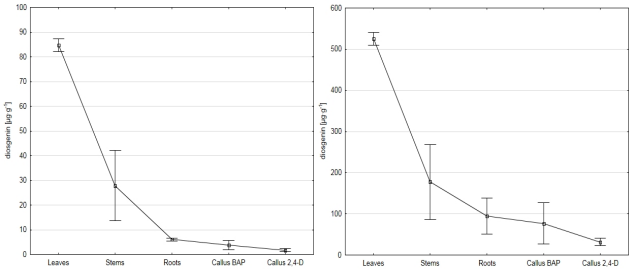

Fig 3: Content of diosgenin in fresh (*left*) and dry (*right*) weight of various parts of fenugreek plant and calli. Squares denote standard error, and dashed lines represent 95% confidence intervals.

Amount of diosgenin in stems and roots was respectively 3- and 5-fold lower compared to the leaves (i.e. 177 and 94 µg·g^{-1} dw, respectively). Still, all organs tested contained more diosgenin than callus induced by 2,4-D treatment (31 µg·g^{-1} dw). Slightly higher level of diosgenin was observed in callus induced by BAP treatment when compared to 2,4-D derived callus. The positive effect of BAP on diosgenin expression by affecting its biosynthesis and/or translocation in *Trigonella* has been documented [11].

Reaction of fenugreek plants to growth hormones measured by diosgenin level has been already investigated. Oncina *et al.* [14] reported that diosgenin accumulation in callus cultures of *Trigonella foenum-graecum* amounted to 2 mg·g^{-1} of fresh weight and 25 ppm ethephon treatment almost doubled diosgenin content. Concentrations of 50 ppm and 100 ppm reduced diosgenin levels. Treatment of 15- and 30-day-old fenugreek plants with various growth regulators gibberellic acid (GA), indolile-3-acetic acid (IAA) and ethephon showed that GA gave the greater increase of 77% in 15-day-old plants falling to 68% in 30-day-old plants [15]. Content of diosgenin in control whole plants varied from 0.26 to 1.13 mg·g^{-1} fresh whole plant after 15 and 30 days of growth, respectively.

The accumulation of diosgenin in leaves of fenugreek found in our studies was previously observed by Ortuno *et al.* [11]. Similarly, a lower content of diosgenin was assessed in stems and roots. High variation of diosgenin content between different organs of fenugreek indicates that beside genetic variation responsible for general ability of diosgenin biosynthesis, the highest productivity of this target chemical can be obtained from plants grown in conditions stimulating high share of leaves in total biomass. Moreover, comparison of diosgenin level from whole-plant homogenate will be influenced by relative amounts of leaves to stems. In our studies, the most stable concentration of diosgenin was found

in leaves and 2,4-D derived callus what makes these tissues most interesting for studying induced transcriptome changes. Diosgenin content varied in 15 *Trigonella* species, with the mean amount of diosgenin in the aerial parts of fenugreek equal to 0.08 $mg \cdot g^{-1}$ [16]. These results agree with the mean content of diosgenin in fresh leaves of *in vitro* plants 84.7 $\mu g \cdot g^{-1}$ observed in our study.

As regards the place of diosgenin biosynthesis this situation is not determined yet. The previous studies showed the significant positive correlation between diosgenin levels in analyzed organs and different ages of organs. It supports the theory of its transport from leaves to root and its accumulation in intermediary organs like stem and fruits. However, the possibility of the 'in situ' synthesis of this secondary compound in the same organs must be checked [11]. Akhila and Gupta [17] suggested that diosgenin in *Costus speciosus* is biosynthesised in leaves and then translocated to all organs of the plant. Also glycosidation of diosgenin takes place in all the parts of the plant and diosgenin glycosides are stored in the rhizomes, seeds, and flowers.

4. Conclusion

To conclude the highest content of diosgenin was produced by leaves. Other organs showed significantly lower content of diosgenin. Leaves can be used as a place for the accumulation of saponins.

There are no linear dependence between the content of diosgenin in fresh or dried materials and weigh of fresh or dried samples, respectively. Level of diosgenin in various organs is not due to random differences.

Amount of diosgenin in leaves changes during plant development. The highest content of diosgenin occurs at about 21st and 38th day of growth.

Extreme levels of diosgenin content were found in fenugreek plants grown on MS media supplemented 3% sucrose, that suggests an important role of carbohydrates for diosgenin synthesis. McCown's woody plant medium showed medium performance for the production of diosgenin in whole-plant. Testing of different levels and sources of carbon may result in optimization of diosgenin production.

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