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## Method Article

# An *Agrobacterium rhizogenes* mediated hairy root transformation protocol for fenugreek



Constantine Garagounis\*, Maria-Eleni Georgopoulou,  
Konstantina Beritza, Kalliope K. Papadopoulou

University of Thessaly, Department of Biochemistry and Biotechnology, Biopolis, Larisa 41500, Greece

## A B S T R A C T

- This work describes a protocol for hairy root transformation of the medicinal crop legume fenugreek (*Trigonella foenum-graecum* L.). Hairy root plant transformation mediated by *Agrobacterium rhizogenes* is an established method for the rapid genetic transformation of various dicotyledonous plants. We have adapted a hairy root transformation protocol from the model legume *Medicago truncatula* for use in this metabolically rich non-model crop legume. Considering the great variety and abundance of phytochemicals in fenugreek and its established use in traditional medicine, we aim for this method to become a resource for metabolic pathway identification and for production of valuable specialised metabolites via metabolic engineering approaches.
- Development rapid transformation (2.5–3 weeks) of fenugreek roots via *A. rhizogenes*.
- Marker gene cassette with suitable promoter for visual detection of transformed fenugreek roots

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## A R T I C L E I N F O

**Method name:** Hairy root transformation of fenugreek (*Trigonella foenum-graecum* L.)

**Keywords:** *Trigonella foenum-graecum*, Hairy root transformation, Legume metabolic engineering

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\* Corresponding author.

E-mail address: [kgkaragkounis@bio.uth.gr](mailto:kgkaragkounis@bio.uth.gr) (C. Garagounis).

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## Specifications table

Subject Area:	Please Select Subject Area from dropdown list
More specific subject area:	Legume genetic transformation for applications in biotechnology and synthetic biology
Method name:	Hairy root transformation of fenugreek ( <i>Trigonella foenum-graecum</i> L.)
Name and reference of original method:	[1] Boisson-Dernier, A., Chabaud, M., Garcia, F., Becard, G., Rosenberg, C. & Barker, D. G. (2001) <i>Agrobacterium rhizogenes</i> -transformed roots of <i>Medicago truncatula</i> for the study of nitrogen-fixing and endomycorrhizal symbiotic associations, <i>Molecular Plant Microbe Interactions</i> . <b>14</b> , 695–700.
Resource availability:	All resources used in this protocol are commercially available, <i>Agrobacterium rhizogenes</i> strains (ARqua1 and LBA9402), the marker gene plasmid for fenugreek, pLJUB:GFP:tNOS, and reasonable amounts of fenugreek cv Co seeds are available upon request.

## Background

Fenugreek is a medicinally important non-model crop legume, which produces a wide variety of specialised metabolites, the most notable amongst these being diosgenin [2]. Diosgenin is of great pharmacological relevance, as it constitutes the precursor compound in the production of pharmaceutical hormones via hemisynthesis. A biosynthetic pathway has been proposed for diosgenin but has not yet been validated genetically in fenugreek as the native host of this pathway, due to the lack of a viable transformation protocol [3]. Many other compounds of interest are also produced in fenugreek [4,5], and the elucidation of their biosynthetic pathways will greatly improve our ability to unravel this plant's great pharmacological significance. We recently used this proposed method for hairy root transformation to validate the functionality of a legume promoter and to probe the activity of heterologous transcription factors in fenugreek [6]. Two previous reports of fenugreek transformation have not been exploited in the relevant literature, either because they were not reproducible or because pitfalls with the use of commonly used DNA promoters had not at the time been identified and solved [2,7]. Thus the development of a robust transformation protocol for fenugreek is critical for biosynthetic pathway characterization.

## Materials required

This protocol assumes you have already acquired and validated by sequencing the desired T-DNA constructs in binary vectors, and transformed these into appropriate *Agrobacterium rhizogenes* strains. We have validated this protocol with *A. rhizogenes* ARqua1 and LBA9402 strains, and a previous publication suggests K599 is also a suitable strain [7]. Additionally, this protocol assumes that you have access to standard molecular biology equipment for bacterial culture and manipulation.

*To carry out this protocol you will need:*

- Fenugreek (*Trigonella foenum-graecum*) seeds,
- Sterile forceps and scalpels,
- Large petri dishes for plant culture, either square (10 × 10 cm) or round (12 cm diameter) plates,
- 250 mL or larger conical flasks with autoclavable bungs or covered with aluminium foil (*only if hairy root liquid culture is to be undertaken*),
- Half-strength MS medium [2.2 g/L Murashige & Skoog salts including vitamins (from Duchefa Biochimie, Belgium), 1% (w/v) sucrose, 0.05% (w/v) MES (2-(N-**morpholino**)ethanesulfonic acid), 0.6% (w/v) agar, pH adjusted to 5.7 using KOH],

*or*

- Full strength McCown's Woody Plant medium [8] (from Duchefa Biochimie, Belgium) supplemented with 3% (w/v) sucrose, 0.05% (w/v) MES (pH 5.7),
- Hoagland's nutrient solution [9] [Full strength Hoagland's composition: MgSO<sub>4</sub> 2 mM, KH<sub>2</sub>PO<sub>4</sub> 1 mM, FeEDTA 0.1 mM, KNO<sub>3</sub> 10 mM, Ca(NO<sub>3</sub>)<sub>2</sub> 4H<sub>2</sub>O 5 mM, H<sub>3</sub>BO<sub>3</sub> 46 mM, MnCl<sub>2</sub> 9 mM, ZnCl<sub>2</sub> 0.8 mM, CuCl<sub>2</sub> 0.2 mM, H<sub>2</sub>MoO<sub>4</sub> 0.1 mM].

- Antibiotics as needed for selection of binary vectors, suppression of bacterial growth after co-cultivation, and if applicable antibiotic or herbicide for transformed root selection (see “Protocol” section for details).
- Reagents and equipment for standard PCR

And/or

- Dissecting microscope equipped with a UV lamp and suitable filters for fluorescence detection, or other similar system capable of fluorescent marker detection.
- All chemicals should be molecular biology/tissue culture grade.

## Protocol

The protocol is separated into two sections, one relates to *Agrobacterium* preparation and the other to germination and transformation of fenugreek seedlings. Both sections are executed in parallel as described in the protocol flowchart (Fig. 1)

### *Agrobacterium* preparation

#### Day 1

- 1) Streak out all *Agrobacterium rhizogenes* strains harbouring the desired binary vectors on solid LB medium with appropriate antibiotic selection. Grow 24–48 hrs as necessary at 28°C.
  - Antibiotic selection for the two strains tested with this protocol are: streptomycin at a minimum of  $100\mu\text{g mL}^{-1}$  for ARqua1, and rifampicin at  $50\mu\text{g mL}^{-1}$  for LBA9402. Additional selection must be used as appropriate for these strains transformed with the desired binary vector.

#### Day 2–3

- 2) Inoculate liquid LB medium, supplemented with appropriate antibiotics, with freshly grown *Agrobacterium* strains from step 6. Grow to saturation (plateau phase, may be verified by measurement of OD<sub>600</sub>, which usually ranges from 1.8–2.2, but this is not crucial), with selection, for ~24 hrs at 28°C. These cultures can be kept at 4°C for 1–2 days without a significant effect on downstream steps (Fig. 2A).
  - Usually 5 mL culture per strain is enough, but if a very large number of transformants is desired inoculate a larger volume or multiple cultures.

#### Day 3–4

- 3) On the day prior to infection, spread 500μL of the resulting saturated culture on LB medium plates, with selection, incubate overnight at 28°C overnight (16–24 hrs), with the lid-side up instead of inverted, as the amount of liquid will not be completely absorbed on the medium. This will result in formation of a carpet-like slurry of bacteria (Fig. 2B).
  - One such plate is sufficient to infect 30–40 seedlings so calculate the number of *Agrobacterium* plates with each construct you will need to infect the desired number of seedlings as follows: [Number of plates = (Number of infected seedlings desired/30) + one plate].

### *Fenugreek* preparation and transformation

#### Day 1

- 1) Prepare the desired number of fenugreek seeds, depending on the desired number of transformants, in an appropriate tube (2, 15, 50 mL eppendorf/falcon-type tubes).
  - An appropriate tube should have enough space for the desired number of seeds plus at least five times their volume to ensure enough space for the sterilisation solution.
- 2) Surface sterilise the seeds by covering with multiple volumes (5–10 vol, e.g., for ~1 mL seeds use 5 mL sterilization solution) of sterilization solution [10% (v/v) commercial bleach in water] and incubate for 10–15 min at room temperature with occasional mixing (Fig. 3A).

### Fenugreek transformation protocol flowchart

#### Fenugreek germination & infection

##### Steps 1-5 (Day 1):

Surface sterilize and germinate seeds

##### Steps 7-9 (Day 3-4):

Infect explants  
Place on plates  
Keep in dark for 2 days

##### Step 9 (Day 6):

Move infected explants to normal light cycle (16/8 hr light/dark)

##### Step 10 (Day 11):

Transfer explants to medium with antibiotic to eliminate Agrobacterium

##### Step 11 (Days 11-16):

Transformed roots begin to emerge 12 days post infection, remove roots emerging prior to this with sterile scalpel

##### Step 12a or b (>Day 20):

Transfer plants with roots to pots (Step 12a) or to plates/flasks for in vitro growth (Step 12b)

#### Agrobacterium preparation

##### Step 1 (Day1):

Streak out desired Agrobacterium strains, grow 24-48 hr

##### Step 2 (Day 2-3):

Inoculate liquid LB with fresh colonies from Step 1, grow 24 hr.

##### Step 3 (Day 3-4):

**Spread 1mL saturated** Agrobacterium culture on solid LB agar plates, grow 16-24 hr to form biofilm/slurry. Use for explant infection.

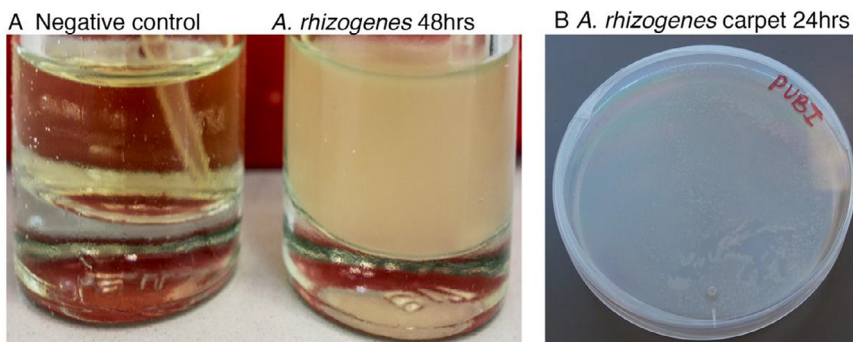
Week 1

Week 2

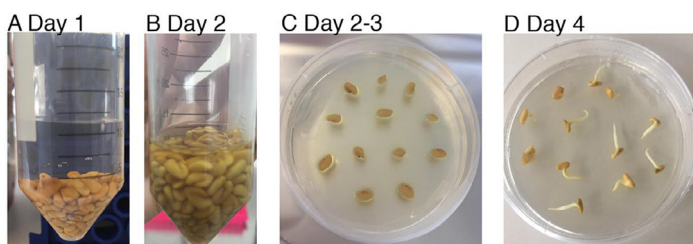
Week 3

**Fig. 1.** Flowchart of the fenugreek hairy root transformation protocol. See Protocol main text for detailed instructions.

- 3) Remove sterilization solution by pouring off (do not worry about removing it completely at this stage).
- 4) Rinse 5–6 times with 5–10 vol of sterile dH<sub>2</sub>O each time.
- 5) Cover the seeds with sterile water in the same tube and place in the dark at 4°C (you can wrap the seeds in aluminium foil, or place in a dark box in a fridge or cold room) for 24–48 hrs.
  - During this step seeds will imbibe water and increase in volume compared to their starting size (Fig. 3B).



**Fig. 2.** Preparation of *Agrobacterium rhizogenes* for fenugreek explant infection. A) Liquid LB cultures supplemented with appropriate antibiotics for binary vector selection are inoculated with an *A. rhizogenes* colony harbouring the desired binary vector (negative control is untransformed *A. rhizogenes*) and grown for 48 hr B) 1 mL of saturated *A. rhizogenes* culture (from A) is spread on an LB plate, again with antibiotic selection, and incubated for a further 24 hr to generate a slurry.



**Fig. 3.** Surface sterilization and germination of fenugreek seeds for hairy root transformation. A) After surface sterilization as described in steps 2–4 seeds are covered with an approximately and equal volume of sterile water. B) After a 24 hr incubation (step 5) seeds swell and increase in size. C) Seeds are placed on agar-water or MS-agar plates (step 6). D) Radicles emerge within the next 24–48 hr.

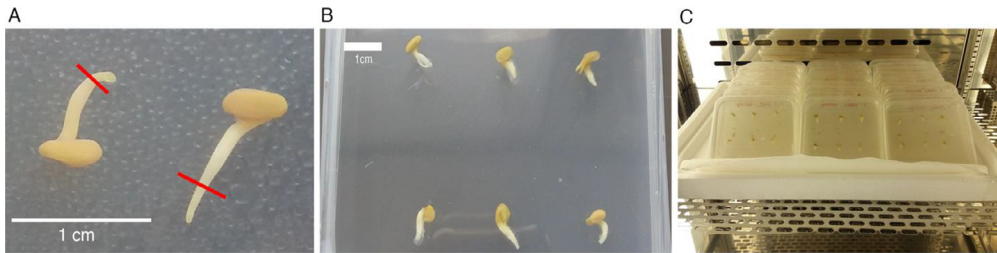
### Day2 – 3

- 6) After this imbibition period transfer the seeds to petri dishes on 1% (w/v) agar-water, or half-strength MS, 1% (w/v) agar medium, under sterile conditions (in a laminar flow hood) place them in plant growth incubator at 20–22°C, in the dark for 24 hrs, or until radicles emerge (sometimes it takes 48 hrs for all seeds to germinate) (Fig. 3C,D). There is no difference in the germination rate or a visible effect on growth after 48 hrs of germination on agar water or MS plates. Both can be used as available without an adverse effect on downstream steps.

### Day3–4

- Attention: The following two steps (7, 8) are carried out in a laminar flow-hood!

- 7) To infect fenugreek, place germinated seedlings (Fig. 3D) in the lid of a petri dish and excise the radicle with a sterile scalpel ~3 mm from the tip (Fig. 4A). This can be done to 3–5 seedlings in a batch, gently squeeze the hypocotyl with forceps just above the cut site to increase the wound surface and lightly dip in the *Agrobacterium* slurry generated previously.
- 8) Place the infected explants in rows in large square (10 × 10 cm) or round (12 cm diameter) plates, no more than 6–8 explants per plate (Fig. 4B), both because the seedling grow quite rapidly and in order to minimize contamination. Using 1% sucrose, and even 0%, allows root regeneration, but 3% sucrose results in significantly faster root growth [2]. Repeat this for all explants and constructs.



**Fig. 4.** Infection and co-cultivation of fenugreek seedlings with *Agrobacterium rhizogenes*. Explants are prepared by removing the radicle ~3 mm from the root tip (A, red lines), scraping a small amount of *Agrobacterium* lawn from plates (Fig. 2B) and explants are placed on co-cultivation medium in large plates (B) and incubated in a growth chamber at a near vertical angle (C).

- 9) Seal the plates with parafilm but make a large incision at the top to enable gas exchange, place them near vertical in a growth chamber at 20–22°C (Fig. 4C). Keep them in the dark for 2 days, and subsequently expose them to a standard light-cycle (16 h light/8 h dark works well).

#### **Day 11:**

- 10) Transfer the explants to new plates with half-strength MS medium plus ampicillin 400  $\mu\text{gml}^{-1}$  to eliminate *Agrobacterium* growth. Once again seal plates with parafilm and make an incision for gas exchange. Return to growth chamber as before. Grow the plants for a further two weeks and monitor regularly for callus growth and root emergence.
  - Ampicillin or carbenicillin can be used for beta-lactam sensitive *Agrobacterium* strains, cefotaxime or ceftazidime at 300  $\mu\text{gml}^{-1}$  can be used for beta-lactam resistant strains.
  - In many hairy root protocols it is standard to add antibiotic or herbicide to the medium at this stage, in order to select transformed roots and callus cells. However, selectable markers under the control of typically used promoters, such as pCAMV35S and pNOS do not function well in fenugreek roots. We have constructed and validated a fluorescent marker gene under control of a suitable promoter (data in co-submitted manuscript: PLAPHY-D-20-00667) and aim to provide a selection gene cassette for use in this protocol in the near future.

#### **Day 16**

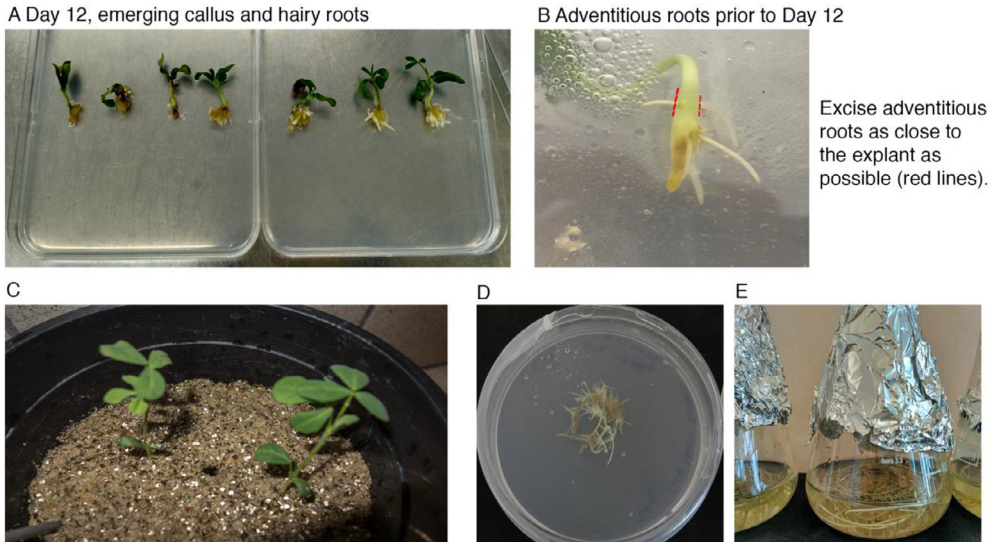
- 11) Transformed regenerated roots will begin to emerge at about twelve days post-infection (Fig. 5A). Roots emerging prior to this time point, especially from points on the hypocotyl much above the cut site made in step 9, are almost invariably adventitious roots and are not transformed. Remove these roots with a sterile scalpel and forceps under a laminar flow hood, as they emerge (Fig. 5B).
  - In future, functional selection cassettes will eliminate the need for this step.

#### **Day 20 onwards**

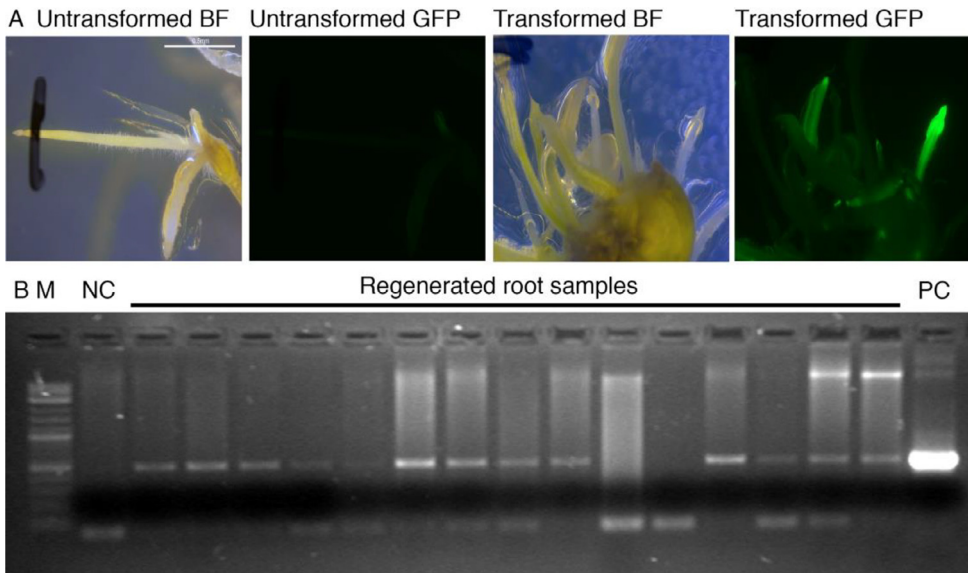
- 12a) Once regenerated roots grow sufficiently, composite seedlings can be transferred to pots with 2:1 sand:vermiculite and watered with half-strength Hoagland's nutrient solution in a growth chamber under the same conditions as before (Fig. 5C).

Or

- 12b) Alternatively, callus and emerging roots can be separated from the hypocotyl and shoot tissue with a sterile scalpel and transferred to new plates with MS medium plus antibiotic selection to suppress bacterial growth (Fig. 5D). After one or two weeks of growth on solid medium in plates, roots with a small section of callus can be placed in liquid MS medium, with 1–3% (w/v) sucrose, in sterile conical flasks, covered with aluminium foil and maintained in the dark on a rotary shaker (30–45 rpm) in a 20–22°C growth chamber (Fig. 5E). Roots grown in tissue culture



**Fig. 5.** Callus formation, root regeneration and hairy root tissue culture of infected fenugreek explants. (A) Callus forms around infection sites and roots are clearly visible at 12 days post infection. (B) Roots emerging prior to this time point and above the infection site should be removed, as they are invariably untransformed. (C) Plants with regenerated hairy roots after transfer to potting mix for growth as composite plants (transformed hairy roots with a wild type shoot). (D) Solid medium, and (E) liquid medium hairy root cultures.



**Fig. 6.** Verifying transformation of regenerated hairy roots using: (A) marker gene (GFP) fluorescence and (B) PCR to target the T-DNA construct with which explants were infected. BP: bright field, M: Molecular weight marker, NC: negative control (wild type fenugreek genomic DNA), and PC: positive control (pure binary vector used to transform hairy roots). Images in A are from a binocular dissecting microscope equipped with a UV source lamp and appropriate filters for GFP detection. The arrow indicates the expected amplicon.

will need to be divided and sub-cultured under sterile conditions every two weeks to one month depending on culture vessel size and experimental requirements. *Agrobacterium* will eventually be eliminated after a few weeks of growth with antibiotic, but antibiotic selection can be maintained constantly to suppress bacterial contamination.

## Method validation

In order to identify transformed hairy roots we routinely rely on two methods. The first is through the use of a fluorescent marker, transformed roots expressing GFP can be visualised on a dissecting microscope equipped with a mercury UV lamp and appropriate filters. This is non invasive and can be used to remove non-transformed roots before downstream manipulations are undertaken (Fig. 6A). The second method, is invasive and requires the extraction of DNA from individual regenerated hairy roots followed by PCR to amplify a suitable region of the T-DNA used to infect the specific explants (Fig. 6B). Since this is a destructive method it should be carried out only when sufficient root biomass is accumulated so that individual primary roots will provide enough tissue both for DNA extraction and other desired manipulations.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgments

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## References

- [1] A. Boisson-Dernier, M. Chabaud, F. Garcia, G. Becard, C. Rosenberg, D.G. Barker, *Agrobacterium rhizogenes*-transformed roots of *Medicago truncatula* for the study of nitrogen-fixing and endomycorrhizal symbiotic associations, *Mol. Plant Microbe Interact.* 14 (2001) 695–700.
- [2] A. Merkli, P. Christen, I. Kapetanidis, Production of diosgenin by hairy root cultures of *Trigonella foenum-graecum* L, *Plant Cell Rep.* 16 (1997) 632–636.
- [3] B. Christ, C. Xu, M. Xu, F.S. Li, N. Wada, A.J. Mitchell, X.L. Han, M.L. Wen, M. Fujita, J.K. Weng, Repeated evolution of cytochrome P450-mediated spiroketal steroid biosynthesis in plants, *Nat. Commun.* 10 (2019) 3206.
- [4] Z. Benayad, C. Gomez-Cordoves, N.E. Es-Safi, Characterization of flavonoid glycosides from fenugreek (*Trigonella foenum-graecum*) crude seeds by HPLC-DAD-ESI/MS analysis, *Int. J. Mol. Sci.* 15 (2014) 20668–20685.
- [5] P. Zandi, S.K. Basu, L.B. Khatibani, M.O. Balogun, M.O. Aremu, M. Sharma, A. Kumar, R. Sengupta, X. Li, Y. Li, S. Tashi, A. Hedi, W. Cetzal-Ix, Fenugreek (*Trigonella foenum-graecum* L.) seed: a review of physiological and biochemical properties and their genetic improvement, *Acta Physiol. Plant.* 37 (2014).
- [6] C. Garagounis, K. Beritza, M.-E. Georgopoulou, P. Sonawane, K. Haralampidis, A. Goossens, A. Aharoni, K.K. Papadopoulou, A hairy-root transformation protocol for *Trigonella foenum-graecum* L. as a tool for metabolic engineering and specialised metabolite pathway elucidation, *Plant Physiol. Biochem.* 154 (2020) 451–462.
- [7] Z. Shahabzadeh, B. Heidari, R.F. Hafez, Induction of transgenic hairy roots in *Trigonella foenum-graecum* co-cultivated with *Agrobacterium rhizogenes* harboring a GFP gene, *J. Crop Sci. Biotechnol.* 16 (2014) 263–268.
- [8] B.H. McCown, G. Lloyd, Woody plant medium (WPM)-a mineral nutrient formulation for microculture of woody plant species, *Hortic. Sci.* 16 (1981) 453.
- [9] D.R. Hoagland, Arnon, The water-culture method for growing plants without soil, *Circular (California Agricultural Experiment Station)* (1950) 347.