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An Optimized Protocol for *Agrobacterium rhizogenes*-Mediated Genetic Transformation of *Citrullus colocynthis*

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Abstract

Introduction: *Citrullus colocynthis* is considered as a precious herb due to its medicinal and nutritional values and also for its ability to produce valuable bioactive compounds such as cucurbitacin E and quercetin. The hairy root systems are valuable tools for scaling-up of secondary metabolites and for introducing new beneficial traits into herbs. The present research has aimed to develop a protocol for hairy root culture of *C. colocynthis* using *Agrobacterium rhizogenes*.

Materials and Methods: After the establishment of the hairy root system, factors such as explant type, bacterial strain, pre-culture period, co-cultivation period, and the use of acetosyringone that often affect the efficient transformation of herbs were optimized. Four *A. rhizogenes* strains (MSU440, A4, A13 and ATCC 15834) and three types of explant (leaf, excised shoot and hypocotyl) were tested. Furthermore, the insertion of transgene into the genome of *C. colocynthis* was confirmed by polymerase chain reaction analysis.

Results: The highest transformation frequency was obtained after the infection of excised shoots by MSU440. Co-cultivation for 48 hours resulted in enhanced transformation frequency, while the results of this research showed that the protocol is better not to include the pre-culturing step. In addition, the presence of acetosyringone in bacterial culture and co-cultivation medium significantly increased the success of *C. colocynthis* transformation.

Conclusions: This study describes an efficient protocol for hairy roots culture of *C. colocynthis* which can be used for scaling-up of the plant active phytochemicals or for genetic manipulations of the plant.

Keywords: *Agrobacterium*, Acetosyringone, Colocynth, Hairy Root, PCR, Transformation

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Introduction

Citrullus colocynthis (L.) Schrad. (Cucurbitaceae) known as colocynth or bitter apple is an herbal medicine used in Ayurvedic and Unani medicine.[1,2] In traditional Iranian medicine, the dried pulp of colocynth has been popularly used as an anti-diabetic medication. Recently, several animal and clinical studies have shown notable anti-diabetic activity of colocynth.[3,4] Other pharmacological effects of colocynth that were reported in previous literature include antioxidant, anticancer, lipid-lowering, insecticide, antimicrobial and anti-inflammatory effects. The most medically important compounds isolated from colocynth structurally belong to cucurbitacins (A, B, C, D, I, J, K and L), phenolic acids (e.g. ferulic acid, caffeic acid, chlorogenic acid) and flavonoids (e.g. quercetin, kaempferol and catechin).[5]

Due to its medicinal (fruit) and nutritional (seed) values, ability for production of bioactive chemical constituents and resistance to viruses, diseases and drought, colocynth has attracted biotechnologists' attention. Several research groups have attempted to develop protocols for the regeneration or genetic transformation of colocynth or fortify the production of its bioactive compounds.[6,7] For instance, Tanveer et al[8] established colocynth callus cultures as a source of quercetin. They used different combinations of plant growth regulators to establish callus cultures from the leaf and internode explants of *in vitro* grown seedlings. Then, they observed that the combination of 2 mg/L benzyl adenine (BA) and 3 mg/L α-naphthaleneacetic acid (NAA) could remarkably promote the quercetin production. Hegazy et al[9] revealed that stem-derived callus induced on Murashige Skoog (MS) medium using combination of 2 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D) and 4 mg/L kinetin generated notable contents of cucurbitacins. El-Baz et al[10] attempted to optimize culture medium contents in order to increase the production of phenolic and flavonoid contents. They reported that colocynth calli cultured in media supplemented with 2,4-D

and kinetin produced more phenolic and flavonoid contents in comparison to those cultured in media supplemented with BA and NAA. Krishna and Shasthree[11] developed a protocol for the induction and proliferation of adventitious roots from different explants of colocynth on MS media which were fortified with different concentrations of auxins and cytokinins. Dabauza et al[12] established genetic transformation in the cotyledon explants of colocynth using an *Agrobacterium tumefaciens* strain LBA4404 bearing the binary vector pBI121, holding genes coding for the β-glucuronidase (GUS; reporter) and the neomycin phosphotransferase (nptII; marker). To establish genetic transformation in two colocynth genotypes, Ntui et al[13] used *A. tumefaciens* strain EHA101 carrying either plasmid pIG121-Hm harboring genes coding for GUS, hygromycin phosphotransferase (hpt) and nptII or plasmid pBBRacdS harboring the same genes along with a gene coding for 1-aminocyclopropane-1-carboxylate (ACC) deaminase.

The present research has aimed to develop and establish *a Agrobacterium*-mediated hairy root culture of colocynth. Similar to normal roots of many plants, hairy root lines produce different kinds of natural compounds. Rapid, unrestricted and hormone-independent growth, and biochemical and genetic stability are among the most important advantages of hairy root culture compared to callus and suspension cultures.[14,15] In addition to generating a lasting source for the production of valuable phytochemicals, transformation by *A. rhizogenes* is a valuable tool for engineering medicinal herb cells through transferring additional genes and Ri plasmid into them.

Materials and Methods

Seed Sterilization and Germination

Initially, seeds were de-coated to release dormancy. For surface sterilization, the de-coated seeds were immersed in commercial bleach solution containing 2% (v/v) sodium hypochlorite for 7 minutes, followed by being rinsed 5 times in sterilized water. The seeds were placed on Murashige Skoog (MS) basal medium[16] supplemented with 0.7% (w/v) agar (pH 5.8). Seeds were germinated in a 16/8 h light/dark photoperiod at 25 ± 1°C. After 3 weeks of culture, *in vitro* plantlets were obtained and used as a source of explants for hairy root induction.

Bacterial Strains and Culture Condition

Four strains of *A. rhizogenes* (A4, ATCC15834, MSU440 and A13) were used for hairy root induction. The strains were grown overnight in liquid Luria–Bertani (LB) medium supplemented with 50 mg/L rifampicin at 28°C and 160 rpm in the dark. After 24 hours, the bacteria were harvested by centrifugation at 3500 rpm for 12 minutes and re-suspended in liquid MS medium (inoculation medium).

Induction and Establishment of Hairy Roots

The explants (hypocotyls, stems and leaves) were wounded with a sterile scalpel and were pre-incubated on ½ MS solid medium in the dark for one day prior to infection. Pre-cultured explants were soaked in *Agrobacterium* culture (OD 0.6-0.8) containing acetosyringone (AS) 100 μM for 5 minutes. To remove the excess bacteria, the soaked explants were placed on sterile blotting paper and then incubated on optimized co-cultivation medium (MS medium containing 100 μM AS solidified with 0.8% agar) under dark conditions at 25 ± 1°C. After co-cultivation, the explants were transferred to ½ MS based solid medium containing 30 mg/L sucrose and cefotaxime (200 mg/L) to arrest the growth of Agrobacteria and then, they were incubated at the dark at 25 ± 1°C. The control explants were cultured on MS basal medium without *Agrobacterium* infection. After the emergence of hairy roots in mother explants, they were maintained by sub-culturing of 3-4 cm long pieces of roots on ½ MS solid medium containing 200 mg/L cefotaxime every 4 weeks. Hairy roots were propagated in 100 mL of liquid MS medium in 250 mL Erlenmeyer flasks on an orbital shaker at 110 rpm in the dark at 25 ± 1°C.

In this study, the effects of factors such as *Agrobacterium* strain and explant type, pre-culture period (0, 24 and 48 hours), co-cultivation period (24, 48 and 72 hours), absence and presence of AS (100 μM) during incubation and co-cultivation on induction rate of hairy root were evaluated.

GUS Histochemical Assay

To detect GUS expression, cultured roots were treated with X-Gluc (5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid) solution according to the research by Jefferson et al.[17] GUS-expressing cells were detected under light microscope which could be associated to the blue spots emerged as a result of enzymatic cleavage of X-glucuronide. Furthermore, non-transformed roots were tested to indicate whether the root tissue of colocynth possessed endogenous GUS activity or not.

Polymerase Chain Reaction Analysis

Genomic DNAs of both transformed and non-transformed roots (0.5 g fresh weight) were isolated using the Cetyl trimethyl ammonium bromide (CTAB) method.[18] PCR analysis was used to investigate the presence of *rolB* gene in hairy roots. The PCR was performed with the following primers: 5′-GCTCTTGCAGTGCTAGATTT-3′ (forward primer) and 5′-GAAGGTGCAAGCTACCTCTC-3′ (reverse primer). The primers were used to amplify a 423-bp fragment of the *rolB* gene. Using a Bio-Rad (I-Cycler) Thermal Cycler, PCR reaction was carried out in a 25 μL volume of reaction mixtures containing 12.5 μL of Taq DNA Polymerase (NGene Biotech. Co.), 1 μL of each primer, 9.5 μL of H_2O and 1 μL of DNA. The PCR program was comprised of an initial denaturing step of 5 minutes at 94°C followed by 30 cycles of 1 minute at 94°C, 30 seconds at 58°C and 45 seconds at 72°C and a final extension step of 10 minutes at 72°C. The PCR products were separated by Electrophoresis on 1% agarose gel in 0.5 TBE buffer, stained with safe stain and were then visualized under UV.

Analysis of Results

The experiment was carried out with a completely randomized design with three replicates. The results were expressed as transformation frequency percentage which is defined as the number of explants induced hairy roots divided by the total

number of explants infected with *A. rhizogenes* and multiplied by 100. To compare the treatment groups, analysis of variance was used, followed by the Turkey test.

Results and Discussion

Susceptibility of the Explants and the Virulence of *Agrobacterium* Strains

In previous studies, many cucurbits were transformed using *A. rhizogenes*.[13,19] The effect that *Agrobacterium* strain and the type of explant had on the transformation efficiency of cucurbits was well documented.[20-22] To achieve an optimized condition for the establishment, virulence of four *Agrobacterium* strains (MSU440, A4, A13, ACTT 15834) were compared against three types of colocynth explants (leaf, excised shoot and hypocotyl). According to the results (Figure 1), all the studied strains were virulent against the leaf and excised shoot explants. However, hypocotyl explants seemed to be resistant to A4 transformation (transformation frequency: 0%). Excised shoots of colocynth were highly and significantly more susceptible to *Agrobacterium* infection compared to leaves and hypocotyls. In previous studies on cucurbits, excised shoots were rarely used for hairy root induction. Moreover, the susceptibility of hypocotyl explants to *Agrobacterium* infection has mostly been less than that of other explant types.[23-25] Nevertheless, there are instances such as *Citrullus lanatus* which its hypocotyls have showed higher rate of transformation by ACTT 15834 in comparison to its cotyledon and root explants.[21]

The highest transformation frequency was obtained with the infection of the excised shoots by MSU440 (60 ± 0.58%; Figure 2) and ACTT 15834 (50 ± 0%), respectively. There were no significant differences ($P \leq 0.05$) among the virulence of MSU440, A4 and ACTT 15834, whereas the virulence of A13 to excised shoots were significantly lower than the others. The strain A4 has been successfully used for hairy root induction in *Cucumis sativus*.[26] The ACTT 15834 also has efficiently induced hairy roots in *C. lanatus* and *Cucumis melo*.[21,22] A relatively suitable rate of transformation has been obtained after the infection of *Cucurbita pepo* with MSU440.[20]

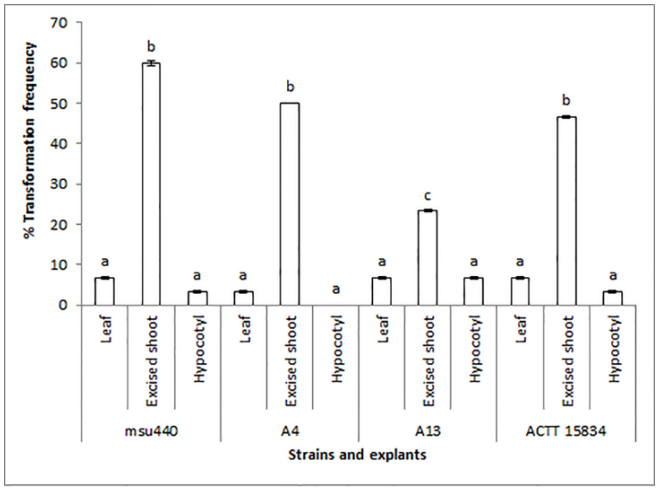

Figure 1. Effect of Different *Agrobacterium* Strains and Plant Explant on Transformation Frequency of *Citrullus colocynthis*. Data represent mean values of three replicates ± SE. Different letters represent statistically significant differences ($P \leq 0.05$).

Pre-culture Period

In this study, wounded excised shoots were pre-cultured on ½ MS solid medium during different periods of time (0, 24 and 48 hours). Results (Figure 3) indicated that in all studied strains, there was no significant difference between transformation frequencies of groups that were not pre-cultured (0 hours) and those that were pre-cultured for 24 hours. In addition, prolongation of pre-culture period to 48 hours led to a significant decrease in the transformation frequencies. The range of transformation frequencies in non-precultured explants varied from 23 ± 0.33% to 60 ± 0.57%. It decreased to the range of 23 ± 0.33% to 46 ± 0.3% after 24 hours of pre-culturing and diminished to the range of 10± 0% to 13± 0.33% after a pre-culturing period of 48 hours. Overall, it seems that pre-culturing does not only enhance hairy root induction in colocynth, but also has a negative influence on it.

Co-cultivation Period

Groups of excised shoot explants were separately co-cultivated with four strains of *Agrobacterium* for 24, 48 and 72 hours. As shown in Figure 4, the effect of co-cultivation period on the transformation frequency was remarkable. In a similar pattern, co-cultivation of all four strains for 48 hours led to a significant increase in transformation frequency, while no significant differences were found between transformation frequencies of groups co-cultured for 24 hours and those co-

Figure 2. Induction of Hairy Root on Wound Sites of Excised Shoots Using MSU440 Strain.

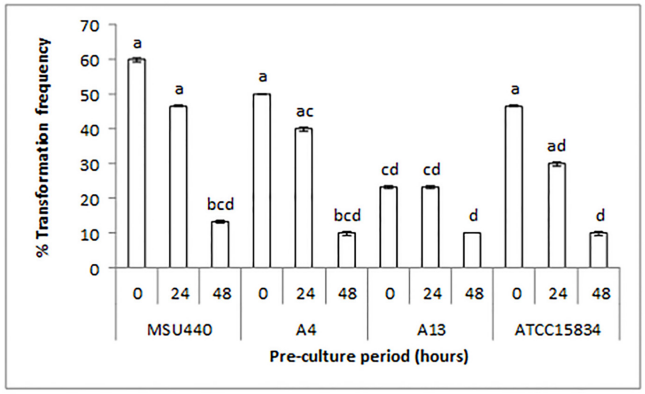

Figure 3. Effect of Different Pre-culture Periods on Transformation Frequency of *Citrullus colocynthis*. Data represent mean values of three replicates ± SE. Different letters represent statistically significant differences ($P \leq 0.05$).

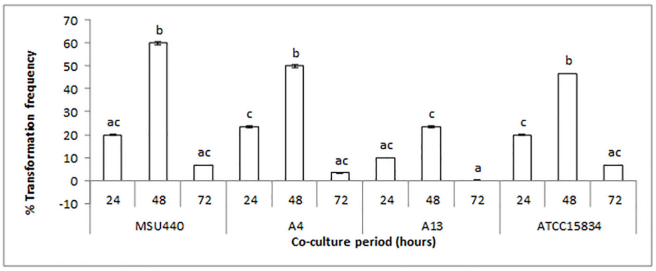

Figure 4. Effect of Different Co-cultivation Periods on Transformation Frequency of *Citrullus colocynthis*. Data represent mean values of three replicates ± SE. Different letters represent statistically significant differences ($P \leq 0.05$).

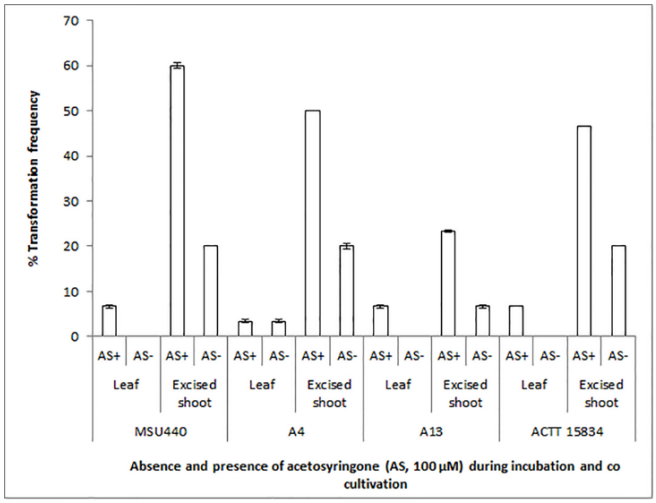

Figure 5. Effect of Acetosyringone Supplementation in Bacterial Culture and Co-cultivation Medium on Transformation Frequency of *Citrullus colocynthis*. AS+ indicates presence and AS- indicates absence of acetosyringone during incubation and co-cultivation. Data represent mean values of three replicates ± SE.

cultured for 72 hours. The lowest transformation frequencies were obtained after 72 hours of co-cultivation due to the overgrowth of agrobacteria and explant cell death. In a study on *C. lanatus*, hairy root was successfully established after 48 hours of co-cultivation on MS medium in the dark.[21] Similarly, to establish hairy root culture of *Momordica charantia*, its explants were co-cultivated for 48 hours on MS medium supplemented with 5 mg/L AS in the dark.[23]

The Effect of Acetosyringone during Incubation and Co-cultivation

Acetosyringone (AS) is a simple phenol which induces the virulence (vir) genes and subsequently transfers T-DNA fragments of *Agrobacterium* Ri plasmid into the plant cells.[27] In this study, the effect of AS was investigated on the transformation frequency of colocynth in pair groups (Figure 5). In the groups of excised shoots which were infected by MSU440, A4, or ACTT 15834, the absence of AS resulted in a significant decrease in the transformation frequencies ($P \leq 0.05$). However, in other groups, there was no significant difference between AS-treated groups and similar groups that were not treated with AS. Regardless of the statistical differences, AS was proved to be necessary for virulence of MSU440, A13, and ACTT 15834 against the leaf explants, while it did not exhibit any effect on virulence of A4 against the leaf explants. The enhancement effect of AS on transformation efficiency of *C. lanatus* was previously indicated.[28] This compound also increased the virulence of the strains ACTT 15834 and 8196 on Muskmelon (*Cucumis melo*).[22]

Confirmation of transformation

To confirm transformation, the hairy root lines were subjected to PCR. By using primers designed based on *rolB* gene, the PCR assay successfully showed the presence of the gene in the cultured lines (Figure 6A). Besides, GUS histochemical assay was conducted on transformed and non-transformed lines. After histochemical staining, blue spots were developed in the entire transformed root lines (Figure 6B), while no spots were observed on the non-transformed root line.

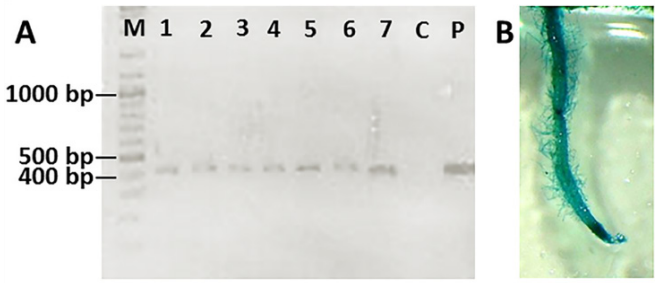

Figure 6. Confirmation of Transformation in the Hairy Root Lines of *Citrullus colocynyhis*. A) a PCR amplification of 423-bp fragment of the *rolB* gene. Lane M molecular weight marker (1 kb ladder Fermentase); lanes 1–7 samples (transformed roots obtained after *A. rhizogenes* infection), lane C negative control (non-transformed root); lane P positive control (Ri plasmid). B) GUS expression in the hairy root of *C. colocynyhis*.

Conclusions

The present study aimed to establish an efficient protocol for hairy root culturing of *C. colocynthis*. The highest rate of hairy root induction was obtained using excised shoot explant and MSU440 strain. Co-cultivation for 48 hours resulted in enhanced transformation frequency while pre-culturing is not recommended. The AS supplementation in bacterial culture and co-cultivation medium positively increased the transformation frequency. This protocol provides a background for future studies to introduce new beneficial traits into *C. colocynthis* via gene transferring. In addition, the established hairy root may be considered as a source of valuable secondary metabolites. Therefore, further research is required to isolate and scale up its compounds.

Authors' Contributions

MB conducted experiments and collected the laboratory data. AS and SJ made contributions to the study design. AS conducted PCR analysis and GUS assay, and SJ supervised the work, drafted the manuscript and conducted the data analysis.

Conflict of Interest Disclosures

The authors declare they have no conflicts of interest.

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References

1. Gruenwald J, Healthcare T, Brendler T, Jaenicke C. PDR for Herbal Medicines. Thomson; 2007.
2. Khare CP. Indian Medicinal Plants: An Illustrated Dictionary. New York: Springer Science & Business Media; 2008.
3. Rahimi R, Amin G, Ardekani MR. A review on Citrullus colocynthis Schrad.: from traditional Iranian medicine to modern phytotherapy. J Altern Complement Med. 2012;18(6):551-554. doi:10.1089/acm.2011.0297.
4. Heydari M, Hashempur MH, Ostovar M, Shams M. Citrullus colocynthis and its potential role against diabetes and its complications. In: Watson RR, Preedy VR, eds. Bioactive Food as Dietary Interventions for Diabetes. 2nd ed. Academic Press; 2019:495-507. doi:10.1016/B978-0-12-813822-9.00032-1.
5. Hussain AI, Rathore HA, Sattar MZ, Chatha SA, Sarker SD, Gilani AH. *Citrullus colocynthis* (L.) Schrad (bitter apple fruit): a review of its phytochemistry, pharmacology, traditional uses and nutritional potential. J Ethnopharmacol. 2014;155(1):54-66. doi:10.1016/j.jep.2014.06.011.
6. Teixeira da Silva JA, Hussain AI. *Citrullus colocynthis* (L.) Schrad. (colocynth): Biotechnological perspectives. Emir J Food Agric. 2017;29(2):83-90. doi:10.9755/ejfa.2016-11-1764.
7. Sultana B., Ashraf R. Watermelon (*Citrullus lanatus*) Oil. In: Ramadan M, ed. Fruit Oils: Chemistry and Functionality. Cham: Springer; 2019. doi:10.1007/978-3-030-12473-1_39.
8. Tanveer H, Mirza SA, Asi MR. Appraisal of an important flavonoid, quercetin, in callus cultures of *Citrullus colocynthis*. Int J Agric Biol. 2012;14(4):528-532.
9. Hegazy AK, Mohamed AA, Ali SI, Saker MM. Enhancement of callus induction and cucurbitacin content in *Citrullus colocynthis* L. (Schrad) using plant growth regulators. J Ala Acad Sci. 2010;81(1):23-35.
10. El-Baz FK, Mohamed AA, Ali SI. Callus formation, phenolics content and related antioxidant activities in tissue culture of a medicinal plant colocynth (*Citrullus colocynthis*). Nova Biotechnologica. 2010;10(2):79-94.
11. Krishna DR, Shasthree T. Adventitious rooting and proliferation from different explants of *Citrullus colocynthis* (L.) Schrad an endangered medicinally important cucurbit. Asian J Biotechnol. 2015;7(2):88-95. doi:10.3923/ajbkr.2015.88.95.
12. Dabauza M, Bordas M, Salvador A, Roig LA, Moreno V. Plant regeneration and *Agrobacterium*-mediated transformation of cotyledon explants of *Citrullus colocynthis* (L.) Schrad. Plant Cell Rep. 1997;16(12):888-892. doi:10.1007/s002990050340.
13. Ntui VO, khan RS, Chin DP, Nakamura I, Mii M. An efficient *Agrobacterium* tumefaciens-mediated genetic transformation of "Egusi" melon (*Colocynthis citrullus* L.). Plant Cell Tissue Organ Cult. 2010;103(1):15-22. doi:10.1007/s11240-010-9748-y.
14. Sharma P, Padh H, Shrivastava N. Hairy root cultures: a suitable biological system for studying secondary metabolic pathways in plants. Eng Life Sci. 2013;13(1):62-75. doi:10.1002/elsc.201200030.
15. Halder M, Roychowdhury D, Jha S. A critical review on biotechnological interventions for production and yield enhancement of secondary metabolites in hairy root cultures. In: Srivastava V, Mehrotra S, Mishra S, eds. Hairy Roots. Singapore: Springer; 2018. doi:10.1007/978-981-13-2562-5_2.
16. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant. 1962;15(3):473-497. doi:10.1111/j.1399-3054.1962.tb08052.x.
17. Jefferson RA. Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol Biol Report. 1987;5(4):387-405. doi:10.1007/bf02667740.
18. Murray MG, Thompson WF. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 1980;8(19):4321-4325. doi:10.1093/nar/8.19.4321.
19. Rekha K, Thiruvengadam M. Secondary Metabolite Production in Transgenic Hairy Root Cultures of Cucurbits. In: Jha S, ed. Transgenesis and Secondary Metabolism. Cham: Springer; 2017:267-293. doi:10.1007/978-3-319-28669-3_6.
20. Ilina EL, Logachov AA, Laplaze L, Demchenko NP, Pawlowski K, Demchenko KN. Composite *Cucurbita pepo* plants with transgenic roots as a tool to study root development. Ann Bot. 2012;110(2):479-489. doi:10.1093/aob/mcs086.
21. Kajikawa M, Morikawa K, Abe Y, Yokota A, Akashi K. Establishment of a transgenic hairy root system in wild and domesticated watermelon (*Citrullus lanatus*) for studying root vigor under drought. Plant Cell Rep. 2010;29(7):771-778. doi:10.1007/s00299-010-0863-3.
22. Mohiuddin AK, Abdullah ZC, Chowdhury K, Kamal C, Kulaveerasingam H, Napis S. Enhanced Virulence Gene Activity of Agrobacterium in Muskmelon (*Cucumis melo* L.) cv.'Birdie'. Ann Bot. 2011;3(2):71-79. doi:10.15835/nsb.3.2.5877.
23. Swarna J, Ravindhran R. Agrobacterium rhizogenes - Mediated hairy root induction of *Momordica charantia* Linn. and the detection of charantin, a potent hypoglycaemic agent in hairy roots. Res J Biotechnol. 2012;7(4):227-231.
24. Thiruvengadam M, Praveen N, Maria John KM, Yang YS, Kim SH, Chung IM. Establishment of *Momordica charantia* hairy root cultures for the production of phenolic compounds and determination of their biological activities. Plant Cell Tissue Organ Cult. 2014;118(3):545-557. doi:10.1007/s11240-014-0506-4.
25. Yoon JY, Chung IM, Thiruvengadam M. Evaluation of phenolic compounds, antioxidant and antimicrobial activities from transgenic hairy root cultures of gherkin (*Cucumis anguria* L.). S Afr J Bot. 2015;100:80-86. doi:10.1016/j.sajb.2015.05.008.
26. Anuar MR, Ismail I, Zainal Z. Expression analysis of the 35S CaMV promoter and its derivatives in transgenic hairy root cultures of cucumber (*Cucumis sativus*) generated by *Agrobacterium rhizogenes* infection. Afr J Biotechnol. 2011;10(42):8236-8244. doi:10.5897/AJB11.130.
27. Zhou ML, Zhu XM, Shao JR, Tang YX, Wu YM. Production and metabolic engineering of bioactive substances in plant hairy root culture. Appl Microbiol Biotechnol. 2011;90(4):1229-1239. doi:10.1007/s00253-011-3228-0.
28. Kavitah G, Taghipour F, Huyop F. Investigation of factors in optimizing agrobacterium-mediated gene transfer in *Citrullus lanatus* cv. round dragon. J Biol Sci. 2010;10(3):209-216. doi:10.3923/jbs.2010.209.216.