Influence of different *Agrobacterium rhizogenes* strains on hairy root induction and analysis of phenolic and flavonoid compounds in marshmallow (*Althaea officinalis* L.)

Parisa Tavassoli¹ · Akbar Safipour Afshar¹

Received: 8 November 2017 / Accepted: 27 July 2018 / Published online: 31 July 2018
© Springer-Verlag GmbH Germany, part of Springer Nature 2018

Abstract

Hairy roots were induced in *Althaea officinalis* using *Agrobacterium rhizogenes*, strains A4, A13, ATCC15834, and ATCC15834_(GUS). The leaf, petiole and shoot explants of marshmallow were used for the hairy roots induction. When hairy roots appeared, cultures were established in MS (Murashige and Skoog) liquid medium without growth regulators. Hairy roots in explants appeared 5–12 days after inoculation. Maximum transformation frequency of 83% was observed on shoot explants with ATCC15834 strain. Among the strains, ATCC15834_(GUS) strain showed better potential in the mass production of hairy roots in the hormone-free liquid medium after 50 days of culturing. The highest total phenolic and flavonoids content was found at 1.57 ± 0.1 mg/g dry weight in A13 strain and 3.47 ± 0.3 mg/g in A4 strain, respectively. Secondary metabolite content of hairy roots was found to be strain-specific.

Keywords *Agrobacterium rhizogenes* · *Althaea officinalis* · *rolB* · Secondary metabolites · Transgenic roots

Introduction

Marshmallow (*Althaea officinalis* L.), is a medicinal plant of Malvaceae family, from Europe and Western Asia. This plant has been well-known for its healing properties since ancient times. The entire plant possesses antitussive, antiviral, and antimicrobial properties. *A. officinalis* is a main source of mucilage which is useful for respiratory tract diseases like bronchitis, tracheitis, pertussis, and bronchial asthma (Khidyrova et al. 2012). Its roots and leaves include asparagine, althein, flavonol glycosides, pectin, quercetin and phenolic acid components (Shah et al. 2011; Benbassat et al. 2014).

Plant phenolics and flavonoids have attracted a lot of interest because of powerful biological activity and various applications in medicine (Granato et al. 2018). Several studies have focused on the production, purification and enhancement of these compounds in plants (Pistelli et al. 2010; Guerriero et al. 2018). Moreover, the extraction of

phytochemicals from plant tissues is subject to limitations due to the impact of ecological conditions on their growth and yield (Panda et al. 2017).

Recent years, *Agrobacterium rhizogenes* have been used for hairy roots production, a beneficial approach due to their stable growth and enhanced capability of synthesizing secondary metabolites in hormone-free media (Tian 2015). Further, these transformed roots exhibit the characteristic features such as rapid growth, frequent branching, genetic stability and fast mass production (El-Esawi et al. 2017).

Previous studies revealed that hairy roots induced by different bacterial strains vary in growth, morphology, and production of secondary metabolites (Gupta et al. 2016; Thwe et al. 2016). Therefore, based on plant species, a proper *Agrobacterium* strain must be selected. Different plasmid harbors by the strains cause differences in virulence and morphology of hairy roots (Thwe et al. 2016). Agropine-type strains are the most virulent or hypervirulent and often used in hairy root culture establishment (Lee et al. 2010). However, to date, there are not many reports on the transformation of *A. officinalis* via different *A. rhizogenes* strains for hairy root induction except one preliminary study (Ionkova 1992) and a study by Drake et al. (2013), wherein anti-HIV microbicide ‘cyanovirin-N’ production from hairy roots (induced by LBA 9402 strain) was reported. Therefore, in

✉ Akbar Safipour Afshar
Asafshar@iau-neyshabur.ac.ir

¹ Biology Department, Neyshabur Branch, Islamic Azad University, Neyshabur, Iran

the present study, we aimed to induce hairy roots of *A. officinalis* using different strains of *A. rhizogenes* and following screening of the hairy roots for production of higher levels of flavonoids and phenolic compounds.

Materials and methods

Plant material

Seeds of *A. officinalis* were surface sterilized with alcohol (70% v/v) for 30 s and sodium hypochlorite solution (60% v/v) for 2 min and finally washed with sterilized water for 3 times. Sterile seeds were germinated on MS basal medium (Murashige and Skoog 1962) with 30 g/L sucrose and 5 g/L agar, the pH of the medium was adjusted to 5.8 with 1N NaOH or 1N HCl prior to sterilization at 121 °C for 20 min. The seeds were incubated at 25 °C, a 16/8 h (light/dark) photoperiod in growth chambers.

Bacterial strains and culture conditions

A. rhizogenes strains A4, A13, ATCC15834, and ATCC15834 (carrying the GUS gene) were used for infections. A single clone of *A. rhizogenes* was selected and cultured on 25 mL Luria–Bertani (LB) medium (10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl, pH = 7.0). Bacterial cultures were collected at 5000 rpm for 5 min and resuspended at a cell density A600 = 0.5 in MS liquid medium (containing sucrose and 100 µM acetosyringone) and were shaken for 1 h in the rotary shaker before inoculation.

Transformation procedure

Two different procedures were used for inoculation of explants. In the first method, 8–15-day-old shoots and also leaf and petiole explants of 10–30 days old were inoculated using an insulin syringe. In the second procedure, different seedling parts, including shoot, leaf and petiole were isolated from in vitro grown seedlings and were immersed for about 5 min in liquid bacterial cultures then blotted on sterile filter paper. All the samples transferred to the MS basal medium supplemented with 30% sucrose, 0.7% agar (pH 5.8) for co-cultivation. After 2 days of co-culture at 25 °C in the dark, explants were transferred to MS medium containing cefotaxime antibiotic so as to kill the residual *Agrobacterium*. In the presence of infection, the cultures were washed with 250 mg/L of cefotaxime solution, and blot-dried on sterile filter paper then transferred to MS medium supplemented with 30% sucrose, 0.7% agar (pH 5.8), and 500 mg/L of cefotaxime. Cefotaxime concentration was then reduced in subsequent subcultures from 500 to 100 mg/L and finally,

cultures free of *A. rhizogenes* were transferred to MS media plates. After the appearance of hairy roots and to obtain the root lines, single roots were picked off and were cultured on hormone-free MS medium in the dark at 25 °C. After several subcultures on fresh solid medium, the roots were transferred to MS liquid medium containing 30 g/L sucrose, kept in a rotary shaker at 80 rpm and 25 °C in darkness, and subcultured routinely every 2 weeks.

DNA isolation and PCR analysis

Total genomic DNA was isolated from putative transgenic and control hairy root lines by using the CTAB (hexadecyltrimethyl ammonium bromide) DNA isolation method (Moyo et al. 2008). The DNA samples were then used in PCR analysis for detecting the presence of *rolB* gene (780 bp) and GUS gene (320 bp) in transgenic hairy root cultures. The primer set for the *rolB* gene was: 5'-ATGGATCCCAAATTGCTATTCACCA-3' and 5'-TTAGGCTTCTTTCATTTCGGTTTACTGCAGC-3' and the primer set for GUS gene was: 5'-GGTGGGAAAGCGCGTTACAAG-3' and 5'-TGGATTCCGGCATAGTTAAA-3' (Rahnama et al. 2008). The PCR reaction was carried out in 20 µl volume with 20 ng (1 µl) DNA, 100 ng (1 µl) of forward and reverse primer; 2 µl (1 mM) dNTPs; 0.7 µl (2.5 U) Taq DNA polymerase; 2 µl 10x Reaction Buffer; 1 µl (4 mM) MgCl₂, 11.3 µl H₂O. PCR reaction comprised 30 cycles. The amplification cycle for *rolB* gene consisted of denaturation for 1 min at 94 °C, primer annealing for 1 min at 54 °C, and primer extension for 1 min at 72 °C and final extension for 10 min at 72 °C. Cycling parameters for GUS gene amplification was similar except that the annealing temperature was 62 °C. PCR products were visualized after electrophoresis on a 1% agarose gel stained with ethidium bromide under UV irradiation. Plasmid DNA from *A. rhizogenes* strains was isolated by alkaline lysis method (Green and Sambrook 2012) and used as positive control. The genomic DNA from untransformed *A. officinalis* root was used as a negative control in PCR analysis.

GUS histochemical assay

Hairy roots were tested for histochemical GUS expression by soaking in X-Gluc solution (Jefferson 1987). Such that 1-cm-long hairy roots from each individual line were excised and transferred into histochemical assay buffer containing 1.0 M phosphate buffer, 1.0 mM ferricyanide, 10% Triton X-100, 0.5 mM EDTA and 0.1 M 5-Bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc). The samples were incubated for 16 h at 37 °C in the dark. After staining roots and washing several times in 70% ethanol, GUS expression exerted as a blue color was visualized in the hairy root lines.

Determination of total phenolic content

The total phenolic content was determined as described by Benbassat et al. (2014) using Folin–Ciocalteu reagent. 100 mg samples of hairy roots are grounded in 95% ethanol and the resulting mixtures were incubated for 24 h in darkness. Then centrifuged (UNIVERSAL 320, Hettich, Germany) at 6000 rpm for 10 min and the supernatants were collected. 0.5 mL of the Folin–Ciocalteu reagent was added to 0.5 mL of each extract. The solution was mixed well, and after 5 min, 1 mL 20% solution of sodium carbonate was added. Then, the solution was again mixed and finally, the volume was diluted to 10 ml with distilled water. The mixtures were kept at room temperature for 2 h and the absorbance was read at 765 nm (T80, PG Instruments, UK). The data were expressed as pyrogallol equivalent. All analyses were carried out in triplicate and averaged.

Determination of total flavonoid content

Total flavonoid content was determined following a method by Park et al. (2008). In a test tube, 4 ml of methanolic extracts, 0.15 ml of NaNO_2 (0.5 M) and 0.15 ml of $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ (0.3 M) were mixed. After 5 min, 1 ml of NaOH (1 M) was added. The solution was mixed well and the absorbance was measured at 506 nm. The standard curve for total flavonoids was made using rutin standard solution

(0–100 mg/l) under the same procedure. The total flavonoids were expressed as milligrams of rutin equivalents per g of the dried fraction.

Statistical analysis

All the experiments were performed in triplicates. The data were analyzed using analysis of variance (ANOVA) with Statistical Analysis System (SAS) Version 9.2 (SAS Institute, Cary, NC). Significant differences using means from triplicate analyses ($p < 0.05$) were determined by Duncan's multiple range test.

Results and discussion

Hairy roots induction

Hairy roots in explants inoculated with strains A13 (Fig. 1c) and ATCC15834_(GUS) (Fig. 1d) about 5 days and the explants inoculated with strain A4 (Fig. 1a, b) appeared about 12 days after inoculation. Our results showed that roots induced by four *A. rhizogenes* strains did not differ in morphological characteristics and differed only at the time of appearance. The leaves injured with a sterile scalpel were utilized as the control. Hairy roots were never observed in simply wounded leaves, affirming that development of transformed

Fig. 1 Hairy roots of *A. officinalis* transformed with different strains of *A. rhizogenes* 21 days after inoculation (injection by syringe); shoot (a) and leaf explant (b) with A4 strain, leaf explant (c) with A13 strain and petiole explants (d) with ATCC15834_(GUS) strain. (a: 2×) (b: 0.6×) (c: 0.3×) (d: 0.5×)

roots came about because of a morphogenetic response not to physiological stresses (Lee et al. 2010).

The efficacy of different bacterial strains in inducing hairy roots in different explants was investigated. The highest transformation frequency was observed by strain ATCC15834 in shoot explant at $83 \pm 5.2\%$ after 5 days of co-cultivation. Strains A4 and A13 induce rooting in 65 ± 1.54 and $45 \pm 2.3\%$ shoot explants, respectively. In leaf explants of *A. officinalis* maximum rooting frequency was $60 \pm 6.3\%$, infected with strain ATCC15834. The induction of hairy roots in petiole explants by ATCC15834, A4 and A13 strains was 76.2 ± 4.1 , 60.35 ± 2.2 and $55.75 \pm 3.4\%$, respectively (Fig. 2).

Many factors of plants such as explants type, cultivar, culture conditions and *Agrobacterium*–host interactions could influence the successful transformation (Colling et al. 2010). Similarly, shoots are the explants of choice for hairy root induction using *A. rhizogenes* in *Artemisia pallens* (Pala

et al. 2016), *Papaver bracteatum* Lindl. (Rostampour et al. 2009) and *Dracocephalum moldavica* (Weremczuk-Jezyna et al. 2013).

One of the main factors to achieving hairy root induction is the choice of a bacterial strain (Sharifi et al. 2014). Reports on the use of strain ATCC15834 were presented in many plants, including *Semecarpus anacardium* (Panda et al. 2017), *Origanum vulgare* (Habibi et al. 2016), *Hypericum* spp. (Zubricka et al. 2015), *Artemisia aucheri* (Sharafi et al. 2014) and *Nepeta pogonosperma* (Valimehr et al. 2014). In most cases, ATCC15834 strain had better performance and proved to be more competent than the others one.

In this study, two methods were used for inoculation explants of marshmallow; direct injection with a syringe and immersion in bacterial suspensions. The majority of shoots and leaf explants were infected by injecting bacteria produced hairy roots (Fig. 1c, d), but in the immersion method, only strain A13 produced hairy roots on leaf explants. Compared to bacteria suspension, insulin syringe provided a lower level of wounded and faced with the less influx of bacteria. Similarly, a study was performed to induce hairy roots of *Gentiana scabra*, the results showed that stem explants directly injected by *A. rhizogenes* induced the formation of hairy roots from all inoculated explants and failed to form in control samples (Huang et al. 2014).

On the hormone-free medium isolated roots exhibited a hairy-root phenotype similar to that described: vigorous–rapidly grew, high branching and loss of gravity response. Morphologically transgenic roots (on solid MS medium) revealed no differences in growth and development (Fig. 3a, b) in agreement with Samadi et al. (2014) and Moghadam et al. (2013) studies.

In many plant species, fast growth and high lateral branching are characteristics of transgenic hairy roots resulting from *A. rhizogenes* (Runo et al. 2012). Integration position and copy number of T-DNA(s) in the plant genome may influence the properties of hairy roots (Tenea et al. 2008). The induction of *vir* genes in bacteria by signal molecules

Fig. 2 Comparison of four *A. rhizogenes* strains and explant type after 5 days of co-cultivation periods on transformation frequency of *A. officinalis* in the injection method. Bars represent the mean $\pm$ SD of three independent experiments. Different letters above columns denote statistically significant differences between groups based on the LSD test ($p < 0.05$)

Fig. 3 Hairy roots were examined and photographed under a stereomicroscope (SZX2-ILLB, Japan); leaf explant (a) and shoots (b) of marshmallow (a: 6 $\times$) (b: 8 $\times$)

or *Agrobacterium* Co-cultivation with wounded tissue or media that contains the signal molecules, all these factors would be useful in genetic transformation of some recalcitrant plants (Elfahmi and Chahyadi 2014). Since acetosyringone enhanced *Agrobacterium*-mediated transformation frequency (Mishra and Ranjan 2008) and reduced the time for induction of hairy roots, so in order to increase the efficiency of gene transfer, it was added to the *Agrobacterium* suspension medium and co-cultivation medium of explants. Results observed with acetosyringone suggest that this compound is effective to improve genetic transformation in many of species (Nagella et al. 2013).

Establishment of hairy root cultures

Hairy roots induced by the four strains of *A. rhizogenes* were subcultured to fresh solid MS medium (Fig. 4a) and after sufficient growth hairy roots of *A. officinalis* were moved to MS liquid medium (Fig. 4b, c).

We observed the rapid growth of hairy roots in the hormone and antibiotic-free liquid MS medium. Particularly, the profuse growth of the hairy roots obtained from ATCC15834_(GUS) strain. In other words, hairy roots of strains A4, A13 and ATCC15834 were white, slender, while the transgenic hairy roots of strain ATCC15834_(GUS) were the higher growth rate. These roots are thick and have produced many lateral branches. Furthermore, newly formed hairy roots were white, which later became brown. In agreement with our study, Chandran and Potty (2008) reported that the hairy roots of *Ipomoea batatas* (two strains, A4 and ATCC15834) were white, slender, branched and brittle; the hairy roots of *Canavalia* sp. were thick and produced a lot of lateral branches. In another study, hairy roots of *Tylophora indica* within 3–4 weeks gradually changed from white to yellowish-white, then to reddish-brown within 6–8 weeks (Chaudhuri et al. 2005). It has been reported that

the medium type can affect the growth and proliferation of hairy roots and appropriate condition in liquid culture could increase hairy root biomass. Besides, the hairy root cultures formed in liquid medium showed a more rapid growth compared to hairy roots in solid medium (Huang et al. 2014).

Histochemical staining for GUS activity

Histochemical staining for β -glucuronidase activity can demonstrate the stable genetic transformation in induced hairy roots. Therefore, the putative transformants were checked for the activity of the reporter gene through GUS assay. In this study, the blue color was observed only in ATCC15834_(GUS) transformed roots (Fig. 5a, c, d) whereas control roots (Fig. 5b) did not show GUS activity. These results indicate that the GUS gene in the T-DNA had already been integrated into the genome of the transgenic *A. officinalis* hairy root cultures. Similar results were reported earlier (Liu et al. 2011).

Molecular analysis

Verification of transgenic nature of the hairy roots was done with PCR amplification of a portion of the *rolB* gene and a portion of GUS gene. A 780-bp (Fig. 6 left) and 320-bp (Fig. 6 right) fragment amplification were observed in all the hairy root lines. No amplification was detected in negative control as well as in the genomic DNA of non-transformed normal roots (Fig. 6). These results showed the integration of T-DNA (transferred-DNA) into the plant genome and the roots were free of bacteria. The results obtained indicate the same results of Li et al. (2015) and Moghadam et al. (2013). Among the genes of *rol*, *rolB* plays a critical role in pathogenicity, while *rolA*, *rolC* and *rolD* contribute to the root induction (Sujatha et al. 2013).

Fig. 4 Rapidly growing hairy roots culture on solid (a) and in liquid (b, c) culture media, in the dark without plant growth regulators (a: 0.3×) (b: 0.3×) (c: 0.5×)

Fig. 5 GUS histochemical assay showing gene expression in transgenic roots of *A. officinalis* (a) while no expression can be detected in the non-transgenic root (b). Hairy root tissue transformed with the

GUS gene (c, d) and photographed under a stereomicroscope (SZX2-ILLB, Japan) (a, b: 0.5×) (c: 6×) (d: 4×)

Fig. 6 Molecular analysis in transgenic hairy roots of *Althaea officinalis*. (Left) rolB PCR amplification: marker (1 kb ladder Fermentas); lanes 1–2, DNA from hairy roots (780 bp); lanes 3–4, roots from a non-transformed root (negative control); lane 5, plasmid DNA (pos-

itive control). (Right) GUS PCR amplification: marker (1 kb ladder Fermentas); lanes 1, DNA from hairy roots (320 bp); lanes 2–3, roots from a non-transformed root (negative control); lane 4, plasmid DNA (positive control)

Total phenolic and flavonoid contents

The contents of phenolic and flavonoid compounds were measured in transformed hairy roots and non-transformed roots. We found substantial differences in phenolic and flavonoid contents between transformed and non-transformed roots ($p < 0.01$). The highest total phenolic content (1.57 ± 0.1 mg/g) observed in hairy roots of A13 strain, which was 4.7-fold more than the untransformed roots, while ATCC15834 and ATCC15834_(GUS) strains had the lowest content of phenolic and flavonoid compounds compared with the other strains. Statistical analysis for phenolic content indicated that there were significant differences among strains and control sample ($p < 0.01$). Strains A4 (0.74 ± 0.15 mg/g) and ATCC15834 (0.59 ± 0.07 mg/g) were not significantly different from each other (Fig. 7a). Furthermore, A4 strain showed the highest (3.47 ± 0.3 mg/g)

flavonoid content, which was threefold higher than control. The statistical analysis for flavonoid content showed significant differences among strains of A4 (3.47 mg/g), A13 (2.56 ± 0.17 mg/g) and control sample (1.15 ± 0.2 mg/g), while there were no significant differences in two strains ATCC15834 (1.65 ± 0.08 mg/g) and ATCC15834_(GUS) (1.71 ± 0.12 mg/g) (Fig. 7b). The accumulations of higher levels of phenolics and flavonoids, in hairy roots, could be due to the effect of RolB that up-regulate the genes involved in secondary metabolite production (Bulgakov et al. 2018) as reported in many other plant species (Weremczuk-Jeżyna et al. 2013; Thiruvengadam et al. 2014; El-Esawi et al. 2017). Previously, many reports demonstrated hairy roots induced by different strains of *A. rhizogenes* vary in secondary metabolite production (Gupta et al. 2016; Thwe et al. 2016). They suggested that the secondary metabolite production is strain-specific. Similarly, Gupta et al. (2016)

Fig. 7 Comparison of the total phenolic (a) and total flavonoid (b) content in control and hairy roots obtained by *A. rhizogenes* strains. Bars represent the mean $\pm$ SD of three independent measurements. Different letters above columns denote statistically significant differences between groups based on the LSD test ($p < 0.05$)

and Skala et al. (2015) reported that A4 was efficient for the production of secondary metabolites. Our results showed that despite the mass production of hairy roots by strain ATCC15834_(GUS), their phenolic and flavonoid contents were not higher than the other strains. Therefore, these results suggest no correlation between regulation of growth rate and secondary metabolite synthesis in hairy roots.

Conclusions

Our results demonstrated an efficient *A. rhizogenes*-mediated transformation protocol for the foundation of *A. officinalis* hairy root cultures using four different *A. rhizogenes* strains: A4, A13, ATCC15834 and ATCC15834_(GUS). Among the four different strains of *A. rhizogenes*, ATCC15834_(GUS) had potential ability to mass production of hairy root, especially in the MS liquid medium. However, hairy roots generated by *A. rhizogenes* are a potential source for plant secondary metabolites, but production of certain metabolites at the commercial level could be the next important step.

Acknowledgements This work was financially supported by Neyshabur Branch, Islamic Azad University Neyshabur, Iran.

Author contributions PT performed experiments, analyzed data and drafted the paper; ASA designed and supervised the experiments, analyzed the data and drafted the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Benbassat N, Yoncheva K, Hadjimitova V, Hristova N, Konstantinov S, Lambov N (2014) Influence of the extraction solvent on antioxidant activity of *Althaea officinalis* L. root extracts. Cent Eur J Biol 9(2):182–188
- Bulgakov VP, Vereshchagina YV, Bulgakov DV, Veremeichik GN, Shkryl YN (2018) The rolB plant oncogene affects multiple signaling protein modules related to hormone signaling and plant defense. Sci Rep 8(1):2285
- Chandran RP, Potty V (2008) Induction of hairy roots through the mediation of four strains of *Agrobacterium rhizogenes* on five host plants. Indian J Biotechnol 7(1):122–128
- Chaudhuri KN, Ghosh B, Tepfer D, Jha S (2005) Genetic transformation of *Tylophora indica* with *Agrobacterium rhizogenes* A4: growth and tylophorine productivity in different transformed root clones. Plant Cell Rep 24(1):25–35
- Colling J, Groenewald JH, Makunga NP (2010) Genetic alterations for increased coumarin production lead to metabolic changes in the medicinally important *Pelargonium sidoides* DC (Geraniaceae). Metab Eng 12(6):561–572
- Drake PM, de Moraes Madeira L, Szeto TH, Ma JK (2013) Transformation of *Althaea officinalis* L. by *Agrobacterium rhizogenes* for the production of transgenic roots expressing the anti-HIV microbicide cyanovirin-N. Transgenic Res 22(6):1225–1229
- El-Esawi MA, Elkelish A, Elansary HO, Ali HM, Elshikh M, Witczak J, Ahmad M (2017) Genetic transformation and hairy root induction enhance the antioxidant potential of *Lactuca serriola* L. Oxid Med Cell Longev 2017:5604746
- Elfahmi SS, Chahyadi A (2014) Optimization of genetic transformation of *Artemisia annua* L. using *Agrobacterium* for Artemisinin production. Pharmacogn Mag 10(Suppl 1):S176–S180
- Granato D, Shahidi F, Wrolstad R, Kilmartin P, Melton LD, Hidalgo FJ, Miyashita K, van Camp J, Alasalvar C, Ismail AB, Elmore S (2018) Antioxidant activity, total phenolics and flavonoids contents: should we ban in vitro screening methods? Food chem 264:471–475
- Green MR, Sambrook J (2012) Molecular cloning: a laboratory manual, vol 1. Cold Spring Harbor Laboratory Press, New York
- Guerriero G, Berni R, Muñoz-Sánchez JA, Apone F, Abdel-Salam EM, Qahtan AA, Alatar AA, Cantini C, Cai G, Hausman JF, Siddiqui KS (2018) Production of Plant Secondary Metabolites: Examples, Tips and Suggestions for Biotechnologists. Genes. <https://doi.org/10.3390/genes9060309>
- Gupta R, Pandey P, Singh S, Singh DK, Saxena A, Luqman S, Bawankule DU, Banerjee S (2016) Advances in Boerhaavia diffusa hairy root technology: a valuable pursuit for identifying strain sensitivity and up-scaling factors to refine metabolite yield and bioactivity potentials. Protoplasma 253(4):1145–1158
- Habibi P, de Sa MF, da Silva AL, Makhzoum A, da Luz Costa J, Borghetti IA, Soccol CR (2016) Efficient genetic transformation

- and regeneration system from hairy root of *Origanum vulgare*. *Physiol Mol Biol Plants* 22(2):271–277
- Huang SH, Vishwakarma RK, Lee TT, Chan HS, Tsay HS (2014) Establishment of hairy root lines and analysis of iridoids and secoiridoids in the medicinal plant *Gentiana scabra*. *Bot Stud* 55(1):17. <https://doi.org/10.1186/1999-3110-55-17>
- Ionkova I (1992) Alternative ways for the production of biologically active substances from *Althaea officinalis* var. *russalka*. *CR Acad Bulg Sci* 45:137–140
- Khidyrova N, Van E, Shakhidoyatov RK, Bobakulov KM, Abdullaev N, Shakhidoyatov KM (2012) Polyprenols from leaves and stems of the plant *Althaea officinalis*. *Chem Nat Compd* 48(3):358–360
- Lee SY, Kim SG, Song WS, Kim YK, Park N, Park SU (2010) Influence of different strains of *Agrobacterium rhizogenes* on hairy root induction and production of alizarin and purpurin in *Rubia akane* Nakai. *Rom Biotechnol Lett* 15:5405–5409
- Li B, Wang B, Li H, Peng L, Ru M, Liang Z, Yan X, Zhu Y (2015) Establishment of *Salvia castanea* Diels f. *tomentosa* Stib. hairy root cultures and the promotion of tanshinone accumulation and gene expression with Ag, methyl jasmonate, and yeast extract elicitation. *Protoplasma* 253(1):87–100
- Liu DH, Ren WW, Cui LJ, Zhang LD, Sun XF, Tang KX (2011) Enhanced accumulation of catharanthine and vindoline in *Catharanthus roseus* hairy roots by overexpression of transcriptional factor ORCA2. *Afr J Biotechnol* 10(17):3260
- Mishra BN, Ranjan R (2008) Growth of hairy-root cultures in various bioreactors for the production of secondary metabolites. *Biotechnol Appl Biochem* 49(1):1–10
- Moghadam YA, Piri K, Bahramnejad B, Habibi P (2013) Methyl Jasmonate and Salicylic acid effects on the dopamine production in hairy cultures of *Portulaca oleracea* (purslane). *Bull Env Pharmacol Life Sci* 2(6):89–94
- Moyo M, Amoo S, Bairu M, Finnie J, Van Staden J (2008) Optimising DNA isolation for medicinal plants. *S Afr J Bot* 74(4):771–775
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15(3):473–497
- Nagella P, Thiruvengadam M, Jung S, Murthy H, Chung I (2013) Establishment of *Gymnema sylvestre* hairy root cultures for the production of gymnemic acid. *Acta Physiol Plant* 35(10):3067–3073
- Panda BM, Mehta UJ, Hazra S (2017) Optimizing culture conditions for establishment of hairy root culture of *Semecarpus anacardium* L. *3 Biotech* 7(1):21. <https://doi.org/10.1007/s13205-017-0608-x>
- Park YS, Jung ST, Kang SG, Heo BG, Arancibia-Avila P, Toledo F, Drzewiecki J, Namiesnik J, Gorinstein S (2008) Antioxidants and proteins in ethylene-treated kiwifruits. *Food Chem* 107(2):640–648
- Pistelli L, Giovannini A, Ruffoni B, Bertoli A, Pistelli L (2010) Hairy root cultures for secondary metabolites production. In: *Bio-Farms for Nutraceuticals*. Springer, Boston, pp 167–184
- Rahnama F, Hasanloo T, Shams MR, Sepehrifar R (2008) Silymarin production by hairy root culture of *Silybum marianum* (L.) Gaertn. *Iran J Biotechnol* 6(2):113–118
- Rostampour S, Sohi HH, Jourabchi E, Ansari E (2009) Influence of *Agrobacterium rhizogenes* on induction of hairy roots and benzylisoquinoline alkaloids production in Persian poppy (*Papaver bracteatum* Lindl.): preliminary report. *World J Microbiol Biotechnol* 25(10):1807–1814
- Runo S, Macharia S, Alakonya A, Machuka J, Sinha N, Scholes J (2012) Striga parasitizes transgenic hairy roots of *Zea mays* and provides a tool for studying plant-plant interactions. *Plant methods* 8(1):20. <https://doi.org/10.1186/1746-4811-8-20>
- Samadi A, Jafari M, Nejhad NM, Hosseini F (2014) Podophyllotoxin and 6-methoxy podophyllotoxin Production in Hairy Root Cultures of *Lium mucronatum* ssp. *mucronatum*. *Pharmacogn Mag* 10(38):154–160
- Shah S, Akhtar N, Akram M, Shah PA, Saeed T, Ahmed K, Asif H (2011) Pharmacological activity of *Althaea officinalis* L. *J Med Plants Res* 5:5662–5666
- Sharafi A, Sohi HH, Mirzaee H, Azadi P (2014) In vitro regeneration and *Agrobacterium* mediated genetic transformation of *Artemisia aucheri* Boiss. *Physiol Mol Biol Plants* 20(4):487–494
- Sharifi S, Sattari TN, Zebarjadi A, Majd A, Ghasempour H (2014) The influence of *Agrobacterium rhizogenes* on induction of hairy roots and ss-carboline alkaloids production in *Tribulus terrestris* L. *Physiol Mol Biol Plants* 20(1):69–80
- Skała E, Kicel A, Olszewska MA, Kiss AK, Wysokińska H (2015) Establishment of hairy root cultures of *Rhaponticum carthamoides* (Willd.) Iljin for the production of biomass and caffeic acid derivatives. *Biomed Res Int*. <https://doi.org/10.1155/2015/181098>
- Sujatha G, Zdravković-Korać S, Čalić D, Flamini G, Kumari BR (2013) High-efficiency *Agrobacterium rhizogenes*-mediated genetic transformation in *Artemisia vulgaris*: hairy root production and essential oil analysis. *Ind Crops Prod* 44:643–652
- Tenea GN, Calin A, Gavrila L, Cucu N (2008) Manipulation of root biomass and biosynthetic potential of *Glycyrrhiza glabra* L. plants by *Agrobacterium rhizogenes* mediated transformation. *Roumanian Biotechnol Lett* 13(5):3922–3932
- Thiruvengadam M, Praveen N, Kim EH, Kim SH, Chung IM (2014) Production of anthraquinones, phenolic compounds and biological activities from hairy root cultures of *Polygonum multiflorum* Thunb. *Protoplasma* 251(3):555–566
- Thwe A, Arasu MV, Li X, Park CH, Kim SJ, Al-Dhabi NA, Park SU (2016) Effect of different *Agrobacterium rhizogenes* strains on hairy root induction and phenylpropanoid biosynthesis in tartary buckwheat (*Fagopyrum tataricum* Gaertn). *Front Microbiol* 7:318
- Tian L (2015) Using hairy roots for production of valuable plant secondary metabolites. *Adv Biochem Eng Biotechnol* 149:275–324
- Valimehr S, Sanjarian F, Sohi HH, Sharafi A, Sabouni F (2014) A reliable and efficient protocol for inducing genetically transformed roots in medicinal plant *Nepeta pogonosperma*. *Physiol Mol Biol Plants* 20(3):351–356
- Weremczuk-Jeżyn I, Grzegorzczak-Karolak I, Frydrych B, Królicka A, Wysokińska H (2013) Hairy roots of *Dracocephalum moldavica*: rosmarinic acid content and antioxidant potential. *Acta Physiol Plant* 35(7):2095–2103
- Zubricka D, Misianikova A, Henzelyova J, Valletta A, De Angelis G, D'Auria FD, Simonetti G, Pasqua G, Cellarova E (2015) Xanthones from roots, hairy roots and cell suspension cultures of selected *Hypericum* species and their antifungal activity against *Candida albicans*. *Plant Cell Rep* 34(11):1953–1962