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RESEARCH ARTICLE

A Comparative Analysis of the Hairy Root Induction Methods in *Hypericum perforatum*

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ABSTRACT: *Hypericum perforatum* is a medicinal plant which Hypericin, Hyperforin and phenolic compounds are its active secondary metabolites. Hairy root induction by *Agrobacterium rhizogenes* in this plant is difficult and has low efficiency. In the present study two inoculation methods, immersion in bacterial suspension and direct injection of *A. rhizogenes* has been compared. For this purpose, the best conditions for *H. perforatum* hairy root induction including *A. rhizogenes* strains (A4, LBA9402, NCPPB2656), plant explants (Stem, Apical bud, leaves), co-cultivation media (MS, ½MS, B5, and ½B5) and Acetosyringone (AS) concentration (0 and 100 µM) were specified and used for comparative analysis. It was found that strain A4, Stem explants, ½MS co-cultivation medium without AS constitute the best conditions for hairy root induction of *H. perforatum*. Transgenic nature of the potential hairy roots was confirmed using PCR and specific *rol*B and *rol*C genes primers. The results showed that the efficiency of applying direct injection method is four times higher than immersion in bacterial suspension in *H. perforatum* hairy root induction. In general, the results indicate that direct injection can be the method of choice to successful hairy root induction in *H. perforatum*.

KEYWORDS: *Agrobacterium rhizogenes,* frequency of Transformation, Induction method, hairy root

INTRODUCTION

Hypericum perforatum (Hypericaceae family) is a medicinal plant rich of valuable secondary metabolites including, Hyperforin ($C_{35}H_{52}O_5$), Hypericin ($C_{30}H_{16}O_8$) and pseudo-Hypericin ($C_{30}H_{16}O_9$) [27]. Hypericin is present in different species of *Hypericum*, while considerable amount of Hyperforin is only found in *H. perforatum* [44]. This plant has sedative and astringent properties, and has been used traditionally for the treatment of excitability, neuralgia, fibrositis, sciatica, menopausal neurosis, anxiety and depression [4]. Other pharmacological activities, including anti-viral, anti-cancer, anti-bacterial, nervous protection, anti-inflammatory, analgesic, diuretic, anti-malarial, and wound-healing are documented for extracts of *H. perforatum* [48]. Currently, several pharmaceutical-grade preparations of *H. perforatum* are commercially available for treating depression and other mood disorders [23].

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Environmental conditions (light, temperature, soil water, soil fertility and salinity) can significantly affect processes associated with growth and development of the plants, even their ability to synthesize secondary metabolites, eventually leading to the change of overall yield and profile of phytochemicals [47]. An alternative approach for rapid and selective production of bioactive secondary plant metabolites is applying hairy root cultures [40]. *Agrobacterium rhizogenes* is a gram-negative soil bacterium that can induce hairy root phenotype, generally without induction of plant defense response [24]. Hairy roots usually produce higher amounts of secondary metabolites than normal plants or cell/callus cultures and are characterized by rapid growth, genetic stability, and biosynthetic stability [28]. Sensitivity of plant species to *A. rhizogenes* infection and transformation is different and largely depends on plant tissue and induction method [42]. Besides, plant defense system is another important controlling factor which is triggered by response to agrobacterium infection and induce necrosis and programmed cell death in plant cells [31]. *H. perforatum* is sensitive to *A. rhizogenes* infection and its defense machinery reduces the viability of bacteria during co-cultivation [22]. It has shown that addition of antioxidants and ethylene inhibitors to the co-cultivation medium cannot prevent the decadence of plant tissues during bacterial infection, so it is a large obstacle to inducing hairy roots and metabolite biosynthesis in *H. perforatum* [17, 18]. Taken these reasons into account, in the present study two induction methods, namely, immersion in suspension and direct injection were applied and their respective efficiencies in order to induce hairy roots in *H. perforatum* were evaluated.

MATERIALS AND METHODS

Plant material

H. perforatum seeds were purchased from Pakan Bazr Co., Isfahan, Iran. Seeds were surface sterilized with 1% NaClO for 10 min, and then thoroughly washed with distilled water. The sterilized seeds were germinated on MS medium [26] containing B5 vitamins [19], 0.8% Agar and 2% Sucrose (pH was adjusted to 5.8 prior to Autoclaving) at 25$^{\circ C}$ and a 16/8-h photoperiod. The seedlings per jar were thinned and finally three seedlings were kept in each jar. Two months old Seedlings were used for preparing leaf and stem explants and direct injection of bacterial suspension.

Bacterial culture and inoculum preparation

In order to induce hairy roots of *H. perforatum*, three strains of *A. rhizogenes,* A4 and LBA9402, agropine type [8, 12] and NCBBP2656, cocomopine type [46] were used. Bacteria were grown in MYB medium (containing 40 mg/l rifampicin) at 28^{oC} to OD_{600}= 0.6. The bacterial suspension was centrifuged at 4500 rpm for 10 min at 4^{oC} and the pellet was then gently suspended in 50 ml of ½MS medium. The resultant suspension was used for *H. perforatum* hairy root induction [30].

Hairy root induction conditions

To determine optimum conditions for *H. perforatum* hairy root induction in immersion method various factors were assayed. The transformation ability of different *A. rhizogenes* strains, A4, NCPPB2656, and LBA9402 on leaf, stem and apical bud explants of *H. perforatum* were investigated. After determining the suitable strain and explant, the effect of various co-cultivation media (including B5, ½B5, MS and ½MS) supplemented with 100 µM acetosyringone (AS) or without it on hairy root induction were evaluated. According to the results, the best strain, explant, co-cultivation medium, and AS concentration were selected and used for further comparative experiments.

Hairy root induction by immersion method

In this method, the explants were agitated in Agrobacterium suspension for 15 minutes on a rotary shaker. After that, the extra bacteria were removed by filter paper and then the leaf and stem explants were transferred to hormone-free co-cultivation media and were incubated at 25±2^{oC} in darkness. After 72 h explants were sub-cultured on ½MS supplemented with 500 mg/l cefotaxime. The antibiotic concentration was gradually decreased in subsequent sub-cultures. After co-cultivation, emergence of potential hairy roots was evaluated on a daily basis for three weeks. The hairy roots were transferred to hormone-free ½MS liquid medium and were subsequently shaken at 120 rpm on a rotary shaker at 25^{oC}.

Hairy root induction by direct injection method

For this method, the bacterial suspension (OD_{600}= 0.6) was directly injected by a 30g syringe into stems of two months old seedlings until a drop of cell suspension appear on the injection sites (20 injection sites per

seedling). Treated plants were incubated in darkness at 25±2°C for 72 h. Then, in order to remove bacteria, the treated plants were taken out of the medium and washed with 0.85% sodium chloride solution containing 500 mg/l cefotaxime. Subsequently, the plants were sub-cultured in ½MS solid medium containing 500 mg/l cefotaxime. After one week, emergence of hairy roots was evaluated and noted down on a daily basis for three weeks. The hairy roots were transferred to hormone-free ½MS medium and were subsequently shaken at 120 rpm at 25°C.

Molecular confirmation of hairy roots

Genomic DNA of potential hairy roots and A. rhizogenes were extracted by a CTAB [15] and Alkaline lysis [36] methods, respectively. The quality and quantity of extracted gDNA was determined using electrophoresis and spectrophotometry. PCR reaction was conducted in 25 µl solution containing PCR buffer, 0.2 mM of each dNTPs, 0.3 µM of *rol*B (gctcttgcagtgctagattt and gaaggtgcaagctacctctc) and *rol*C (ctcctgacatcaaactcgtc and tgcttcgagttatgggtaca) specific primers, 100 ng of template DNA, and one unit of *Taq* DNA polymerase. The thermal conditions were set as 94˚C for 3 min followed by 35 cycles of denaturation (94˚C, 30s), annealing (56˚C, 30s), and extension (72oC, 45s), after that a 7 min cycle at 72˚C for final extension. PCR products were analyzed on 1% agarose gel and were visualized by ethidium bromide staining.

Statistical analysis

For all treatments, three independent experiments were done under the same conditions based on a completely randomized design. Each experiment was included three replicas, with 20 explants or 20 injections per seedling. Transformation frequency (%) was calculated after three weeks by the below equation:

$$T(\%) = \frac{\text{No. of explants inducing hairy roots}}{\text{Total No. of explants infected with A. rhizogenes}} \times 100$$

The data were subjected to analysis of variance (ANOVA) using SPSS program, version 16.0 and the means were compared by the least significant difference (LSD) test at 0.05 level of probability. Values were represented as mean ± SE.

RESULTS

According to the results of an immersion method as primary experiment, the best conditions for hairy root induction including, bacterial strain, explant type, co-cultivation medium and concentration of AS in co-cultivation medium were determined. These conditions were used to compare efficiency of two hairy root induction methods, namely immersion and direct injection, in H. perforatum.

The effect of bacterial strain and explant type on hairy root induction

As shown in Fig. 1, the transformation ability of bacterial strains is influenced by explant type. Among A4 and LBA9402 treated explants, the highest number of hairy roots was observed in stem explants, however their difference was statistically significant ($p<0.05$). In addition to stem explants, number of hairy roots in A4 treated leaf explants was high and comparable with LBA9402 treated stem explants. On the other hand, the smallest number of hairy roots was observed in terminal bud explants in all bacterial treated explants. NCPPB2652 strain was showed the lowest transformation ability and there was no significant difference among explants ($p<0.05$). Therefore, the A4 strain and stem explant were chosen as the most potent strain and the most suitable explant in comparative experiments, respectively.

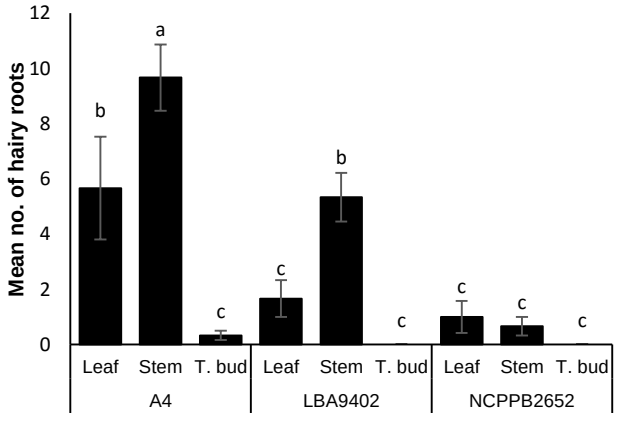

Figure 1. The effect of explant type (leaf, stem and terminal bud) and bacterial strains (A4, LBA 9402 and NCPPB 2652) on the number of *Hypericum perforatum* hairy roots. Results are represented as mean of three replicates ± SE. Columns with different letters indicate significant differences at $P \leq 0.05$ by LSD's test.

The effect of co-cultivation medium and AS concentration on hairy root induction

A4 strain and stem explants were used to evaluate the effect of acetosyringone (0 and 100 µM) on hairy root induction in various co-cultivation media (MS, ½MS, B5, and ½B5). According to the results (Fig. 2), there was no significant difference between plants treated with different concentrations of AS in MS, B5, and ½B5 co-cultivation media ($p<0.05$). But, a remarkable negative impact of AS was observed in ½MS co-cultivation medium. Consequently, AS was not used in further hairy root induction experiments. The greatest number of hairy roots (10.3) was observed in ½MS co-cultivation medium and therefore this medium was selected for co-cultivation and sub-culturing of hairy roots in comparative experiments.

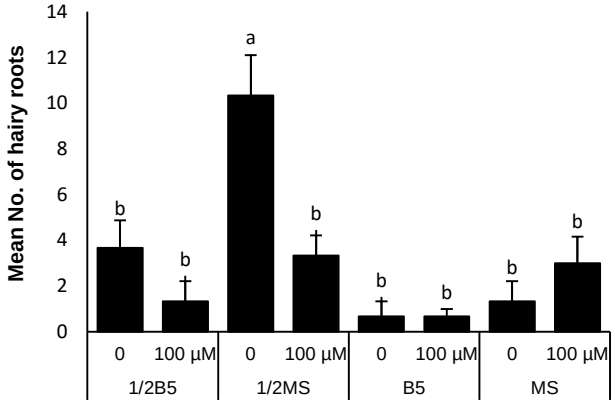

Figure 2. The effect of Acetosyringone (0 and 100 µM) and co-cultivation medium (MS, ½MS, B5, and ½B5) on *Hypericum perforatum* hairy root induction. Results are represented as mean of three replicates ± SE. Columns with different letters indicate significant differences at $P \leq 0.05$ by LSD's test.

Figure 3. Emergence of hairy roots in *Hypericum perforatum* after injecting bacterial suspension is indicated by arrows.

Efficiency comparison of H. perforatum hairy root induction methods

There was no significant difference in hairy root initiation and the first hairy roots in both methods were observed 4 days after inoculation (Fig. 3). Until 21 days the emergence of new hairy roots was recorded, that its trend in immersion and direct injection methods is shown in Fig. 4a. After three weeks the mean number of hairy roots in both inoculation methods was compared and a significant difference between them was observed ($p<0.05$). The direct injection method efficiency in inducing *H. perforatum* hairy roots was four times greater than immersion method (Fig. 4b).

Molecular confirmation of transgenic Hairy roots

Transgenic state of different hairy root clones was confirmed by PCR and the presence of *rol*B and *rol*C genes of bacterial T-DNA in their genome was evaluated. As shown in Fig. 5 all of hairy root clones show amplicons with 420 bp and 650 bp representing the *rol*B and *rol*C genes, respectively. In normal plant, none of these amplicons were observed.

DISCUSSION

In the present research two hairy root induction methods in *H. perforatum* were compared. Results showed that stem explants display high sensitivity to *A. rhizogenes* contamination so that this explant type is suitable for developing hairy roots in *H. perforatum*. The efficiency of immersion in bacterial suspension method was 13% while, in direct injection, it was 57%. Vinterhalter et al. (2006) reported hairy root induction efficiency of 21% in stem explants of *H. perforatum* using immersion method that is a bit greater than results obtained in the present study [45]. Bivadi et al. (2014) concluded that stem explants are better than leaf explants for hairy root induction in *H. perforatum*, which is in accordance with our results [9]. In another experiment the rate of hairy root induction by ATCC15834 in leaf and root explants of *H. perforatum* reported 13% and 25%, respectively. It should, however, be noticed that for achieving better results they were added Indole-3-acetic acid (IAA) and zeatin to induction medium [13]. High transformation rate (33%) in root explants of *H. perforatum* was also reported [41].

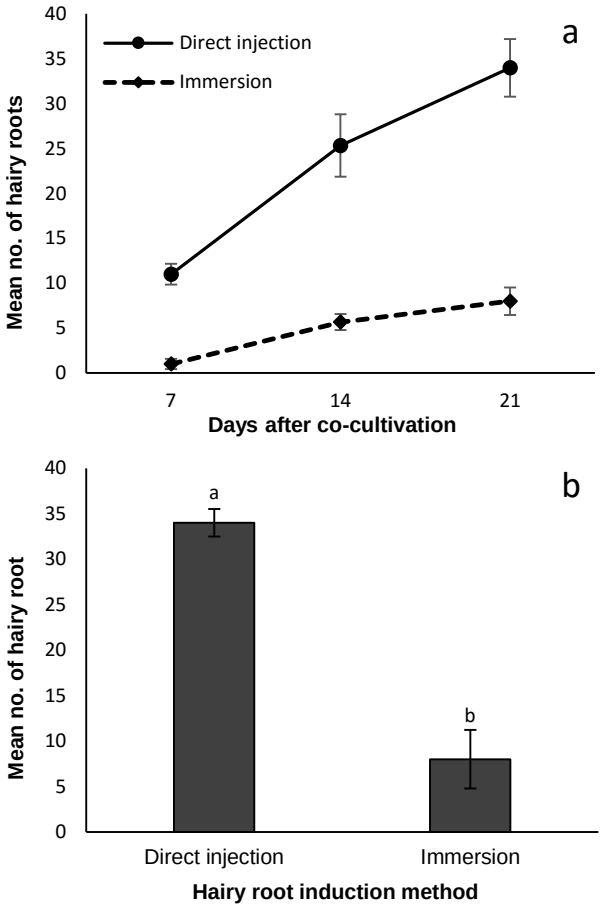

Figure 4. a) Mean number of hairy roots in direct injection and immersion methods until 21 days after co-cultivation. b) The hairy root induction efficiency in direct injection and immersion methods. Results are represented as mean of three replicates ± SE. Columns with different letters indicate significant differences at $P \leq 0.05$ by LSD's test.

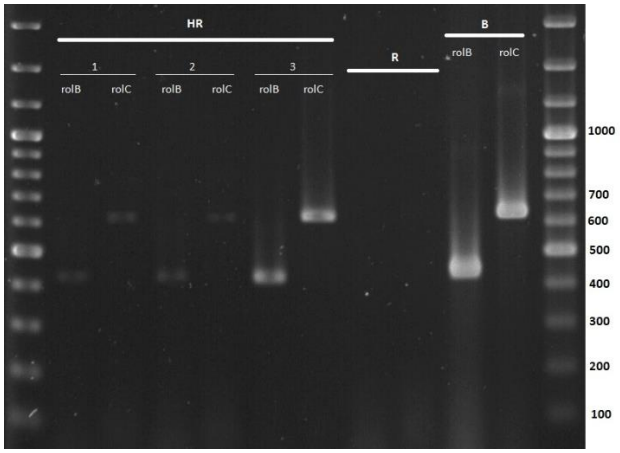

Figure 5. Molecular confirmation of transgenic state of different hairy root clones (HR1, HR2 and HR3) of *H. perforatum* using *rol*B and *rol*C gene specific primers. R: DNA extracted from root of normal plant as PCR template (negative control). B: DNA of *A. rhizogenes* as PCR template (positive control).

Selecting the suitable and effective *A. rhizogenes* strain is important for efficient production of hairy root mass as well as secondary metabolites and depends on plant species. In the present study, the transformation efficiency of various *A. rhizogenes* strains in hairy root induction of *H. perforatum* was investigated. Results indicate a significant difference among strains and, accordingly, A4 was chosen as the best strain for inducing hairy roots. This strain has been used for hairy root induction of various medicinal plants such as *Scutellaria baicalensis, Gentiana macrophylla, Aesculus hippocastanum* and *Catharanthus roseus* [5, 38, 39, 49]. Hairy root induction in leaf and root explants of *H. perforatum* by ATCC15834 [6] and of root explants by A4 strain has been reported [41]. Bivadi et al. (2014) investigated the effect of different *A. rhizogenes* strains (ATCC15834, A4, and 11325) on hairy root induction of *H. perforatum*, and introduced ATCC15834 as the best strain [9]. The efficiency of *A. rhizogenes* strains in inducing hairy root in different plants has been investigated. Pakdin et al. (2013) investigated efficiency of four *A. rhizogenes* strains in *Valeriana officinalis* and identified A4 as the best strain [30]. In addition, it was shown that the ability of hairy root induction in *Capsicum* species by various *A. rhizogenes* strains differed significantly, ATCC13333 and ATCC15834 were identified as the best strains in *C. frutescens* while ATCC43056 and ATCC43057 were identified as the best strains in *C. annuum* [35]. In another study, SA79 strain was found to cause maximum transformation frequency in *Bacopa monnieri* compared with R1000, SA79, MTCC532, and MTCC2364 strains [2]. These differences in hairy root induction capability of different *A. rhizogenes* strains can be explained in terms of pathogenicity or host specificity [32].

Some phenolic compounds like AS are known to induce transcription of pathogenic genes in *vir* region of Agrobacterium [29]. This compound promotes Agrobacterium-mediated infection and enhances the efficiency of transformation [21]. Presence of AS (100 μM) in the co-cultivation media has no significant effect or significantly reduced the transformation ability of A4 in the case of ½MS medium. Di Guardo et al. (2003) and Vinterhalter et al. (2006) reported successful hairy root induction in *H. perforatum* in the absence of AS [13, 45]. However, in another study on this plant 200 μM AS has been applied, but its effect on hairy root induction is not mentioned [18]. Several studies have reported the

positive effect of AS on increasing the frequency of transformation [1, 3]. It has been showed that, secretion of phenolic compounds by plant tissues can be sufficient for *vir* genes activation and the application of exogenous acetosyringone may not be necessary for bacterial induction [33]. Efficiency reduction of hairy root induction in *Torenia fournieri* [37] and *Pisum sativum* [11] by applying AS have been reported.

In the present study, various co-cultivation medium with different compositions were used and their effect on hairy root induction and growth were evaluated. ½MS was found to be the best medium for inducing hairy roots in *H. perforatum*, while MS medium significantly was reduced the number of hairy roots. On the other hand, although the number of hairy roots in ½B5 medium was slightly higher than B5 and MS media, but their difference was not statistically significant ($p<0.05$). It seems that high concentration of macro and micro elements in MS medium prohibit hairy root induction in *H. perforatum*. It has reported that ½MS is a better medium for *H. perforatum* hairy root induction than MS medium [9], that is consistent with our results. However, it has mentioned that B5 medium is well as ½MS medium [9], whilst it showed the least efficiency in hairy root induction in our experiments. Genotype, physiological requirements, growth conditions, and bacterial strain are crucial in determining medium for hairy root induction [25]. MS medium was identified as the most suitable for Chicory's hairy root induction in a study which investigated the effects of various media [34].

A. rhizogenes uses the host cell's factors and processes during transformation and usually does not provoke plant defense response [43]. Nonetheless, plants are able to modify gene expression and initiate defense mechanism in response to *A. rhizogenes* and thereby control contamination and transformation [14]. Agrobacterium adaptation is largely depending on plant species and it can influence transformation efficiency [20]. In the present study, the efficiency of hairy root induction in different explants of *H. perforatum* in immersion method was low that is in contrasts with Vinterhalter et al. (2006) and Bivadi et al. (2014) results [9, 45]. Franklin et al. (2008) showed that *H. perforatum* and *A. rhizogenes* incompatibility cannot be explained by apoptosis or apoptotic process [18]. On the other hand, antibacterial components of plant, mainly Hypericin and Hyperforin, targets Gram-positive bacteria and therefore, do not affect *A. rhizogenes* viability in co-cultivation [18]. It has showed that accumulation of phenolic compounds such as salicylic acid, is one of key defense strategies that plants use against bacterial invasion [10]. Various internal and external factors including trauma, physical damage, drought, and pathogenic invasion affect the synthesis and accumulation of phenolic material [7]. Oxidation of explants and plant cell death during Agrobacterium-mediated transformation has been reported to be caused by generation of phenolic compounds (Parrot et al. 2002). Explants were heavily damaged physically during immersion method and thus were exposed to bacterial invasion. Severe physical damage and presence of bacteria lead to increased phenolic compound production and thereby activation of plant defense mechanisms. In the present study, the immersed explants exhibit necrosis and brown color that is in consistent with Franklin et al. (2007) which reported these symptoms on stipule, Petiole, stem and root explants of *H. perforatum* after bacterial induction [17]. In direct injection of bacterial suspension, less physical damage is present and fewer cells are involved in plant defense mechanisms. In another research, sterile *H. perforatum* plants were scared and inoculated with *A. rhizogenes*, hairy roots were successfully emerged without any problems such as production of phenolic compounds or inefficient transformation. Hairy root induction of *Pinus halepensis* was investigated with three different methods (immersion in suspension, direct injection of bacterial suspension, and scarring and contaminating with bacterial suspension) and results showed that transformation highly depends on explants type and inoculation method. This study also reported successful hairy root induction despite embryonic sensitivity to *A. rhizogenes* contamination [42]. Hairy root induction was also investigated in case of *Dracocephalum kotschyi* Boiss using direct injection of LBA9402 strain suspension in leaf and root explants [16]. This study found that direct injection of bacterial suspension is a satisfactory hairy root induction method in Agrobacterium-mediated resistant plants such as *H. perforatum.* The method is easy, yields high amount of transformants and does not leads to plant defense reactions and can improve frequency of transformation by reducing plant-pathogen interaction.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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REFERENCES

[1] Aggarwal, D., Jaiswal, N., Kumar, A. and Reddy, MS. 2013. Factors affecting genetic transformation and shoot organogenesis of Bacopa monnieri (L.) Wettst. J Plant Biochem Biotechnol, 22:382-391.

[2] Bansal, M., Kumar, A. and Reddy, MS. 2014. Influence of Agrobacterium rhizogenes strains on hairy root induction and 'bacoside A'production from Bacopa monnieri (L.) Wettst. Acta Physiol Plant, 36:2793-2801.

[3] Barik, DP., Mohapatra, U. and Chand, PK. 2005. Transgenic grasspea (Lathyrus sativus L.): factors influencing Agrobacterium-mediated transformation and regeneration. Plant Cell Rep, 24:523-531.

[4] Barnes, J., Anderson, L. A., and Phillipson, J. D. 2001. St John's wort (Hypericum perforatum L.): a review of its chemistry, pharmacology and clinical properties. J Pharm Pharmacol, 53(5):583-600.

[5] Batra, J., Dutta, A., Singh, D., Kumar, S. and Sen, J. 2004. Growth and terpenoid indole alkaloid production in Catharanthus roseus hairy root clones in relation to left-and right-termini-linked Ri T-DNA gene integration. Plant Cell Rep, 23:148-154.

[6] Bertoli, A., Giovannini, A., Ruffoni, B., Guardo, AD., Spinelli, G., Mazzetti, M. and Pistelli, L. 2008. Bioactive constituent production in St. John's wort in vitro hairy roots. Regenerated plant lines. J Agric Food Chem, 56:5078-5082.

[7] Bhattacharya, A., Sood, P. and Citovsky, V. 2010. The roles of plant phenolics in defence and communication during Agrobacterium and Rhizobium infection. Mol Plant Pathol, 11:705-719.

[8] Birot, AM., Bouchez, D., Casse-Delbart, F., Durand-Tardif, M., Jouanin, L., Pautot, V. and Vilaine, F. 1987. Studies and uses of the Ri plasmids of Agrobacterium rhizogenes [transformation, T-DNA]. Plant Physiol Biochem [France].

[9] Bivadi, V., Zakaria, RA., Zare, N. and Yazdani, B. 2014. Effects of different tissue culture conditions in Hairy roots induction in Hypericum perforatum L. Int J Agric Crop Sci, 7:646.

[10] Boller, T. and He, SY. 2009. Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. Science, 324:742-744.

[11] De Kathen, A. and Jacobsen, HJ. 1990. Agrobacterium tumefaciens-mediated transformation of Pisum sativum L. using binary and cointegrate vectors. Plant Cell Rep, 9:276-279.

[12] De Paolis, A., Mauro, ML., Pompom, M., Cardarelli, M., Spano, L. and Costantino, P. 1985. Localization of agropine-synthesizing functions in the T R region of the root-inducing plasmid of Agrobacterium rhizogenes 1855. Plasmid, 13:1-7.

[13] Di Guardo, A., Cellarova, E., Koperdáková, J., Pistelli, L., Ruffoni, B., Allavena, A. and Giovannini, A. 2003. Hairy root induction and plant regeneration in Hypericum perforatum L. J Genet Breed, 57(3):269-278.

[14] Ditt, RF., Nester, E. and Comai, L. 2005. The plant cell defense and Agrobacterium tumefaciens. FEMS Microbiol Lett, 247:207-213.

[15] Doyle, J. and Doyle, JL. 1987. Genomic plant DNA preparation from fresh tissue-CTAB method. Phytochem Bull, 19:11-15.

[16] Fattahi, M., Nazeri, V., Torras-Claveria, L., Sefidkon, F., Cusido, RM., Zamani, Z. and Palazon, J. 2013. A new biotechnological source of rosmarinic acid and surface flavonoids: Hairy root cultures of Dracocephalum kotschyi Boiss. Ind Crop Prod, 50:256-263.

[17] Franklin, G., Oliveira, M. and Dias, ACP. 2007. Production of transgenic Hypericum perforatum plants via particle bombardment-mediated transformation of novel organogenic cell suspension cultures. Plant Sci, 172:1193-1203.

[18] Franklin, G., Conceição, LF., Kombrink, E. and Dias, ACP. 2008. Hypericum perforatum plant cells reduce Agrobacterium viability during co-cultivation. Planta, 227:1401-1408.

[19] Gamborg, OL., Miller, R. and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res, 50:151-158.

[20] Georgiev, MI., Pavlov, AI. and Bley, T. 2007. Hairy root type plant in vitro systems as sources of bioactive substances. Appl Microbiol Biotechnol, 74:1175.

[21] Giri, A. and Narasu, ML. 2000. Transgenic hairy roots recent trends and applications. Biotechnol Adv, 18:1-22.

[22] Hou, W., Shakya, P. and Franklin, G. 2016. A perspective on Hypericum perforatum genetic transformation. Front Plant Sci, 7:879.

[23] Klemow, K. M., Bartlow, A., Crawford, J., Kocher, N., Shah, J., and Ritsick, M. 2011. Herbal Medicine: Biomolecular and Clinical Aspects. CRC Press, 2(11): 211-228.

[24] Lima, JE., Benedito, VA., Figueira, A. and Peres, LEP. 2009. Callus, shoot and hairy root formation in vitro as affected by the sensitivity to auxin and ethylene in tomato mutants. Plant Cell Rep, 28:1169-1177.

[25] Mehrotra, S., Kumar Kukreja, A., Singh Khanuja, SP. and Nath Mishra, B. 2008. Genetic transformation studies and scale up of hairy root culture of Glycyrrhiza glabra in bioreactor. Electron J Biotechnol, 11:69-75.

[26] Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant, 15:473-497.

[27] Murch, SJ., KrishnaRaj, S. and Saxena, PK. 2000. Tryptophan is a precursor for melatonin and serotonin biosynthesis in in vitro regenerated St. John's wort (Hypericum perforatum L. cv. Anthos) plants. Plant Cell Rep, 19:698-704.

[28] Namdeo, AG., Jadhav, TS., Rai, PK., Gavali, S. and Mahadik, KR. 2007. Precursor feeding for enhanced production of Secondary metabolites: A review. Pharmacogn Rev, 1(2):227.

[29] Nilsson, O. and Olsson, O. 1997. Getting to the root: the role of the Agrobacterium rhizogenes rol genes in the formation of hairy roots. Physiol Plant, 100:463-473.

[30] Pakdin Parizi, A., Farsi, M., Nematzadeh, GA. and Mirshamsi, A. 2015. Impact of different culture media on hairy roots growth of Valeriana officinalis L. Acta agric Slov, 103(2): 299-305.

[31] Parrott, DL., Anderson, AJ. and Carman, JG. 2002. Agrobacterium induces plant cell death in wheat (Triticum aestivum L.) Physiol Mol Plant Pathol, 60:59-69.

[32] Porter, JR. and Flores, H. 1991. Host range and implications of plant infection by Agrobacterium rhizogenes. Crit Rev Plant Sci, 10:387-421.

[33] Rao, MVR. and Rao, GJN. 2007. Agrobacterium-mediated transformation of indica rice under Acetosyringone-free conditions. Plant Biotechnol, 24:507-511.

[34] Sara, K., Jafar, Z., Gorbanalli, N. and Ehsan, S. 2012. Optimization of hairy root culture establishment in Chicory plants (Cichorium intybus) through inoculation by Agrobacterium rhizogenes. J Agri Biotech, 4:61-75.

[35] Setamam, NM., Sidik, NJ., Rahman, ZA. and Zain, CRCM. 2014. Induction of hairy roots by various strains of Agrobacterium rhizogenes in different types of Capsicum species explants. BMC Res Notes, 7:414.

[36] Sinnett, D., Richer, C. and Baccichet, A. 1998. Isolation of stable bacterial artificial chromosome DNA using a modified alkaline lysis method. Biotechniques, 24:752-754.

[37] Tao, J. and Li, L. 2006. Genetic transformation of Torenia fournieri L. mediated by Agrobacterium rhizogenes. S Afr J Bot, 72:211-216.

[38] Tiwari, RK., Trivedi, M., Guang, ZC., Guo, GQ. and Zheng, GC. 2007. Genetic transformation of Gentiana macrophylla with Agrobacterium rhizogenes: growth and production of secoiridoid glucoside gentiopicroside in transformed hairy root cultures. Plant Cell Rep, 26:199-210.

[39] Tiwari, RK., Trivedi, M., Guang, ZC., Guo, GQ. and Zheng, GC. 2008. Agrobacterium rhizogenes mediated transformation of Scutellaria baicalensis and production of flavonoids in hairy roots. Biol Plant, 52:26-35.

[40] Toivonen, L. 1993. Utilization of hairy root cultures for production of secondary metabolites. Biotechnol Prog, 9:12-20.

[41] Tusevski, O., Stanoeva, JP., Stefova, M., Kungulovski, D., Pancevska, NA., Sekulovski, N. and Simic, SG. 2013. Hairy roots of Hypericum perforatum L.: a promising system for xanthone production. Cent Eur J Biol, 8:1010-1022.

[42] Tzfira, T., Yarnitzky, O., Vainstein, A. and Altman, A. 1996. Agrobacterium rhizogenes mediated DNA transfer in Pinus halepensis Mill. Plant Cell Rep, 16:26-31.

[43] Tzfira, T. and Citovsky, V. 2006. Agrobacterium-mediated genetic transformation of plants: biology and biotechnology. Curr Opin Biotechnol, 17:147-154.

[44] Vasconsuelo, A. and Boland, R. 2007. Molecular aspects of the early stages of elicitation of secondary metabolites in plants. Plant Sci, 172:861-875.

[45] Vinterhalter, B., Ninković, S., Cingel, A. and Vinterhalter, D. 2006. Shoot and root culture of Hypericum perforatum L. transformed with Agrobacterium rhizogenes A4M70GUS. Biol Plant, 50:767-770.

[46] Weller, SA., Stead, DE. and Young, JPW. 2006. Recurrent outbreaks of root mat in cucumber and tomato are associated with a monomorphic, cucumopine, Ri-plasmid harboured by various Alphaproteobacteria. FEMS Microbiol Lett, 258:136-143.

[47] Yang, L., Wen, K. S., Ruan, X., Zhao, Y. X., Wei, F., and Wang, Q. 2018. Response of plant secondary metabolites to environmental factors. Molecules, 23(4): 762.

[48] Yu, ZZ., Fu, CX., Han, YS., Li, YX. and Zhao, DX. 2006. Salicylic acid enhances jaceosidin and syringin production in cell cultures of Saussurea medusa. Biotechnol Lett, 28:1027-10

[49] Zdravković-Korać, S., Muhovski, Y., Druart, PH., Ćalić, D. and Radojević, L. 2004. Agrobacterium rhizogenes-mediated DNA transfer to Aesculus hippocastanum L. and the regeneration of transformed plants. Plant Cell Rep, 22:698-704.

مقایسه روش‌های القای ریشه موئین در گیاه گل راعی (*Hypericum perforatum*)

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چکیده

گل راعی (*Hypericum perforatum*) گیاهی دارویی با متابولیت‌های ثانویه هایپریسین، هایپرفورین و ترکیبات فنولی می‌باشد. القای ریشه‌های موئین با آگروباکتریوم ریزوژنز در این گیاه مشکل بوده و کارایی پایینی دارد. در تحقیق حاضر دو روش تلقیح مختلف شامل غوطه‌وری در سوسپانسیون و تزریق مستقیم باکتری آگروباکتریوم ریزوژنز با هم مقایسه شده است. برای این منظور، شرایط بهینه برای القای ریشه موئین در گل راعی از نظر سویه باکتری (A4, LBA9402, NCPPB2656) ریزنمونه گیاهی (ساقه، جوانه انتهایی و برگ)، محیط همکشتی (MS, ½MS, B5, and ½B5) و غلظت استوسیرینگون (۰ و ۱۰۰ میکرومولار) تعیین و برای آزمایشات مقایسه‌ای مورد استفاده قرار گرفت. براساس نتایج به دست آمده سویه A4، ریزنمونه ساقه و محیط همکشتی ½MS بدون استوسیرینگون بهترین شرایط القای ریشه موئین در گیاه گل راعی بودند. ماهیت تراریخته ریشه‌های موئین احتمالی با استفاده از PCR و آغازگرهای اختصاصی ژن‌های *rol*B و *rol*C تایید شد. بر اساس نتایج آزمایشات مقایسه‌ای کارایی القای ریشه موئین در گیاه گل راعی با روش تزریق مستقیم باکتری آگروباکتریوم ریزوژنز چهار برابر بیشتر از غوطه‌وری در سوسپانسیون باکتری بود. بنابراین تزریق مستقیم می‌تواند روش انتخابی برای القای موفقیت آمیز ریشه موئین در گیاه گل راعی باشد.

کلمات کلیدی: آگروباکتریوم ریزوژنز، فرانوانی ترنسفورماسیون، روش القا، ریشه موئین