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Genetically Transformed Root-Based Culture Technology in Medicinal Plant *Cosmos bipinnatus*

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Abstract

Background: *Cosmos bipinnatus* is an important medicinal plant with antioxidative, antigenotoxic, anti-inflammatory, and anti-proliferative effects on several cancer cell lines. It has great potential for development as a promising cancer chemo-preventive agent. Hairy root-based culture technology is a new sustainable production platform for producing specific pharmaceutical secondary metabolites.
Objectives: The current study developed and introduced a reliable transformation system for *C. bipinnatus* by optimization of aspects important in transformation frequency using *Agrobacterium rhizogenes*.
Methods: Five bacterial strains, including ATCC 15834, ATCC 31798, A7, MAFF-02-10266, and MSU440, 2 explant types (leaf and stem), and 2 co-cultivation media (full MS and ½ MS) were examined. Genomic DNA was extracted using a modified CTAB protocol from putative transgenic root lines and the control root. Transgenic hairy root lines were approved by means of Polymerase Chain Reaction (PCR) using specific *rol*B gene primers.
Results: The highest ratio of genetically transformed root induction was found from leaf explants using *A. rhisogenes* strains ATCC15834 and MSU440 (72% to 73%). When ½ MS medium was used as a co-cultivation medium, a significant increase in transformation frequency (84%) was observed.
Conclusions: The MSU440 *Agrobacterium* strain and ½ MS co-cultivation medium could significantly improve genetic transformation efficiency for establishment of hairy root-based cultures for *C. bipinnatus*.

Keywords: Hairy Root, *Agrobacterium rhizogenes*, *Cosmos bipinnatus*

1. Background

Apigenin 7-O-glucoside is amongst very important secondary metabolites, which is produced by *Cosmos bipinnatus*. *Cosmos bipinnatus* has been used in phytomedicine for numerous diseases, such as jaundice, spasmodic fever, and anti-inflammatory activity (1). Jang et al. (2008) reported antioxidative and antigenotoxic activity for *C. bipinnatus* extracts (2). They suggested that it has important antioxidant activity and protecting effect against oxidative DNA damage. Olajuyigbe and Ashafa (2014) reported the chemical composition of essential oils of *C. bipinnatus* and its antibacterial activity (3). Zheng et al. (2005) suggested that the hepatoprotective activity of apigenin is due to its antioxidant properties, performing as an ROS scavengers (4). Sohn et al. (2013) reported that a sesquiterpene lactone isolated from the roots of *C. bipinnatus* demonstrates an anti-inflammatory effect (1). It exhibits anti-inflammatory, anti-mutagenic, antiviral, and purgative effects (5, 6).

Pharmaceutical secondary metabolites have been used for the cure of numerous illnesses and are becoming a significant research area for drug discovery (7-11). Choi et al. (2009) reported that it may be a very useful anticancer drug candidate for chemotherapy and cancer prevention (12). Way et al. (2004) revealed anti-proliferative properties of apigenin on some cell lines of human breast cancer (13). It strongly inhibited tumor cell invasion in a breast tumor cell line (14). Zheng et al. (2005) reported that apigenin inhibited the growth of HeLa cells by inducing apoptosis (15). Wang et al. (2000) reported that the use of apigenin in colon carcinoma cell lines caused G_2/M cell cycle arrest and cell growth inhibition (16). Wang et al. (1999) re-

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ported that apigenin is very effective in inducing apoptosis on human leukemia cells (17). In another study, Budhraja et al. (2011) reported that Akt signaling pathways are possible objectives for inducing apoptosis in leukemia cells, using apigenin (18). Li et al. (2007) reported that it could inhibit lung cancer cell proliferation (19). Gupta et al. (2001) assessed the growth inhibitory properties of apigenin on human prostate cancer (20). Shukla and Gupta (2010) demonstrated that apigenin could support the improvement of cardiovascular disorders, excite immune system and deliver some defense alongside skin, thyroid, endometrial, gastric, liver, and adrenal cortical cancers (21). Patel et al. (2007) suggested that in the future, apigenin owns high possibility for progress as a capable cancer chemopreventive factor (22).

Root-based culture technology has become a sustainable production platform for producing specific pharmaceutical secondary metabolites. *Agrobacterium rhizogenes* are gram negative bacteria, which induce hairy roots in plants. The T-DNA from its Ri plasmid can be transferred to the plant genome. T-DNA encodes the enzymes for phytohormone auxin control and biosynthesis of cytokinin. Root-inducing (Ri) plasmid contains *rol* (root loci) genes harbored by *A. rhisogenes*. The *rol* genes are integrated into the genome of the host plant, producing a hairy root. The *rol* genes are believed to influence transformed roots growth and development, and prompt synthesis of secondary metabolite via turning on the transcription defense genes. The *rol*B gene is very crucial for genetically transformed root production (23). Genetically transformed roots are mostly used as a transgenic tool for secondary metabolites production and evaluation of key genes in secondary metabolite pathway studies. Hairy roots are both genetically and biochemically stable over long culture periods in comparison to cell suspension cultures. The biosynthesis of secondary metabolites is induced by increasing plant defense genes expression. Fast growth of hairy roots offers an additional benefit to use as a continuous source for valuable secondary metabolite production in the absence of growth regulators (which are usually expensive) in a sterile culture medium (24-26). For increasing the production of pharmaceutical compounds in hairy root cultures, the identification of key genes, which are involved in regulating secondary metabolic pathway stages can aid in secondary metabolic engineering. Fortunately, over the past decades, much information about the biochemistry and genetics of biosynthetic pathways involved in plant natural products creation has been illustrated (27, 28). Gene-coding committed enzymes and strategic transcription factors should be used for increasing preferred secondary metabolites production by overexpressing them in genetically transformed root cultures (25, 29). Genetically transformed root culture as a green factory has biotechnological potential for producing high-value plant-derived metabolites and recombinant pharmaceutical proteins (30).

Numerous features effect the frequency of *A. rhizogenes* mediated transformation. Sharafi et al. (2014a) used liquid inoculation and solid co-cultivation media in absence of macro elements, such as KH_2PO_4, NH_4NO_3, KNO_3 and $CaCl_2$, to transform medicinal plant *Dracocephalum kotschyi* with high efficiency (31). In another study, Henzi et al. (2000) indicated that the usage of acetosyringone and arginine in the agar solidified co-cultivation medium, which revealed major enhancement in transformation rate of *Brassica* by *Agrobacterium* (32).

2. Objectives

The present study evaluated 4 strains of *A. rhizogenes*, 2 co-cultivation media and explants for improvement of an efficient protocol for genetically transformed roots induction from *C. bipinnatus*. This study, as the first report, demonstrated a simple, well-organized, and reliable transformation method for *C. bipinnatus*.

3. Methods

3.1. Seed Sterilization and Germination

Cosmos bipinnatus seeds (provided from Chiba university, Japan) were sterilized in 70% v/v ethanol followed by 25% (w/v) sodium hypochlorite for 12 minutes and germinated on hormone-free MS medium containing 3% (w/v) sucrose and 0.7% agar (Phytotechnology Co., USA) at 24 $\pm$ 1°C under a 16-hour photoperiod regime with fluorescent light in a culture room. Germination was started within 5 days and 4-week-old in vitro grown seedlings were used for hairy root induction.

3.2. Agrobacterium Rhizogenes Strains Preparation

Five strains of *A. rhizogenes* (ATCC 15834, ATCC 31798, A7, MAFF-02-10266, and MSU440) were used. From each strain, a single colony was inoculated in 15 mL of liquid Luria-Bertani medium, pH 7.2, to an optical density of 0.7, at 28°C, 160 rpm on a rotary shaker incubator for 24 hours. The bacterial suspensions were centrifuged at 4000 rpm for 10 minutes. The pellets were suspended in 25 mL MS liquid medium complemented with 150 μM acetocyringone after sterilization using 0.22 μm syringe filters (Sartorius, USA) (inoculation medium) at 28°C in the dark.

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3.3. Co-Cultivation and Hairy Root Production

Leaf and stem explants from 1-month-old plants were cultured for one day on MS medium supplemented with 1 mgL^{-1} 6-Benzyl-Aminopurine (BA). The explants were dipped in bacterial inoculation medium (immersion method) for 8 minutes and blotted on sterile filter paper. The inoculated explants were incubated in a co-cultivation medium containing MS or ½ MS salts and vitamins accompanied by 45 mgL^{-1} sucrose and 100 μM acetocyringone at 24°C in the dark.

After 48 hours, the explants moved to phytohormone-free MS media supplemented with 350 mgL^{-1} cefotaxime to remove the bacteria. Similarly, some explants as controls were processed yet without any bacterial inoculation. Explants were sub-cultured every week until bacterial colonies vanished. The concentration of antibiotics was decreased in subsequent sub-culture. After 3 weeks, hairy roots appeared on the explants and were cut out from the explants and moved to a liquid MS medium containing antibiotics. After 6 subcultures, hairy roots were transferred to an antibiotic-free MS medium. Hairy root cultures were grown in Erlenmeyer flasks with constant shaking (121 rpm) at 24°C in the dark.

3.4. Detection of rolB Gene in Transgenic Roots by Polymerase Chain Reaction

The genomic DNA was isolated from the genetically transformed roots (100 mg) of each clone and control roots based on the CTAB method. The pellets of DNA were air dried, dissolved in 50 μL of water, and then stored at -20°C. Isolated DNA was used in PCR analysis for detecting the *rolB* gene. Fragments of the *rolB* gene were PCR-amplified by the following primer pairs: 5'-GCTCTTGCAGTGCTAGATTT-3' and 5'-GAAGGTGCAAGCTACCTCTC-3'.

The PCR was performed as follows: at 94°C for 5 minutes, 30 cycles in 3 steps (94°C for 1 minute (denaturation), 58°C for 1 minute (annealing) and 72°C for 1 minute (elongation)), and 72°C for 10 minutes for final extension. The PCR amplified DNA were run by electrophoresis on 0.8% agarose gel , stained with EtBr and visualized by a UV trans-illuminator.

3.5. Statistical Analysis

The experiments were laid on a CRD with 3 replications and 10 explants cultivated in each Petri dish. After analysis of variance, the means were compared by the Duncan test using the SPSS software version 16.

4. Results

4.1. Effect of Agrobacterium rhizogenes Strains and Co-Cultivation Medium

Seeds were germinated in 5 days and leaf explants from 4-week old *C. bipinnatus* seedlings were used for co-cultivation (in vitro flowering occured for this plant after 7 to 8 weeks) (Figure 1A, B). Five different *A. rhizogenes* strains (ATCC 15834, ATCC 31798, A7, MAFF-02-10266, and MSU440) were examined for transformation ability. In all tests, acetosyringone was used to prompt *A. rhizogenes* T-DNA transduction. Hairy roots of *C. bipinnatus* originated from stem and leaf explants after 3 weeks (Figure 2A-E).

The obtained hairy roots were moved to liquid ½ MS medium for further growth after 1 month. The color of media after inoculating the hairy roots turned yellow, which indicated that secondary metabolites are secreted in the culture medium (Figure 3). Also, the hairy roots showed plagiogeotropism (lack of geotropism) in liquid medium as expected (Figure 3). In this period, roots propagated by three means: meristematic cell propagation at the tip, cell elongation, and branching via side root production (33).

All of the bacterial strains used in this study resulted in hairy root induction at the wound site of explants and in some cases caused the formation of tumorigenic calli. Tumor induction appeared on the explants by all strains with differing rates (data not shown). The results revealed that the leaf explants were the best explant for *A. rhizogenes* mediated transformation in *C. bipinnatus* (Figure 4). The leaf explants were considerably vulnerable to infection by each strain of *A. rhizogenes*. The highest rate of transformation occurred in MSU440 and ATCC15834 in the leaf explants with 72% and 73%, respectively, within 3 weeks; while, the MAFF-02-10266 strain transfected only 30% of the excised leaf explants (Figure 4). Stem explants showed lower rate of genetically transformed root generation in comparison to leaf explants. To determine the optimal co-cultivation medium for *A. rhizogenes* mediated transformation of *C. bipinnatus*, full MS and ½ MS co-cultivation media were evaluated using leaf explant and *A. rhizogenes* MSU440, which was considered as the best choice in a previous trial. In the full strength, in the MS medium as a co-cultivation medium, the rate of transformation was 69.6% and in the ½ MS co-cultivation medium, this was 85.6% (Figure 5).

4.2. Polymerase Chain Reaction Analysis

Total Genomic DNA was extracted from putative transgenic hairy root lines and control root line with the CTAB protocol (26). The PCR analysis of hairy roots and controls resulted in the amplification of expected fragment in hairy root lines similar to that of the positive control, while there was no extension in the DNA isolated from normal roots

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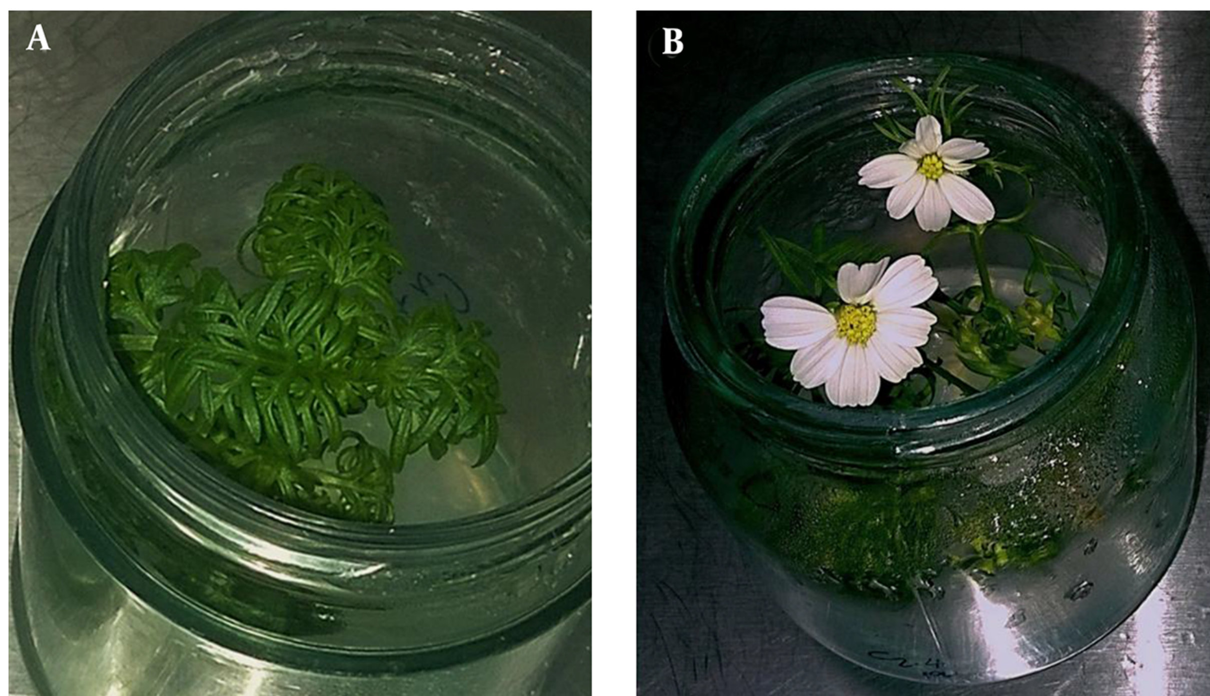

Figure 1. A, Four Weeks Old *C. bipinnatus* Plants in MS Medium; B, In Vitro Flowering After 7 Weeks

(Figure 6). The PCR with *rolB* primers resulted in *rolB* fragment amplification in all putative transgenic hairy root lines. Five transgenic hairy root lines among 55 obtained lines are presented in Figure 6. The PCR analysis using *virG* primers was done to approve that the obtained genetically transformed roots were not contaminated by *A. rhizogenes*, and *virG* amplification was not observed (data not shown). This result shows transformation of T-DNA in genetically transformed root lines.

5. Discussion

This study, for the first time, established a reliable protocol for induction of hairy roots of the important *C. bipinnatus*. Secondary metabolites, probably apigenin, were released in ½ MS liquid culture medium after inoculation with hairy roots compared to control as indicated by the color of the medium (Figure 3). Former reports showed that *A. rhizogenes* strain and form of explants could influence hairy root initiation (26, 32, 34). In some explants, tumorigenic calli (galls) were induced. Tumorigenic calli formed at the infection spot of explants might be because of T_R-DNA transferring *iaa*M and *iaa*H genes, which are accountable for auxins biosynthesis (23). This might be prompted by the special effects of endogenous hormone combined with the interface between explant and bacterial strain in reaction to the *rol* gene products (35). As tumorigenic calli is not appropriate for large scale productions, the results indicated that strain MSU440, inducing high frequency of genetically transformed roots and low amount tumorigenic calli, is more capable than the other bacterial strains used in this study. Some studies described that a proper application of acetosyringone in the culture medium through hairy root induction phase, supported hairy root production (32, 34). The difference in virulence, morphology, and growth level could be partially described by the diversity of plasmids harbored by the bacterial strains. Also, some studies described that a proper application of acetosyringone in the culture medium through hairy root induction phase, supported hairy root production (32, 34).

This research suggests that a medium with lacking mineral compounds is a better co-cultivation medium to achieve the highest rate of transformation. Prior studies indicated that *vir* G expression can be triggered by low levels of PO_4 and proposed that PO_4 deficiency could be a helpful signal to prompt genetic transformation. Similar results were described in transformation by *A. tumefaciens* in *Lilium* (36) and genetically transformed root generation of *Papaver bracteatum*, *Nepeta pogonosperma*, *Dracocephalum kotschyi*, and *Artemisia aucheri* (26, 31, 34, 37).

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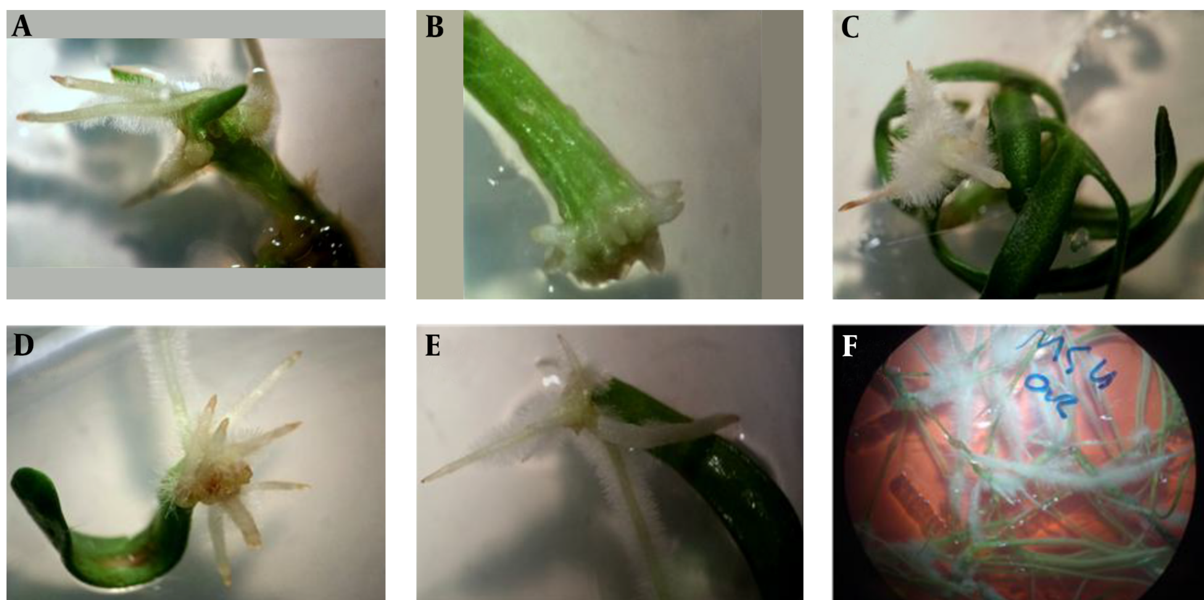
Figure 2. *A. rhizogenes* Mediated Transformation of *C. bipinnatus*



A, hairy root induction on leaf explant after 3 weeks using strain ATCC 15834; B, hairy root induction on leaf explant after 3 weeks using strain ATCC 31798; C, hairy root induction on leaf explant after 3 weeks using strain MSU440; D, hairy root induction on leaf explant using strain MAFF-02-10266; E, hairy root induction on leaf explant using strain A7; F, Hairy root growth in agar solidified ½ MS medium.

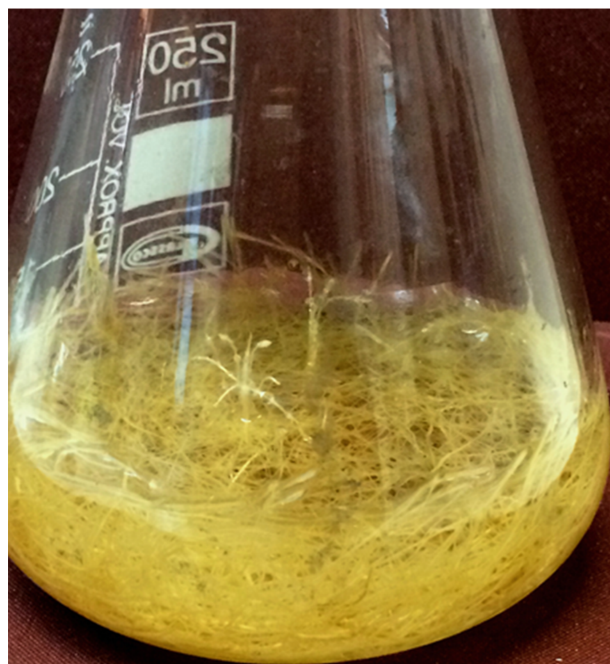


Figure 3. Hairy Root Culture in Liquid ½ MS Medium After One Month

Among the different strains evaluated for induction of transformed hairy roots in *D. kotschyi*, the MSU440 and ATCC15834 strains showed the highest rates of hairy root induction in each tested co-cultivation medium (31). In *N. pogonosperma*, the highest rate of hairy root induction was found using strain MSU440 followed by strains A13 and ATCC15834, respectively (26). In the present study, as the transformation efficiency was significantly increased by decreasing MS salts, the results suggest that extra macro-elements in co-cultivation media have inhibitory effects on *A. rhizogenes* mediated transformation of *C. bipinnatus*. Further studies on the role of these components are necessary to clarify how T-DNA transfer is controlled by mineral compounds.

When the produced metabolites are veiled into the culture medium, a catching system can improve their biosynthesis, opening the way to design uninterruptedly producing culture systems (23). The use of genetically transformed root could have a decent potential in exploring the molecular regulation of genes encoding apigenin biosynthetic enzymes. The hairy root culture system is a potential method for the production of apigenin, since it has several good potentials, such as fast growth frequency, easy culture and genetic manipulation, and an increased capability to synthesize by elicitation (23). This objective is additional accompanying with the rise

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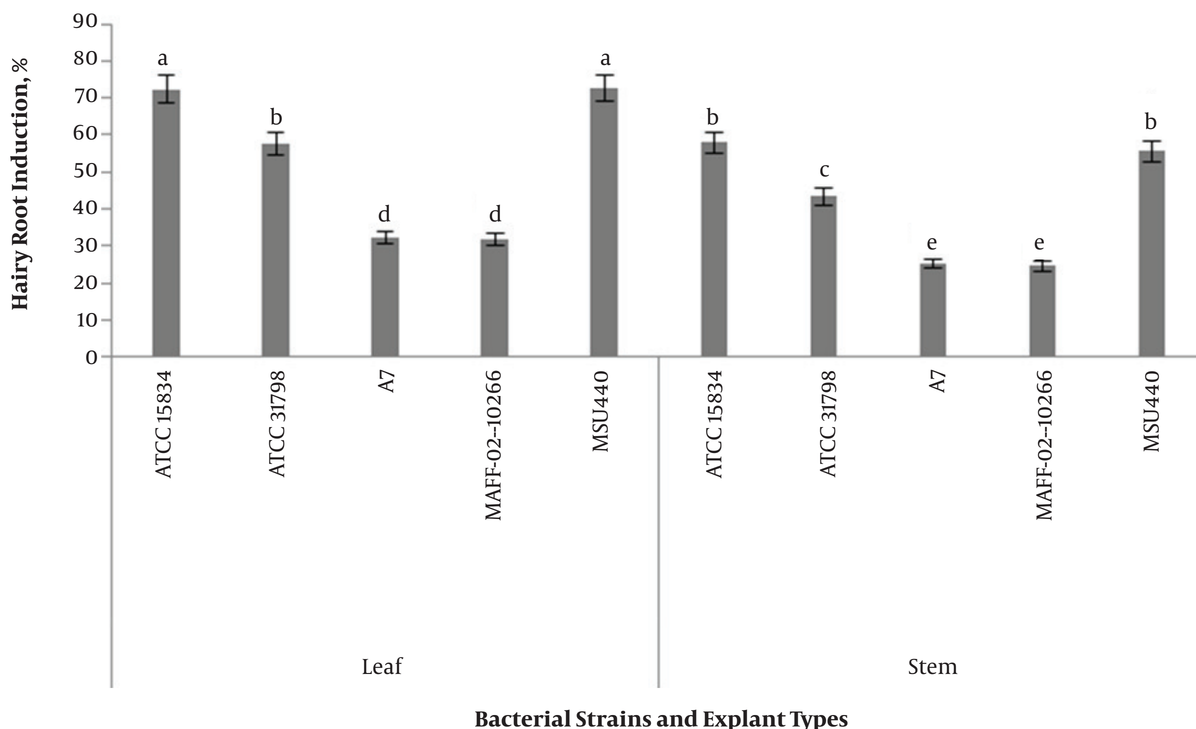


Figure 4. Effect of different *A. rhizogenes* strains on frequency of hairy root induction in *C. bipinnatus*; the data were obtained as a mean of three replications. The different letters denote a statistically significant difference at $P \leq 0.05$, as determined by Duncan's multiple range test. Vertical lines represent standard errors

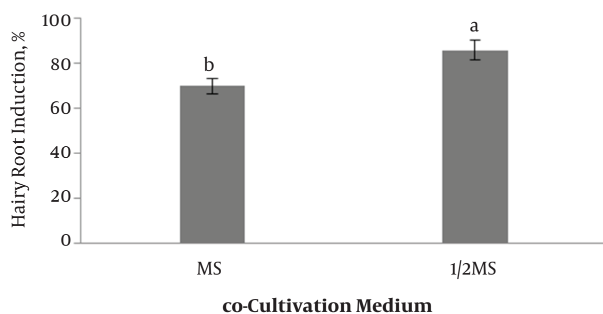
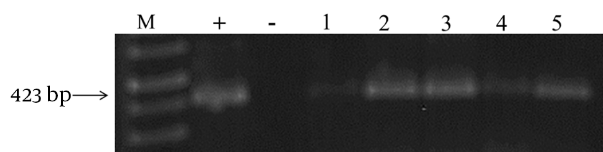


Figure 5. Effect of co-cultivation media on frequency of hairy root induction in *C. bipinnatus*; the data were obtained as a mean of three replications. The different letters denote a statistically significant difference at $P \leq 0.05$, as determined by Duncan's multiple range test. Vertical lines represent standard errors.

Figure 6. Molecular Analysis of Hairy Roots



A, PCR analysis for detection of the *rolB* gene in hairy root lines of *C. bipinnatus*; M: Molecular size marker (1 kb ladder); 1 - 5: hairy root lines, (-): negative control (non-transformed root); (+): positive control (Ri plasmid).

of private companies interpreting this technology to marketable scales. The attention of the industry to genetically transformed roots will probably lead to more extensive research devoted to this model, to adapt it to different scales and various objectives. Advances in plant transcriptomics and metabolomics combined with modeling of metabolic fluxes via *in silico* methods and genetic engineering will allow genetically transformed root cultures to develop a great and maintainable phytochemical production scheme.

The current study was dedicated to developing an effective procedure for high frequency transformation of *C. bipinnatus* through genetically transformed roots induction. This improved transformation method succeeded by adapting to the co-cultivation medium. Also, different strains of *A. rhizogenes* and types of *C. bipinnatus* explants were valued. The maximum ratio of hairy root induction was found from leaf explants using MSU440 and ATCC15834 bacterial strains (72% to 73%). A signifi-

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cant increase in transformation frequency (84%) was observed when ½ MS medium was used as the co-cultivation medium. This is the first report considering the influence of different factors on genetic transformation of *C. bipinnatus* by *A. rhizogenes*.

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References

1. Sohn SH, Yun BS, Kim SY, Choi WS, Jeon HS, Yoo JS, et al. Anti-inflammatory activity of the active components from the roots of Cosmos bipinnatus in lipopolysaccharide-stimulated RAW 264.7 macrophages. *Nat Prod Res*. 2013;**27**(11):1037–40. doi: 10.1080/14786419.2012.686906. [PubMed: 22607357].
2. Jang IC, Park JH, Park E, Park HR, Lee SC. Antioxidative and antigenotoxic activity of extracts from cosmos (Cosmos bipinnatus) flowers. *Plant Foods Hum Nutr*. 2008;**63**(4):205–10. doi: 10.1007/s11130-008-0086-8. [PubMed: 18758962].
3. Olajuyigbe O, Ashafa A. Chemical Composition and Antibacterial Activity of Essential Oil of Cosmos bipinnatus Cav. Leaves from South Africa. *Iran J Pharm Res*. 2014;**13**(4):1417–23. [PubMed: 25587332].
4. Zheng PW, Chiang LC, Lin CC. Apigenin induced apoptosis through p53-dependent pathway in human cervical carcinoma cells. *Life Sci*. 2005;**76**(12):1367–79. doi: 10.1016/j.lfs.2004.08.023. [PubMed: 15670616].
5. Thiery-Vuillemin A, Nguyen T, Pivot X, Spano JP, Dufresnne A, Soria JC. Molecularly targeted agents: their promise as cancer chemopreventive interventions. *Eur J Cancer*. 2005;**41**(13):2003–15. doi: 10.1016/j.ejca.2005.06.005. [PubMed: 16098739].
6. Kuo ML, Lee KC, Lin JK. Genotoxicities of nitropyrenes and their modulation by apigenin, tannic acid, ellagic acid and indole-3-carbinol in the Salmonella and CHO systems. *Mutat Res*. 1992;**270**(2):87–95. doi: 10.1016/0027-5107(92)90119-m.
7. Nomani A, Nosrati H, Manjili HK, Khesalpour L, Danafar H. Preparation and Characterization of Copolymeric Polymersomes for Protein Delivery. *Drug Res (Stuttg)*. 2017;**67**(8):458–65. doi: 10.1055/s-0043-106051. [PubMed: 28561240].
8. Manjili HK, Sharafi A, Danafar H, Hosseini M, Ramazani A, Ghasemi MH. Poly (caprolactone)-poly (ethylene glycol)-poly (caprolactone)(PCL-PEG-PCL) nanoparticles: a valuable and efficient system for in vitro and in vivo delivery of curcumin. *RSC Advances*. 2016;**6**(17):14403–15.
9. Danafar H, Sharafi A, Kheiri Manjili H, Andalib S. Sulforaphane delivery using mPEG-PCL co-polymer nanoparticles to breast cancer cells. *Pharm Dev Technol*. 2017;**22**(5):642–51. doi: 10.3109/10837450.2016.1146296. [PubMed: 26916923].
10. Izadi A, Manjili HK, Ma'mani L, Moslemi E, Mashhadikhan M. Sulforaphane loaded PEGylated Iron oxide-gold core shell nanoparticles: A promising delivery system for cancer therapy. *American Int J Contemporary Sci Res*. 2015;**2**(5):84–94.
11. Kheiri Manjili H, Jafari H, Ramazani A, Davoudi N. Anti-leishmanial and toxicity activities of some selected Iranian medicinal plants. *Parasitol Res*. 2012;**111**(5):2115–21. doi: 10.1007/s00436-012-3059-7. [PubMed: 22875395].
12. Choi EJ, Kim GH. Apigenin Induces Apoptosis through a Mitochondria/Caspase-Pathway in Human Breast Cancer MDA-MB-453 Cells. *J Clin Biochem Nutr*. 2009;**44**(3):260–5. doi: 10.3164/jcbn.08-230. [PubMed: 19430615].
13. Way TD, Kao MC, Lin JK. Apigenin induces apoptosis through proteasomal degradation of HER2/neu in HER2/neu-overexpressing breast cancer cells via the phosphatidylinositol 3-kinase/Akt-dependent pathway. *J Biol Chem*. 2004;**279**(6):4479–89. doi: 10.1074/jbc.M305529200. [PubMed: 14602723].
14. Lindenmeyer F, Li H, Menashi S, Soria C, Lu H. Apigenin acts on the tumor cell invasion process and regulates protease production. *Nutrition Cancer*. 2001;**39**(1):139–47. doi: 10.1207/S15327914nc391_19.
15. Zheng QS, Sun XL, Xu B, Li G, Song M. Mechanisms of apigenin-7-glucoside as a hepatoprotective agent. *Biomed Environ Sci*. 2005;**18**(1):65–70. [PubMed: 15861781].
16. Wang W, Heideman L, Chung CS, Pelling JC, Koehler KJ, Birt DF. Cell-cycle arrest at G2/M and growth inhibition by apigenin in human colon carcinoma cell lines. *Molecular Carcinogenesis*. 2000;**28**(2):102–10.
17. Wang IK, Lin-Shiau SY, Lin JK. Induction of apoptosis by apigenin and related flavonoids through cytochrome c release and activation of caspase-9 and caspase-3 in leukaemia HL-60 cells. *European J Cancer*. 1999;**35**(10):1517–25. doi: 10.1016/s0959-8049(99)00168-9.
18. Budhraja A, Gao N, Zhang Z, Son YO, Cheng S, Wang X, et al. Apigenin induces apoptosis in human leukemia cells and exhibits anti-leukemic activity in vivo. *Mol Cancer Ther*. 2012;**11**(1):132–42. doi: 10.1158/1535-7163.MCT-11-0343. [PubMed: 22084167].
19. Nosrati H, Sefidi N, Sharafi A, Danafar H, Kheiri Manjili H. Bovine Serum Albumin (BSA) coated iron oxide magnetic nanoparticles as biocompatible carriers for curcumin-anticancer drug. *Bioorg Chem*. 2018;**76**:501–9. doi: 10.1016/j.bioorg.2017.12.033. [PubMed: 29310081].
20. Gupta S, Afaq F, Mukhtar H. Selective growth-inhibitory, cell-cycle deregulatory and apoptotic response of apigenin in normal versus human prostate carcinoma cells. *Biochem Biophys Res Commun*. 2001;**287**(4):914–20. doi: 10.1006/bbrc.2001.5672.
21. Shukla S, Gupta S. Apigenin: a promising molecule for cancer prevention. *Pharm Res*. 2010;**27**(6):962–78. doi: 10.1007/s11095-010-0089-7. [PubMed: 20306120].
22. Kheiri Manjili H, Ghasemi P, Malvandi H, Mousavi MS, Attari E, Danafar H. Pharmacokinetics and in vivo delivery of curcumin by copolymeric mPEG-PCL micelles. *Eur J Pharm Biopharm*. 2017;**116**:17–30. doi: 10.1016/j.ejpb.2016.10.003.
23. Georgiev MI, Pavlov AI, Bley T. Hairy root type plant in vitro systems as sources of bioactive substances. *Appl Microbiol Biotechnol*. 2007;**74**(6):1175–85. doi: 10.1007/s00253-007-0856-5. [PubMed: 17294182].
24. Danafar H. Study of the composition of polycaprolactone/poly (ethylene glycol)/polycaprolactone copolymer and drug-to-polymer ratio on drug loading efficiency of curcumin to nanoparticles. *Jundishapur J Nat Pharm Prod*. 2017;**12**(1). e34179. doi: 10.17795/jjnpp-34179.
25. Sharafi A, Hashemi Sohi H, Mousavi A, Azadi P, Dehsara B, Hosseini Khalifani B. Enhanced morphinan alkaloid production in hairy root cultures of Papaver bracteatum by over-expression of salutaridinol 7-o-acetyltransferase gene via Agrobacterium rhizogenes mediated transformation. *World J Microbiol Biotechnol*. 2013;**29**(11):2125–31. doi: 10.1007/s11274-013-1377-2. [PubMed: 23681746].
26. Valimehr S, Sanjarian F, Sohi HH, Sharafi A, Sabouni F. A reliable and efficient protocol for inducing genetically transformed roots in medicinal plant Nepeta pogonosperma. *Physiol Mol Biol Plants*. 2014;**20**(3):351–6. doi: 10.1007/s12298-014-0235-5. [PubMed: 25049462].
27. Staniek A, Bouwmeester H, Fraser PD, Kayser O, Martens S, Tissier A, et al. Natural products - modifying metabolite pathways in plants. *Biotechnol J*. 2013;**8**(10):1159–71. doi: 10.1002/biot.201300224. [PubMed: 24092673].
28. Mirzaee H, Sharafi A, Hashemi Sohi H. In vitro regeneration and transient expression of recombinant sesquiterpene cyclase (SQC) in Artemisia annua L. *South African J Botany*. 2016;**104**:225–31. doi: 10.1016/j.sajb.2015.10.005.

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29. Sharafi A, Sohi HH, Mousavi A, Azadi P, Khalifani BH, Razavi K. Metabolic engineering of morphinan alkaloids by over-expression of codeinone reductase in transgenic hairy roots of Papaver bracteatum, the Iranian poppy. *Biotechnol Lett*. 2013;**35**(3):445–53. doi: 10.1007/s10529-012-1080-7. [PubMed: 23160738].
30. Huet Y, Ekouna JP, Caron A, Mezreb K, Boitel-Conti M, Guerineau F. Production and secretion of a heterologous protein by turnip hairy roots with superiority over tobacco hairy roots. *Biotechnol Lett*. 2014;**36**(1):181–90. doi: 10.1007/s10529-013-1335-y. [PubMed: 24078130].
31. Sharafi A, Sohi HH, Azadi P, Sharafi AA. Hairy root induction and plant regeneration of medicinal plant Dracocephalum kotschyi. *Physiol Mol Biol Plants*. 2014;**20**(2):257–62. doi: 10.1007/s12298-013-0217-z. [PubMed: 24757330].
32. Henzi MX, Christey MC, McNeil DL. Factors that influence Agrobacterium rhizogenes -mediated transformation of broccoli (Brassica oleracea L. var. italica). *Plant Cell Reports*. 2000;**19**(10):994–9. doi: 10.1007/s002990000221.
33. Wyslouzil BE, Waterbury RG, Weathers PJ. The growth of single roots of Artemisia annua in nutrient mist reactors. *Biotechnol Bioengineering*. 2000;**70**(2):143–50.
34. Sharafi A, Sohi HH, Mousavi A, Azadi P, Razavi K, Ntui VO. A reliable and efficient protocol for inducing hairy roots in Papaver bracteatum. *Plant Cell, Tissue and Organ Culture (PCTOC)*. 2012;**113**(1):1–9. doi: 10.1007/s11240-012-0246-2.
35. Sudha CG, Sherina TV, Anu Anand VP, Reji JV, Padmesh P, Soniya EV. Agrobacterium rhizogenes mediated transformation of the medicinal plant Decalepis arayalpathra and production of 2-hydroxy-4-methoxy benzaldehyde. *Plant Cell, Tissue and Organ Culture (PCTOC)*. 2012;**112**(2):217–26. doi: 10.1007/s11240-012-0226-6.
36. Azadi P, Otang NV, Supaporn H, Khan RS, Chin DP, Nakamura I, et al. Increased resistance to cucumber mosaic virus (CMV) in Lilium transformed with a defective CMV replicase gene. *Biotechnol Lett*. 2011;**33**(6):1249–55. doi: 10.1007/s10529-011-0550-7. [PubMed: 21287228].
37. Sharafi A, Sohi HH, Mirzaee H, Azadi P. In vitro regeneration and Agrobacterium mediated genetic transformation of Artemisia aucheri Boiss. *Physiol Mol Biol Plants*. 2014;**20**(4):487–94. doi: 10.1007/s12298-014-0248-0. [PubMed: 25320471].

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