

Full Length Research Paper

Thin layer chromatography (TLC) technique in the investigation of artemisinin production in *Artemisia annua* L. medicinal plant hairy roots

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***Artemisia annua* L. is an important medicinal plant, which is used for the treatment of malaria. Artemisinin is the active secondary metabolite in this plant and it is a sesquiterpen. This plant was studied for artemisinin production in hairy root induced by *Agrobacterium rhizogenes*. Polymerase chain reaction (PCR) for *rolB* gene confirmed the transformed hairy roots induced by *A. rhizogenes*. Then, thin layer chromatography (TLC) was employed for investigating the characteristics and quality of artemisinin in extracts of hairy roots and control roots of *A. annua*. Result of TLC showed the presence of artemisinin in the induced hairy roots.**

Key words: *Artemisia annua*, *Agrobacterium rhizogenes*, hairy root, thin layer chromatography (TLC) technique and artemisinin.

INTRODUCTION

Artemisia annua L. (Worm wood) an important medicinal plant of the family Asteraceae, its contains an antimalaria sesquiterpene endoperoxide, artemisinin, which is effective against both chloroquine-resistant and chloroquine-sensitive strains of plasmodium falciparum, and is thus useful for the treatment of malaria (Mannan et al., 2008; Putalun et al., 2007).

The only commercial source of the drug is extracted from field-grown leaves and flowering topes of this plant. But the compound occurred in low yield in plant 0.01 to 0.6% w/w of tissue dry weight and also chemical synthesis is economically unattractive (Baldi and Dixi, 2008; Namdeo et al., 2006). Therefore, enhancement of artemisinin production, either in tissue culture or in the breeding *A. annua* is the aim of many research groups.

The neoplastic roots produced by *Agrobacterium rhizogenes* infection, is characterized by high growth rate, genetic stability and producing higher levels of secondary metabolites (Arsenault et al., 2008; Giri et al., 2001; Tzfira and Citovsky, 2006).

Although, the concentration of artemisinin in hairy roots transformed with *A. rhizogenes* remained low (Giri et al., 2001). The productivity of artemisinin many depend on the choice of transformed root clones, basal media and culture conditions (Putalun et al., 2007).

The TLC analysis is an important technique that has been used in many cases, such as, agricultural products, foods, beverages, and plant constituents for many years (Sherma, 2000). Early investigations used TLC for the determination of the artemisinin production in *A. annua* plant (Rimada et al., 2009).

Hairy root induction in the Iranian *A. annua* was not reported. In this study we induced hairy root in Iranian clone of *A. annua* and investigated the artemisinin production in this hairy root by TLC technique.

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MATERIALS AND METHODS

Plant material

Seeds of *A. annua* were collected from the botanic garden in the Noshahr Province in Iran, seeds were surface-sterilized by immersion in NaClO 2% (v/v) for 10 min and EtOH 70% (v/v) for 1 min, followed by washing 3 times with sterile water. MS medium was used for germination sterile seeds. These cultures were placed in the growth chamber condition with (16:8) h, photoperiod (light: dark) and $25 \pm 1^\circ\text{C}$ (Rahnama et al., 2008).

Agrobacterium strain and culture

The *A. rhizogenes*, strain AR15834 was used in this experiment. Single clone of this Agrobacterium were cultured into LB medium and obtained suspension with optical density at 600 nm ($\text{OD}_{600} = 0.7$).

Establishment and culture of transformed root

Leaves from the 2 week old seedlings were used for transformation with *A. rhizogenes*. Leaf explants were then infected by immersing them into an *A. rhizogenes* suspension for 10 min. The leaf explants were blotted with filter paper to remove excess Agrobacteria. Sterile LB medium without bacteria was applied to the explants as a control. After 48 h of co-cultivation at $25 \pm 1^\circ\text{C}$ in (16 : 8) h light and dark, respectively, the explants were transferred onto MS Medium supplemented with 500 mgL^{-1} cefotaxime and placed in the growth chamber at $25 \pm 1^\circ\text{C}$ in light : dark for (16 : 8) h, to induced hairy roots.

Roots that appeared 7 to 10 days after infection were cultured separately on solid MS medium supplemented with 500 mgL^{-1} cefotaxime. Root of 4 cm sized were excised and immediately to 50 ml liquid 1/2 MS basal media containing cefotaxime (500 mgL^{-1}) in 250 ml Erlenmeyer flask.

Hairy roots were made bacteria free by transferring to fresh medium containing cefotaxime every 14 days. The bacteria free hairy roots were maintained in Erlenmeyer flask containing phytohormone free 50 ml liquid 1/2 MS medium (Rahnama et al., 2008; Weathers et al., 2004). The roots were checked for *A. rhizogenes* contamination by culturing hairy roots sample on LB medium.

PCR analysis of hairy roots

Genomic DNA of the hairy roots were extracted by using CTAB method (Cai et al., 1997). Natural Plant (non-transformed) genomic DNA and plasmid DNA from *A. rhizogenes* were used as negative and positive controls, respectively. Isolated DNA was analyzed by polymerase chain reaction (PCR) for *roB* gene (Sambrook et al., 1989). The primer was designed according to Rahnama et al. (2008). PCR was performed in 35 thermal cycles (denaturation at 94°C for 1 min, primer annealing at 53°C for 1 min, and primer extension at 72°C for 1 min) for *roB* (Forward primer 5'-ATGGATCCCAAATTGCTATTCCCCACGA-3' and Reverse primer 5'-TTAGGCTTCTTCATTCGGTTTACTGCAGC-3').

Artemisinin extraction

In order to extract the artemisinin we followed the method reported by Smith et al. (1997) with the following modifications. In this method hairy roots and control roots were harvested and carefully washed with deionized water and blotted dry in room temperature.

0.2 g of powdered dried roots was extracted twice with 3 ml of scintanalyzed toluene in an ultrasonic bath for 30 min in ice-cold water. Then the extracts were centrifuged (OSK 173 Model) at 4000 rpm for 15 min and the supernatants were dried under nitrogen and storage in -20°C for later TLC analysis (Smith et al., 1997).

Thin-layer chromatography (TLC)

For TLC analysis the artemisinin standard (Sigma Aldrich) were first dissolved in acetonitrile (HPLC grade), for producing 1000 ppm artemisinin concentration, then the dried extracts was dissolved in 1 ml of acetonitrile.

Extracts were spotted manually on a silica gel TLC plate, by micro pipet. Plates were put in a glass TLC-tank and eluted with the mobile phase acetone : normal hexane ether mixture in the proportion 3:10 (v/v) for 3 min, plates were developed to a height of 3 cm, later plates were air dried at room temperature and spots were visualized by placing in a iodine tank. All solvents were of HPLC grade and from Merck (Germany). Artemisinin was identified by comparing the Intensity color of the Artemisinin standard (Sigma Aldrich) spot, with the other extracts.

RESULTS AND DISCUSSION

Hairy root induction

A. rhizogenes strain AR15934 could induce hairy root formation in 2 week seedling explants of *A. annua*. While frequency of root production in the non-infection explants were about 4.76% (Figure 1). Hairy root lines were isolated and maintained on free hormone solid MS medium. The roots showed fast growth, 1 to 2 month after infection.

Confirmation of *A. annua* hairy roots by polymerase chain reaction (PCR) analysis

By using DNAs from the hairy roots as template and the non-transformed roots as control, PCR products amplified with *roB* primers, could be detected (fragment with 780 bp). These results indicated that the *roB* gene (780 bp) from the TL-DNA of Ri plasmid of *A. rhizogenes* AR15834 were integrated into the genome of *A. annua* hairy root (Figure 2).

Artemisinin production analysis by TLC technique

We used TLC technique for determination of present artemisinin in both extracts of hairy roots and control roots and we observed that each extract produced only one spot for each injection site. These spots were placed on the one level and they were adequate with the artemisinin standard spot level. There were differences between intensity color of these spots.

Artemisinin standard (1000 ppm) color spot was darker than the other spots (spots of extracts) and this mean is the amount of artemisinin in the extract of the hairy roots

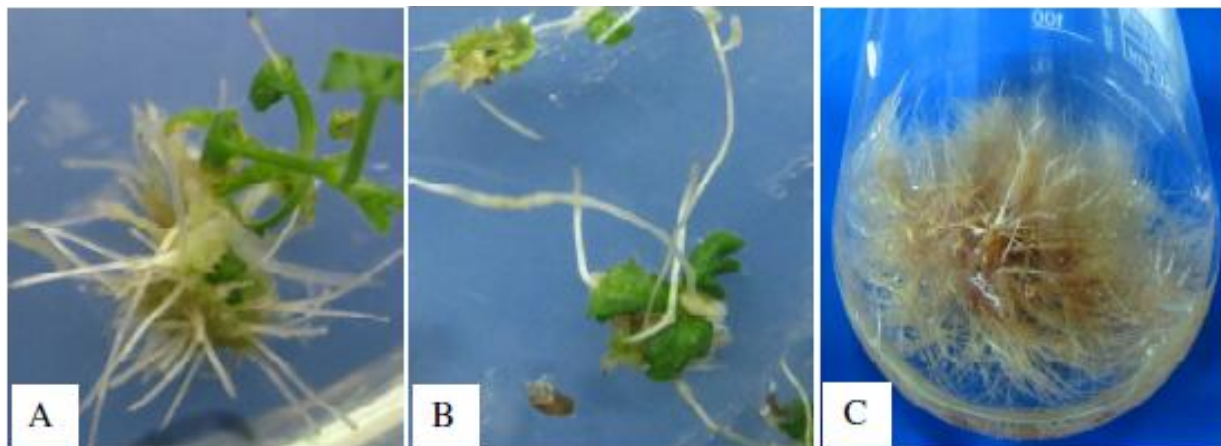


Figure 1. (A and B) Induce hairy root in 2 week seedling of *A. annua* leaf explants; C) hairy root culture of *Artemisia annua* in the liquid medium.

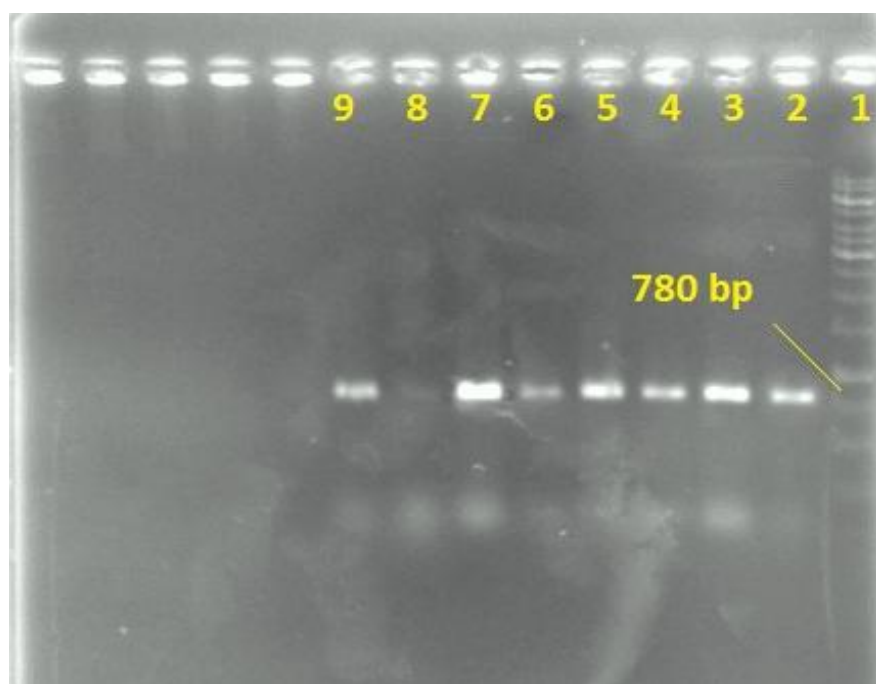


Figure 2. PCR analysis of hairy roots. PCR analysis was performed using primers for the TL-*ro/B* gene. The *ro/B* gene size is 780 bp. Lane 1: marker DNA (1 Kb); lanes 2 to 7: *A. annua* hairy roots; lane 8: *A. annua* control root; lane 9: *A. rhizogenes* plasmid DNA.

and the control roots is lower than the standard artemisinin concentration (Figure 3). However, when we compared intensity color between hairy roots extract spots with control root, it showed that hairy roots extract spots were darker than the control roots extracts. On the other hand, inducing hairy roots increased the amount of artemisinin (Figure 3).

Conclusions

According to these results, the *A. rhizogenes*, strain AR15834, could induce hairy roots in *A. annua* leaf explants. Hairy root induction in the medicinal plants was reported by many researchers, such as *Saussurea involucrate* (Fu et al., 2005), *Silybum marinum* (Rahnama

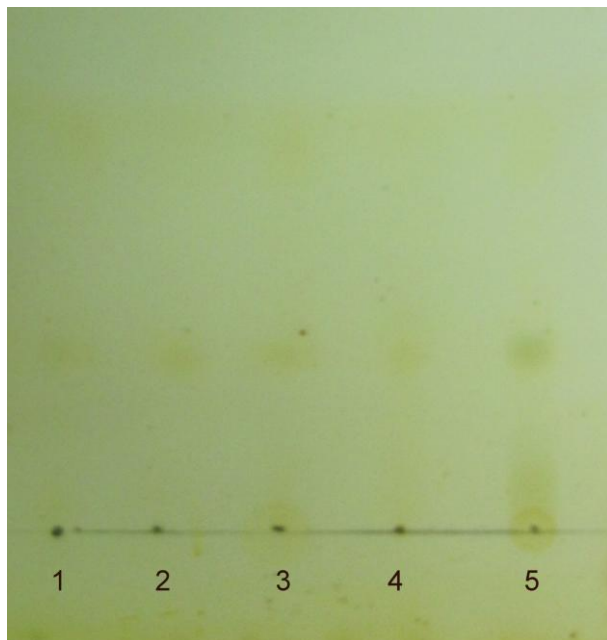


Figure 3. Thin layer chromatography (TLC) plate. Lanes 1 and 2: control roots; lanes 3 and 4: hairy roots; lane 5: Artemisinin standard (Sigma Aldrich).

et al., 2008), *Hyoscyamus muticus* (Zolala et al., 2007), *Glycyrrhiza glabra* (Mehrotra et al., 2008).

After TLC analysis we showed the presentation of artemisinin in both of hairy roots and control roots. But spots of hairy roots extract were darker than the control. Thus, inducing hairy roots were effective for artemisinin production. Production of higher levels of secondary metabolites or amount comparable in the hairy roots to that of intact plants was reported by many different scientists (Bandyopadhyay et al., 2007; Mirjalili et al., 2009; Moyano et al., 1999). However, rapid growth rate and genetic stability of these transform roots, are very important factors that can be used for culture in bioreactor system and finally this system culture can use such as semi-industrial or industrial method for artemisinin production or other secondary metabolites and drug from medicinal plants (Giri and Narasu, 2000; Hu and Du, 2006).

REFERENCES

- Arsenault PR, Wobbe KK, Weathers PJ (2008). Recent advances in artemisinin production through heterologous expression. *Curr. Med. Chem.*, 15(27): 2886.
- Baldi A, Dixi VK (2008). Yield enhancement strategies for artemisinin production by suspension cultures of *Artemisia annua*. *Bioresour. Technol.*, 99: 4609-4614.
- Bandyopadhyay M, Jha S, Tepfer D (2007). Changes in morphological phenotypes and withanolide composition of Ri-transformed roots of *Withania somnifera*. *Plant Cell Rep.*, 26: 599-609.
- Cai D, Kleine S, Kifle HJ, Horloff NN, Sandal KA, Marcker RMK, Lankhorst EMJ, Salentijn W, Lange WJ, Stiekema V, Wyss FMW, Jung C (1997). Positional cloning of a gene for nematode resistance in sugarbeet. *Science*, 275: 832-834.
- Fu CX, Zhao DX, Xue XF, Jin ZP, Ma FS (2005). Transformation of *Saussurea involucreta* by *Agrobacterium rhizogenes*: hairy root induction and syringin production. *Proc. Biochem.*, 40: 3789-3794.
- Giri A, Ravindra ST, Dhingra V, Narasu ML (2001). Influence of different strains of *Agrobacterium rhizogenes* on induction of hairy roots and artemisinin production in *Artemisia annua*. *Curr. Sci.*, 81: 4-25.
- Giri A, Narasu L (2000). Transgenic hairy root: recent trends and applications. *Biotechnol. Adv.*, 18: 1-22.
- Hu ZB, Du M (2006). Hairy root and its application in plant genetic engineering. *J. I. P.B.*, 48(2): 121-127.
- Mannan A, Shaheen N, Arshad W, Rizwana QA, Zia M, Mirza B (2008). Hairy roots induction and artemisinin analysis in *Artemisia dubia* and *Artemisia indica*. *Afr. J. Biotechnol.*, 7(18): 3288-3292.
- Mehrotra S, Kukreja AK, Khanuja SPS, Mishra BN (2008). Genetic transformation studies and scale up of hairy root culture of *Glycyrrhiza glabra* in bioreactor. *Electron. J. Biotechnol.*, ISSN: 0717-3458.
- Mirjalili MH, Moyano E, Bonfill M, Cusido RM, Palazn J (2009). Steroidal Lactones from *Withania somnifera*, an Ancient Plant for Novel Medicine. *Molecules*, 14: 2373-2393.
- Moyano ES, Fornale B, Palazzo J, Cusido ARM, Moralesa C, Pin MT (1999). Effect of *Agrobacterium rhizogenes* T-DNA on alkaloid production in Solanaceae plants. *Phytochemistry*, 52: 1287-1292.
- Namdeo AG, Mahadik KR, Kadam SS (2006). PHCOG MAG.: Review Article Antimalarial Drug – *Artemisia annua*. *Pharmacogn. Mag.*, 2: 6.
- Putalun W, Luealon W, De-Eknamkul W, Tanaka H, Shoyama Y (2007). Improvement of artemisinin production by chitosan in hairy root cultures of *Artemisia annua* L. *Biotechnol. Lett.*, 29: 1143-1146.
- Rahnama H, Hasanloo T, Shams MR, Sepehrifar R (2008). Silymarin production by hairy root culture of *Silybum marianum* (L.) Gaertn. *I.J.B.*, 6: 2.
- Rimada RS, Gatti WO, Jeandupeux R, Cafferata I (2009). Isolation, characterization and quantification of artemisinin by NMR from Argentinean *Artemisia annua* L. *B.L.A.C.P.M.A.*, 8(4): 275-281.
- Sambrook J, Fritsch EF, Maniatis T (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Laboratory Press, ColdSpring, Harbor, NY.
- Sherma J (2000). Thin-layer chromatography in food and agricultural analysis. *J. Chromatogr. A*, 880: 129-147.
- Smith T, Weathers PJ, Cheetham R (1997). Effect of gibberlic acid on hairy root culture of *Artemisia annua*: growth and artemisinin production. *In vitro Cell Dev. Biol.*, 33: 75-79.
- Tzfira T, Citovsky V (2006). *Agrobacterium*-mediated genetic transformation of plants: biology and biotechnology. *Curr. Opin. Biotechnol.*, 17: 147-154.
- Weathers PJ, Jesus-Gonzalez LD, Kim YJ, Souret FF, Towler MJ (2004). Alteration of biomass and artemisinin production in *Artemisia annua* hairy roots by media sterilization method and sugars. *Plant Cell Rep.*, 23: 414-418.
- Zolala J, Farsi M, Gordan HR, Mahmoodnia M (2007). Producing a High Scopolamine Hairy Root Clone in *Hyoscyamus muticus* through Transformation by *Agrobacterium rhizogenes*. *J. Agric. Sci. Technol.*, 9: 327-339.