

Full Length Research Paper

Enhanced production of artemisinin by hairy root cultures of *Artemisia dubia*

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The current research was to enhance the artemisinin concentration in hairy root cultures, produced by transformation of *Artemisia dubia* with *Agrobacterium rhizogenes* LBA9402. These cultures were exposed to gibberellic acid and salicylic acid, separately, to determine growth rate and artemisinin production. Gibberellic acid increased the growth rate of hairy roots at its 0.001 mg/L concentration while salicylic acid increased the growth rate at its 0.138 mg/L concentration. Maximum artemisinin concentration that is, 80±3 µg/g of DW was measured at 0.01 mg/L of gibberellic acid which was 91% increase in comparison with control, followed by artemisinin that is, 58±4 µg/g of DW at its 0.001 mg/L concentration that was 38% increase with respect to control. Maximum artemisinin concentration that is, 79±3 µg/g of DW was measured when hairy roots were exposed to 13.8 mg/L of salicylic acid and second highest concentration 73±3 µg/g was obtained at 1.38 mg/L of salicylic acid. The results showed that both substances increased growth rate and artemisinin production.

Key words: *Agrobacterium rhizogenes*, *Artemisia dubia*, artemisinin, gibberellic acid, salicylic acid, hairy root cultures.

INTRODUCTION

Artemisia species has been used for a long time in traditional Chinese medicine for the treatment of fevers, skin diseases and malaria. Studies showed that this genus has antibacterial (Kaul et al., 1976), antimalarial (Zafar et al., 1990) antihepatitic (Gilani and Jambaz, 1995) activities etc. Artemisinin is the well known secondary metabolite isolated from *Artemisia* species. This secondary metabolite is responsible for many of these properties. It is, naturally, present in aerial parts of many *Artemisia* species (Mannan et al., 2010a).

Low yield of artemisinin from natural sources (Mannan et al., 2010a, 2011) and its uneconomical synthesis has triggered the utilization of various techniques for its enhancement in *Artemisia annua* (Mannan et al., 2010b) as well as in other *Artemisia* species (Mannan et al., 2008; Zia et al., 2007). Now a days, several laboratories developed hairy root cultures of *A. annua*. These hairy

root cultures contain various genes, transforming from various species of *Agrobacterium rhizogenes* (Giri et al., 2001; Weathers et al., 1994). In each case, levels of artemisinin depend upon the clone and culture conditions of the transformed roots. Smith et al. (1997) and Pu et al. (2009) obtained high concentration of artemisinin from *A. annua* hairy roots on treatment with different plant regulators.

Precursor and elicitation treatments proved to be an effective way to enhance plant secondary metabolites. The precursors such as sodium acetate (Zeng et al., 2003) and mevalonic acid (Woerdenbag et al., 1993) as well as elicitors like jasmonic acid (Walker et al., 2002), salicylic acid (Donnell et al., 1996) and gibberlic acid (Woerdenbag et al., 1993) have been investigated to enhance secondary metabolites production. Effect of single elicitor on a particular cell line was investigated in most of these studies. Hairy root cultures of *Artemisia dubia*, a member of *Artemisia* genus reported to contain artemisinin (Mannan et al., 2008). The production of artemisinin in these hairy roots lines can be enhanced under treatment of gibberellic acid and salicylic acid.

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Figure 1. Hairy roots emerging from infected portion of stem.

MATERIALS AND METHODS

A. dubia species, already identified in Herbarium of Quaid-i-Azam University, (Islamabad, Pakistan) was growing naturally in green house. Juvenile stem segments of 2 of 3 cm in length were taken, surface sterilized with 0.1% w/v mercuric chloride (HgCl_2) for 6 min, transferred to the shooting medium (MS medium with 2.5 μM 6-benzylaminopurine/L and 0.25 μM α -naphthaleneacetic acid/L) of pH 5.7 in growth room, at about 25°C with 24 $\mu\text{mol m}^{-2} \text{s}^{-1}$ cool white fluorescence light of 16 h photoperiod. After a few days, shoots were emerged from the stem portion and sub-cultured, regularly, on shooting medium at 2-weeks interval. For root initiation, sub-cultivated shoots were shifted to rooting medium ($\frac{1}{2}$ MS with 0.025 mg NAA/L).

Bacterial cultures of *A. rhizogenes* strain LBA9402 were prepared in yeast mannitol broth (YMB), solidified with 0.5% (w/v) agar and supplemented with 50 mg kanamycin/L in the petriplate. A single colony of overnight grown bacterial cultures was picked with sterile scalpel and stem portion of *A. dubia* was infected by incision. After a few days, hairy roots emerged from the infected portion of stem and started increasing in length. These hairy roots were excised and cultivated on the B_5 selection medium (containing sucrose 30 g/L, gelrite 2 g/L and Cefotaxime 600 mg/L) at about 25°C in dark. Then, hairy roots were transferred to simple B_5 medium. Roots cultures of untransformed plants were used as control.

Genomic DNA was extracted from hairy roots cultures along with control roots by following CTAB method and the plasmid DNA was isolated by following alkaline lysis method. Quality and quantity of DNA were determined by spectrophotometric analysis. Identification of *rol A* gene was carried out by PCR analysis followed by agarose gel (1.5% w/v) electrophoresis and UV-Tran illuminator.

Hairy roots were allowed to grow on B_5 medium of 5.7 pH at about 25°C in 24 $\mu\text{mol m}^{-2} \text{s}^{-1}$ cool white fluorescence light. Three concentrations, each of gibberellic acid (0.1, 0.01 and 0.001 mg/L) as well as of salicylic acid (13.8, 1.38 and 0.138 mg/L), were used in B_5 medium in separate set of experiments. The hairy roots grown on different concentrations of gibberellic acid and salicylic acid

along with controls were harvested and their artemisinin was extracted. Quantification of artemisinin was carried out by using Agilent 1200 series HPLC system with diode array detector (G1315B-DAD) (Mannan et al., 2010a). The significance of results was analyzed statistically by using analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Sterilized stem segments of *A. dubia* were grown on shooting medium, for the production of shoot cultures. Then shoot cultures were rooted, successfully. Hairy roots were produced from different parts of these rooted cultures (Giri and Narasu, 2000). According to the reports of many laboratories, hairy roots are, genetically and biosynthetically, stable cultures with fast growth rate. Simple maintenance and vast biosynthetic ability are the advantages that make the hairy roots efficient source for plant secondary metabolites production (Krolicka et al., 2001).

In current study, hairy roots were emerged from the infected portion of stem after 1 to 2 weeks of infection. The produced hairy roots were of different morphology (Figure 1). After transferring to B_5 media, hairy roots proliferated rapidly and increased in mass. Similar habits of hairy roots were observed by Giri et al. (2001) when they used same strain of *Agrobacterium* for production of hairy roots in *A. annua*, another species of the genus. These hairy roots were thin and whitish than control roots and their proliferation rate was much higher. Similar results were published by Weathers et al. (2004) from hairy root cultures of *A. annua*. Christey (2001) reported that *rol* genes are responsible for the characteristic

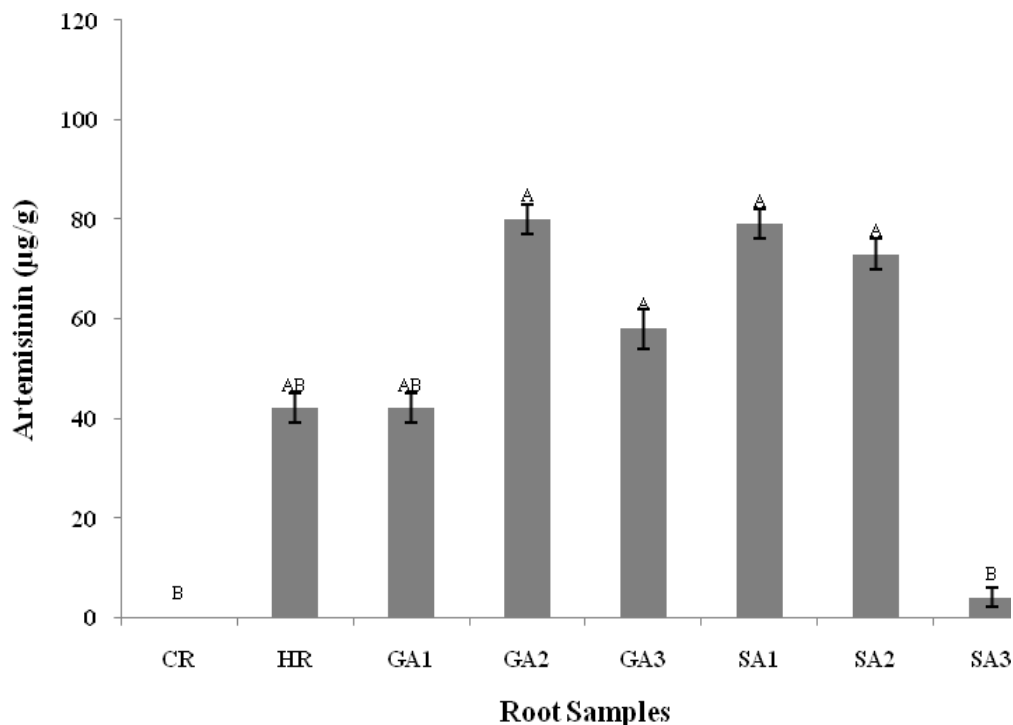


Figure 2. Artemisinin concentration in control roots (CR), hairy roots (HR), gibberellic acid treated roots (GA1 = 0.1, GA2 = 0.01, GA3 = 0.001 mg/L) and salicylic acid treated roots (SA1 = 13.8, SA2 = 1.38, SA3 = 0.138 mg/L).

phenotypic properties of hairy roots. Transformation of *rol A* gene in hairy roots was confirmed through PCR analysis. This gene was present in hairy root lines and in plasmid but not in control roots.

When hairy roots were grown with different concentrations of gibberellic acid, their growth rate was increase at 0.001 mg/L concentration of gibberellic acid. The concentration of 0.01 mg/L of gibberellic acid did not affect the normal growth rate while an inhibition in growth rate was observed at its 0.1 mg/L. The present findings agreed with findings of Smith et al. (1997) and Bais et al. (2002). They reported that higher concentration of gibberellic acid inhibited the growth rate of *A. annua* hairy roots while lower gibberellic acid concentrations showed effective growth response.

Gibberellic acid also effected production of artemisinin in hairy root cultures. Maximum artemisinin concentration that is, 80 ± 3 µg/g of DW was detected at 0.01 mg/L of gibberellic acid which was 91% increase with respect to control. Second highest artemisinin concentration of hairy roots was 58 ± 4 µg/g of DW when it was exposed to 0.001 mg/L of gibberellic acid that was 38% increase in quantity with respect to control. Other gibberellic acid treatment did not affect artemisinin quantity with respect to control. A negligible or no artemisinin was present in untransformed roots (Figure 2). A previous report also claimed

that artemisinin content increases with increasing the concentration of phytohormone that is, gibberellic acid in the culture medium (Weathers et al., 2005). It is claimed that concentration of artemisinic acid in biosynthetic pathway of artemisinin is 8 to 10 times higher than artemisinin (Abdin et al., 2003). Zhang et al. (2005) proposed that the rate limiting step seems to be between artemisinic acid and artemisinin. When gibberellic acid was applied to *A. annua*, artemisinic acid converts to artemisinin. Oxidative burst played a role in stimulating the conversion of artemisinic acid to artemisinin (Zheng et al., 2010).

When these hairy roots were exposed to the three different concentrations of salicylic acid, 0.138 mg/L concentration of salicylic showed good growth rate response. Otherwise, an increase in concentration of salicylic acid growth rate of hairy roots decreased. Previously, similar results on growth rate of hairy roots were reported by Bais et al. (2002) while working on effect of different concentrations of salicylic acid on growth rate of plants.

Salicylic acid also affected the synthesis of artemisinin in hairy root cultures when they exposed to different concentrations of salicylic acid. Maximum artemisinin concentration that is, 79 ± 3 µg/g of DW was measured when hairy roots were exposed to 13.8 mg/L of salicylic

acid. Second highest artemisinin concentration that is, 73 ± 3 $\mu\text{g/g}$ of DW was obtained when hairy roots were exposed to 1.38 mg/L of salicylic acid. In former case, an increase in artemisinin was 87% while in later case; increase was 73% with respect to control, respectively (Figure 2). Results of present study were similar to the results of other laboratories (Baldi and Dixit, 2008; Pu et al., 2009; Guo et al., 2010). They used salicylic acid concentrations to enhance artemisinin production in hairy root cultures of *Artemisia* species. Recently, it has been reported that when salicylic acid was exposed to the transgenic *A. annua* species, expression level of ADS gene increased with the emission of singlet oxygen (Guo et al., 2010).

Conclusion

Overall results revealed that gibberellic acid and salicylic acid have a positive role in growth and secondary metabolites production of hairy root cultures. Optimum concentration of these secondary plant hormones can be used to enhance Artemisinin production.

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