

Full Length Research Paper

Hairy roots induction and artemisinin analysis in *Artemisia dubia* and *Artemisia indica*

Abdul Mannan^{1*}, Nazia Shaheen¹, Waheed Arshad¹, Rizwana Aleem Qureshi², Muhammad Zia³ and Bushra Mirza¹

¹Department of Biochemistry, Quaid-i-Azam University, Islamabad, Pakistan. ²Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan. ³Department of Biotechnology, Quaid-i-Azam University, Islamabad, Pakistan.

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Transformation of two *Artemisia* species (*Artemisia dubia* and *Artemisia indica*) was carried out by using two *Agrobacterium rhizogenes* strains LBA 9402 and 8196 for hairy roots production. Induction of hairy roots was higher in both *Artemisia* species when infected with LBA9402 as compared to 8196. When biomass of roots along with their artemisinin content was evaluated, a significant effect of *Agrobacterium* strain on fresh weight of roots was observed. While artemisinin content was significantly affected by both plant species as well as *Agrobacterium* strains used for infection. However roots of *A. dubia* infected either with LBA9402 or 8196 produced maximum artemisinin (0.603% and 0.753, respectively). When transformed roots were cultured in liquid medium, highest root fresh weight as well as artemisinin content (3.9 g and 0.042%, respectively) was observed in hairy roots of *A. indica* induced by *Agrobacterium* 8196.

Key words: *Agrobacterium rhizogenes*, hairy roots, transformation, *artemisia*.

INTRODUCTION

Malaria, one of the world's most serious parasitic diseases, caused by *Plasmodium*, has at least 500 million cases globally every year, resulting in more than one million deaths (Ro et al., 2006). The biggest challenge faced in the fight against malaria is the multi-drug resistance of *Plasmodium* strains to the widely used anti-malarials such as chloroquine, mefloquine and sulfadoxine-pyrimethamine (Liu et al., 2006). However artemisinin has been reported to have marked activity against both chloroquine-resistant and chloroquine-sensitive strains of *Plasmodium falciparum* and is useful in treatment of cerebral malaria (Luo and Shen, 1987). Presently most common commercial sources of artemisinin are field-grown leaves and flowering tops of *Artemisia annua* L that are subject to seasonal and somatic variation. Today, the search for increased productivity follows two trends: the search for new species with better yield and a further development and application of methods for genetic improvement (Levy et al., 2001).

Although presence of artemisinin is supposed to be in *Artemisia* genus, however, there are two reports describing presence or production of artemisinin in species other than *A. annua* (Arab et al., 2006; Zia et al., 2007). Chemical synthesis of artemisinin is known to be difficult and expensive therefore search of other *Artemisia* species containing artemisinin and the plant source such as callus (He et al., 1983), shoot (Fulzele et al., 1991) and hairy root cultures (Qin et al., 1994) are attractive alternates.

Hairy roots are induced by infection of *A. rhizogenes* and produce secondary metabolites like the whole plant (Uozumi and Kobayashi, 1994) and may provide a means to produce these commercially valuable products on a large scale (Flores and Curtis, 1992; Wilson, 1997). Hairy root clones of *A. annua* L. if routinely subcultured in shake flasks produce artemisinin for more than 5 years (Weathers et al., 1994; Weathers et al., 1996).

Artemisia dubia and *Artemisia indica* (Asteraceae) are perennial herbs found in different hilly areas of Pakistan. These have been employed by local people successfully to alleviate chronic fever, dyspepsia and hepatobiliary ailments (Said, 1984). Phytopharmacological evaluation of this genus shows the presence of antimalarial (Zafar et al., 1990), anti-inflammatory (Soomers et al., 1965), anti-

*Corresponding author. E-mail: abdulmannan_ka@yahoo.com
Tel: 0092-51-90643108.

Table 1. Performance of different strains of *A. rhizogenes* on hairy root induction of *A. indica* and *A. dubia*

Strain x Species	Response (%)	Mean number of hairy roots	Ranking
<i>A. rhizogenes</i> LBA9402 x <i>A. indica</i>	100	24.3	A
<i>A. rhizogenes</i> LBA9402 x <i>A. dubia</i>	100	23.6	A
<i>A. rhizogenes</i> 8196 x <i>A. dubia</i>	85	15.2	B
<i>A. rhizogenes</i> 8196 x <i>A. indica</i>	80	14.7	B
<i>A. indica</i> Un-infected	60	2.5	C
<i>A. dubia</i> Un-infected	65	2.5	C

pyretic (Ikram et al., 1987), antibacterial (Kaul et al., 1976), antifungal (Maruzzella et al., 1960), anthelmintic, antihepatic (Gilani and Janbaz, 1995), and antidiabetic activities (Tan et al., 1999).

The objectives of the present work were to study hairy root induction and artemisinin production by two *Artemisia* species (*A. dubia* and *A. indica*) transformed by two *A. rhizogenes* strains 9402 and 8196.

MATERIALS AND METHODS

A. dubia and *A. indica* seeds were collected from Donga gali and Ayubia pipeline track, NWFP, Pakistan, respectively. Seeds were disinfected with 70% (v/v) ethanol for 30 s, surface sterilized with 0.1% mercuric chloride solution for 3 - 4 min, washed 3 to 4 times with sterilized distilled water and transferred to plain agar medium containing 3% sucrose solidified with 0.8% agar. Seed germination started after 3 days under controlled condition of temperature (25±2°C), light (1000 lux) and photoperiod (16/8 h light dark cycle).

A. rhizogenes strains 9402 and 8196 containing Ri plasmid were grown overnight at 28°C in 50 ml of liquid YMB (Yeast Mannitol Broth) and MYB (Malt Yeast Broth) medium, respectively, in 100 ml flask on shaker at 120 rpm. For co-cultivation 10 days old seedlings were cut at hypocotyls region, the roots were discarded while the stem portions were dipped in *Agrobacterium* culture (O.D. 1.0) containing acetosyringone 100 µM for 20 min. To remove the access of bacteria these soaked stems were blotted on sterile blotting paper and transferred to MS medium containing 100 µM acetosyringone solidified with 0.8% agar. After 3 days, the infected stems were washed with Cefotaxime solution (500 µg/ml), blotted on filter paper and transferred to MS medium for ten days containing Cefotaxime (500 µg/ml) and solidified with 0.8% agar.

In the first experiment, rooted plants were then transferred to solid half MS medium (Murashige and Skoog, 1962) for root proliferation. After 10, 20 and 30 days, roots of un-infected (control) and infected seedlings were separated, weighed and analyzed for artemisinin content. In second experiment, 3 - 4 cm root tips were cut from seedlings and incubated on 100 ml each of half and full strength MS liquid medium containing 3% sucrose. After 30 days, transformed hairy roots as well as untransformed roots as control were weighed and their artemisinin content was analyzed.

Artemisinin was extracted in toluene as described by Kim et al. (2001). After extraction of artemisinin from 1 g hairy roots, toluene was evaporated and dried extract was solubilised in 400 µL methanol and 1600 µL of 0.2% NaOH. Mixture was hydrolyzed for 45 min at 50°C. Reaction was stopped by adding 1600 µL of 0.2 M acetic acid and placed in ice water. To make the final volume 4 ml, 400 µL of methanol was added. For spectrophotometric analysis, samples were again dried in freeze drier and dissolved in mixture of 5 ml ethanol and 0.3 ml of trifluoro acetic acid (10% v/v). Artemisinin (Aldrich, HPLC grade) standard solutions were also

prepared in same mixture of ethanol and TFA. Spectrophotometric analysis was performed at 218 nm by using Agilent 8453 UV/Vis Spectrometer with DAD Detector.

Statistical analysis

To analyze transformation efficiency, thirty explants of each *Artemisia* species were infected with *Agrobacterium* strain separately, while other experiments were conducted in triplicate. The data was analyzed by MSTAT and LSD was measured at 5% probability level.

RESULTS AND DISCUSSION

In the present study, *Artemisia* species (*A. dubia* and *A. indica*) were successfully transformed by *Agrobacterium rhizogenes* Ri T-DNA and increased artemisinin content was detected in transformed roots as compared to control. Search for new *Artemisia* species with artemisinin content has gained interest in the last few years. Besides *A. annua*, *Artemisia absinthium* and *Artemisia sieberia* are also reported to contain some level of artemisinin especially in aerial parts (Arab et al., 2006; Zia et al., 2007). In some other species of *Artemisia* including *A. dubia* and *A. indica* collected from Northern areas of Pakistan, artemisinin has been detected (unpublished data presented in International Symposium on Medicinal Plants, NARC Pakistan, 2004).

Efficiency of transformation

A significant response of hairy root production was observed in both species depending upon *Agrobacterium* strain. Highest number of roots was observed in both *Artemisia* species when infected with *Agrobacterium* strain 9402 (Table 1). The same *Agrobacterium* strain has also been reported to produce 100% transformants in *A. annua* (Giri et al., 2001). A mean number of hairy roots of 14.7 and 15.2 were observed in *A. indica* and *A. dubia* respectively when infected with *A. rhizogenes* strain 8196. While untransformed plants did not produce remarkable number of roots (2.5 mean numbers of roots in both species). According to other reports too, efficiency of transformation depends upon *Agrobacterium* strain, plant species and explant type (Van-de-Velde et al., 2003).

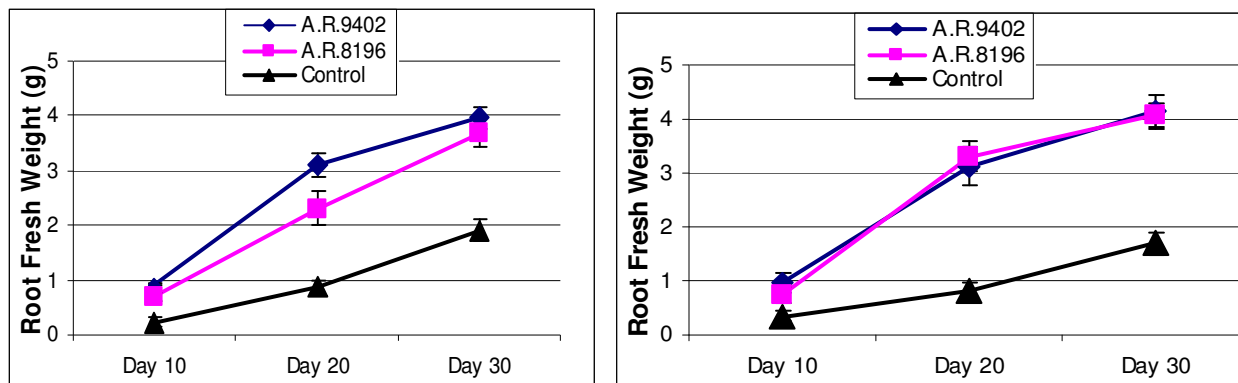


Figure 1. Fresh weight of *A. indica* (A) and *A. dubia* (B) roots induced by *A. rhizogenes*, cultured on ½ MS Agar medium at different time intervals. (A.R. *Agrobacterium rhizogenes*).

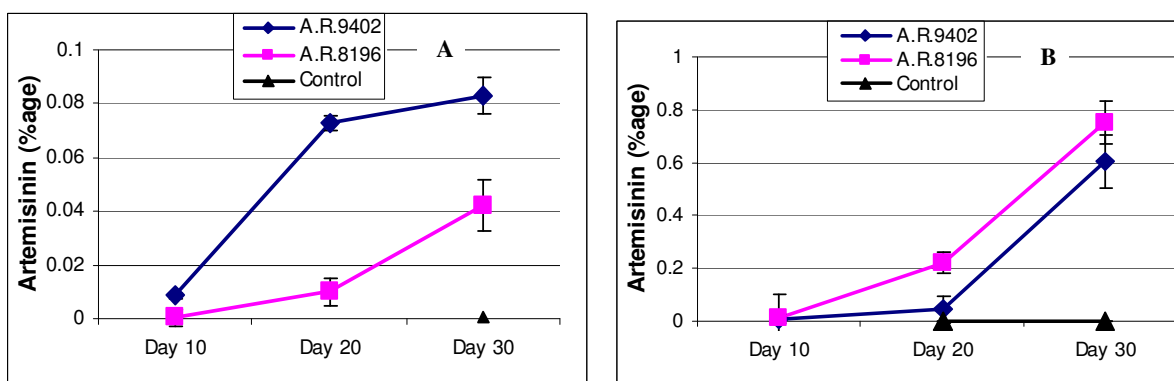


Figure 2. Artemisinin content in *A. indica* (A) and *A. dubia* (B) roots induced by *A. rhizogenes*, cultured on ½ MS Agar medium at different time intervals. (A.R., *Agrobacterium rhizogenes*).

Root weight and artemisinin content in different time intervals

Results of root weight and artemisinin content when grown on ½ MS solid medium after 10, 20 and 30 days are presented in Figure 1 and 2. Statistical analysis revealed that *Agrobacterium* strain and culture days significantly affect fresh weight of hairy root while effect of plant specie was non significant at 5% probability level. *A. indica* and *A. dubia* hairy roots in response to *A. rhizogenes* 9402 infection attained maximum weight after 30 days i.e. 4.16 and 3.96 g respectively as compared to control (Figure 1). It has been suggested that transformed roots grow faster than normal and hairy roots production varies from plant to plant (Kovalenko et al., 2004). Hairy roots of *A. indica* and *A. dubia* attained a weight of 4.06 and 3.7 g respectively after 30 days of infection with *A. rhizogenes* strain 8196. It was observed that *A. indica* roots grew faster in 10 to 20 days period as compared to 20 to 30 days period after infection either with *A. rhizogenes* 9402 or 8196. A weight difference of 2.56 g was observed between 10 to 20 days while only

0.75 g increase was observed in 20 to 30 days in *A. indica* hairy roots after infection by *A. rhizogenes* 8196. Figure 1 also describes that a steady increase in fresh weight was observed in *A. dubia* hairy roots in response to 8196 with 1.6 and 1.4 g increase from 10 to 20 days and 20 to 30 days period, respectively.

A meaningful increase in artemisinin content was observed in transformed roots compared to control; especially in the case of *A. dubia* infected with *A. rhizogenes* strain of either 9402 or 8196 (Figure 2). An earlier report shows that that *A. rhizogenes* mediated transformation increased the level of secondary metabolites in plants (Kovalenko et al., 2004). Figure 2 describes that there is a gradual increase in artemisinin content. *Agrobacterium* strain and plant species significantly affected production of artemisinin while effect of time period was non significant at alpha level 0.05. Untransformed roots of *A. indica* showed only 0.001% artemisinin after 30 days of growth while 0.001% and 0.0026% artemisinin content was observed after 20 and 30 days of culture in *A. dubia*, respectively. There was sudden increase (0.0644%) in artemisinin content from day 10 to day 20 (Figure 2a) while

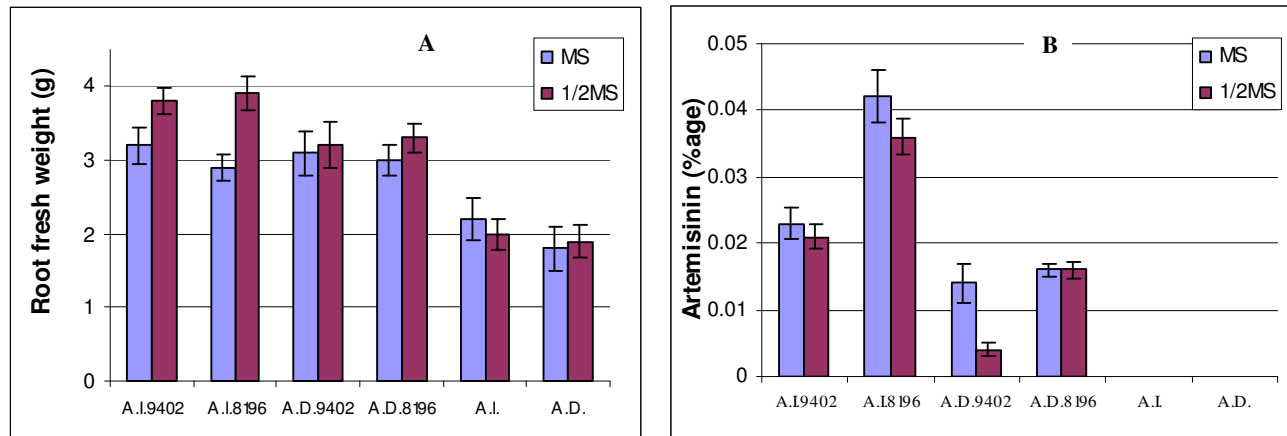


Figure 3. Fresh weight of roots (A) and artemisinin content (B) in *A. indica* and *A. dubia* roots grown on half and full MS liquid medium (A.I., *Artemisia indica*; A.D., *Artemisia dubia*).

from day 20 to day 30 only 0.01% increase was observed in *A. indica* hairy roots in response to *A. rhizogenes* 9402 infection. The reverse pattern was observed in case of 8196 *Agrobacterium* strain for same *Artemisia* species. There was highest increase in artemisinin % in day 20 to 30 period as compared to day 10 to 20 period. In case of *A. dubia* there was drastic increase in artemisinin content from day 20 to day 30 in hairy roots produced by either *Agrobacterium* strain. Maximum artemisinin (0.753%) was observed after 30 days of culture produced by *A. rhizogenes* 8196 in *A. dubia* while same plant species showed 0.603% artemisinin by *Agrobacterium* strain 9402. Artemisinin production (0.42%) has been reported in hairy roots culture of *A. annua* (Weathers et al., 1997). Primary metabolites are more pronounced in log phase; where as secondary products are produced in significant quantities only after the culture has reached the stationary phase (Giri et al., 2001). Figure 2 also describes that *A. dubia* showed higher artemisinin content than *A. indica*. While *A. rhizogenes* strain 9402 seems better to be a choice for *A. indica* than 8196 and vice versa for *A. dubia*.

Analysis of artemisinin content in roots grown on full and half MS solid medium

Transformed and untransformed roots of both *Artemisia* species were cultured in full and half MS liquid medium and analyzed for artemisinin content after 30 days. Statistical analysis showed significant effect of *Agrobacterium* strain on fresh weight of hairy roots while the effect of plant species and medium was non-significant. Figure 3a shows better growth of hairy roots on half MS medium irrespective to plant species and *Agrobacterium* strain. *A. indica* hairy roots in response to *Agrobacterium* strain 9402 and 8196 grew faster on 1/2MS medium and

attained fresh weight 3.8 and 3.9 g, respectively. There was not much difference in fresh weight of *A. dubia* hairy roots either grown on 1/2 MS or full MS medium. A range between 3.0 to 3.3 g of fresh weight was observed by these roots produced either by *A. rhizogenes* strain 9402 or 8196 (Figure 3a). Untransformed roots of both *Artemisia* species grown on 1/2 and full MS medium did not attain fresh weight as transformed roots.

Promising results were observed for artemisinin content in the roots grown on full and half MS liquid medium. But statistically these results are non significant with respect to *Agrobacterium* strain, plant species and culturing medium. Figure 3b describes that more artemisinin content was observed in the roots cultured on full MS medium as compared to those of half MS medium. Maximum artemisinin (0.042%) was obtained in the roots of *A. indica* in response to *Agrobacterium* 8196 when cultured on MS medium followed by same plant species and same strain cultured on 1/2 MS medium (0.036%). In a previous report, 0.001% artemisinin was found in hairy roots of *A. annua* transformed with *A. rhizogenes* strain 8196 (Jaziri et al., 1995). Hairy roots of both plant species in response to both *Agrobacterium* strains showed presence of artemisinin content while no artemisinin was detected in untransformed roots of both *Artemisia* species either cultured on half or full MS medium (Figure 3b). The results agree with the earlier findings that hairy root culture produce many fold increased secondary metabolites in comparison with control (Kovalenko and Maliuta, 2003; Hu and Du, 2006).

Our overall results reveal that artemisinin, the promising antimalarial compound can be successfully produced in the hairy roots of *Artemisia* species other than *A. annua*. From the results it can also be inferred that transformed roots while intact with plant produce more artemisinin than those of being cultured in liquid medium.

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