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Comparative Artemisinin analysis in *Artemisia dubia* transformed with two different *Agrobacteria* harbouring *rol ABC* genes

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Abstract

Artemisinin, a potent antimalarial drug and a major constituent of *Artemisia*, is effective against quinine resistant strains of *Plasmodium*. The aim of the present study was to enhance artemisinin production in the roots of *Artemisia dubia* through transformation with *rol* genes by using *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes. Agrobacterium tumefaciens* strain LBA4404 containing pRT99 and *Agrobacterium rhizogenes* strain LBA8196 and 9402 harboring *rol ABC* genes were used for the transformation experiments. PCR and Southern hybridization confirmed the T-DNA integration events in *Artemisia dubia* roots. The transformed roots proliferated two fold more as compared to untransformed roots. After 30 days of roots proliferation, the HPLC analysis showed higher concentration of artemisinin (36.581 µg/g DW) in roots of *Artemisia dubia* transformed with *A. rhizogenes* (strain 9402) compared to that of roots transformed with *A. tumefaciens* (0.855 µg/g DW) while no artemisinin was observed in control roots.

Keywords: *Agrobacterium tumefaciens,* Hairy root cultures, HPLC analysis, Southern blotting, Transformation.
Abbreviations: DW- Dry weight; PCR- Polymerase chain reaction; NAA- Naphthalene acetic acid; CTAB- Cetyl trimethyl ammonium bromide; CSPD**-** Chemiluminescent substrate for alkaline phosphatase detection .

Introduction

Artemisinin is one of the most potent antimalarial agents (Ridder et al., 2008). It is a sesquiterpene lactone that contains an endoperoxide moiety or internal oxygen bridge, which forms carbon-based free radicals when exposed to uni-iron to which its antimalarial properties are attributed. Artemisinin is not only effective against malaria but different types of cancer, parasitic diseases like Schistosomiasis, viral hepatitis B and some animal diseases are also known to be treated by artemisinin (Efferth 2009; Ferriera and Gonzalez, 2008). Naturally, artemisinin accumulates in glandular trichomes, leaves, small green stems, flowers buds and seeds of *Artemisia* (Abdin et al., 2003; Arsenault et al., 2010). Artemisinin is most commonly reported in *Artemisia annua.* However, other members of this genus including *Artemisia dubia* are also reported to contain this secondary metabolite (Mannan et al., 2010). Although biosynthesis of artemisinin seems to be restricted to the green parts of the plants, and is not synthesized in roots of field-grown plants or pollens, yet it can be produced by transformed roots (Liu et al., 2006; Mannan et al., 2008). The soil-borne plant pathogen *A. rhizogenes* containing the *rol* genes responsible for transformed hairy root formation at the site of infection causes certain biochemical changes in the plant metabolism. Hairy root cultures produced after transformation with *A. rhizogenes* are known to produce secondary metabolites consistently at improved levels (Park et al., 2010)**.** Shkryl et al. (2008) reported transformed calli of *Rubia cordifolia* expressing *rol* genes and producing higher levels of anthraquinones compared to control calli. *Rol B* gene is apparently the most powerful inducer of secondary metabolism and when expressed in *Vitis amurensis*, resulted in increased production of resveratrol; an important stilbene that prevents carcinogenesis at tumor initiation, promotion and progression (Kiselev et al*.,* 2007). Furthermore, the *rol C* gene is reported to be capable of stimulating the production of tropane alkaloids, pyridine alkaloids, indole alkaloids, ginsenosides and anthraquinones in transformed plants and plant cell cultures (Bulgakov et al*.,* 2002; Bulgakov et al., 2003; Bulgakov, 2005). Numerous investigations have shown that *rol* genes play a major role in the pathway that leads to high levels of secondary metabolites in hairy root cultures of most transformed plant species (Bulgakov, 2008) and considering the fact that chemical synthesis of artemisinin is not yet economically feasible because of its complexity and low yield, transformation seems to be the most appropriate choice to improve its production. Hence, the aim of the investigation reported here was to increase and compare artemisinin production in the roots of *Artemisia dubia* transformed with *rol ABC* genes by using *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* with the non transformed plants.

Results

Agrobacterium tumefaciens- mediated transformation

Explants incubated for 5 minutes in *A. tumefaciens* culture gave the maximum (70%) regeneration response on selection medium. Further increase in the length of incubation period resulted in overgrowth of *Agrobacterium* culture and subsequent decrease in the regeneration efficiency as shown in Fig. 1. After three days of co-cultivation, infected explants

Table 1. Root length (cm), fresh weight (g) and dry weight (g) of *Artemisia dubia* transformed with *A. tumefaciens* and *A. rhizogenes* in comparison with control.

Plants	Time interval for analyzing roots	Root length (cm) ± S.E	Fresh wt. (g) ± S.E	Dry wt. (g) ± S.E
Transformed roots (*Agrobacterium tumefaciens)*	Day 10	3 ± 0.09	0.8 ± 0.003	0.3 ± 0.07
	Day 20	5.6 ± 0.25	1.3 ± 0.006	0.8 ± 0.001
	Day 30	7 ± 0.33*	2.3 ± 0.01*	1.5 ± 0.05*
Transformed roots *(Agrobacterium rhizogenes)*	Day 10	2.5 ± 0.16	0.5 ± 0.01	0.2 ± 0.003
	Day 20	6 ± 0.33	1.5 ± 0.03	0.9 ± 0.03
	Day 30	7.5 ± 0.09*	2.9 ± 0.03*	1.8 ± 0.06*
Roots of Control plant	Day 10	1 ± 0.33	0.3 ± 0.07	0.1 ± 0.007
	Day 20	2.5 ± 0.16	1.0 ± 0.003	0.4 ± 0.008
	Day 30	4 ± 0.57	1.3 ± 0.003	1.1 ± 0.008

*The bold and asterisk values represents the improved and statistically significant performance ($P < 0.05$) of the transgenic lines in comparison with control for the different parameters studied after one month analysis. Values are the mean of three replicates ± S.D.

were transferred to selection medium. After two weeks, transgenic shoots were obtained (Fig. 2, a,b). When the length of the transgenic shoots reached 3-4 cm, they were shifted to half MS medium containing 0.025 mg/l of Naphthalene acetic acid for rooting (Fig. 2c). The rooting response in transformed plants was found to be 50%.

Agrobacterium rhizogenes- mediated transformation

For *A. rhizogenes* mediated transformation, small stem explants carrying a bud were excised from green house grown plants and cultured on MS medium. New plants appeared from bud after one month; these plantlets were cut and then transferred to half MS medium. The subculturing of these plantlets to half MS medium resulted in the propagation of multiple plantlets from a single bud. The four-week old plants on half MS medium were infected with *A. rhizogenes strain* LBA9402; hairy roots appeared after six days of infection whereas no hairy roots were produced with the strain LBA8196. *In-vitro* grown plantlet of *Artemisia dubia* and induction of hairy roots from infected stems are shown in Fig. 3.

Molecular analysis

For molecular analysis of transformed plants/roots, PCR was performed for *rol A, B* and *C* genes and the amplified products (308 bp, 779 bp, 540 bp respectively) were observed to confirm transformation. Same size amplified product was also obtained from the plasmid DNA of *Agrobacterium* strains. *Rol A, B* and *C* genes were detected in all transformed lines (T1, T2, T3, R1, R2), as well as in plasmid DNA but not in control roots (Fig. 4). Hybridization bands were detected in Southern blots with the *rol A* probe in the transgenic roots. In all the transformants, the inserted copy number was one except line T2 in which two copies of the inserted gene were observed (Fig. 5). The results confirmed the integration of the *Agrobacterium* T-DNA in the genome of *Artemisia dubia* transgenic lines.

Evaluation of transformed root cultures

After transformation, the roots from the *rol ABC* transformants and the control plants were transferred to solid B5 medium for further proliferation. The roots were harvested after ten days interval in triplicate and analyzed for biomass production (Table 1). A large variation in biomass of fresh roots was observed in transformants. The fresh weight from day 10 to day 30 increases from 0.8 g to 2.3 g showing rapid multiplication of roots. In this time period, average biomasses for *A. tumefaciens* transformed roots were 1.2 g ± 0.04 as compared to 1.6 g ± 0.031 obtained for *A. rhizogenes* transformed roots. Untransformed roots of *Artemisia dubia* attained average biomass of only 0.73 g ± 0.007.

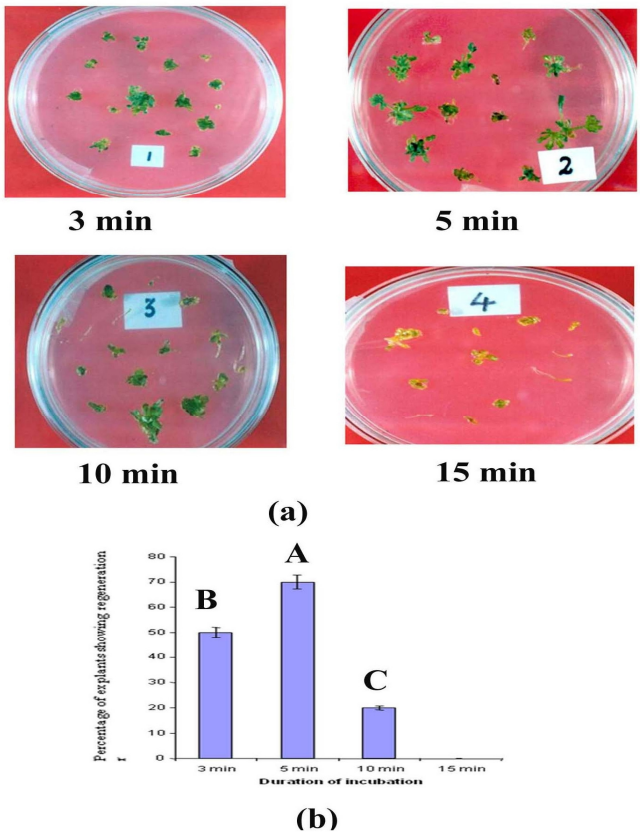

Fig 1. Effect of *Agrobacterium tumefaciens* incubation period on regeneration efficiency of *Artemisia dubia*. The visual (a) and graphical (b) representation of regeneration response in *Artemisia dubia* at various time intervals is shown in the figure. Vertical bars indicate ± standard deviation of the mean (n = 6 petri dishes with 15 explants each). Different letters indicate significant differences at $P \leq 0.05$ (LSD test).

Analysis of artemisinin content

Artemisinin was analyzed through HPLC after extraction following the methodology of Towler and Weathers, 2007. Since, maximum biomass of transformed and control roots

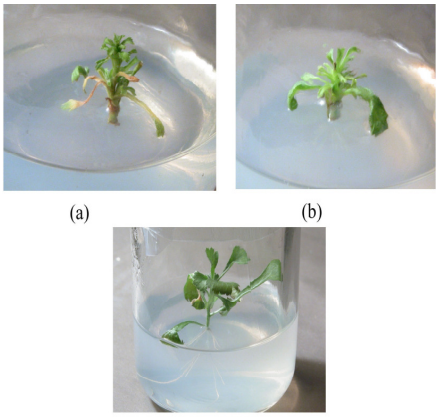

Fig 2. Shooting and rooting response of *Artemisia dubia* plants transformed with *A. tumefaciens* containing *rol ABC* genes. Shooting of transformants is obtained from (a) leaf and (b) stem explants. Rooting (c) is obtained on half MS medium.

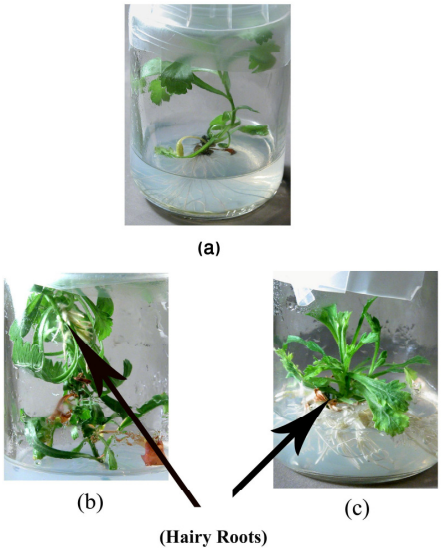

Fig 3. In-vitro grown plantlet of *Artemisia dubia* (a) and generation of hairy roots (b,c) from infected stems. Arrow points the hairy roots.

was observed on 30th day therefore, these transgenic and control roots with improved fresh weight were subjected to HPLC analysis for determination of the artemisinin content. Experiment was conducted in three replicates with maximum artemisinin content observed in roots transformed with *A. rhizogenes* (36.581 µg/g DW) followed by the roots transformed with *A. tumefaciens* (0.855 µg/g DW), whereas no artemisinin was detected in control roots (Fig. 7).

Discussion

In this report, successful transformation of *Artemisia dubia* with *rol ABC* genes by using *A. tumefaciens* and *A. rhizogenes* was carried out for artemisinin production. First of all, transformation of *Artemisia dubia* was achieved by using *A. tumefaciens* carrying *rol ABC* genes. An incubation time of five minutes was found to be the best giving 70% transformation efficiency. Furthermore, three days of co-cultivation gave maximum transformation response (4-5%). Longer co-cultivation periods do not help in increasing the transformation efficiency as it results in the overgrowth of the *Agrobacterial* strains on the explants which then becomes difficult to control. This is the first report of successful transformation of *Artemisia dubia;* however the selection and regeneration conditions used are in accordance to the previous reports stated for *Artemisia annua* (Liu et al., 2003; Jorsobe et al*.,* 2003; Bettini et al., 2003; Morgan et al., 2004; Han et al., 2005). For transformation with *A. rhizogenes,* small stem (2-4cm) portions having a bud were transferred to MS medium after sterilization. New plants appeared from bud after one month and hairy roots appeared in six days after infection with *A. rhizogenes* strain LBA9402, while *A. rhizogenes* strain LBA8196 did not produce any hairy roots. Nin et al., (1997) also infected different genotypes of *Artemisia absinthium* shoots with two different *A. rhizogenes* strain 1855 and LBA9402. The frequency of transformation obtained with strain LBA9402 was genotype dependent as compared to strain 1855. Furthermore, Giri et al., (2001) transformed *Artemisia annua* with different *A. rhizogenes* strains such as A4, K599, LBA9402, 9365, 9340 and reported variable virulence among these strains for induction of hairy roots with the best transformation response obtained with LBA9402. Zebarjadi et al., (2011) infected a medicinal plant, *Valeriana officinalis* L with two *A. rhizogenes* strains and reported better performance of LBA9402 as compared to AR15834 strain with the 90% production of the hairy roots. These reports are in agreement with our results that LBA9402 is more effective in generating *Artemisia dubia* transformants as compared to LBA8196 and the effect of *A. rhizogenes* strains are plant genotype dependent. Even though LBA8196 did not produce any transformed roots in *Artemisia dubia,* yet the possibility of this strain to produce transformants in other species of *Artemisia* cannot be excluded. The transformation in *Artemisia dubia* was assessed by PCR and Southern analysis. All the transformants tested gave positive PCR results for the *rol A, B* or *C* genes. Southern blots also detected the copy number in independent transgenic lines with the single T-DNA insertion being the most frequent. Han et al., (2005) and Zhang et al., (2009) also reported the optimization of *Agrobacterium* transformation systems in *Artemisia annua* and confirmed the integration of the target genes by Southern blot analysis. After transformation, the transformed and non-transformed roots were shifted to solid B5 medium for further proliferation. In the present investigation, the transformed roots grew faster as compared to non transformed roots. Transformation of *Artemisia dubia* also affected production of artemisinin as transformed roots produced more artemisinin compared to non-transformed roots. Highest artemisinin was detected in roots transformed with *A. rhizogenes* (0.0036581% DW) as compared to artemisinin content in roots transformed with *A. tumefaciens* containing *rol ABC* genes (0.0000855% DW). Difference in the artemisinin content detected in roots transformed with *A. tumefaciens* or *rhizogenes* suggests the possible influential role of *rhizogene* genes other than the targeted *rol ABC* genes. However, elucidation of the key genes that could improve artemisinin production at maximum is still required.

Materials and methods

Plant material

Artemisia dubia seeds were collected from the plants growing in Donga gali, Ayubia pipeline (NWFP), Pakistan.

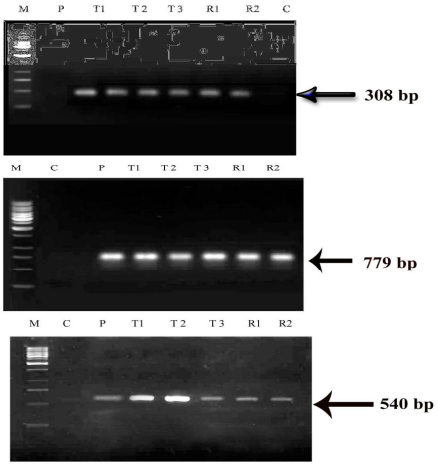

Fig 4. PCR analysis of transgenic *Artemisia dubia* harbouring rol genes. Amplified PCR product of *rol A* (a), *B* (b) and *C* (c) genes are shown in the figure. T1-T3 represents the plants transformed by *A. tumefaciens* and R1-R2 represents the plants transformed by *A. rhizogenes*. Lane P represents the plasmid DNA. Lane C refers to the non transformed control plants. Lane M corresponds to 1 kbp Ladder (Fermentas).

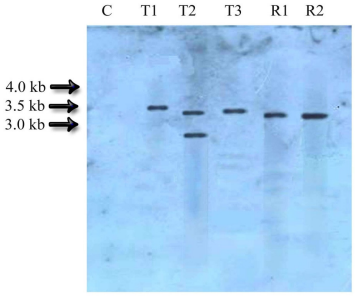

Fig 5. Southern blot analysis of PCR-positive plants showing the integration of *rol A* gene into the *Artemisia dubia* genome. C untransformed control plant, T1-T3 represents the plants transformed by *A. tumefaciens* and R1-R2 represents the plants transformed by *A. rhizogenes*. PCR product of *rol A* gene from plasmid was used as the probe.

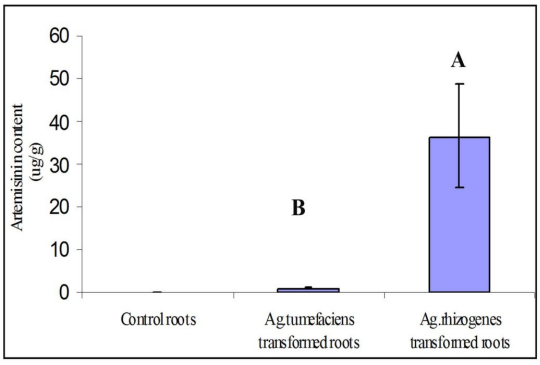

Fig 6. Graphical representation of comparative artemisinin contents in roots transformed by *Agrobacterium rhizogenes, Agrobacterium tumefaciens* and control roots. Vertical bars indicate ± standard deviation of the mean (n = 3). Different letters indicate significant differences at $P \leq 0.05$ (LSD test).

Seed sterilization and germination

Seeds were disinfected with 70% (v/v) ethyl alcohol for 30 seconds; surface sterilized with 0.1% (w/v) Mercuric chloride ($HgCl_2$) solution for 2 minutes, washed 3 to 4 minutes with sterile distilled water and transferred to half MS medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose solidified with 0.8% (w/v) agar. The seedlings were grown in growth room at 26°C ± 2 with 16 / 8 hours light dark cycle and light intensity maintained at 1000 lux.

Bacterial Strains

Agrobacterium tumefaciens strain LBA4404 and *Agrobacterium rhizogenes* strain LBA9402 and LBA8196 harbouring *rol* ABC genes were used for the inoculation. *A. tumefaciens* strain LBA4404 containing pRT99 was grown overnight in 50 ml of liquid malt yeast broth (MYB; 5 g/l yeast extract, 0.5 g/l casein hydrolysate , 8 g/l mannitol, 2 g/l ammonium sulfate, 5 g/l sodium chloride, pH 6.6). *A. rhizogenes* strains LBA9402 and 8196 containing Ri plasmid were grown overnight in 50 ml of liquid yeast mannitol broth (YMB; 0.5 g/l Di-potassium hydrogen phosphate, 2g/l magnesium sulphate, 0.4 g/l yeast extract, 10 g/l mannitol, 0.1 g/l sodium chloride, pH 7.0). Prior to infection, the bacterial strains were grown overnight in liquid medium at 28°C on a rotary shaker at 100 rpm in the dark.

Agrobacterium tumefaciens- mediated transformation

Approximately 0.5-1 cm long stem and leaf explants were excised from the in-vitro grown seedlings and were cultured on plain MS medium. After 2-3 days of preculturing, these explants were dipped in *A. tumefaciens* culture containing 50 mg/l kanamycin with different time durations blotted on sterilize filter paper and cocultivated on MS medium containing 200 µM acetosyringone and 0.8% agar. After 3 days of co-cultivation, the infected explants were washed with cefotaxime solution (500 µg/L), dried and transferred to selection MS medium containing 0.1 mg/l BAP (benzyl aminopurine), 20 mg/l kanamycin and 500 mg/l cefotaxime. These explants were maintained in growth room at 26°C ± 2 with 16h of photoperiod, illumination of 45 uE $m^{-2}s^{-1}$ and 60% relative humidity. The explants were then transferred to fresh selection medium weekly during the first month. Afterwards, subcultures were made every two weeks. After eight weeks, the concentration of cefotaxime was reduced to 50 mg/l. For rooting, the developed shoots cut off segments were cultured on half MS medium containing 0.025 mg/l naphthalene acetic acid (NAA) solidified with 0.1% gelrite.

***Agrobacterium rhizogenes*-mediated transformation**
In-vitro plant production and sterilization

For in-vitro plant production, small stem (2-4cm) portion having a bud were taken from green house growing *Artemisia dubia* plants. These stem portions were disinfected with 70% ethanol (in sterilized water) for 4 min and solution of commercial bleach (20 ml) + sterilized water (80 ml) + tween-20 (100 µl), for 15 min with gentle shaking, followed by two minutes washing with sterile distilled water for 5 times and transferred to MS medium. New plants appeared from the bud after one month; these plantlets were cut and transferred to half MS medium.

Hairy roots induction

The four-weeks old plants were infected with *A. rhizogenes* strain LBA9402 and 8196. The stem portions were infected with 24 hours old single colony of *A. rhizogenes* with the help of scalpel. Hairy roots appeared after six days of infection.

Molecular analysis

Polymerase Chain reaction

For molecular analysis, genomic DNA was isolated by using CTAB method of Doyle and Doyle, (1990) from transformed and untransformed plants, and plasmid DNA was also isolated by using alkaline lysis method from both *A. tumefaciens* and *A. rhizogenes*. PCR analysis was performed using a programmed DNA thermal cycler (Biometra, USA). The *rol A* gene forward 5'-AGAATGGAATTAGCCGGACTA-3' and reverse primer 5'-GTATTAATCCCGTAGG TTTGTT-3', the *rol B* forward 5'-GCTCTTGCAGTGCTAGATTT-3' and reverse primer 5'-GAAGGTGCAAGCTACCTCTC-3',the *rol C* gene forward 5'-GAAGACGACCTGTGTTCTC-3' and reverse primer 5'-CGTTCAAACGTTAGCCGATT-3' were used for PCR analysis. The PCR reaction was carried out in 25 µl final reaction volume containing 50 ng DNA template with the following thermal cycling conditions: 35 cycles of 5 minutes at 94ºC, 1 minute at 53- 55°C and 1 minute at 72ºC. Agarose gel (1.5% w/v) electrophoresis was carried out to analyze 10 ul aliquot of PCR product.

Southern blotting

Southern blot analysis of PCR-positive plants was performed by DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Cat. No. 11585614910) according to the manufacturer's instructions. For southern blotting, 20 µg of genomic DNA was digested with EcoRI and electrophoresed on 0.8% agarose gel. The DNA was denatured by alkaline solution and transferred to positively charged nylon membranes according to the standard procedure of Sambrook et al., (1989). PCR products of *rol A* gene from plasmid was used as the probe. The probe was labeled using digoxigenin (DIG)-11-dUTP with DIG High Prime DNA Labeling reagents (Roche, Mannheim, Germany). Hybridization was carried out at 42–45°C followed by immunological detection on X-ay film using CSPD substrate according to the manufacturer's instructions.

Evaluation of transformed root cultures

Root tips 3-4 cm in size from both *A. tumefaciens*- mediated transformed plants and *A. rhizogenes*- mediated transformed roots were cut and placed on solid B5 medium. Growth rate was determined by visual examination and increase in fresh weight and dry weight was recorded for thirty days with the interval of 10 days. Roots of untransformed shoots served as a control. These roots were then harvested and dried in Vacuum oven for 3-days at 60ºC and then analyzed for artemisinin content by high-performance liquid chromatography (HPLC).

Analysis of artemisinin content

Artemisinin was extracted from roots by using method described by Kim et al., (2001) and HPLC analysis was carried out by using Agilent 1200 series HPLC system with diode array detector (G1315-DA), following the methodologies of Towler and Weathers, (2007). Samples were analyzed by using Zorbax SB C18 column (150 x 4.6 mm x 5 µm). Flow of mobile phase through column (stationary phase) was 1 ml/min. and injection volume was 20 µl. Artemisinin was detected at 260 nm and analyzed against standard calibration curve.

Statistical analysis

The data were subjected to analysis of variance and mean comparisons among the transgenic and control lines were made using least significant difference at the 0.05 probability level. The statistical software program MSTATC version 2.00 (East Lansing, MI, USA) was used for the experimental analysis.

Conclusion

Conclusively, this paper represents the comparison of the artemisinin contents observed in the roots of *Artemisia dubia* transformed with two different *Agrobacterial* strains. *A. rhizogenes* proved to be a significant candidate for improving the artemisinin content in *Artemisia dubia* transformants as compared to the transgenic plants transformed with *A. tumefaciens* or the control plants. *A. rhizogenes* mediated transformed hairy roots of *Artemisia dubia* can be an effective source for enhanced production of artemisinin. Furthermore, they can make a significant contribution to our understanding of secondary metabolite production.

Acknowledgements

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