Short Communication

Genetic transformation in *Artemesia annua* L. for hairy root induction and enhancement of secondary metabolites

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Abstract

Hairy root induction was attempted in *Artemesia annua* for enhancing the production of the secondary metabolite artemisinin, a powerful antimalarial drug. Leaf segments were regenerated on MS medium with 0.5 mg l^{-1} BAP alone or on 0.1 mg l^{-1} NAA and 2 mg l^{-1} BAP. *Agrobacterium rhizogenes* strain MTCC 2364 showed very fast growth on YEB, YEP, NA, *Xanthomonas* and LBA media and since the growth was poor on YEM media, this medium was selected for growing the bacteria to obtain single cell colonies. For the *Agrobacterium* strains ATCC 15834 and A4, both NA and YEB were good. Confirmation of transformation was done through opine analysis using high-voltage paper electrophoresis and PCR assay . Southern hybridization was also carried out further for confirming the presence of *rol* B gene of TL-DNA in the transformed roots. ATCC 15834 was the most efficient *Agrobacterium* strain to induce the hairy roots in *Artemisia*. When estimated using TLC, there was increase in artemisinin content of ATCC 15834 induced hairy roots grown in media elicited with the homogenate (from 0.0039 per cent FW to 0.0042 per cent FW).

Keywords: *Agrobacterium*; *Artemesia*; Artemisinin; Genetic modification; Hairy root; Secondary metabolite; Transformation

Artemisia annua L., belonging to Asteraceae family, contains a potent antimalarial drug called artemisinin, which is effective against both chloroquine-resistant and sensitive strains of *Plasmodium falciparum* (Davis et al., 2005). The plant had been used since ancient times for the treatment of malaria, cancer, viral hepatitis B, parasitic diseases like Schistosomiasis and fever by Chinese (Efferth, 2009; Ferriera and Gonzalez, 2008). Artemisinin production in *Artemesia annua* is usually in the range of 0.01 to 0.5 percent. Artemisinin has been detected from aerial parts of the plants mostly in leaves and inflorescence with low levels in stems and none in pollen or roots (Abdin et al., 2003; Arsenault et al., 2010).

Every year more than 50 percent of the world population is exposed to malaria and there are three million deaths due to it. Death due to malaria is occurring in increasing number because of frequent failure of conventional treatment using drugs such as chloroquinone and sulphadoxine against *Plasmodium falciparum*. The low concentration of artemisinin in *Artemisia annua* and its restricted biosynthesis in green parts of the plant becomes a bottleneck for the commercialization of the drug (Soleimani, 2012). The only commercial source of this compound is the plant *Artemisia annua,* which is short in supply and from which extraction is expensive. Being unstable at high temperature due to the presence of

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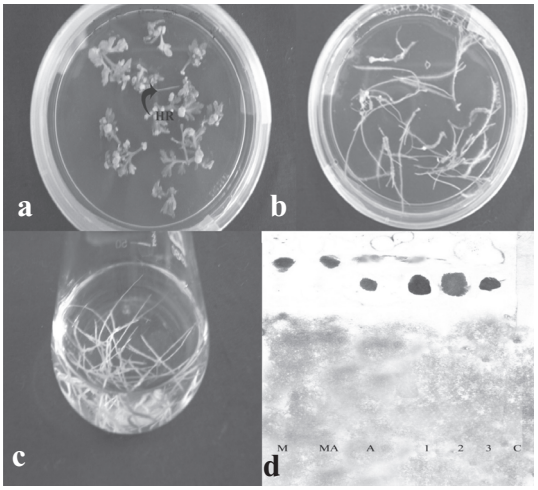

Figure 1. (a) Hairy roots emerging from leaf explants (b) Establishment of hairy root (c) Growth of hairy roots in MS liquid medium (d) Hairy roots (lane 1,2,3) showing presence of agropine(A) while normal roots(C) showing absence of agropine.

endoperoxide bridge, the chemical synthesis of the compound is uneconomical and highly complex (Abdin et al., 2003). Hence, enhancement in the production of artemisinin in cell or tissue culture is desirable.

Hairy roots induced by naturally occurring bacteria *Agrobacterium rhizogenes* have been extensively explored for the production of secondary metabolites in many plants. The hairy roots are fast growing and genetically stable and can also be successfully cultured in large scale bioreactors (Giri and Narasu, 2000). There has been a growing demand to standardize the *in vitro* regeneration protocol in *Artemesia annua* L. and to enhance artemisinin content from cell and hairy root cultivation.

Various explants like leaf segments, petiole segments, shoot tips, nodal segments, inflorescence bits and roots from *Artemesia annua* plants (from GKVK college, University of Agricultural Sciences, Bangalore) were used for the study. Surface sterilization of various explants like shoot tip,nodal segments,leaf segments with $HgCl_2$ was tried

Table 1. Artemisinin content of experimental samples

Sl. No.	Sample	Artemisinin content (µg g^{-1}) ± SD
A	Field grown plant	
1	Root	00.00 ± 0.00
2	Shoot	79.50 ± 0.01
3	Inflorescence	94.50 ± 0.04
4	Leaves	10.00 ± 0.32
B	*In vitro* plant	
1	Shoots	20.00 ± 0.22
2	Roots	00.00 ± 0.00
C	Transformed shoot	
1	A4	36.00 ± 0.69
2	ATCC 15834	19.00 ± 0.01
3	MTCC 2364	18.00 ± 0.99
D	Transformed root	
1	A4	17.00 ± 0.22
2	MTCC 2364	00.00 ± 0.00
3	ATCC 15834	39.00 ± 0.14
E	Rooted *in vitro* shoot	88.00 ± 0.32

with various concentration(0.05 to 2 per cent) and time period (30 seconds to 5 minutes). The *in vitro* regeneration protocol was standardized using these explants. The leaf segments, shoot, buds and nodal segments obtained from *in vitro* grown plantlets were tested for their ability to induce hairy roots.

Three strains of *Agrobacterium rhizogenes* viz., A4, MTCC 2364 and ATCC 15834, of agropine family (obtained from ILRA, France)

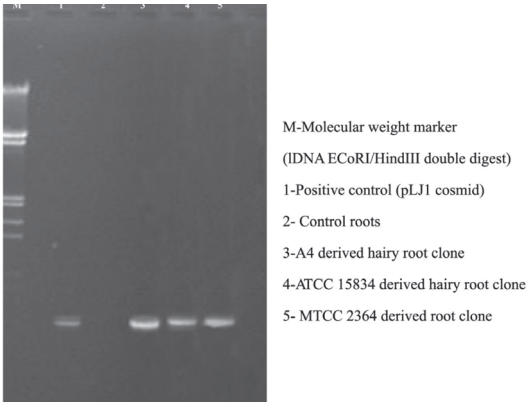

Figure 2. PCR analysis of hairy roots for *rol* B

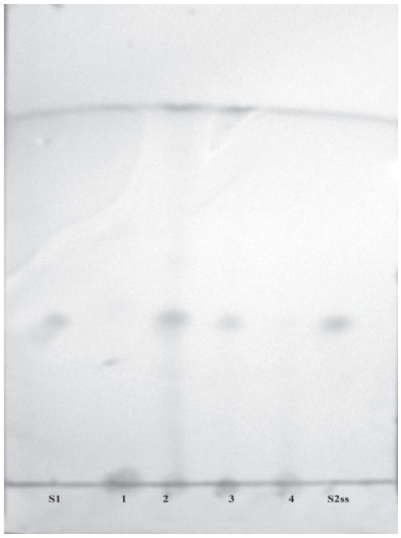

Figure 3. TLC analysis of artemesinin.54 S1 and S2 are standards, 2,3,4 are hairy roots showing presence of artemesinin where as artemesinin is absent in normal roots (1)

were used for the inoculation. *A. rhizogenes* strains were cultured on four different media such as Luria Bertani Agar (LBA), Yeast Extract Peptone, Yeast Extract Mannitol (YEM), Yeast Extract Broth (YEB), *Xanthomonas* and Nutrient Agar (NA) media. The strains were tested for resistance to antibiotics cefotaxime, ampicillin and carbenicillin.

The explants from *in vitro* rooted plantlets were cultured on MS solid medium (Murashige and Skoog 1962) in petriplates for two days prior to their infection with bacteria. These explants were then infected using Direct Inoculation Method (DIM) and Suspension culture inoculation Method (SM). Uninfected explants were placed on growth regulator free MS medium as control in both cases. The infected explants were co-cultured in dark at 26 ± 2°C for 1 to 4 days. Transformation percentage obtained and response of different explants under different pre-cultures and different co-cultivation periods were recorded.

Explants were then co-cultivated on MS medium containing 100 μ*M* acetosyringone. After co-cultivation, the infected explants were washed thoroughly with MS liquid medium containing 500 mg l^{-1} cefotaxime, dried and then transferred to solid MS medium containing 500 mg l^{-1} cefotaxime. The explants were further cultured at 26 ± 2°C under diffused light. The number of hairy roots per transformed explant, the transformation percentage and the number of normal roots per explant were recorded 25 days after infection.

Induced hairy roots were placed on full and half strength MS solid and liquid medium containing 250 mg l^{-1} cefotaxime to test its effect on root growth. The transformation was confirmed by morphological features, opine analysis (modified procedure given by Dessaux et al., 1991), PCR analysis of *rol* B genes, and Southern hybridization. The artemisinin content in different explants and hairy roots was estimated using thin layer chromatography (TLC) method modified from Gupta et al. (1996). The hairy roots derived from ATCC 15834 derived root clones were subjected to enhancement studies using techniques such as addition of osmoregulators, precursor feeding and elicitation.

The *in vitro* method for regeneration of *Artemesia annua* was standardized. Surface sterilization of leaf and inflorescence segments with 0.1 per cent $HgCl_2$ for 1 min. was found to be optimum for culture establishment. Maximum regeneration of leaf segments (90%) was obtained on MS medium with 0.5 mg l^{-1} BAP alone or on 0.1 mg l^{-1} NAA and 2 mg l^{-1} BAP. The *A. rhizogenes* strains were cultured on YEB, YEM, YEP, NA, *Xanthomonas* and LBA media. All the culture media favoured the growth of *A. rhizogenes*

strain. Strain MTCC 2364 showed very fast growth in all the media tested except YEM. So YEM was selected for growing MTCC 2364, so as to obtain single cell colonies. Both NA and YEB were found best for growing ATCC 15834 and A4. The optimum temperature for the growth of all the strains was observed to be 26 ± 2°C. Among the different antibiotics tested, cefotaxime (500 mg l^{-1}) was found to be effective for the elimination of *A. rhizogenes* strains from the explant tissues. Among the three *Agrobacterium rhizogenes* strains tested, all strains produced hairy roots in *A. annua*. ATCC 15834 was the most efficient in inducing hairy roots compared to other two.

Morphological analysis revealed that most of the roots were positively geotropic. However some showed negative geotropism. Lateral roots were more in hairy roots. Confirmation of transformation using Opine analysis showed spots corresponding to agropine in hairy roots produced by ATCC 15834 and MTCC 2364. No spot was produced by control roots. Also no spot was produced at positions corresponding to mannopine and mannopinic acid.

Confirmation of the transformation by detection of opines using high-voltage paper electrophoresis was found to be successful. Further confirmation was done by PCR. PCR analysis of hairy roots was carried out to demonstrate the presence of TL-DNA with *rol* B in the transformed roots. Amplification was obtained with Rol BF2R2 primer sets which confirmed the presence of *rol* B gene in the transformed roots as well as positive control. Amplification with Rol BF2R2 primer set corresponding to *rol* B gene produced a 205 bp band for the transformed roots and for the cosmid pLJ1. Southern hybridization was carried out further for confirming the presence of *rol* B gene of TL-DNA in the transformed roots.

Thin Layer Chromatography method was employed for the estimation of artemisinin. Artemisinin was detected in shoot, inflorescence, leaves from the plants grown in the field, *in vitro* shoots, transformed shoots and hairy roots induced by A4 and ATCC 15834. No artemisinin was detected in roots from plants grown in the field, *in vitro* roots and hairy roots induced by MTCC 2364. Rooting of *in vitro* shoots enhanced the artemisinin content. Artemisinin was highest in the inflorescence followed by *in vitro* rooted shoots.

The addition of osmoregulant PEG (6000g) was found to affect the hairy root growth in no way. There was increase in artemisinin content of ATCC 15834 induced hairy roots grown in media elicited with the homogenate (from 0.0039 per cent FW to 0.0042 per cent FW). Addition of yeast extract to media resulted in no specific change in growth and artemisinin content of ATCC 15834 induced hairy roots.

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