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# EFFICIENT PROTOCOL FOR REGENERATION OF TRANSFORMED *ARTEMISIA ANNUA* PLANTS FROM HAIRY ROOTS

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## ABSTRACT

Elite genotypes from high yielding variety of *Artemisia annua* L. (Jeevanraksha, CIMAP Lucknow) were regenerated form nodal explants and maintained in MS culture medium with BAP and NAA. The leaves of tissue culture grown plants were inoculated with *Agrobacterium rhizogenes* strain LBA9402 containing the selective marker gene NPT II. Hairy roots emerged from the leaves, 8-10 days after inoculation and were maintained in MSO liquid/ solid medium with kanamycin as the selective agent. Hairy root line was selected on the basis of high artemisinin content. Regeneration from hairy roots was obtained in half strength MS solid medium containing a combination of cytokinins and auxins (BAP, Kinetin, NAA and IBA). Transgenic plants showed typical transgenic morphology and profuse growth of plageotropic roots. Transformed hairy root cultures and transgenic plants can be used as an experimental system for studies related to induction of secondary metabolite synthesis.

**KEYWORDS:** **Artemisinin, sesquiterpene lactone, antimalarial, Agrobacterium rhizogenes, Benzylaminopurine (BAP), Naphthaleneacetic acid (NAA), Indolebutyric acid (IBA)**

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## INTRODUCTION

Artemisinin also known as qinghaosu is a sesquiterpene lactone, naturally formed in the shoots of *Artemisia annua* L. Artemisinin has proved to be an effective and safe antimalarial agent, exhibiting excellent activities against *Plasmodium falciparum* parasites that are resistant to commonly used antimalarial drugs.[1]Artemisinin and its derivatives are also found to be effective against Schistosomiasis caused by parasite *Schistosoma japonica, S. mansoni* and *S. haematobia* causing 1-1.5 million disabilities each year.[2] Oral administration of artemisinin has also been found to prevent and delay the development of 7, 12-dimethylbenz (a) anthracene (DMBA) induced breast cancer in rat.[3] Artemisinin reacts with iron and forms cytotoxic free radical. It is selectively more toxic to cancer than normal cells because cancer cells contain more intracellular free iron. Covalent tagging of artemisinin to transferrin is reported to increase its selectivity and toxicity towards cancer cells in vitro. Artemisinin- transferring conjugate significantly retarded the growth rate of breast tumors in rat and could be developed into a potent therapeutic agent of cancer in humans.[4] Artemisinin is also reported to be effective against human leukemia, colon cancer and lung carcinomas.[5,6] According to WHO, artemisinin based combination therapy (ACT) is the only reliable treatment option for multidrug resistant *Plasmodium falciparum* malaria.[7] Low yield of artemisinin in *A. annua* (0.01-0.8%, in some genotypes 1.5%) is a serious limitation to meet the annual targets of the drug for ACT treatments. Research studies are conducted in many laboratories to enhance artemisinin content in cell or tissue cutures of *A. annua* L. Biotechnological approaches are also being explored to increase artemisinin content. Transformed root and shoot cultures of *A. annua* were established by infection with *Agrobacterium rhizogenes* and *A. tumifaciens*. Hairy roots were induced from *A. annua* L. by transformation with *Agrobacterium rhizogenes*.[8,9] Transformed hairy root cultures of *A. annua* L were produced by co-culture method using leaf segments of *A. annua* and *A. rhizogenes* strain NCIB 8196 or MAFF 03-01724.[10] Hairy roots from *A. annua* L. leaf blade pieces and petiole segments were induced by *A. rhizogenes* strain 1602 and a clone was obtained with high content of artemisinin (1.195 mg/g dry weight).[11] *Agrobacterium tumifaciens* mediated transformation system was develoed for *A. annua* L. plants with high transformation rates (75% regenerants harbouring foreign genes).[12] Studies have been conducted in different laboratories to elucidate the biochemical pathway of artemisinin and its regulation with an aim to improve artemisinin content of *A. annua* L.[13,14,15] Conversion of farnesyl diphosphate to amorpha 4-11-diene is the first committed step in the biosynthesis of artemisinin, but little is known about the intermediates and enzymes that catalyze the pathway of artemisinin biosynthesis beyond amorpha- diene to artemisinic acid.[13] Twelve genes related to artemisinin biosynthetic pathway have been cloned from *A. annua* L. Researches in the field of transgenics aim to increase artemisinin content in the plants and to increase the supply of artemisinin and its derivatives for ACT treatments. Increase in the yield of artemisinin would significantly bring down the cost of malaria treatment and would help in reducing the incidence of malaria.

## MATERIALS & METHODS

***Tissue culture of field grown Artemisia annua plants***

Seeds of high yielding variety of *A. annua* L. (Jeevanraksha, CIMAP Lucknow)[16] were sown and plants grown to maturity in the field. Field grown plants ( fig 1) were screened for artemisinin content at preflowering and flowering stage. Nodal explants from field grown *A. annua* plants, having high artemisinin content (0.5% and above) were selected for tissue culture. *Artemisia annua* plants were surface sterilized with 0.1% (w/v) mercuric chloride for 2 min, rinsed thoroughly with sterile distilled water 7–8 times and cultured on MS basal medium ( table 1, fig 2,3,4 ) ) with BAP (0.2mg/l) and NAA (0.05mg/l).

**Table 1**
***Murashige and Skoog Medium for tissue culture of Artemisia annua***

| Composition | Concentration |
|---|---|
| MS A | 10 ml/l |
| MS B | 10 ml/l |
| MS C | 5 ml/l |
| MS D | 5ml/l |
| MS E | 5ml/l |
| MS F | 5 ml/l |
| Sucrose | 10 g/l |
| Myo Inositol | 100 mg/l |
| Casein hydrolysate | 0.5 mg/l |
| RT Vitamins | 2 ml/l |
| Biotin | 5 ml/l |
| Agar agar | 8 g/l |
| BAP | 0.2 mg/l |
| NAA | 0.05 mg/l |
| pH | 5.7 |

***Agrobacterium rhizogenes culture and infection of Artemisia annua leaves***

*Agrobacterium rhizogenes* strain LBA 9402 containing NPT II gene for kanamycin resistance was maintained in YMB medium ( table 2) with 50µg/ ml kanamycin. For transformation single cell colony of *A. rhizogenes* LBA 9402 was inoculated in YMB liquid medium and kept on rotary shaker. The bacterial culture was used after 24

hours for inoculation in axenically grown *A.annua* leaves. The leaf tissue was wounded with a syringe loaded with bacterial culture and incubated in MSO medium in dark at 5ºC for 24 hours. The leaves were then transferred to MS basal medium containing the selective agent kanamycin (50µg/ ml) and an antibiotic sporidex (1000 µg/ ml) to eliminate excess agrobacterial growth. Hairy roots emerged from the leaves 8-10 days after inoculation ( fig 5 ). These hairy root cultures were maintained in MSO medium with kanamycin (50µg/ ml) as the selective agent and kept at 25 ± 2°C in dark ( fig 6 ). The neoplastic (cancerous) roots produced by *A. rhizogenes* infection are characterized by high growth rate, genetic stability and growth in hormone free media. Root emergence from non transformed explants (control) was not observed.

**Table 2**
***YMB medium for culture of Agrobacterium rhizogenes***

| Composition | Concentration |
|---|---|
| $K_2HPO_4$ | 0.5 g/l |
| $MgSO_4$ | 2.0 g/l |
| NaCl | 0.1 g/l |
| Mannitol | 10.0 g/l |
| Yeast extract | 0.4 g/l |
| Kanamycin | 50 µg/ ml |
| Agar agar | 15.0 g/l |
| pH | 7.0 |

***Regeneration of transgenic Artemisia annua plants from hairy roots***

MS media with varying sucrose levels and different combinations and concentrations of auxin and cytokinin were tested for regeneration of transgenic plants. Single combination of cytokinin and auxin were not able to induce regeneration. Regeneration of transgenic *A. annua* plants from hairy roots was obtained in a media containing a combination of cytokinins and auxins (BAP + Kinetin + NAA + IBA). The selective agent kanamycin (50µg/ ml) was maintained in all the culture media throughout plant development (table 3, fig 7,8). Transformed cells and hairy root lines containing NPT II gene encode enzyme neomycin phosphotransferase (NPT) and thus inactivate the antibiotic kanamycin present in the medium, but normal plant cells are very sensitive to kanamycin and are killed in a medium containing kanamycin.

**Table 3**
***Media composition (MS Media) for regeneration of transformed Artemisia annua plants from hairy roots***

| Composition | Concentration |
|---|---|
| MS A | 5.0 ml/l |
| MS B | 5.0 ml/l |
| MS C | 2.5 ml/l |
| MS D | 2.5 ml/l |
| MS E | 2.5 ml/l |
| MS F | 2.5 ml/l |
| Sucrose | 20 g/l |
| Myo inositol | 100 mg/l |
| Casein hydrolysate | 0.5 g/l |
| RT vitamins | 2 ml/l |
| Biotin | 5 ml/l |
| Kanamycin | 50µg/ ml |
| BAP | 2 mg/l |
| Kn | 3 mg/l |
| NAA | 0.05 mg/l |
| IBA | 0.05 mg/l |
| pH | 5.7 |

## RESULTS & DISCUSSION

The study reports for the first time the hormone combination and concentration for the production of transgenic *A. annua* plants from hairy roots. Regeneration from hairy roots was obtained in half strength MS solid medium containing a combination of cytokinins and auxins (BAP 2mg/l, Kinetin 3mg/l, NAA 0.05mg/l & IBA 0.05mg/l). Transgenic plants showed typical transgenic morphology and profuse growth of plageotropic roots (fig 8). Conversion of farnesyl diphosphate to amorpha 4-11- diene is the first committed step in the biosynthesis of artemisinin.[7] Two genes of artemisinin biosynthetic pathway have now been identified – amorpha 4-11-diene synthase and sesquiterpene cyclase or synthase.[9] Transformed hairy root cultures and transgenic plants can be used as an experimental system for studies related to induction of secondary metabolite synthesis.

**Figure 1**
***Field grown plant of A. annua***

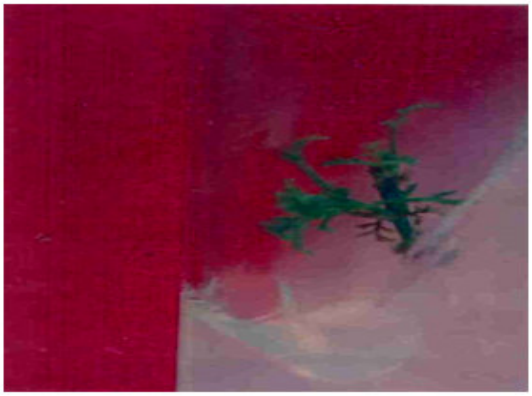

**Figure 2**
***Internodal explant in medium***

**Figure 3**
***Growth of tissue culture explant***

**Figure 4**
***Tissue culture raised plants***

**Figure 5**
***Induction of hairy roots***

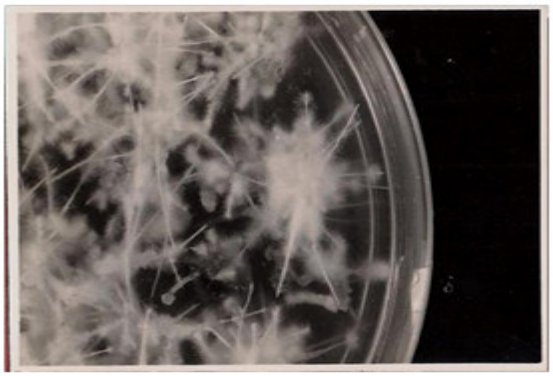

**Figure 6**
***Hairy root culture***

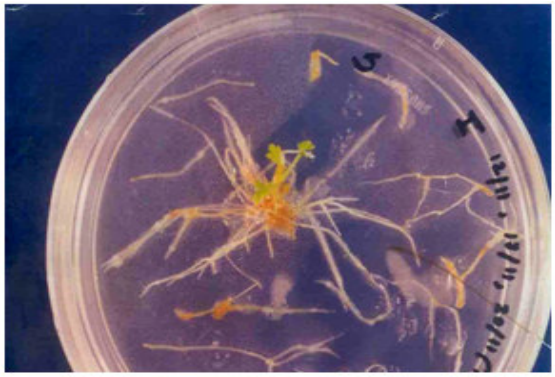

**Figure 7**
***Regeneration of plant from hairy root***

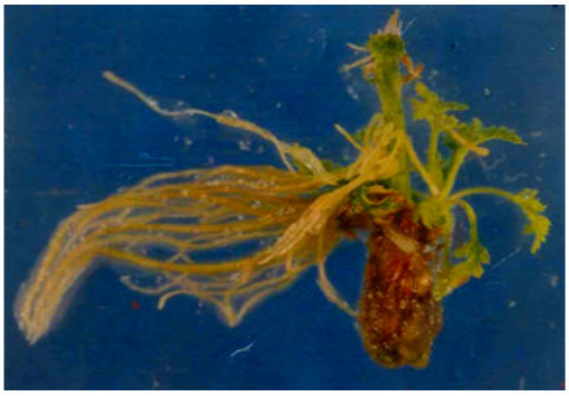

**Figure 8**
***Transgenic A. annua plant***

## CONCLUSION

It would be economically interesting to produce transgenic plants of *Artemisia annua* which ensure a constant high production of artemisinin by over-expressing a key enzyme in the biosynthetic pathway of artemisinin or by inhibiting an enzyme of another pathway, competitive for the precursors. The 'Hairy root' system can be used as a tool for plant breeding and improvement, via the transfer of desirable genes and for studies of gene expression with respect to changes in phenotype and secondary metabolism.

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## CONFLICT OF INTEREST

Conflict of interest declared none.

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