SUMMARY AND CONCLUSIONS

Malaria is still a major health problem in many developing countries. The parasite responsible for the vast majority of fatal malaria infections is *Plasmodium falciparum*. The first effective anti-malarial drug, quinine was isolated from the bark of *Cinchona*. Since, then malaria has been treated with quinoline based drugs such as chloroquine, mefloquine, etc. Unfortunately, most *Plasmodium* strains including *P. falciparum* have become resistant to most of the antimalarials including chloroquine, mefloquine, etc. To combat this problem, WHO has recommended the use of artemisinin and its derivatives in artemisinin based combination therapy (ACT).

Artemisinin, a sesquiterpene lactone, is a potent antimalarial isolated from *Artemisia annua* L. Its derivatives are effective against both drug resistant and sensitive strains of *Plasmodium* sp. including *P. falciparum*, the causal organism of cerebral malaria. Besides its antimalarial activity, it has also been shown to be effective against a variety of cancer cell lines including breast cancer, human leukemia, colon cancer and small cell-lung carcinomas, schistosomiasis, hepatitis-B and leishmaniasis. Due to its current use in artemisinin based-combination therapy (ACT), its global demand is increasing continuously. But, the relatively low yield of artemisinin in *A. annua* L. plants and unavailability of economically viable synthetic protocols are the major bottlenecks for its commercial production and clinical use. Efforts, therefore, are being made to enhance the production of artemisinin both *in vivo* and *in vitro*. Chemical synthesis of artemisinin is very complex and uneconomical. Breeding of high artemisinin yielding plants as well as the manipulation of culture conditions, growth media, and hormone levels to increase the yield of artemisinin in cell culture have not been successful. It is, therefore, essential to look for alternative strategies, which are economically viable for commercial production of artemisinin.

Mass scale cultivation of *A. annua* L. hairy roots by plant tissue culture technology may be an alternative route for production of artemisinin. A comprehensive investigation of the hairy root system of *A. annua* L. would help
in developing a viable process for the production of artemisinin. Keeping in view these facts, the present study was undertaken.

The silent features of the findings obtained in this study are as follows:

- Different hairy root lines (380 hairy root clones) were initiated after 5-6 days by infecting the *A. annua* L. explants with *A. rhizogenes* LBA 9402.

- Out of these, only nine hairy root lines (A1, A2, A3, A4, A5, A6, A7, A8 and A9) were selected on the basis of higher specific growth rates (0.001±0.0005-0.07±0.003) and artemisinin content (0.10±0.005-0.66±0.03 mg g⁻¹) in comparison to other hairy root lines.

- The presence of a fragment of 750 kb of rol B gene in PCR product confirmed the transformed nature of the selected hairy root lines.

- Southern hybridization was carried out to confirm the integration of the rol B gene and to find out the copy number of the rol B gene in the genomic DNA of hairy root lines. The hairy root lines A1, A4, A8, A9 were found to have single copy of the rol B gene, while A2, A3, A5, A6, A7 showed two copies. Moreover, these transgenic events were found to be independent with each other and the transgene was located at different positions in the genomes of the hairy root lines, as the size of DNA fragments were different from each other.

- A1 root line was selected for further study on the basis of highest specific growth rate (0.07±0.003) and maximum artemisinin content of (0.66±0.03) among the induced root lines.

- Maximum biomass (8.4±0.42 g l⁻¹) and artemisinin production (5.6±0.28 mg l⁻¹) were obtained with media:flask volume ratio of 0.16.

- The highest specific growth rate (0.07±0.003 d⁻¹) of hairy roots and maximum artemisinin production (6.2±0.31 mg l⁻¹) from the hairy root culture of *A. annua* L. were obtained in 25 days.

- Total 30 metabolites were identified by GC-MS. The major metabolites and related compounds detected were camphor, caryophylene, fatty acids and farnesene (45.03%), followed by metabolites from artemisinin biosynthetic pathway (8.57%) and the precursor of sterol biosynthesis,
squalene (SQ) (1.28%) in transformed hairy root line. Though compounds related to artemisinin biosynthetic pathway were not detected in normal roots, compounds from essential oil pathway (21.86% farnesene, 16.86% caryophylene oxide and 16.54% spathulenol) were found.

- Sucrose was found to be a better carbon source than glucose for hairy root cultivation because of maximum artemisinin productivity (0.248±0.01 mg l⁻¹ d⁻¹) obtained in liquid culture.
- Higher artemisinin content (7.1±0.35 mg l⁻¹) was obtained under 16/8 h light/dark photoperiod as compared to that obtained during complete dark regime (5.5±0.23 mg l⁻¹). Hence, 16/8 h light/dark photoperiod was selected for optimized cultivation conditions.
- Inoculum concentration of 3 g l⁻¹ on dry weight basis gave maximum growth index (2.9±0.1) and artemisinin production (7.6±0.31 mg l⁻¹), when compared to that obtained with 1, 5, 7 and 9 g l⁻¹ inoculum. Hence, 3 g l⁻¹ inoculum was selected as the optimized inoculum concentration for liquid culture of hairy roots.
- Maximum growth index (2.7±0.1) and artemisinin production (5.6±0.28 mg l⁻¹) were obtained when 25 days old inoculum was used in liquid culture of the selected hairy root line.
- Maximum biomass (10.1±0.51 g l⁻¹) and artemisinin content (5.8±0.29 mg l⁻¹) were achieved at 80 rpm shaker speed. Hence, this was selected as the optimum speed for liquid culture studies of hairy root.
- B5 medium was modified to get maximum biomass and artemisinin production. Statistically optimized B5 medium composition for the production of maximum biomass (12.8±0.61 g l⁻¹) and artemisinin production (8.5±0.41 mg l⁻¹) was as follows:
  - Sucrose – 40 g l⁻¹
  - Sodium phosphate – 0.19 g l⁻¹
  - Potassium nitrate – 3.1 g l⁻¹
  - Ammonium nitrate- 1.65 g l⁻¹
• Magnesium sulphate – 0.41 g l\(^{-1}\)
• Calcium chloride – 0.15 g l\(^{-1}\)
  
  • Statistically optimized pH and temperature for the production of maximum biomass (10.0±0.49 g l\(^{-1}\)) and artemisinin yield (6.6±0.31 mg l\(^{-1}\)) were as follows:
  
  o pH: 5.8
  o Temperature: 25±1°C

  • Maximum biomass (16.4±0.9 g l\(^{-1}\)) and artemisinin production (11.1±0.5 mg l\(^{-1}\)) were obtained in shake flask liquid culture under the optimized cultivation conditions in 20 days.

  • Maximum biomass (30.4±1.5 g l\(^{-1}\)) and artemisinin production (24.1±1.2 mg l\(^{-1}\)) were obtained in 20 days of hairy root culture by supplementing the optimized B5 medium with 100 μM MeJA, 3% v/v CH of P. indica, 0.50 mg l\(^{-1}\) FPP, 20 mg l\(^{-1}\) micanazole, 1.0 mg l\(^{-1}\) IAA and 0.025 mg l\(^{-1}\) GA\(_3\) under optimized cultivation conditions.

• Relative expression profiles of 7 genes encoding key enzymes (ADS, ALDH, HMGR, CYP71AV1, DXS, DXR, DBR2) of MVA, MEP and artemisinin biosynthetic pathways were studied by qPCR. All treatments [1(hairy root culture with methyl jasmonate and Piriformospora indica together), 2 (hairy root culture with Piriformospora indica), 3 (hairy root culture with methyl jasmonate), 4 (hairy root culture with miconazole), 5 (hairy root culture with farnesyl pyrophosphate), 6 (hairy root culture with GA\(_3\))] had led to increased expression of these genes, and consequently, increased production of artemisinin. Our results showed that hmgr was up-regulated in hairy roots with all the treatments in comparison to control hairy root cultures. It was therefore, concluded that increased artemisinin production under these treatments could be due to higher expression of the genes and increased cellular levels of the key enzymes encoded by the genes involved in regulatory genes of MVA, MEP and artemisinin biosynthetic pathways.
The results of our study clearly indicate that the artemisinin production using hairy root culture of *A. annua* L. plants might be an alternative and effective strategy to increase yield of artemisinin and its production throughout the year. This effort will be beneficial in supplementing the production and supply of artemisinin from plant source; thus helping to reduce the cost and achieve higher targeted production of artemisinin needed for artemisinin based combination therapies (ACTs). It will ultimately lead to combat the drug resistant malaria in developing countries like Indian sub continent and large swaths of Africa. In order to make commercial application of hairy root cultures for artemisinin production feasible, effective scale-up of hairy roots in bioreactors is however, required. That is a great challenge at the moment. The efficiency of the scaling up systems still needs optimization before industrial exploitation becomes viable.