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Full Length Research Paper

Influence of *Agrobacterium rhizogenes* on induction of hairy roots for enhanced production of artemisinin in *Artemisia annua* L. plants

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Artemisinin production from plant tissue cultures and induction of hairy roots *in vitro* have been considered to be a promising alternative, which offer a high degree of genetic stability, grow rapidly and produce the higher spectrum of secondary metabolites than wild type plants. Hairy root cultures developed from infection of different explants of *in vitro* germinated *Artemisia annua* L. plant with *Agrobacterium rhizogenes* LBA 9402 strain were selected on the basis of high artemisinin content and growth. Integration of the TL-DNA (*rol* gene) region of the pRi plasmid was confirmed by polymerase chain reaction (PCR) analysis of the gene located in this region. The effect of different environmental factors like temperature, pH, cultivation media and carbon source on growth and artemisinin production were studied in shake flask cultures. Detailed batch growth and production kinetics with sugar consumption profile were also established. Maximum volumetric productivity of 390 µg L^{-1} day^{-1} was obtained in hairy root cultures.

Key words: *Agrobacterium*, *Artemisia annua* L., artemisinin, hairy root cultures.

INTRODUCTION

Half of the world's population is at risk of malaria, and an estimated 243 million cases led to 863,000 deaths in 2008 (WHO, 2009). *Artemisia annua* L. (Asteraceae), an important medicinal plant, contains an antimalarial compound called artemisinin, which is effective against both chloroquine-resistant and sensitive strains of *Plasmodium falciparum* (Davis et al., 2005). The low concentration (0.01 to 1% dry weight) of artemisinin in *A annua* L. is a serious problem in the commercialization of drug (Liu et al., 2006). At present, commercial artemisinin is extracted from wild type plants, which are subjected to seasonal and environmental limitation (Wang et al., 2004). According to WHO (2005), *A. annua* L. plants are currently the only source of the drug and even modest annual targets of 20 to 22 tons $year^{-1}$ of artemisinin are not being achieved. The commercial sources of most artemisinin are from field grown leaves and flowering tops of *A. annua*, which are subjected to seasonal and somatic variation and infestation of bacteria, fungi, and insects that can affect the functional medicinal content of this plant (Wallart et al., 2000). The physiological and cell culture studies were carried out to improve the yield of artemisinin, but were mostly unsatisfactory (Bhattacharya et al., 2008). The chemical synthesis of artemisinin is also possible, but it is complicated and economically unviable due to the poor yield (Abdin et al., 2003).

Recent reports have highlighted the use of biotechnological approaches such as metabolic engineering and genetic modification of microbes and plants as a feasible alternative for the semi-synthesis of artemisinin

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Abbreviations: ANOVA, Analysis of variance; **CCD,** central composite design; **CTAB,** cetyltrimethylammonium bromide; **DCW,** dry cell weight; **DW,** dry weight; **FW,** fresh weight; **HPLC,** High performance liquid chromatography; **MS,** Murashige and Skoog medium; **PCR,** polymerase chain reaction; **RSM,** Response surface Methodology; **YMB,** Yeast mannitol broth.

and its precursors (Weathers et al., 2005); *Saccharomyces cerevisiae* had been engineered to produce artemisinic acid (a precursor of artemisinin) at a significantly higher level than in *A. annua* (Ro et al., 2006). The complete synthesis of artemisinin outside the source plant is however, not achieved as yet and has to rely either on biotransformation using plant extract (Bharel et al., 1998; Chang et al., 2000) or semi-synthesis (Chang and Keasling, 2006) to obtain end product. 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. (Alam et al., 2010; Alam and Abdin, 2011; MaujiRam et al., 2010; Nafis et al., 2011).

The accumulation of artemisinin by plant tissue cultures *in vitro* occurs in calli, cell suspension, shoot and hairy root cultures (Weathers et al., 1994; Cai et al., 1995). Hairy root cultures of *A. annua* L. with rapid growth and high productivity of artemisinin, therefore, represent a potential source of commercial artemisinin (Weathers et al., 1994; Cai et al., 1995). Variation among hairy root lines developed from the same plant, but different plant parts due to differences in their biosynthetic capabilities is well reported for production of secondary metabolites (Kim et al., 2002). Selection of high yielding root line is one of the most important steps to develop a commercial process for production of secondary metabolites by hairy root cultures (Baldi et al., 2007). Therefore, enhancement of artemisinin production, through tissue culture of *A. annua* L., is the aim of many research groups.

In this paper, development and selection of high artemisinin producing hairy root cultures of *A. annua* is reported. A detailed comparison of transformation frequency of various strains of *Agrobacterium rhizogenes* with *A. annua* L. is given. The influence of different environmental parameters like temperature, pH, cultivation medium, media components and carbon sources are also studied on growth and artemisinin accumulation in these cultures.

MATERIALS AND METHODS

Plant material and culture conditions

The seeds of *Artemisia annua* L. were obtained from IPCA Laboratories Pvt. Ltd. Ratlam, M.P., India. Seeds were washed in 1% Savlon (Johnson and Johnson, USA), surface-sterilized using 70% ethanol treatment for 1 min, and rinsed thrice with sterile double distilled water. This was followed by treatment with 0.01% w/v mercuric chloride for 2 min and washed with sterile double distilled water for 4 to 5 times. For germination, sterilized seeds were then placed on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 3% sucrose and 1% agar at 25 ± 2°C in 16 h day^{-1} photoperiod provided by a light intensity of 16.2 µmol $m^{-2}s^{-1}$.

Initiation of cultures

The explants were taken from *in vitro* germinated plants. Different chemotypes of *A. annua* L. were tested for hairy roots initiation by *A. rhizogenes* mediated transformation. Explants from 20 to 25-day old *in vitro* germinated plants were used for culture initiation studies. Hairy root cultures were induced by infecting different explants (leaves, stems and petiole) with *A. rhizogenes* strains (LBA 9402, LBA 920, LBA 301, MTCC 532, NRRL B193, A4). MTCC-532 was obtained from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. NRRL B193 and A4 strains were obtained from Northern Regional Research Laboratory (NRRL), USA and American Type Culture Collection (ATCC), USA respectively. LBA 9402, LBA 920 and LBA 301 were from our personal collection. 20 explants were used for each transformation experiment. All explants were cultured on sterile Petri dishes comprising solid MS basal medium containing 3% sucrose. The pH was adjusted to 5.8 ± 0.2.

Co-cultivation and transformation

The explants were infected and co-cultivated with *A. rhizogenes* strain for induction of hairy roots. Bacterial colonies were cultured for 2 days on solid Yeast mannitol broth (YMB) at 25 ± 2°C. Two loopful bacteria were then allowed to grow in YMB for 24 h at 25 ± 2°C. Inoculum (2% v/v) of this culture was re-inoculated in YMB and grown till an $O.D_{600}$ of 1.0 was achieved. The suspension was centrifuged at 7,000 *g* for 10 min. The supernatant was discarded and the pellet was re-suspended in 5 ml of the fresh YMB. This concentrated culture was used for infection of plant materials. The explants were kept in disposable sterile Petri plates, pricked manually with 24 gauge metal needle (~5 wounds cm^{-2}), dipped in *A. rhizogenes* culture and incubated for 5 min.

YMB without bacteria applied to the explants served as control. The infected explants were pre-incubated for co-cultivation at 25 ± 2°C for 48 h on MS basal medium and were incubated at 25 ± 2°C in 16 h day^{-1} photoperiod regime. Hairy root cultures were then transferred to fresh MS medium containing 500 mg L^{-1} cephotaxime and 500 mg L^{-1} ampicillin (HiMedia, Mumbai, India) to check the overgrowth of bacteria. Axenic cultures were obtained by subsequent subculture to fresh MS medium containing the antibiotic agent for 6th day-interval. The hairy root cultures were observed for *Agrobacterium* contamination on YMB after each subculturing.

Factors affecting growth parameters and artemisinin content of transformed *A. annua* L. hairy root lines

Effect of different cultivation medium

Five commonly used standard plant cell cultivation media viz. MS, B5, White, Nitsch and Street (Gamborg et al., 1968; Bhojwani and Rajdan, 1996) were tested for their effect on growth and artemisinin production in hairy root cultures of *A. annua* L. The hairy root culture from selected root line was initiated by transferring fresh hairy roots equivalent to 3.0 g L^{-1} DW into 250 ml Erlenmeyer flask containing 50 ml of liquid B5 medium. The cultures were incubated on a gyratory shaker at 80 rpm and 25°C under 16 h day^{-1} photoperiod regime. The flasks, in triplicate, were harvested after 25 days to analyze DW and artemisinin content.

Use of carbon sources

To study the growth and production kinetics of *A. annua* L. hairy root cultures, 3 g L^{-1} hairy roots on DW basis were inoculated in 50 ml of Gamborg's B5 medium without phytohormone. The cultures were incubated at 80 rpm and 25°C under 16 h day^{-1} photoperiod regime. Individual flasks, in triplicate, were harvested at a regular interval of 5 day and analyzed for dry weight (DW), residual sugar,

Table 1. Initiation and selection of transformed (hairy) root cultures of *A. annua* L.

Explant	*A. rhizogenes* strain	Transformation Frequency (%)	Initiation time (day)	Artemisinin Content (mg g^{-1}DW)
Leaves	LBA 9402	100	5 to 6	0.66 ± 0.017
	NRRL B 193	40	8 to 9	0.3 ± 0.023
	A4	25	9 to 11	0.01 ± 0.002
Stem	LBA 9402	80	5 to 6	0.05 ± 0.003
	NRRL B 193	0	-	-
	A4	10	9 to 11	0.09 ± 0.004

pH, conductivity and artemisinin content.

Measurement of dry cell weight and residual sugar

To determine DW, the flasks, in triplicate were harvested. Hairy roots were collected, blotted on a filter paper and dried at 40 ± 2°C until constant weight was achieved. Residual carbon source, glucose and sucrose, were estimated in spent medium by dinitrosalicylic acid method Miller (1959) and phenol-sulphuric acid method (Dubois et al., 1956), respectively.

Effect of temperature and pH

A 2^2 factorial Central Composite Design with four axial points, four quadrant points and five replicates at the centre point was used to carry out a total of 13 treatments for pH and temperature optimization. The experimental design protocol for RSM was developed using Design-Expert software (version 5.0.9) (Stat-Ease Corporation, USA). The Erlenmeyer flasks were harvested after 25 days for biomass (DCW) (g L^{-1}) and artemisinin content (mg L^{-1}) analysis.

Media optimization studies using statistical designs

Plackett–Burman design was used to study the influence of major nutrients on growth and artemisinin production in suspension culture of hairy roots. Five variables (sucrose, potassium nitrate, potassium dihydrogen phosphate, magnesium sulphate and calcium chloride) were selected. Trial experimental protocols were formulated using the Design-Expert version 5.0.9 software (Stat-Ease Corporation, USA). Each independent variable was tested at two levels; a high (+) and a low (-) level of concentration.

Selection of high yielding hairy root lines

Hairy root cultures of *A. annua* L. were subjected for determination of growth index and artemisinin content.

Determination of growth index (G.I)

Hairy roots of *A. annua* L. were dried at 40 ± 2°C until a constant weight was obtained. The final weight of the roots obtained in this way was considered as dry cell weight (DW). Fresh weight: dry weight ratios (FW: DW) were established for hairy root cultures by allowing a definite weight of roots to dry. Initial DW was calculated from FW: DW ratio by transferring a known amount of roots to respective maintenance medium. Growth indices of different root lines were then calculated by the following formula: (Final DW-Initial DW) / Initial DW.

PCR amplification of transformed hairy root lines

The genomic DNA was isolated from transformed *A. annua* L. hairy root line Al 8 and wild type root of *A. annua* L. by CTAB method (Doyle and Doyle, 1990). Integration of the TL-DNA (*rol* gene) region of the pRi plasmid was confirmed by PCR analysis in transformed lines of *A. annua* L. The specific primers used to amplify *rol* gene were: forward primer, 5′-CAGAATGG-AATTAGCCGGACTA-3′ and the reverse primer, 5′-CGTATTAATCCCGTAGGTTTGTTT-3′. The PCR conditions for amplification of *rol* gene consisted of denaturation at 94°C for 1 min, 2 min annealing at 55°C followed by a 3 min extension at 72°C and a final extension at 72°C for 7 min. The amplicons were analyzed by agarose gel electrophoresis. For the positive control, plasmid from *A. rhizogenes* strain LBA 9402 was used as template during the PCR.

Extraction and quantitative analysis of artemisinin

To analyze the contents of artemisinin, hairy roots were collected and air dried thoroughly. These samples were weighed and stored. 1 g dried sample was powdered and extracted with hexane (10 mL × 3) at 40°C, with the change of hexane after every 24 h. The hexane fractions were pooled and concentrated under reduced pressure. The residue obtained was defatted with acetonitrile. The precipitated fat was filtered out and the filtrate was concentrated under reduced pressure. The residue thus obtained was dissolved in 2 ml methanol (MeOH). Afterward, 20 µL aliquot from the solution was then used for making Q_{260} to determine artemisinin through HPLC (Zhao and Zeng, 1986).

RESULTS AND DISCUSSION

Establishment of hairy root cultures

Various factors like type of explants (Dupre et al., 2000), *A. rhizogenes* strains (Giri et al., 2001; Tiwari et al., 2007), phenolic compounds (Kumar et al., 2006), growth medium (Azlan et al., 2002), bacterial concentration (John et al., 2009), growth hormones (Falasca et al., 2000) and pH (Danesh et al., 2006) showed great influence on root induction. All these factors were optimized for hairy root induction of *A. annua* L. (Table 1).

Table 2. High artemisinin yielding cell line selection of *A. annua* L.

S/N	Root line	Specific growth rate (d^{-1})	Artemisinin content (mg g^{-1})
1	A18	0.07 ± 0.001	0.66 ± 0.017
2	Ap21	0.0005 ± 0.00001	0.02 ± 0.001
3	As101	0.0005 ± 0.00001	0.07 ± 0.003
4	A174	0.003 ± 0.0001	0.01 ± 0.002
5	As116	0.001 ± 0.0001	0.09 ± 0.003
6	Ap21	0.002 ± 0.0001	0.11 ± 0.004
7	Ap45	0.001 ± 0.0001	0.15 ± 0.002
8	As20	0.0001 ± 0.00001	0.25 ±0.003
9	Ap39	0.001 ± 0.0001	0.4 ± 0.029

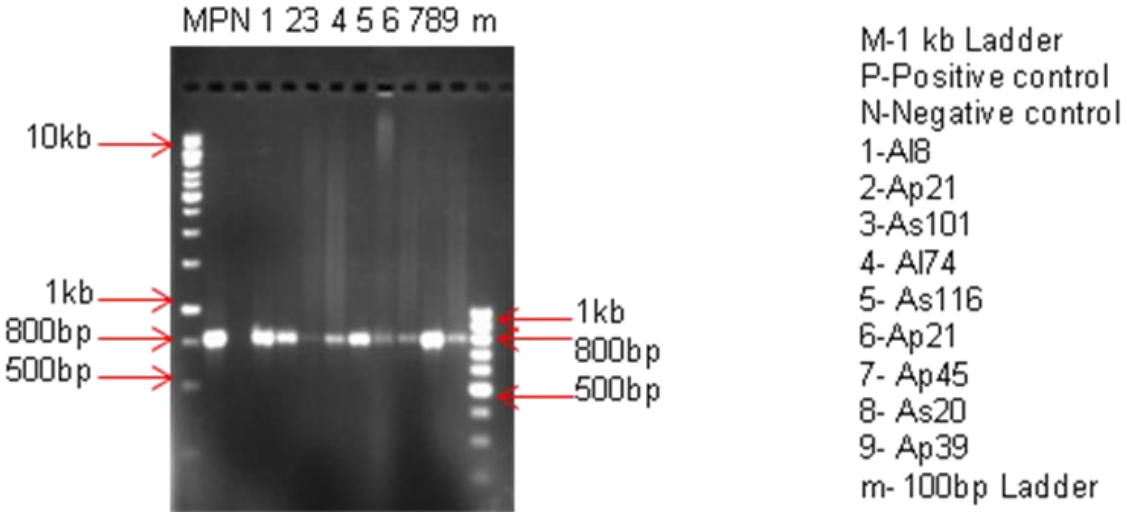

Figure 1. PCR analysis showing the presence or the absence of *rol* gene in selected hairy root lines of *A. annua* L.

A. rhizogenes infectivity was determined in terms of percentage efficiency to induce hairy roots from the explants. The time of exposure of *A. rhizogenes* and co-cultivation period also played an important role in induction of hairy roots.

Infection time of 7 min and co-cultivation period of 48 h were found to be optimum for induction of transformed root cultures. Out of all the six strains of *A. rhizogenes* tested, LBA 301, LBA 920 and MTCC 532 strains were unable to induce hairy roots from any explant tested, while NRRL B 193 strain failed to induce hairy roots from stem explants. The leaves were found to be easier to transform in comparison to stems for NRRL B 193 strain. This may be due to their higher regeneration capacity and better wounding surface as compared to stems. Higher transformation efficiency of leaves was observed with NRRL B193 strain (40%), while stem portions were better transformed with LBA 9402 strain (80%). 100% transformation frequency was observed with LBA 9402 strain in leaves explants. Induction of hairy roots was also faster (5 to 6 days) with LBA 9402 strain irrespective of the explants tested in comparison to NRRL B193 and A4 strains (9 to 11 days). A4 strain exhibited the lowest infectivity of 10 and 25% for stem and leaves explants, respectively. These root lines were tested for artemisinin content (Table 2). Transformed root cultures developed by infection of leaves of *in vitro* germinated *A. annua* L. with LBA 9402 strain was selected as high artemisinin yielding root line (0.66 mg g^{-1} DW).

Molecular validation of T-DNA in transformed hairy roots lines of *A. annua* L.

In agropine Ri plasmids, the T-DNA region consists of two parts; TL-DNA and TR-DNA. These parts are separated by a non-transferable DNA sequence of approximately18 Kb (Paolis et al., 1985). Integration of TL-DNA to plant genome was confirmed by the presence of *rol* gene by PCR analysis as shown in Figure 1. Presence of *rol* gene sequence in the genomic DNA of transformed roots confirmed transformation by its

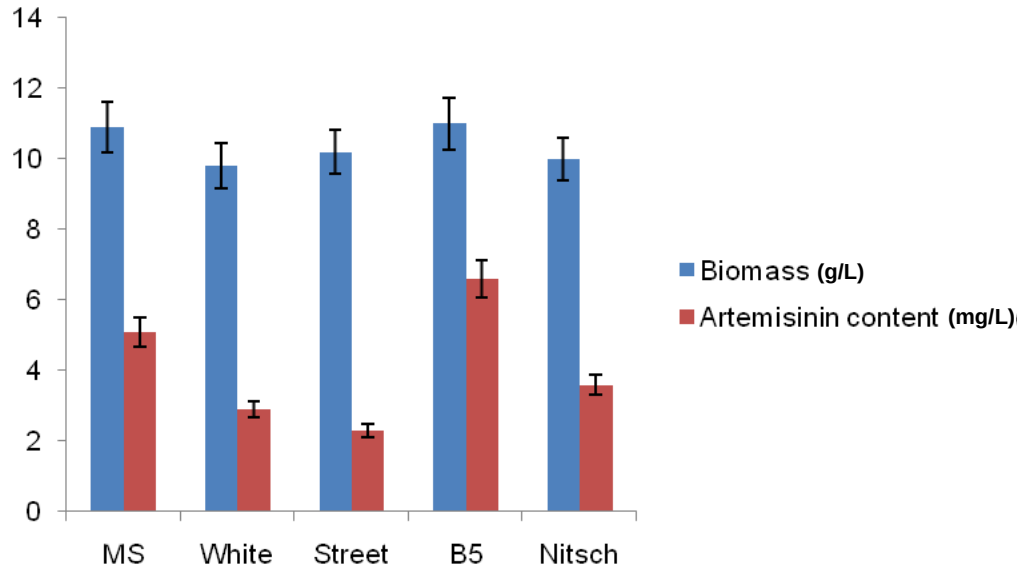

Figure 2. The effect of different culture medium on growth and artemisinin production by hairy root cultures of *A. annua* L. Al8: hairy root line derived from infection of leaf with *A. rhizogenes* strain LBA 9402.

integration in plant genome. No *rol* gene activity was found in normal roots.

Effect of different culture media

Growth of plant cells and production of secondary metabolites in cell cultures depend on concentration and interaction of nutrients present in cultivation medium (Mairet et al., 2009). Hairy roots of *A. annua* L. were cultivated in well-known plant cell cultivation media and their effect on growth and artemisinin production is given in Figure 2. Among the hairy root lines screened, Al8 root line was the fastest growing and Al43 root line was the slowest growing irrespective to the media tested. All root lines when cultivated in B5 medium attained relatively higher biomass in comparison to other media tested. The highest biomass of 11 g L^{-1} on DW basis was obtained when Al8 root line was cultivated in this medium. MS, White, Nitsch and Street media were almost similar to each other with respect to biomass accumulation. In the case of hairy roots, both growth and artemisinin production were lower in MS medium (containing 30 g L^{-1} sucrose). So, Gamborg's B5medium (containing 30 g L^{-1} sucrose) was selected for cultivation of *A. annua* L. hairy roots as maximum artemisinin production of 6.6 mg L^{-1} was found in this medium.

Effect of carbon sources and establishment of growth kinetics

Growth and artemisinin production kinetics along with substrate consumption, pH and conductivity profiles were investigated for hairy root cultures of *A. annua* L. in shake flasks over a period of 35 days (Figure 3). Sucrose was selected as the carbon source in comparison to glucose because of higher biomass and artemisinin concentration achieved for the hairy root culture. Similar results were reported when hairy roots of *Plumbago zeylanica* were grown for plumbagin production (Verma et al., 2002; Sivanesan and Jeong, 2009). Higher biomass of 11.0 g L^{-1} and artemisinin accumulation of 6.6 mg L^{-1} were obtained in 25 days of cultivation with sucrose as carbon source in B5 medium (Figure 4). Predominantly growth associated product formation behavior was observed in this study. When hairy roots were cultivated in glucose, a faster uptake and thus lesser cultivation period of 20 days was observed due to limitation of glucose in the medium but both growth and artemisinin production were lowered in this case (Figure 4). Higher volumetric productivity of artemisinin was obtained with sucrose 264 µg L^{-1} day^{-1} in comparison to glucose (210 µg L^{-1} day^{-1}). Similar trends for conductivity and pH were observed for hairy root cultures of *A. annua* L. cultivation in shake flasks.

The substrate consumption, conductivity and pH profiles during batch cultivation of hairy root cultures are given in Figure 3. Initial decrease in conductivity corresponded with uptake of nutrients from the medium and increase after the 30th day for hairy roots indicated death phase and thus release of intracellular compounds in the medium. A linear relationship between biomass and conductivity of the medium was observed in batch kinetics of hairy roots, which can lead to indirect method of biomass estimation during biomass cultivation. A slow but almost continuous increase in pH was observed throughout the

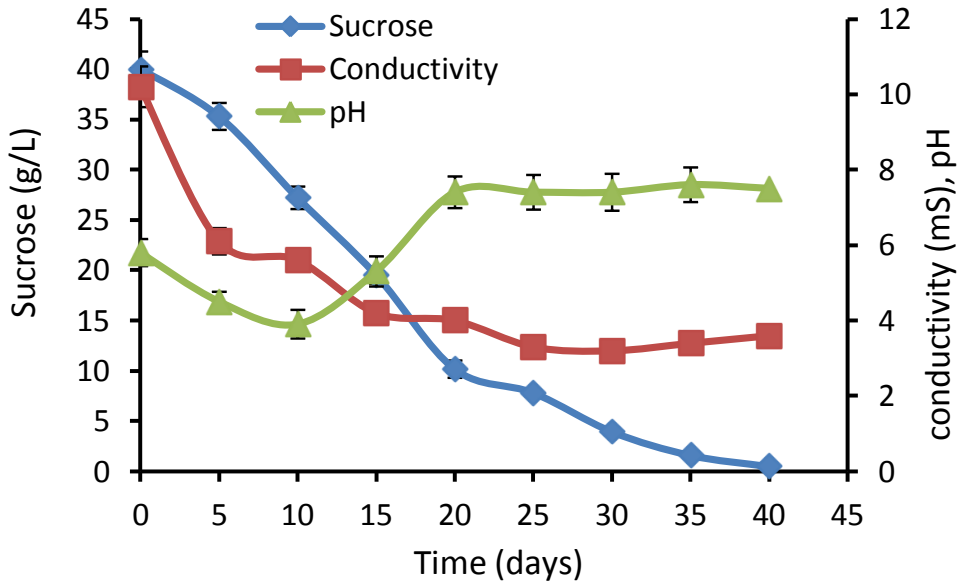

Figure 3. Substrate consumption, conductivity and pH profiles during batch cultivation of transformed root culture of *A. annua* L.

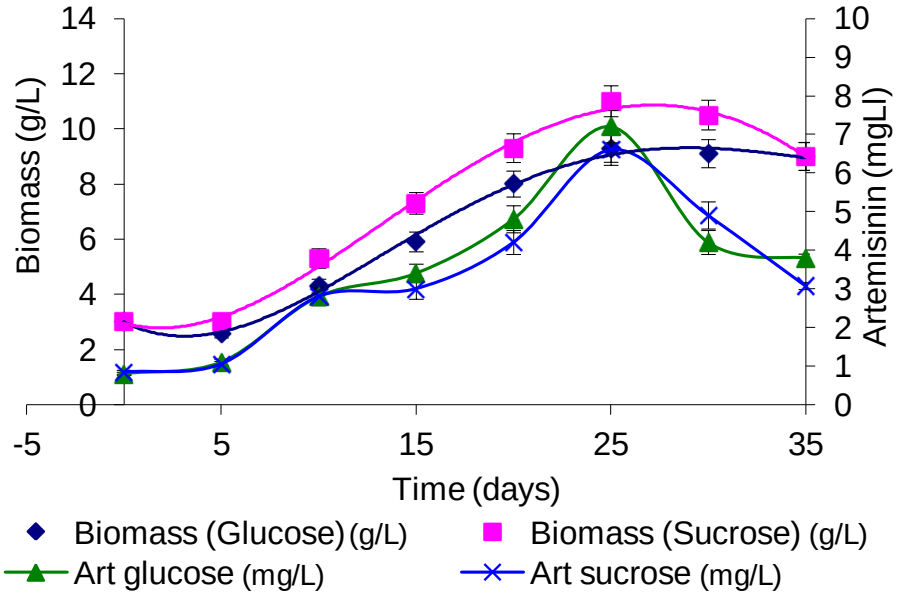

Figure 4. Growth and artemisinin production by transformed roots of *A. annua* L. on different carbon sources.

cultivation period due to change in medium composition. These results are in agreement with earlier report on *L. album* suspension cultures Seidel (2002).

Effect of temperature and pH

Each of the experimental recipes of the CCD design was carried out and responses were fed in the design with respect to biomass and artemisinin content. Data analysis was done by the software and mathematical models were proposed with respect to pH and temperature for growth and artemisinin production. Under optimized conditions of pH (5.7) and temperature (26°C), the model predicted a biomass of 11.05 g L^{-1} and artemisinin content of 6.8 mg L^{-1}, which was experimentally verified

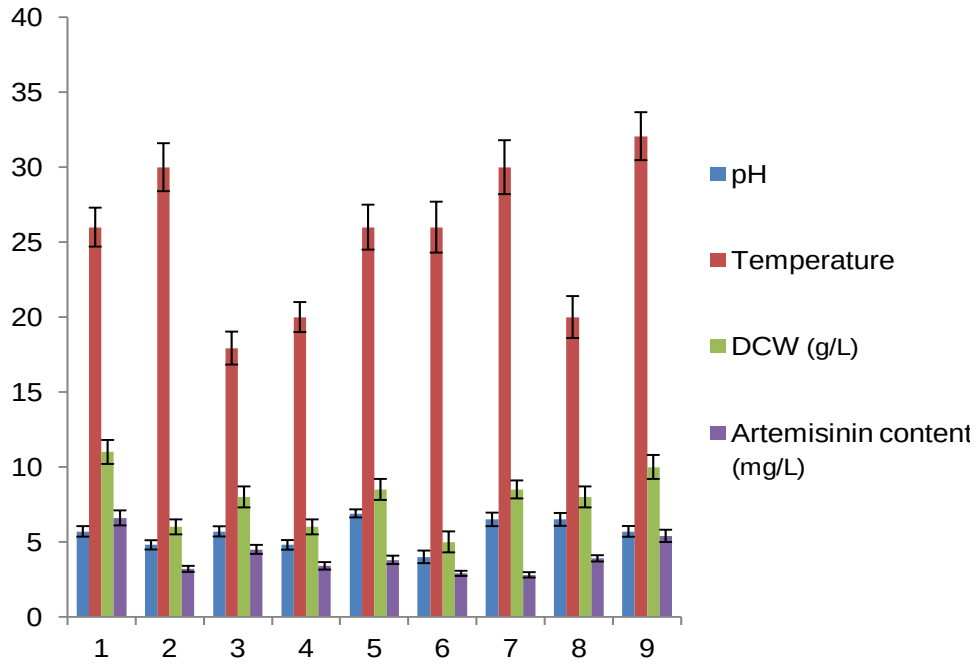

Figure 5. The effect of different pH and temperature on growth and artemisinin production by hairy root cultures of *A. annua*.

(as 11.0 g L^{-1} and 6.6 mg L^{-1}) (Figure 5).

Optimization of media components

To select the significant factors for growth and artemisinin production, data analysis was done by the software, and regression coefficients (or t-values) for each of the selected parameters were calculated by performing ANOVA test. On the basis of values of t-coefficients, it was concluded that sucrose, potassium nitrate, potassium dihydrogen phosphate and magnesium sulphate had a positive effect on growth, while sucrose, potassium nitrate and potassium dihydrogen phosphate had positive effect on artemisinin production. The remaining parameter (calcium chloride) was not significant for either of the two responses in the given concentration range. Hence, those factors which were not significant were taken at an average concentration level in the optimized medium, while concentrations of the significant factors obtained were optimized using Response Surface Methodology. The responses in the form of biomass and artemisinin content for each of the experimental recipe were fed in the design.

A polynomial mathamatical model incorporating the different interactions of low and high concentrations of nutrients in the design was proposed for growth (DCW) and artemisinin production. Optimum values of the medium nutrients (sucrose, 40g L^{-1}; potassium dihydrogen 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}) for the production of maximum biomass and artemisinin content were obtained from the developed contour plots. The model predicted a biomass of 14.5 g L^{-1} and artemisinin content of 8.0 mg L^{-1}, which was experimentaly verified (14.2 g L^{-1} and 7.8 mg L^{-1}, respectively).

Conclusions

This study emphasises on the development and selection of high yielding hairy root lines of *A. annua* L. with special attention to artemisinin production. Genetic confirmation of transformed culture indicate that hairy root cultures have partial integration of Ri-T DNA. Different media compositions and carbon sources have shown great influence on growth and production kinetics. The highest dry cell weight and artemisinin accumulation was observed with sucrose as a carbon source in Gamborg's B5 medium. Maximum volumetric productivity of artemisinin was obtained as 390 µg L^{-1} d^{-1} in hairy root cultures. In order to develop a process for commercial production of artemisinin by hairy root cultures, application of yield enhancement strategies like addition of elictors, precursors and permeability agents are in progress.

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REFERENCES

Abdin MZ, Israr M, Rehman RU, Jain SK (2003). Artemisinin, a novel antimalarial drug: biochemical and molecular approaches for enhanced production. Planta Med. 69: 289-293.

Alam P, Kiran U, Ahmad MM, Kamaluddin, Khan MA, Jhanwar S, Abdin MZ (2010). Isolation, characterization and structural features of amorpha-4,11-diene synthase (ADS3963) from *Artemisia annua* L. Bioinformation, 4: 421-429.

Alam P, Abdin MZ (2011). Over-expression of HMG-CoA reductase and amorpha-4,11-diene synthase genes in *Artemisia annua* L. and its influence on artemisinin content. Plant Cell Rep. 30: 1919-1928.

Azlan GJ, Marziah M, Radzali M, Johari R (2002). Establishment of *Physalis minima* hairy roots culture for the production of Physalins. Plant Cell Tissue Org. Cult. 69: 271-278.

Baldi A, Bisaria VS, Srivastava AK (2007). Biotechnological approaches for production of some plant based chemotherapeutics. In: Kayser O, Quax WJ (ed) Medicinal Plant Biotechnol. - From basic research to industrial applications, Wiley-VCH Verlag, Weinheim, Germany, pp. 117-156.

Bharel S, Gulati A, Abdin MZ, Srivastava PS, Vishwakarma RA, Jain SK (1998). Enzymatic synthesis of artemisinin from natural and synthetic precursors. J. Nat. Prod. 61: 633-636.

Bhattacharya A, Mishra LC, Bhasin VK (2008). *In vitro* activity of artemisinin in combination with clotrimazole or heat-treated amphotericin B against *Plasmodium falciparum*. Am. J. Trop. Med. Hyg. 78: 721-728.

Bhojwani SS, Razdan MK (1996). Plant Tissue Culture: Theory and Practice. Elsevier Publishers: 502, Amsterdam.

Cai GQ, Li GZ, Ye HC, Li GF (1995). Hairy root culture of *Artemisia annua* L. by plasmid transformation and biosynthesis of artemisinin. Chin. J. Biotechnol. 11: 315-320.

Chang MC, Keasling JD (2006). Production of isoprenoid pharmaceuticals by engineered microbes. Nat. Chem. Biol. 2:674-681.

Chang YJ, Song SH, Park SH, Kim SU (2000). Amorpha-4,11-diene synthase of *Artemisia annua*: cDNA isolation and bacterial expression of a terpene synthase involved in artemisinin biosynthesis. Arch. Biochem. Biophys. 383: 178-184.

Danesh YR, Mohammadi GE, Alizadeh A, Modarres SM (2006). Optimizing carrot hairy root production for monoxenic culture of arbuscular mycorrhizal fungi in Iran. J. Biol. Sci. 6: 87-91.

Davis TM, Karunajeewa HA, Ilett KF (2005). Artemisinin-based combination therapies for uncomplicated malaria. Med. J. Aust. 182: 181-185.

Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. Focus, 12: 13-15.

Dubois M, Gilles KA, Hamilton JK, Roberts PA (1956). Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350-356.

Dupre P, Lacoux J, Neutelings G, Mattar LD, Fliniaux MA, David A, Jacquin A (2000). Genetic transformation of *Ginkgo biloba* by *A.tumefaciens*. Physiol. Plant. 108: 413-419.

Falasca G, Reverberi M, Lauri P, Caboni E, Stradis A, Altamura MM (2000). How *Agrobacterium rhizogenes* triggers de novo root formation in a recalcitrant woody plant: an integrated histological, ultra structural and molecular analysis. New Phytol. 145: 77-93.

Gamborg OL, Miller RA, Ojima K (1968). Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell. Res. 50: 151-158.

Giri A, Ravindra ST, Dhingra V, Narasu ML (2001). Influence of different strains of *Agrobacterium rhizogenes* on induction of hairy roots and artemisinin production in *Artemisia annua*. Curr. Sci. 81: 378-382.

John KMM, Joshi SD, Mandal AKA, Kumar SR, Kumar RR (2009). *Agrobacterium rhizogenes*- mediated hairy root production in tea leaves [*Camellia sinensis* (L.) O. Kuntze}. Indian J. Biotechnol. 8: 430-434.

Kim Y, Wyslouzil BE, Weathers PJ (2002). Secondary metabolism of hairy root cultures in bioreactors. *In Vitro* Cell. Dev. Biol. Plant. 38: 1-10.

Kumar V, Sharma A, Prasad NCB, Gururaj BH, Ravishankar AG (2006). *Agrobacterium rhizogenes* mediated genetic transformation resulting in hairy root formation is enhanced by ultrasonication and acetosyringone treatment. Electr. J. Biotechnol. 9: 349-357.

Liu C, Zhao Y, Wang Y (2006). Artemisinin: current state and perspectives for biotechnological production of an antimalarial drug. Appl. Microbiol. Biotechnol. 72: 11-20.

Mairet F, Sierra J, Glorian V, Villon P, Shakourzadeh K, Boitel CM (2009). A new approach to define optimized range of medium composition for enhancement of hairy root production in fed-batch process. Biotechnol. Bioproc. Engineer. 32: 257-265.

MaujiRam, Khan MA, Jha P, Khan S, Kiran U, Ahmad MM, Javed S, Abdin MZ (2010). HMG-CoA reductase limits artemisinin biosynthesis and accumulation in *Artemisia annua* L. plants. Acta Physiol. Plant. 32: 859-866.

Miller GM (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31: 426-428.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Nafis T, Akmal M, MaujiRam, Alam P, Ahlawat S, Mohammad A, Abdin MZ (2011). Enhancement of artemisinin content by constitutive expression of HMG CoA reductase gene in high yielding strain of *Artemisia annua* L. Plant Biotechnol. Rep. 5: 53-60.

Paolis DA, Mauro ML, Pomponi M, Cardarelli M (1985). Localization of agropine-synthesizing functions in the TR region of the root-inducing plasmid of *Agrobacterium rhizogenes*, 1855. Plasmid, 13: 1-7.

Ro DK, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho KA, Eachus RA, Ham TS, Kirby J, Chang MC, Withers ST, Shiba Y, Sarpong R, Keasling JD (2006). Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature, 440: 940-943.

Sivanesan I, Jeong BR (2009). Induction and establishment of adventitious and hairy root cultures of *Plumbago zeylanica* L. Afr. J. Biotechnol. 8: 5294-5300.

Seidel V, Windhovel J, Eaton G, Alfermann AW (2002). Biosynthesis of podophyllotoxin in *Linum album* cell cultures. Planta, 215: 1031-1039.

Tiwari RK, Trivedi M, Guang ZC, Guo GQ, Zheng GC (2007). Genetic transformation of *Gentiana macrophylla* with *Agrobacterium rhizogenes*: growth and production of secoiridoid glucoside gentiopicroside in transformed hairy root cultures. Plant Cell Rep. 26: 199-210.

Verma PC, Singh D, Rahman L, Gupta MM, Banerjee S (2002). *In vitro* studies in *Plumbago zeylanica*: rapid micropropagation and establishment of higher plumbagin yielding hairy root cultures. J. Plant Physiol. 159: 547-552.

Wallaart TE, Pras N, Beekman AC, Quax WJ (2000). Seasonal variation of artemisinin and its biosynthetic precursors in plants of *Artemisia annua* of different geographical origin: proof for the existence of chemotypes. Planta Med. 66: 57-62.

Wang H, Ge L, Ye HC, Chong K, Lie BY, Li GF (2004). Studies on the effects of fpf1 gene on *Artemisia annua* flowering time and on the linkage between flowering and artemisinin biosynthesis. Planta Med. 70: 347-352.

Weathers PJ, Cheetham RD, Follansbee E, Teoh H (1994). Artemisinin production by transformed roots of *Artemisia annua*. Biotechnol. Letts. 16: 1281-1286.

Weathers PJ, Bunk G, McCoy MC (2005). The effect of phytohormones on growth and artemisinin production in *Artemisia annua* hairy roots. *In Vitro* Cell Dev. Biol. Plant. 41: 47-53.

WHO, UNDP/World Bank/WHO (2005). Annual report, Ministry of Health, Family Welfare, Govt. of India.

WHO (2009). World malaria report. Geneva. WHO Library Cataloguing-in-Publication Data.

Zhao SS, Zeng MY (1986). Determination of Qinghaosu in *Artemisia annua* L. by high performance liquid chromatography. Chin. J. Pharm. Anal. 6: 3-5.