

Agrobacterium rhizogenes-mediated hairy root production in tea leaves [*Camellia sinensis* (L.) O. Kuntze]

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Leaves of tea [*Camellia sinensis* (L.) O. Kuntze] plants were transformed with *Agrobacterium rhizogenes* strain (MTCC 532) and hairy roots were induced. Among the different concentrations of acetosyringone tested, 300 μ M/L was found to enhance the transformation frequency upto 70%. Murashige and Skoog (MS) medium supplemented with 30 g/L maltose and indole-3-acetic acid (IAA) at 5 mg/L was found suitable for hairy-root culture and accumulation of phenolic compounds. Confirmatory studies were carried out by PCR analysis using *rol C* gene primer for transformation. Amplification of the specific gene was noted in the transformant at 540 bp. High performance liquid chromatography (HPLC) analysis confirmed the higher levels of catechins and their fractions in roots arising from the transformed tissues. Catechin fractions, viz., EC, ECG, EGC and EGCG were detected both in untransformed leaves and hairy roots produced by *Agrobacterium* infected cells.

Keywords: *Agrobacterium rhizogenes*, *Camellia sinensis*, catechin, hairy root, *rol C* gene, secondary metabolites

Introduction

Tea [*Camellia sinensis* (L.) O. Kuntze] is an economically important plant grown in more than 30 countries. The young shoots of the plant produce important secondary metabolites like polyphenols, catechins and caffeine. The catechins and their fractions are important biomolecules having therapeutic value. The quantity of the catechins produced in tea leaves is dependent upon genotype and environment¹. In many plants, suspension culture, hairy root culture or callus culture were extensively used to produce important secondary metabolites^{2,3}. *Agrobacterium rhizogenes* infected hairy root cultures are fast growing and produce valuable secondary metabolites in large quantity⁴. Further, secondary metabolite synthesis is not strictly limited to those, which are normally produced in the roots of differentiated plants. Previously *A. rhizogenes* wild type strains 15834 (ATCC) and 1855 (NCP) were used to induce hairy roots in *Glycyrrhiza glabra*, *Potentilla alba*, *Ophiorrhiza pumila*^{5,6}. Valuable anti-cancer alkaloids, vinblastine and vincristine, as well

as anti-hypertension agents, ajmalicine and serpentine, were induced in hairy root cultures of *Catharanthus roseus*⁷. Only a few attempts have been made in tea to induce hairy roots using cotyledonary callus and nodal explants⁸. So far no attempt has been made to induce hairy roots using leaf explants of tea. In the present study, authors used *in vitro* tea leaf as explants for the induction of hairy roots and quantified catechin biosynthesis in hairy roots.

Materials and Methods

Explant Preparation

Tea shoots of the clone UPASI-9 were collected from Tea Experimental Farm of UPASI (United Planters Association of Southern India). Leaves were removed from the shoot at their base and 2 cm long segments were made; each containing one node. The nodal segments were washed under running tap water for ½ h and then disinfected with 70% (v/v) ethanol for 1 min and surface sterilized with 0.1% (w/v) HgCl₂ for 3 min, followed by several rinses in sterile distilled water. The surface sterilized explants were cultured on Murashige and Skoog (MS) basal media⁹ supplemented with 3% sucrose, 3 mg/L 6-benzyl adenine (BA) and 0.8% agar. After sprouting, the cultures were transferred to multiplication medium with BA at 5 mg/L concentration and two subcultures

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Abbreviations: EC-epicatechin, ECG-epicatechin gallate, EGC-epigallo catechin, EGCG-epigallo catechin gallate

at 30 d interval were made in the same media. The pH of all the media were adjusted to 5.6 before autoclaving at 121°C for 15 min. Cultures were incubated at 25±1°C under fluorescent light with a 16 h photoperiod (36 µmol m⁻² s⁻¹). After 2 months, the fully exposed leaves were separated from the new shoots grown *in vitro* and used as explants for transformation.

Bacterial Culture Preparation

A. rhizogenes strain MTCC 532 (Microbial Type Culture Collection, IMTECH, Chandigarh), stored in sterile glycerol at -70°C, was cultured on YEB (yeast-extract broth; composition: 1 g/L yeast extract, 5 g/L beef extract, 5 g/L peptone & 0.5 g/L MgSO₄) solid medium for activation of the strain and sub cultured 3 times on the same medium. The bacteria were then transferred into liquid YEB medium and cultured at 25°C for 16 h on an orbital shaker (250 rpm). Bacteria at exponential growth phase were used for transformation studies. The bacterial suspension was transferred to a sterile centrifuge tube and centrifuged at 5000 rpm for 10 min. The biomass of bacteria was suspended in liquid MS media supplemented with 3% sucrose (the OD of bacterial suspensions were between 0.4-0.6 at 600 nm). This suspension was used for the transformation of leaf explants.

Establishment of 'Hairy Root' Culture

For *A. rhizogenes* mediated transformation, 3 to 4 oblique incisions were made on both sides of the midrib of leaf explants and co cultivated with 25 mL *A. rhizogenes* in liquid MS medium in 100 mL conical flask on a shaker (200 rpm) under dark. After 3 h of incubation, the leaf explants were blotted on sterile tissue paper and co-cultivated with adaxial side down on MS basal media containing different concentrations of acetosyringone (100, 200, 300, 400 and 500 µM/L) and solidified with 0.8% bacto agar. Co-cultivation was carried out for 48 h at 25±1°C in the dark. Explants were washed with 50 mL of liquid MS basal media containing 30 g/L sucrose and 250 mg/L cefotaxime (Sigma Chemical Company, USA) and then cultivated with adaxial side down on the same washing medium solidified with 0.8% agar. After 2 subcultures at 2 d interval on the same media, the explants were finally transferred to MS media containing different carbon sources like sucrose, dextrose, maltose and fructose at different concentrations (Table 2), and plant growth regulators

(indole-3 acetic acid & 2,4-dichlorophenoxy acetic acid, Table 3). All the cultures were maintained in the conditions described earlier. Callus developed from the transformed leaf explants within 20 d.

PCR Conformation Study

Plasmid DNA from *A. rhizogenes* strain and DNA from transformed leaf (after 15 d of infection), callus (20 d after infection) and hairy roots were used for PCR amplification. Plasmid DNA was extracted using GeneElute™ HP Plasmid miniprep Kit (Sigma Chemicals Co., USA) and plant DNA was extracted following the method documented by Mariya John¹⁰. Polymerase chain reaction was carried out using *rol C* gene specific primers following the procedure given elsewhere¹¹. For this 50 ng plasmid DNA and DNA from non-transformed leaf tissues were taken as positive and negative controls, respectively. DNA from transformed leaves and callus as well as hairy roots was served as treatments. Each 25 µL reaction mixture contained 1× PCR buffer, 3.5 mM MgCl₂, 25 pmol of each forward (5'-ATGGCTGAAGACGACCTGTT-3') and reverse (5'-TTAGCCGATTGAAAACCTT GCAC-3') primers with 0.2 mM dNTPs and 1 U of *Taq* DNA polymerase (Bangalore Genei Private Ltd. Bangalore, India). Amplification cycle included initial denaturation for 4 min at 94°C, followed by 30 cycles of 45 sec denaturation at 94°C, annealing for 60 sec at 55°C, extension at 72°C for 120 sec and 10 min final extension at 72°C in a programmable peltier thermal cycler (PTC-200, M J Research, USA). After running the samples on 1.2% agarose gel, it was stained with ethidium bromide. Amplified products were visualized under UV light and documented. Calculation of size of the amplified fragment was carried out using software (Total Lab ver.1, Amersham Bioscience, USA).

Quantification of Polyphenol and Catechins

Total polyphenol was estimated by using the procedure of Dev Choudhary and Goswami¹². The individual catechin fractions were analyzed in HPLC (Hewlett Packard series 1100, USA) fitted with phenomenex column according to ISO method¹³ with minor modification. HPLC standards of individual catechins, procured from Sigma Chemical Company, USA were standards for spiking test. Relative distribution of these constituents was expressed in percentage of individual component (w/w) according to ISO method¹³.

Results and Discussion

In the present study, different concentrations of acetosyringone were incorporated into the nutrient medium and the influence on transformation was observed. Among the different (100-500 $\mu\text{M/L}$) concentrations tried, acetosyringone at 300 $\mu\text{M/L}$ enhanced the transformation frequency significantly, which declined both on lower (<300 $\mu\text{M/L}$) or higher concentrations (Table 1). Quadratic relation between acetosyringone concentration and the transformation frequency was significant at 0.5% level (Fig. 1). Acetosyringone is an amino acid derivative which served as a nutrient source for the invading bacterium and enhanced the transformation rate¹⁴. The infecting *Agrobacterium* used this as a nutrient source. Bolton *et al*¹⁵ reported that acetosyringone would induce the *vir* gene of *Agrobacterium* cultures. PCR analysis of the transformed tissues showed amplification at 540 bp in leaf, callus and hairy root samples and in

Table 1—Efficacy of acetosyringone concentration on transformation efficiency

Acetosyringone ($\mu\text{M/L}$)	Total no. of cultures	No. of cultures inducing root	Transformation frequency (%)
Control	20	02	10
100	20	06	30
200	20	08	40
300	20	14	70
400	20	09	45
500	20	08	40
SE \pm		01.29	
CD at $p = 0.05$		03.16	
CV (%)		16.48	

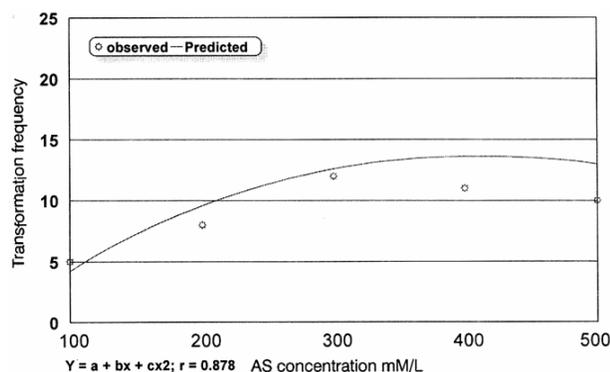


Fig. 1—Quadratic relation between acetosyringone concentration and transformation frequency

positive control (plasmid), indicating the transformation event. But in negative control (non-transformed tissues) there was no amplification (Fig. 2). This confirmed that all hairy roots have developed from transformed callus. Kovalenko & Maliuta¹⁶ also achieved transformation through PCR amplification of *rol C* gene.

Among the different sources of organic carbon tested, maltose at 3% level induced highest percentage of hairy root cultures, followed by dextrose, sucrose and fructose (Table 2). Explants cultured on media supplemented with fructose showed lowest percentage of hairy root cultures. In the absence of carbon source, callus remained recalcitrant and eventually turned brown and dried. Present study confirmed that maltose is a better carbon source for induction of hairy roots, as has also been observed earlier by Giri *et al*¹⁷.

Plant growth regulators play an important role in the induction of hairy roots from cultured explants of

Table 2—Effect of carbon sources and its concentrations on hairy root induction (%)

Carbon source (CS)	Per cent concentration (C)		
	1.0	2.0	3.0
Maltose	05.05	32.30	11.85
Dextrose	06.35	12.65	05.90
Sucrose	02.40	11.05	07.95
Fructose	01.50	04.65	02.10
Statistical significance	SE \pm	CD at $p = 0.05$	CV (%)
Between carbon source (CS)	0.087	0.19	1.4
Between concentrations (C)	0.076	0.16	
Interaction (CS \times C)	0.15	0.33	

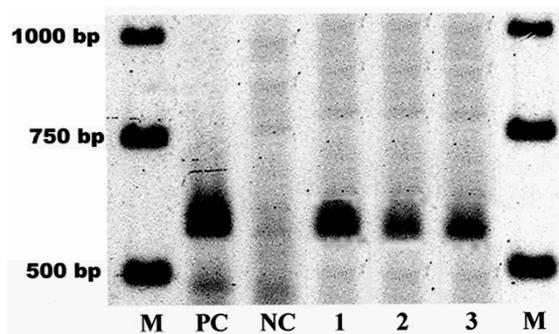


Fig. 2—PCR amplification of DNA extracted from different tissues using *rol C* gene specific primer: M, 1 kb DNA ladder (Fermentas); PC, Positive control (Plasmid DNA); NC, Negative control (DNA from untransformed tissue); Lanes 1-3, DNA from transformed leaf, callus and hairy root samples, respectively.

tea. In general, auxins have the capability of inducing roots. Time required for root induction in transformed callus was 15 and 20 d in IAA and 2,4-D containing medium, respectively; while in case of untransformed callus, it was 31 d for both the media. In the absence of auxin, 35 d was required for root induction in transformed callus and 60 d for untransformed callus. Therefore, IAA containing medium was found to be ideal for the rapid induction of hairy roots followed by 2,4-D (Table 3). Combination of both the hormones showed no significant reduction in hairy root induction-time. Results obtained in the present study are similar to the observation of Kovalenko¹⁸. Root induction was delayed in untransformed calli where root initiation took place only after 2 months, while in transformed calli it took about 35 d for root induction.

The levels of phenolics in hairy roots were quantified and compared with non-transformed leaves. It was found that the synthesis of polyphenols and catechin fractions (EC, ECG, EGC and EGCG) were slightly higher in hairy roots than in untransformed leaves (Table 4). The maximum quantity of catechin fraction was EGCG and the lowest was the EC fraction. Earlier studies of Palazon *et al.*¹⁹ also revealed that transformed hairy roots induced the synthesis of alkaloids more than in the non-transformed tissues.

Wardel and Skoog²⁰ reported that formation of roots on tobacco stem segment *in vitro* depended on their original positions on the plant, presumably due to the presence of a hormonal gradient in plant body. Results of our studies suggest that physiological factor, such as, hormone plays an important role in the formation of hairy roots. Biosynthesis of secondary-metabolite in transformed roots is genetically controlled, but influenced by nutritional and environmental factors. Composition of the culture medium was found to affect growth and secondary metabolite production²¹. The carbon source used as a basal component in the medium for plant tissue growth is one of the major factors that had been investigated by others¹⁷. The present studies also indicated that source and concentration of carbon in the basal medium was one of the important factors for hairy root induction.

This study demonstrated the transformation of tea leaves with *A. rhizogenes* and induction of hairy roots. The study also has demonstrated that the quantity of polyphenols and catechins in hairy roots

Table 3—Effect of plant growth regulators on root induction (d) in leaf derived transformed and untransformed callus

Plant growth regulators (PGRs) concentration	Days required for root initiation		
	Callus from transformed leaves	Callus from untransformed leaves	
Without PGR	35	60	
IAA (5 mg/L)	17	30	
2,4-D (3 mg/L)	21	32	
IAA (5 mg/L) + 2,4-D (3 mg/L)	20	25	
Statistical significance	SE ±	CD at $p=0.05$	CV (%)
Between PGRs (P)	0.81	1.87	3.67
Between days (D)	0.57	1.32	
Interaction (P × D)	1.14	2.64	

Table 4—The phenolic constituents of untransformed fresh leaves and transformed hairy root cultures

Compounds (%)	Untrans-formed leaves	Transformed hairy roots	
Polyphenols	27.89	30.34	
EGCG	13.79	14.98	
ECG	01.25	02.19	
EGC	02.79	02.71	
EC	01.73	01.90	
(+) Catechin	00.83	01.04	
Statistical significance	SE ±	CD at $p=0.05$	CV (%)
Between compounds (C)	0.26	0.58	3.46
Between treatments (T)	0.15	0.33	
Interaction (C × T)	0.37	0.82	

was higher in hairy roots than in untransformed leaf tissue and this could be exploited commercially. However further investigation is required to increase the level of these metabolites in hairy roots as well as to enhance the production of hairy root biomass.

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