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Rhizobium rhizogenes Mediated Hairy Root Transformation and Analysis of Secondary Metabolites in *Phlogacanthus thyrsiflorus* Nees Hairy Roots using GC-MS

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An efficient transformation system was developed for *Phlogacanthus thyrsiflorus,* a medicinal plant of northeast India, using *Rhizobium rhizogenes* strains A4 for infecting shoot tip, nodes and leaf explants derived from aseptically raised plants. The transformation frequency was found dependent on the explants as well as the co-culture periods. Highest transformation frequency of 95.69% was obtained from shoot tip explants co-cultured for 4 hours. An average of 6.37 hairy roots per explant were obtained from shoot tip explants within three weeks of infection. Whereas, transformation frequency along with number of transformed roots were fewer in node and leaf explants. The presence of T_L DNA of *R. rhizogenes* strains A4 in the hairy root genome of *P. thyrsiflorus* was confirmed through PCR based cloning and sequencing in which the sequencing results of the PCR amplicon confirmed the presence of *rol b* and *rol c* genes in the host genome. Sequence results for *rol* genes were found to be 97 percent and 99 percent similar with *rol b* and *rol c* gene present in NCBI database. GC-MS analysis of methanolic extract of the hairy roots reveals the presence of a few medicinally important secondary metabolites.

Keywords: *rol b*, *rol c*, GC-MS, Strain A4

Introduction

Phlogacanthus thyrsiflorus Nees (vernacular name-Titaphul) a gregarious shrub belonging to family Acanthaceae is of high medicinal value. This plant is native to the north-eastern regions of India. In India, traditional healers use the plant parts as cure for various ailments such as allergy, cough, asthma, malarial fever, jaundice, dysentery, rheumatism, tuberculosis[1] etc. *Rhizobium rhizogenes* induced hairy root culture is a well-established method of production of secondary metabolites in many medicinally important plants.[2] With a single preliminary report on hairy root induction in *P. thyrsiflorus*[3], the present work was designed to evaluate the efficacy of this plant more precisely for hairy root induction through sequencing of the PCR product from *P. thyrsiflorus* hairy roots confirming the integration of *rol* genes of *R. rhizogenes* in the host genome. Gas chromatography- mass spectrometry method was used for the analysis of the volatile secondary metabolites present in the transformed roots of *P. thyrsiflorus*.

Materials and methods

Plant material procurement and *in vitro* shoot proliferation

The plant material (*P. thyrsiflorus*) was procured from the medicinal nursery of College of Biotechnology, Birsa Agricultural University, Ranchi, Jharkhand. The identification of the plant was carried out by Botanical Survey of India (No. CNH/Tech.II/2015/243) and the specimen was deposited in the Department of Pharmaceutical Science and Technology, Birla Institute of Technology, Mesra, Ranchi, Jharkhand (Voucher Specimen No.- PhSc-0463/13-14). Aseptic cultures of the plant were established using young, actively growing nodal segments (2–3 cm) from the lateral branches following the protocol of Singh *et al*., 2017.[4] Explant multiplications were achieved using Murashige and Skoog's medium 1962[5] along with 2.0 mgl^{-1} BA, 50.0 mgl^{-1} adenine sulphate and 1.0 mgl^{-1} citric acid. Different explants from *in vitro* grown plantlets were used for hairy root induction in *P. thyrsiflorus.*

Rhizobium rhizogenes strain and hairy root induction

The agropine-type strain A4 of *Rhizobium rhizogenes* was used for inducing hairy root infection in *P. thyrsiflorus* explants. Culture of the strain A4 was maintained in YEB (Yeast extract – Beef extract) broth and 5.0 mgl^{-1} Rifampicin. Shoot tips, nodes and leaves of the 3–4 weeks old aseptically grown plantlets were taken as explants. The explants were co-cultured for 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 h along with the bacteria acclimatized in liquid MS medium. The explants were then washed in MS

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medium supplemented with 250 mgl^{-1} cefotaxime sodium salt. Finally, the cultures were inoculated in MS basal medium and incubated at controlled temperature (25 ± 2°C) and illumination (3000 lux) with 16 h/8 h light/dark periods.

PCR analysis of transgenic root

Genomic DNA isolation of *P. thyrsiflorus* hairy roots was performed using the protocol followed by DNeasy Plant Mini Kit (Qiagen). The primers specific to *rol b* were 5' AACATTCCCACCTCAGCAATACT 3' (Forward) and 5' TCATCATCTGTCGCTGACACC 3' (Reverse). The primers specific to *rol c* were 5' AGGGCCAGTCGATGGATATTG 3' (Forward) and 5' TGTCGGCACTCCTATCGAGAT 3' (Reverse). DNA amplification was carried out in Veriti 96 welled gradient thermal cycler (Applied Biosystems, Foster City, USA).

Cloning and Sequencing of PCR amplicon

The amplified DNA were eluted from the agarose gel using the Silica bead DNA gel extraction kit (Thermo Fisher Scientific, Lithuania). The cloning of eluted DNA was performed using InsTAclone PCR cloning kit #K1214 (Thermo Fisher Scientific, Lithuania). The plasmids were isolated from the transformed bacterial colonies using high pure plasmid isolation kit (Roche Diagnostics GmbH, Mannheim, Germany). Plasmids were then sent to Xcelris labs Ltd., Ahmadabad, for nucleotide sequencing.

Sequence analysis

Forward and reverse sequences obtained through cloning were assembled using CLC Genomics Workbench, Version 11.1 (Qiagen Bioinformatics, Denmark) to get consensus sequences. BLASTn of the consensus were performed with nucleotide database of NCBI to validate the similarity of cloned sequences with the known *rol b* and *rol c* sequences deposited in NCBI database. Finally, these were translated to protein sequences using "translate to protein" tool and Pfam domain search was executed to confirm the presence of Rol B and Rol C domains using CLC genomics workbench.

Preparation of methanolic hairy root extract

The transformed roots of *P. thyrsiflorus* were washed and shade dried. Finely crushed two grams of the samples were macerated in methanol (10 mL × 3), filtered and concentrated in the rotary evaporator. The samples were finally passed through SPE cartridge (Sep- Pak Plus tC 18, Waters corporation, USA).

GC-MS analysis

GC-MS analysis was carried out on Clarus 500 Gas Chromatograph- Mass Spectrophotometer (Perkin Elmer, USA), using electron impact ionization in full scan mode. The column used was an Elite- 5 MS column (Perkin Elmer Instruments, USA) having dimensions 25m × 0.18mm × 0.18µm. The volatile secondary metabolites from *P. thyrsiflorus* hairy root extract were analysed using the National Institute of Standards and Technology (NIST- 14) reference library.

Statistical analysis

The experiments were repeated three times with a minimum of four replicates each. Results obtained were expressed as mean ± standard deviation. A two-way ANOVA was performed to analyse the transformation frequency and rate of hairy root induction.

Results and Discussion

The three different explants of *P. thyrsiflorus*, shoot tips, nodes and leaves were co-cultured with A4 strain of *R. rhizogenes* for nine different time periods. The three explant types exhibited significant difference in the effect of the co- culture periods on both, the transformation frequency and number of induced roots per explants (Tables 1 and 2). Nature of explants and juvenility both influence the transformation process by *Rhizobium*.[1,6,7] In this study, shoot tips and nodes were found more susceptible for root induction after co-culture with bacteria. Within three weeks of infection, highest transformation percentage (95.69%) and number of roots per explants (6.37) were observed in shoot tip explants after 4.0 h co-culture while it was lowest after 1.0 h co-culture (Tables 1 and 2). Leaf explants crumbled within a month of infection as reported earlier in *Tylophora indica*.[8] Transformation frequency has been reported to be affected by changes in co-culture period.[3,9] The two-way ANOVA result for transformation frequency shows that the effects of all the co culture periods along with the three explant types at distinct time interval and their interactions were all statistically significant at $P < 0.0001$. After 21 days of infection with A4 strain of *R. rhizogenes*, maximum hairy roots were observed in the infected shoot tips (6.37/explant) (Table 2, Fig. 1E). Out of different co-culture periods studied, 4.0 h was found to result into maximum numbers of hairy roots per explant both in shoot tips and nodes of *P. thyrsiflorus* (Table 2, Fig. 1). The two-way ANOVA result for

Table 1 — Effect of different co-culture period on transformation frequency (in percentage), induced through A4 strain of *R. rhizogenes* on shoot tip and node explants of *P. thyrsiflorus* Nees

Time of observation (days)	7 Days		14 Days		21 Days	
Co-culture Period (Hours)	Shoot tip	Node	Shoot tip	Node	Shoot tip	Node
1.0	22.14 ± 2.57	0	29.13 ± 5.77	4.46 ± 3.89	35.52 ± 2.09	9.07 ± 4.52
1.5	24.67 ± 5.21	0	33.13 ± 4.47	6.68 ± 0.45	37.74 ± 2.15	11.15 ± 3.98
2.0	35.81 ± 6.19	8.77 ± 3.24	42.50 ± 6.61	11.15 ± 3.98	47.10 ± 9.83	15.46 ± 2.89
2.5	44.58 ± 5.05	11.29 ± 4.39	51.11 ± 1.92	22.14 ± 2.57	58.09 ± 7.33	28.83 ± 2.30
3.0	53.65 ± 9.36	13.67 ± 7.59	64.64 ± 6.01	24.66 ± 5.21	66.73 ± 5.98	37.60 ± 5.46
3.5	66.71 ± 5.81	17.98 ± 4.80	71.17 ± 2.30	31.05 ± 2.39	73.25 ± 1.79	46.67 ± 5.77
4.0	75.77 ± 6.12	24.52 ± 4.30	80.08 ± 5.98	42.20 ± 1.96	95.69 ± 3.73	59.88 ± 2.68
4.5	62.56 ± 7.91	17.68 ± 3.00	69.09 ± 5.78	31.35 ± 5.63	73.55 ± 4.91	48.73 ± 5.35
5.0	49.03 ± 4.86	13.08 ± 5.81	53.19 ± 3.13	26.45 ± 4.91	62.40 ± 5.46	42.04 ± 5.67

*data presented in mean ± SD

Table 2 — Effect of different co-culture period on number of hairy roots/explant, induced through A4 strain of *R. rhizogenes* on shoot tip and node explants of *P. thyrsiflorus*

Time of observation (days)	7 Days		14 Days		21 Days	
Co-culture Period (Hours)	Shoot tip	Node	Shoot tip	Node	Shoot tip	Node
1.0	0.09 ± 0.03	0	0.82 ± 0.08	0.38 ± 0.06	1.11 ± 0.08	0.62 ± 0.10
1.5	0.23 ± 0.05	0	1.16 ± 0.18	0.51 ± 0.08	2.32 ± 0.22	1.06 ± 0.06
2.0	0.24 ± 0.02	0.09 ± 0.09	1.75 ± 0.05	1.29 ± 0.08	2.81 ± 0.32	1.36 ± 0.02
2.5	0.31 ± 0.04	0.16 ± 0.11	2.34 ± 0.14	1.67 ± 0.13	3.78 ± 0.09	1.74 ± 0.22
3.0	0.51 ± 0.15	0.18 ± 0.05	3.12 ± 0.22	1.98 ± 0.10	4.16 ± 0.11	2.37 ± 0.24
3.5	1.16 ± 0.18	0.49 ± 0.13	3.60 ± 0.04	2.34 ± 0.26	5.63 ± 0.18	2.84 ± 0.29
4.0	1.83 ± 0.22	0.62 ± 0.09	5.11 ± 0.42	2.92 ± 0.31	6.37 ± 0.38	3.17 ± 0.34
4.5	1.40 ± 0.04	0.56 ± 0.08	4.14 ± 0.30	2.59 ± 0.42	5.44 ± 0.31	2.79 ± 0.43
5.0	1.25 ± 0.04	0.51 ± 0.19	3.34 ± 0.08	2.27 ± 0.14	4.90 ± 0.34	2.33 ± 0.12

*data presented in mean ± SD

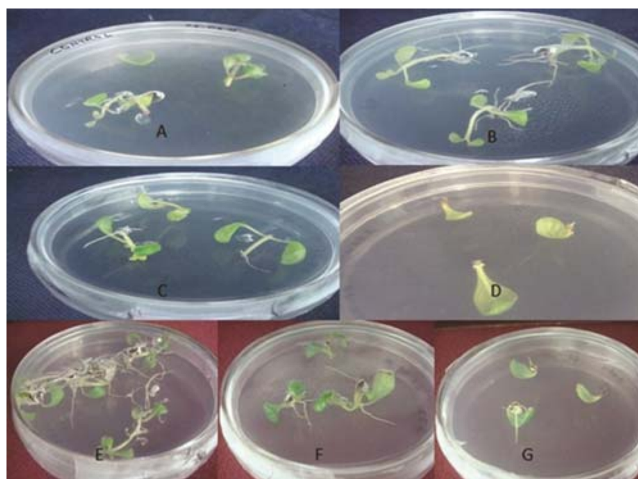

Fig. 1 — Hairy root transformations on different explants of *P. thyrsiflorus* co-cultured for 4 h in *R. rhizogenes* strain A4 after 2 weeks (Control- A, Shoot tip- B, Node- C, Leaf- D) and after 3 weeks (shoot tip- E, Node – F and Leaf- G) of culture

rate of hairy root induction shows that the effects of all the co culture periods along with the three explant types at distinct time interval and their interactions were all statistically significant at $P < 0.0001$. In case of node explants, an average of 3.17 hairy roots per explant were achieved which was much lower than shoot tip explants. No root induction was observed in control experimentation with the three explants inoculated in MS basal medium without infection (Fig. 1A) which dried up after 15 days of inoculation.

DNA isolated from *in vitro* infected roots of *P. thyrsiflorus* was found to be pure (A 260/280- 1.79), of high concentration (982µg) and with high molecular weight (>10Kb). PCR amplification of *rol* genes from hairy roots have been reported in many plant species.[8–10] Repeated success of PCR based techniques has made it almost compulsory step for the confirmation of hairy root transformation. Sequencing of the PCR amplicon for *rol* genes leads to a complete validation of hairy root transformation in the host plant providing an edge to the preliminary work of hairy root transformation in *P. thyrsiflorus*.[3] The PCR amplicon size for *rol b* and *rol c* were around 600 bp each (Fig. 2a-lane A and B). Lane C of Fig. 2a shows the PCR result for no template control reaction. The amplicons were inserted in pTZ57R/T vectors and cloned in *E. coli.* The gel image of the transformed plasmid displayed recombinants

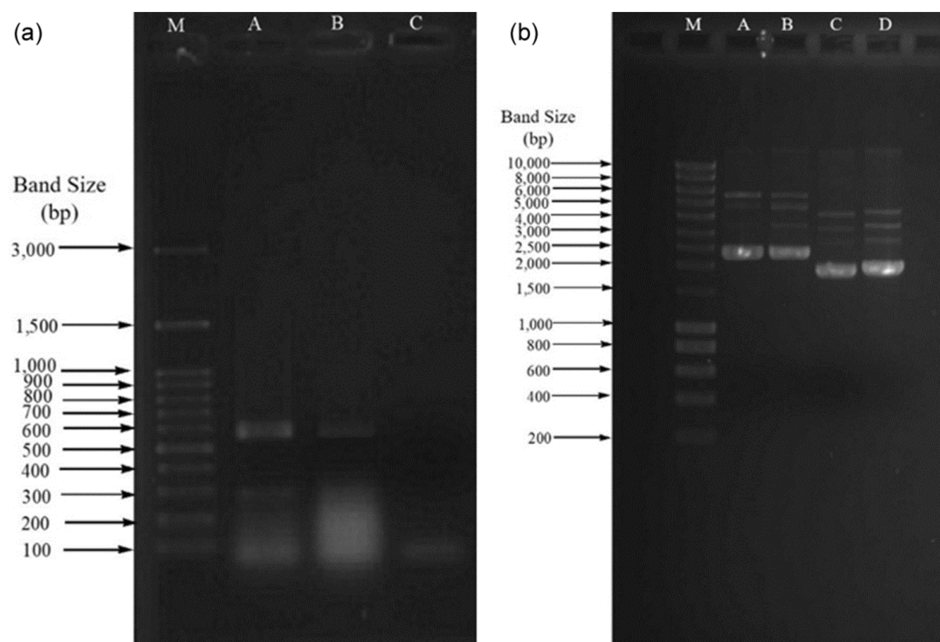

Fig. 2 — (a) Optimized PCR amplification of *P. thyrsiflorus* genomic DNA for *rol b* and *rol c* (Lane M- 3,000 bp DNA marker, A- *rol b* amplicon, B- *rol c* amplicon, C- no template control); (b) Gel image of cloned plasmids (Lane M- 10 kb DNA marker, A- recombinant *rol b*, B- recombinant *rol c*, C and D- non-recombinant transformed plasmids)

Table 3 — Identified compounds in methanolic extract of hairy roots of *Phlogacanthus thyrsiflorus* by GC-MS

Compound	Molecular formula	Molecular weight	RT	Area (%)
2- Amino- 1,3-propanediol	$C_3H_9NO_2$	91	2.52	14.29
Ethanol, 2- butoxy-	$C_4H_6O_3$	102	6.66	39.73
Ethanol, 2- butoxy-	$C_4H_6O_3$	102	7.82	6.90
4H- Pyran- 4-one, 2,3- dihydro- 3,5- dihydroxy- 6- methyl	$C_4H_{10}N_2O$	102	12.69	6.83
2- Methoxy- 4-vinylphenol	$C_9H_{10}O_2$	150	15.35	9.92
1, 5, 5- Trimethyl- 6- methyl-6- methylene- cyclohexene	$C_{10}H_{16}$	136	17.21	7.61
Hexadecane	$C_{16}H_{34}$	226	19.49	2.82
3- (Prop-2- enoyloxy) dodecane	$C_{15}H_{28}O_2$	240	19.59	1.33

with higher molecular weight as compared to non-recombinants, both for the gene *rol b* and *rol c,* in which lane A and B were the recombinant plasmid containing *rol b* and *rol c* genes respectively, whereas lane C and lane D were non- recombinant transformed plasmids in the transformation experiments with *rol b* and *rol c* amplicons respectively (Fig. 2b). The sequencing of the amplicons was carried out through Sanger dideoxy method. Consensus sequences of the forward and reverse sequences were accomplished using CLC genomics workbench. The BLASTn results of the sequences showed 97 per cent and 99 per cent similarities with *rol b* and *rol c* gene present in NCBI database. The Pfam domain search of the protein sequence (obtained through "translate to protein" tool) revealed the presence of tyrosine phosphatase (Rol B domain) and cytokinin- beta- glucosidase (Rol C domain) in overlapped condition. Sequence analysis in conjunction with PCR amplification confirmed the integration of *rol b* and *rol c* genes of *R. rhizogenes* into the host genome. The sequences were submitted with NCBI with code names SMb (for *rol b*) and SMc (for *rol c*) and received the accession numbers KY748277.1 and KY748278.1 respectively.

Gas chromatography- mass spectrometry analysis of methanolic extracts of hairy roots exhibited several compound peaks. These peaks were analysed using NIST 14 reference library. The metabolites present in the hairy roots with their percent composition in the extracts are mentioned in Table 3. Nearly 90 per cent of the compounds were identified using NIST reference library. GC-MS analysis is the most chosen method for the identification of essential oils particularly terpenoids in many medicinally important plants.[11,12] The compounds identified in the methanolic extract of *P. thyrsiflorus* hairy root were different than those

identified in the different plant part extract of the plant except for 4H- Pyran- 4- one, 2,3- dihydro- 3,5- dihydroxy- 6- methyl, which was found in the floral extract of the plant.[13] Ethanol, 2, butoxy- was present in large amount (approximately 47% area). 2- Amino- 1,3- propanediol present in 14% area is used as precursor for antibiotics and immunosuppressant.[14] Another compound called 2- Methoxy- 4-vinylphenol which was present in around 10 per cent in the hairy root extract of *P. thyrsiflorus* is a flavouring agent along with its use as anti- inflammatory agent.[15] Nearly 7% of the extract contain 4H- Pyran- 4- one, 2,3- dihydro- 3,5- dihydroxy- 6- methyl, which has been found to exhibit antimicrobial, anti- inflammatory, anti- cancerous properties.[16] The present study of the secondary metabolites in the methanolic extract of *P. thyrsiflorus* hairy root ensures that the hairy root extract of *P. thyrsiflorus* possess medicinal properties and scaling up of hairy roots can produce these secondary metabolites in large quantity.

Conclusions

Overall observations revealed establishment of hairy roots induction on multiple explants using A4 strain of *R. rhizogenes*. A4 is found virulent producing copious number of hairy roots sturdily for successive generations, which can be used for mass culture of hairy root for phytochemical extraction. Shoot tip explants are best suited for hairy root transformation after 4.0 h bacterial co-culture in this plant. Presence of *rol b* and *rol c* genes of *R. rhizogenes* in the transformed roots was confirmed through PCR study. Further, sequencing of the PCR product from *P. thyrsiflorus* hairy roots has been done for the first time which confirms the integration of *rol b* and *rol c* genes of *R. rhizogenes* in the host genome. The sequences were submitted to the NCBI and received the accession numbers KY748277.1 for *rol b* and KY748278.1 for *rol c*. The GC-MS analysis of the methanolic extract of the hairy roots reveals the presence of a few medicinally important secondary metabolites which argues for the scaling up of these hairy roots for recovering these metabolites in large quantity.

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