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Agrobacterium rhizogenes mediated transformation, initiation and multiplication of hairy roots in *Spilanthes acmella* Murr. (Asteraceae)

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Abstract

Spilanthes acmella is an important medicinal plant that belongs to the family Asteraceae. Traditionally, this plant has been used for years to cure toothaches, stammering, stomatitis and many other ailments. This plant is an important source of various medicinally important secondary metabolites like phenolics, coumarin, spilanthol, scopoletin, triterpenoids etc. The present study was carried out with the objective to transform this plant with Agrobacterium rhizogenes to initiate the hairy roots formation. This study demonstrated the transformation, initiation and multiplication of hairy roots from nodal segments and leaf explants taken from field grown and in vitro developed *S. acmella* plants. The hairy roots produced from different explants were white, slender, showed negative geotropism and lateral branching. PCR analysis of hairy root was performed that confirmed the bacterial transformation. Among the field grown and in vitro grown plants, the explants from the in vitro grown plants gave a high percentage of root induction. Nodal segments from in vitro grown plant gave 90% hairy root induction and leaf segments from in vitro grown plants with petiole gave the highest rate of root induction which was 92%. Nodal segments from field grown plants gave 85% and the leaves from field grown plants gave 80% of hairy root induction. This study offers great potential to establish the protocol for hairy root induction which can be used as an alternative source for the continuous production of this plant's important secondary metabolites and active biocompounds.

Keywords: *Spilanthes acmella*, *Agrobacterium rhizogenes*, hairy roots, transformation, PCR.

1. Introduction

The toothache plant, *Spilanthes acmella* Murr., is a significant medicinal plant that is a member of the Asteraceae family. It can be found all over the world in tropical and subtropical areas. According to Nelofar et al.[1], it has been shown to possess a number of biological properties, including antipyretic, antidiuretic, anti-inflammatory, antioxidant, immunomodulatory, hepatoprotective, anticancer, and antitoothache1. Important secondary metabolites such as coumarin, phenolics, triterpenoids, myrecene, α and β amyrin, spilanthol, and scopoletin have been discovered to be produced by the plant. Spilanthol, an alkamide found in the plant's roots and all of its aerial parts, is the active chemical component.

Hairy roots, produced by the genetic transformation by *Agrobacterium rhizogenes* are gaining importance for production of secondary metabolites *in vitro*.

A. rhizogenes, which carries the root-inducing (Ri) plasmid, infects injured plant tissues to produce hairy root cultures. Like the roots of native plants, they have a similar ability for biosynthesis and can produce secondary metabolites. The benefits of hairy roots culture (HRCs) include significant biomass output, long-term preservation, genetic and biochemical stability, and rapid growth rates independent of phytohormones[2]. More significantly, compared to undifferentiated callus and cell suspension cultures, hairy root cultures frequently collect phytochemicals at higher amounts[3]. Therefore, *A. rhizogenes* may change the targeted medicinal plant into hairy root lines that can produce secondary metabolites and bioactive chemicals with pharmaceutical uses.

2. Materials and Methods

2.1. Establishment of *S. acmella* plants

The seeds of *S. acmella* were procured from Medicinal and Aromatic Plants Research Station, Professor Jayashankar Telangana Agricultural University, Rajendranagar, Hyderabad, sown in soil taken in pots and the plants were grown and maintained (Fig.1). The plant material required for the hairy root induction were collected from these plants.

Figure 1. *Spilanthes acmella*

2.2. Transformation and Initiation of Hairy Roots by *Agrobacterium rhizogenes*

The Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, provided the *Agrobacterium rhizogenes* (MTCC 532), which was then cultivated on solid nutrition agar medium to activate it. A loop full of the bacteria was taken and cultivated on liquid nutrient broth medium in order to induce hairy roots. In an orbital shaker with continuous stirring, *A. rhizogenes* was injected in nutrient broth culture media and allowed to stand at 250 rpm for 16 hours at 25^{0}C. After that, the bacterial suspension was put into a sterile centrifuge tube and spun for ten minutes at 5000 rpm. After that, the residue was suspended in liquid MS media that had been enhanced with 3% sucrose.

MS medium[4] supplemented with 0.8% agar, 3.0% (w/v) sucrose and pH of 5.7 was maintained and used as growth medium for induction of hairy roots from explants.

The concentration of *A. rhizogenes* cultures was standardized for transformation and induction of hairy roots. Different bacterial concentrations having different optical densities (0.2-0.8 OD) at 600 nm were tested for their transformation percentage. The concentration with optical density of 0.6 were found to give the highest transformation percentage and hence only these concentrations were used for hairy root induction.

Hairy roots were induced from the *S. acmella* leaf and nodal explants by transforming them with *A. rhizogenes*. Overnight grown cultures of *A. rhizogenes* were taken and they were seen to have 0.6 optical density (O.D) at 600 nm. The young leaves and nodal segments were collected from in vitro and field grown plants of *S. acmella*. Two types of leaf explants were taken, some bearing petiole and some without petiole. They were cut into small pieces and pricked with a sterile needle and soaked in the bacterial suspension for half an hour. The explants were removed and blotted dry on a sterile blotting paper. Then they were placed onto the co-cultivation medium (MS-medium) in dark for three different time intervals i.e. 24, 48 and 72 hours. After co-cultivation, the leaves were inoculated onto hormone free MS medium containing antibiotic cefotaxime (250 mg/l) to check the non-transformed bacterial growth. The cultures were maintained at 25^{0}C with 16/8 h light and dark period.

For each treatment, 20 leaf and nodal explants were inoculated with *A. rhizogenes* and the experiment was performed in triplicates. Some explants were not treated with the bacteria and maintained as control. The results were expressed in percentage transformation frequency.

2.3 PCR analysis of Hairy roots

The production of hairy roots and the genetic alteration of *A. rhizogenes* were verified by PCR analysis of hairy roots. PCR amplification was

performed using the DNA from hairy roots, plasmid DNA from the *A. rhizogenes* strain (positive control), and DNA from untransformed leaves (negative control). The alkaline lysis method[5] was used to recover plasmid DNA, while the CTAB method[6] was used to extract DNA from hairy roots and non-transformed leaves. Primers specific to rol B and rol C were used in the polymerase chain reaction. The suppliers of the rol B and rol C primers were Xcelris Genomics in Ahmedabad, India. The rol B gene's 3' primer sequence was TTAGGCTT CTTTCTTCAGGTTTACTGCAGC, while its 5' primer sequence was TGGATCCCAAATTGCTATT CCTTCCACGA. In DNA amplification, this amplified the 780 base pair (bp) DNA fragment. The rol C gene's 3' primer sequence was GATTGAAAACTT GCAC, while its 5' primer sequence was ATGGCTGAAGACGACCTGTT TTAGCC. In DNA amplification, this amplified the 540 base pair (bp) DNA fragment. As positive and negative controls, respectively, 50 ng of plasmid DNA from *A. rhizogenes* and DNA from untransformed leaf tissues were used. Hairy root DNA was used as a therapy. Table 1 lists the primers used in this experiment.

Table 1: Primer sequence used for PCR detection of transgene and length of PCR amplified fragment

Gene	DNA Sequence	Length of PCR amplified Fragment (bp)
Rol B	*Forward* TGGATCCCAAATTGCTA TTCCTTCCACGA *Reverse* TTAGGCTTCTTTCTTCA GGTTTACTGCAGC	780
Rol C	*Forward* ATGGCTGAAGACGACC TGTT *Reverse* TTAGCCGATTGAAAACT T GCAC	590

In order to purify DNA, RNA was extracted by treating the sample with DNase-free RNase, which was purchased from Pure-gene in the United States. RNase and other proteins were eliminated by treating them with chloroform:isoamyl alcohol (24:1). To verify the quality and amount of isolated genomic DNA, a Nanodrop spectrophotometer was used. The measured genomic DNA was diluted with TE buffer (10 mM Tris HCl, 1 mM EDTA) to a concentration of about 20 ng/ul, which is suitable for PCR use straight away. The PCR amplification was carried out in a final reaction volume of 30 µL with 1X PCR buffer (Bangalore Genei), 1.5 mM $MgCl_2$, 1 mM each of the four dNTPs, 1.25 U of Taq polymerase (Bangalore Genei), and 0.5 mM each of 5' and 3' primers with 3 µl of the total DNA from transformed roots in order to verify the presence of the rol B and rol C genes. Following a 3-minute initial denaturation at 94^0C, 35 cycles of PCR were conducted at 94^0C for 30 seconds, 550C for 30 seconds, and 72^0C for 1 minute, with a final extension at 72^0C for 7 minutes.

The PCR products were put onto a 1.2% Agarose gel (Himedia, molecular grade) after amplification. The gel was made with 1X TBE buffer that contained 0.5 µg/ml of ethidium bromide. The amplified products underwent 3-3.5 hours of cooling electrophoresis at 100 V. Following separation, the gel was examined using a UV transilluminator and captured on camera.

2.4. Multiplication of hairy roots

The hairy roots from various explants were placed in ½ MS liquid and solid medium that was free of hormones and antibiotics. Under constant darkness, they were kept in flasks on an orbital shaker at 25^0C and 100 rpm. Hairy roots were multiplied by sub culturing in fresh medium every 15 days. The fresh and dry weights of hairy roots were noted for different intervals of time i.e. 7, 15 and 30 days.

3. Results

3.1 Induction of Hairy roots

The hairy roots produced from different explants were white, slender (Plate 1).They showed negative geotropism and lateral branching (Plate 2). All the nodal segments induced hairy roots within 14-16 days of co-cultivation with the bacteria. For nodal segments from field grown plants, the best co-cultivation time was observed to be 72 hours which gave 85% of hairy root induction. The co-cultivation time of 24 and 48 hours gave 40 % and 70% of hairy root induction respectively (Table 2). For nodal segments from in vitro grown plants, the best co-

cultivation time was observed to be 72 hours which gave 90% of hairy root induction. The co-cultivation time of 24 and 48 hours gave 45 % and 68% of hairy root induction respectively. Of the two explants, nodal segments from the in vitro grown plants showed good response (90%) than the field grown plant (85%).

Table2: Evaluation of nodal segments of *S.acmella* for hairy root induction on MS media with 250mg/l cefotaxime

Explants	Co-cultivation time	Explants inoculated	No. of explants responded	Hairy root induction (%) Mean ± SE
Nodal segments from field grown plants	24	20	8	40±0.63
	48	20	14	70±0.52
	72	20	17	85±0.70
Nodal segments from *in vitro* grown plant	24	20	9	45±0.24
	48	20	13	68±0.43
	72	20	18	90±0.75

Table 3: Hairy root induction from leaf explants of *S. acmella* on MS media with 250mg/l cefotaxime

Explants	Co-cultivation time	Explants Inoculated	Explants Responded	Hairy root Induction (%) Mean ± SE
Leaf segments from field grown plants with petiole	24	20	10	50±0.27
	48	20	16	80±0.72
	72	20	7	35±0.63
Leaf segments from *in vitro* grown plant with petiole	24	20	12	60±0.56
	48	20	18	92±0.45
	72	20	11	55±0.66
Leaf segments from field grown plants without petiole	24	20	0	0
	48	20	0	0
	72	20	0	0
Leaf segments from *in vitro* grown plants without petiole	24	20	0	0
	48	20	0	0
	72	20	0	0

Leaf explants with and without petiole region from field grown and in vitro raised plants were tested for hairy root induction. Hairy roots were produced within 12-14 days from leaves containing petiolar region from field grown plants and in vitro grown plants.

For the leaf segments from field plants with petiole, the best co-cultivation time was observed to be 48 hours which gave 80% of hairy root induction. The co-cultivation time of 24 hours gave 50 % of hairy root induction whereas the percentage of hairy root induction reduced to 35% on increasing the co-cultivation time to 72 hours. For the leaf segments from in vitro plants with petiole, the best co-cultivation time was observed to be 48 hours which gave 92% of hairy root induction. The co-cultivation time of 24 hours gave 60 % of hairy root induction whereas the percentage of hairy root induction reduced to 55% on increasing the co-cultivation time to 72 hours (Table 3).The leaf explants without petiole region collected from field grown and also *in vitro* regenerated plants did not show any hairy root induction in any of co-cultivation periods i.e., 28, 48

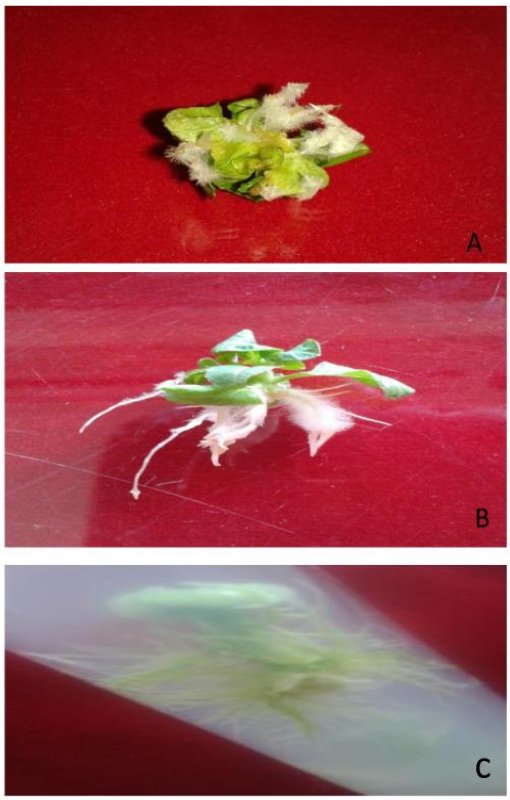


Plate 1: Induction of Hairy Roots from different explants of *Spilanthes acmella* by genetic transformation with *Agrobacterium rhizogenes* (A,B: Leaf explants; C: Nodal segment)

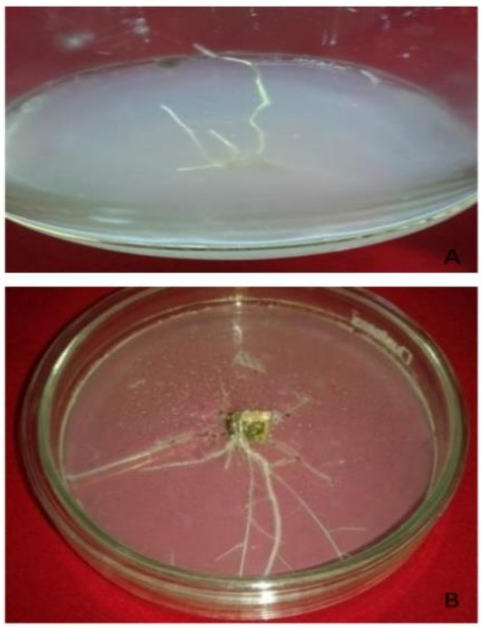


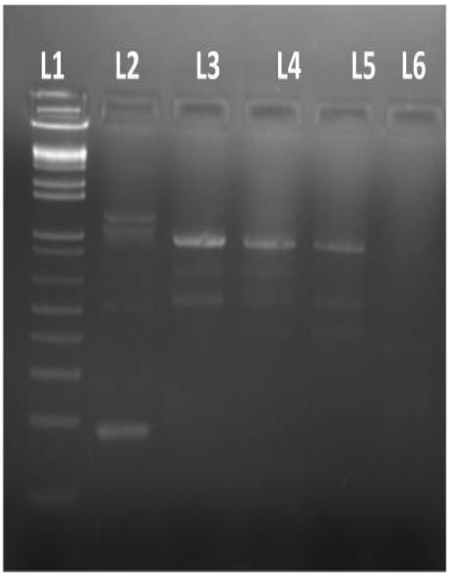
Plate 2: Hairy roots induced from Leaf explants of *S.acmella* showing negative geotropoism and lateral branching. (A: Hairy roots showing negative geotropism; B: Hairy roots showing lateral branching)

and 72 hours. This one of the important observation noticed in the present study

Among the different explants evaluated for hairy root induction, leaf explants (with petiolar region) collected from in vitro grown plants induced high percentage of hairy roots (92 %) with 48 h co-cultivation period. This was followed by nodal explants of in vitro grown plants with 90% hairy root induction at 72 h of co-cultivation.

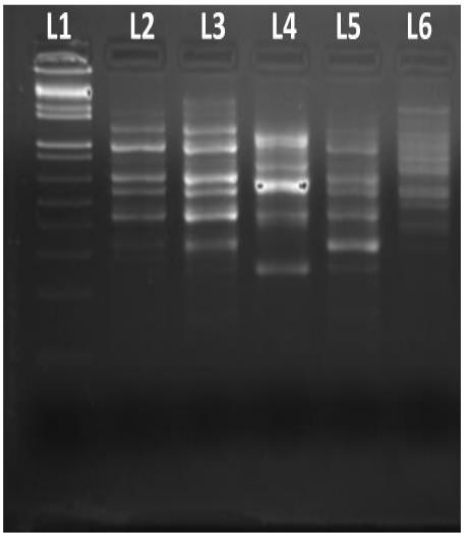
3.2. PCR analysis of Hairy Roots

The transformation event was indicated by PCR analysis of the transformed hairy roots, which revealed amplification of DNA at 540 bp and 780 bp in the hairy root samples and in the positive control (plasmid). However, there was no amplification in the negative control, which consisted of unaltered leaf tissues. This demonstrated that the integration of the rol C and rol B genes of *Agrobacterium rhizogenes* led to the development of hairy roots. As a result of the integration, the altered leaf explants' DNA was amplified at 540 bp and 780 bp, respectively (Fig. 2 and Fig. 3). Hence, the PCR analysis has confirmed that the hairy roots were produced from the leaf explants due to the genetic transformation with *A. rhizogenes*.



L1: ladder
L2: positive control (bacterial DNA)
L3: hairy root (nodal region)
L4: hairy root (leaf segment)
L5: hairy root (*in vitro* leaf)
L6: negative control (non-transformed leaf)

Fig 2: PCR Analysis of Hairy roots to confirm the genetic transformation with *A.rhizogenes* with rol B primers at 780 bp.



L1: 1kb ladder
L2: hairy root (nodal region)
L3: hairy root (leaf segment)
L4: positive control (bacterial DNA)
L5: hairy root (i*n vitro* leaf)
L6: negative control (non-transformed leaves)

Fig 3: PCR Analysis of Hairy roots to confirm the genetic transformation of *A.rhizogenes* with rol C primers at 540 bp

3.3. Growth and Multiplication of hairy roots

To multiply the hairy roots, induced roots were cultured on basal media without any hormonal concentration. To observe the growth, the hairy roots induced from leaf explants were subcultured onto MS and ½ MS solid and MS and ½ MS liquid medium (Plate 3). They grew well in all the media but better growth was observed on MS liquid medium compared to MS solid medium. Hence MS and ½ MS liquid medium was selected for their multiplication.

The hairy roots were grown on MS and ½ MS solid and liquid media without any plant growth regulators. The roots were harvested and fresh and dry weights were noted at different time intervals of culture (7, 15 and 30 days). There was an increase in the fresh and dry weights of hairy roots with an increase in the number of days of incubation (Table 4).

For the hairy roots cultured on MS medium, the fresh and dry weights were 0.54 g and 0.02 g after 7 days of culture. The fresh and dry weights increased to 2.85 g and 0.25 g after 15 days of culture. A maximum of 5.04 g and 0.80 g fresh and dry weights respectively was obtained after 30 days of culture.

For the hairy roots cultured on ½ MS medium, the fresh and dry weights were 0.75 g and 0.13 g after 7 days of culture. The fresh and dry weights increased to 3.41 g and 0.30 g after 15 days of culture. A maximum of 6.05 g and 0.99 g fresh and dry weights respectively was obtained when roots were cultured on ½ MS medium after 30 days of culture.

Of the two different media, more growth of the hairy roots was observed in MS half strength liquid medium without any hormonal supplementation compared to full strength MS basal liquid media.

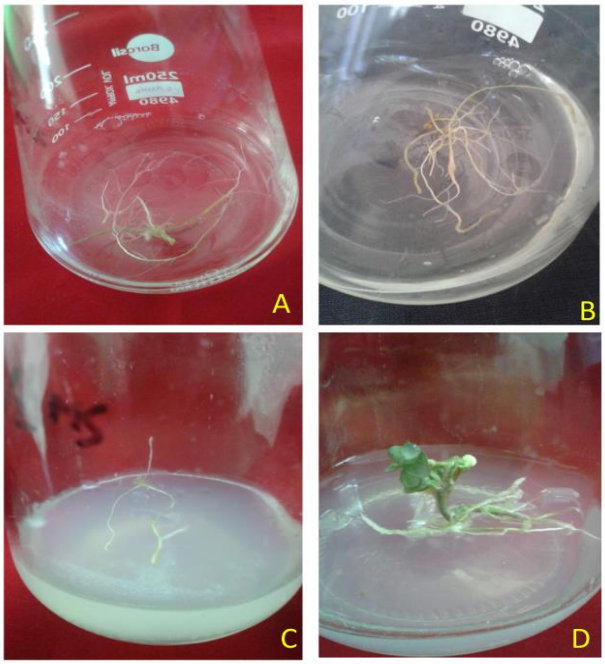


Plate 3: Multiplication of hairy roots from Leaf explants of S. *acmella.* A: MS liquid medium ; B: ½ MS liquid medium; C: MS solid medium; D: ½ MS solid medium

4. Discussion

Hairy root cultures have the potential to produce large amounts of useful secondary metabolites that are used as tastes, colors, and medications. Because of their rapid development in growth hormone-free culture media and genetic stability, genetically modified hairy roots produced by infecting plants with *Agrobacterium rhizogenes* are an excellent source for the synthesis of bioactive compounds[7]. The genetic stability of transformed root cultures is explained by the stable integration of the plasmid into the host plant genome[8].

The study of the genus *Spilanthes*' production of hairy roots is still in its early stages. *A. rhizogenes* strains MTCC 2364 and MTCC 5329 have been reported to produce hairy roots in *Spilanthes paniculata* by infecting the cotyledons and hypocotyl segments[9].

Agrobacterium rhizogenes strain MTCC 532 was used in this investigation to infect *S. acmella* nodal segments and leaf explants in vitro in order to create cultures of altered roots. Compared to other strains 10–13, strain 532 is also successful in inducing hairy roots in cultures of *Plumbago rosea*[10], *Rubia tinctorum*[11], *Arachis hypogaea*[12], and *Withania somnifera*[13].

This work is the first to document the formation of hairy roots from *S. acmella* leaf and nodal segments. Likewise, prior research has documented the induction of hairy roots from nodal segments in plants such as *Arnebia hispidissima*[14], *Berberis aristata*[15], and *Withania somnifera*[16]. According to this study, S. acmella's production of hairy roots is influenced by the kind of explants and the length of co-cultivation. This study is in line with the *Berberis aristata*[15] hairy root induction investigation.

5. Conclusion

This investigation is helpful in establishing *S. acmella*'s hairy roots. In order to produce hairy roots that may be used economically to produce significant secondary metabolites of this plant, these can be multiplied in bioreactors on a big scale. To improve the concentration and secretion of high-value metabolites like spilanthol, hairy roots would be the ideal candidate for metabolic engineering of the secondary metabolite pathways.

6. Authors Contribution

Hajera Sana is responsible for conducting the research work and drafting the manuscript. Prof. A. Sabitha Rani, as the supervisor, provided guidance throughout the study, reviewed the results, and contributed to the finalization of the manuscript.

7. Acknowledgements

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8. Data Availability

Available upon written request from the corresponding author

9. Conflicts of interest

Authors declare no conflicts of interest.

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