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Induction of hairy roots in *Spilanthes acmella* Murr. by genetic transformation with *Agrobacterium rhizogenes* for *in vitro* production of spilanthol

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Abstract

Spilanthes acmella is an important medicinal plant that has been administered as a traditional medicine for years to cure toothaches, stammering, stomatitis and many other ailments. The which resulted in overexploitation and depletion of *S.acmella* from its natural habitat. The plant and its highly valuable medicinal product, spilanthol are not meeting the industrial demand due to less commercial cultivation. Hence, the present study was carried out with the objective to standardize the induction of hairy roots in *S.acmella* by genetic transformation with of spilanthol. This study demonstrated the induction and multiplication of hairy roots from nodal segments and leaf explants taken from field grown and *in vitro* developed *S.acmella* plants.PCR analysis of hairy root was performed that confirmed the bacterial transformation. *S.acmella*. The methanolic extract of hairy roots showed significant amount of spilanthol content (0.134%). This study is the first report of quantification of spilanthol content in hairy roots of *S.acmella,* which offers a great potential for their use as an alternative source for the continuous production of spilanthol **Keywords** : *Spilanthes acmella, Agrobacterium rhizogenes*, hairy roots, PCR, spilanthol, HPLC

Introduction

Spilanthes acmella Murr.,commonly known as toothache plant is an important medicinal plant belonging to family Asteraceae. It is widely distributed in tropical and subtropical regions of the world. It has been reported to have various biological activities like antipyretic, antidiuretic, antiinflammatory, antioxidant, immunomodulatory, hepatoprotective, anticancer and antitoothache (Nelofar et al., 2016). The plant has been found to produce important secondary metabolites like spilanthol, scopoletin, myrecene, α amyrin, β amyrin etc. The active chemical component is spilanthol, an alkamide which is present in roots and all aerial parts of the plant. Spilanthol has high industrial demand for its use in pharmaceutical, cosmetic and toothpaste industry.

Hairy roots, produced by the genetic transformation by *Agrobacterium rhizogenes* are gaining importance for production of secondary metabolites *in vitro*. The hairy root cultures are induced by the infection of wounded plant tissues with *A. rhizogenes*, bearing the root-inducing (Ri) plasmid. They possess comparable biosynthetic capacity for production of secondary metabolites similar to native plant roots. The advantages of hairy roots culture (HRCs) is that they have fast growth rates independent of phytohormones, genetic and biochemical stability, long-term preservation and sizable biomass production (Guillon et al, 2006). More importantly, HRCs often accumulate phytochemicals at higher levels as against undifferentiated callus and cell suspension cultures (Ono and Tian, 2011). Therefore, the transformation of the desired medicinal plant by *A. rhizogenes* could result in hairy root lines, capable of synthesize bioactive compounds with pharmaceutical applications. This method not only facilitates the *de novo* synthesis of novel compounds, but are able to produce metabolites, sometimes even in higher amounts than the intact plants.

S.acmella is quickly getting depleted from its natural habitat, because of its wider applications for commercial use. The plant is not meeting the industrial demand due to less commercial cultivation. The other major limiting factor in large scale propagation *of S.acmella* is low germination and viability of the seed (Pati et al., 2006; Dobranszki and Silva, 2010).

In order to produce high value secondary metabolite such as spilanthol, a constant source of plant material is required which could be utilized as a ready stock to meet the demands of the pharmaceutical industry. The major limitation to the commercial production of spilanthol is less cultivation and supply of *S.acmella* plants. Thus, there is a need to search for alternative methods for spilanthol production. Establishment of hairy roots offers a great potential for rapid and mass production of spilanthol irrespective of seasons, climatic conditions and geographical regions. Hence, the present study has been undertaken with the objective to establish *Spilanthes acmella* as a model system for *in vitro* production of spilanthol through hairy root cultures.

Materials and Methods

Establishment of *Spilanthes acmella* plants for plant material

The seeds of *S.acmella* were procured from Medicinal and Aromatic Plants Research Station, Prof. Jayashankar Telangana Agricultural University, Rajendranagar, Hyderabad, sown in soil in pots and the plants were maintained in the Botanical Garden in University College for Women, Koti, Hyderabad. The plant material required for the hairy root induction were collected from these plants.

Bacterial Culture Preparation

Agrobacterium rhizogenes (MTCC 532) was procured from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh and cultured on solid nutrient agar medium for activation..

Preparation of bacterial suspension

For hairy root induction, a loop full of the bacteria was taken and cultured on liquid nutrient broth medium *Agrobacterium rhizogenes* was inoculated in nutrient broth culture medium and left standing at 250 rpm for 16 hours at 25^0C in an orbital shaker under constant stirring. The bacterial suspension obtained was transferred into a sterilized centrifuge tube and centrifuged at 5000 rpm for 10 minutes. The residue obtained was then suspended in liquid MS media supplemented with 3% sucrose.

Hairy root culture medium

MS medium (Murashige and Skoog, 1962) 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.

Standardization of *Agrobacterium rhizogenes* concentration

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.

Induction of Hairy roots

Hairy roots were induced from the *S.acmella* leaf and nodal explants by transforming them with *Agrobacterium 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 25^0C 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.

$$\%\ \text{Transformation frequency} = \frac{\text{Number of explants inducing hairy roots}}{\text{Total number of explants infected with } A.\ rhizogenes} \times 100$$

Multiplication of hairy roots

The hairy roots produced from different explants were transferred to antibiotic and hormone free ½ MS liquid and solid medium. They were maintained in flasks on orbital shaker at 25^0C at 100 rpm under continuous dark. 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.

PCR analysis of Hairy roots

PCR analysis of hairy roots was done to confirm the genetic transformation of *A.rhizogenes* and induction of hairy roots. The DNA from hairy roots, plasmid DNA from *A.rhizogenes* strain (positive control) and DNA from non transformed leaves (negative control) were used for PCR amplification. Plasmid DNA was extracted using the alkaline lysis method (Birnboim and Doly, 1979) and the DNA from hairy roots and non transformed leaves was extracted following the CTAB method (Doyle and Doyle, 1987). Polymerase chain reaction was carried out using rol B and rol C specific primers. The rol B and rol C primers were purchased from Xcelris genomics, Ahmedabad, India. The 5' primer sequence of *rol* B gene was TGGATCCCAAATTGCTATT CCTTCCACGA and 3' primer sequence was TTAGGCTT CTTTCTTCAGGTTTACTGCAGC. This amplified the DNA fragment of 780 base pair (bp) in DNA amplification.The 5' primer sequence of *rol* C gene was ATGGCTGAAGACGACCTGTT TTAGCC and the 3' primer sequence was GATTGAAAACTT GCAC. This amplified the DNA fragment of 540 base pair (bp) in DNA amplification. For this 50 ng plasmid DNA from *A.rhizogenes* and DNA from non transformed leaf tissues were taken as positive and negative controls respectively. DNA from hairy roots served as treatments. The primers involved in this experiment are given in **Table 1.**

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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 TGGATCCCAAATTGCTATTCCTTCCACGA Reverse TTAGGCTTCTTTCTTCAGGTTTACTGCAGC	780
Rol C	Forward ATGGCTGAAGACGACCTGTT Reverse TTAGCCGATTGAAAACTT GCAC	590

Purification of DNA

RNA was removed by treating the sample with DNase free RNase procured from Pure-gene, USA. Protein including RNase was removed by treating with chloroform: Isoamyl alcohol (24:1).

Quantification of genomic DNA using Nanodrop Spectrophotometer

The quality and quantity of extracted genomic DNA was checked by using Nanodrop spectrophotometer.

Dilution of DNA for PCR

The quantitated genomic DNA was diluted with TE buffer (10 mM Tris HCl, 1mM EDTA) to get a concentration of 20 ng/ul approximately, which can be directly used for PCR.

PCR mix

For checking the presence of *rol* B gene and rol C gene, the PCR amplification was done in a final reaction volume of 30 µL containing 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.

PCR conditions

After initial denaturation at 94^0C for 3 min, PCR was performed for 35 cycles at 94^0C for 30 sec, 55^0C for 30 sec and 72^0C for 1 min followed by a final extension at 72^0C for 7 min **.**

Gel Documentation

Following the amplification, the PCR products were loaded on 1.2% Agarose gel (Himedia, molecular grade), which was prepared in 1X TBE buffer containing 0.5 µg/ml of the Ethidium Bromide. The amplified products were electrophoresed for 3-3.5 hr at 100 V with cooling. After separation the gel was viewed under UV trans-illuminator and photographed by digital camera.

Preparation of the extract

The hairy roots were soaked in methanol (1mg/ml) for 12 h and centrifuged at 5000 rpm for 10 min. The residue was discarded and supernatant was taken and 20ul was injected into the HPLC system.

Preparation of stock solution

Standard spilanthol solution was prepared by dissolving 10 mg in 5 ml methanol and sonicated for 15 min. The solution was diluted up to 10 ml with methanol to obtain a concentration of 1mg/ml.

HPLC analysis of Hairy roots

The standard chemicals required for HPLC were procured from the Merck, Germany (HPLC grade Methanol (99.8% pure), acetonitrile and Milli Q water). Standard spilanthol (98% pure) was purchased from Natural Remedies Pvt. Ltd. (Bangalore, india).

The HPLC analysis was carried out in a Schimadzu HPLC system that consisted of UV detector, a binary pump, 20 ul injection loop and C-18 column. Calibration of the system was done by accurately weighing 0.01 gms caffeine (Merck, Germany) dissolved in 100 ml of HPLC grade water. 20ul of different concentrations made from the caffeine stock solution were injected through the C 18 column. The mobile phase consisted of 93% acetonitrile and 7 % Milli Q water with flow rate 0.5 ml/min was degassed before use. Detection was done at 273 nm.

Standard Spilanthol (20ul) was injected to carry out chromatographic separation on C18 column.

UV detection was performed at 273 nm and standard peak was obtained. Similarly the hairy roots were subjected to HPLC analysis. The percentage of spilanthol in hairy roots was calculated from the peak area obtained from the sample and comparing with the standard spilanthol concentration.

Results

Standardization of *A.rhizogenes* concentration for transformation

Agrobacterium rhizogenes with different O.D values were taken at 600 nm (0.2, 0.4, 0.6, 0.8 O.D) and tested for transformation percentage. Highest percentage of hairy root induction (75%) was observed at 0.6 O.D concentration of bacterium and the same has been selected for all the experiments.

To identify the highly responding explants for hairy root induction, different explants i.e nodal segments and leaf explants from field grown and *in vitro* regenerated plants were tested. All the explants resulted hairy roots production with varying frequencies.

To select the co-cultivation time for hairy root production, different time periods (24, 48, 72 hours) for incubation was tested. The hairy roots produced from different explants were white, slender, highly branched with many lateral branches **(Plate 1).**

Hairy root induction from nodal segments

To induce hairy roots, nodal explants from field grown and *in vitro* grown plants were tested. 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%).

Table 2 : 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**

Induction of Hairy roots from leaf segments

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 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 hrs co-cultivation period. This was followed by nodal explants of *in vitro* grown plants with 90% hairy root induction at 72 hrs of co-cultivation.

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

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

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 2)**. 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 showed negative geotropism and good amount of lateral branching **(Plate 3)**.

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.

Table 4 : Fresh and Dry Weights of Hairy roots induced from leaf explants of *Spilanthes acmella*

Days	**MS media**		**Half MS media**	
	Fresh Weight (g) Mean±SE	**Dry Weight (g) Mean±SE**	**Fresh Weight (g) Mean±SE**	**Dry Weight (g) Mean±SE**
Initial	0.2	-	0.20	-

7	0.54±0.02	0.12±0.01	0.75±0.03	0.21±0.002
15	2.85±0.01	0.25±0.04	3.41±0.02	0.30±0.001

Plate 2: Induction 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

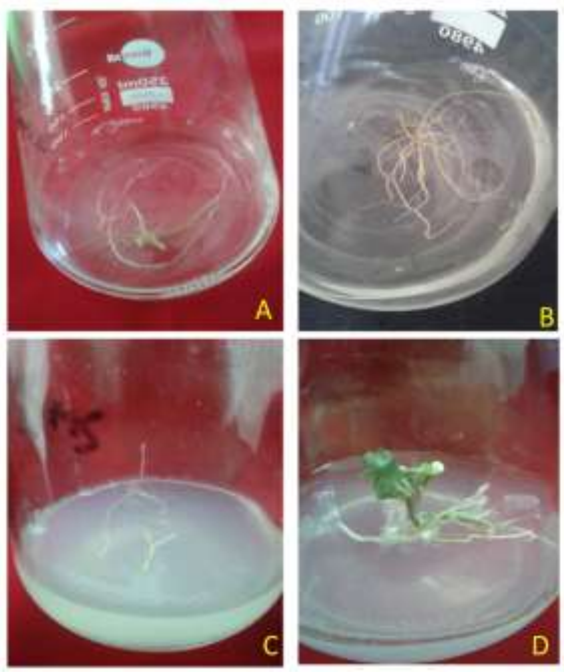
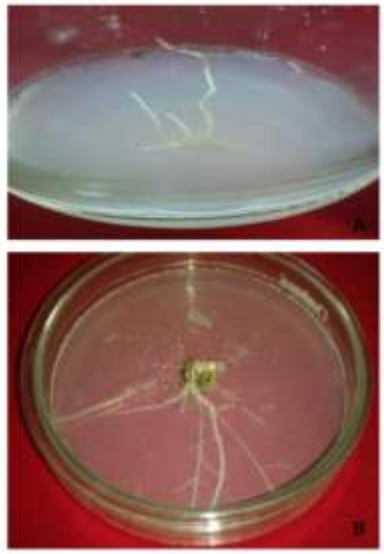


Plate 3 : Multiplication and growth of hairy roots induced from Leaf explants of *S.acmella*
A – Hairy roots showing negative geotropism
B – Hairy roots showing lateral branching



PCR analysis of Hairy Roots

Hairy roots were induced from different explants of *S. acmella* incubated with various concentrations of *Agrobacterium rhizogenes*. After cocultivation, the explants were inoculated onto basal media with antibiotic.

To confirm the genetic transformation of *Agrobacterium rhizogenes*, PCR analysis was done in the hairy roots induced from the *in vitro* and *in vivo* leaf explants and nodal segments.

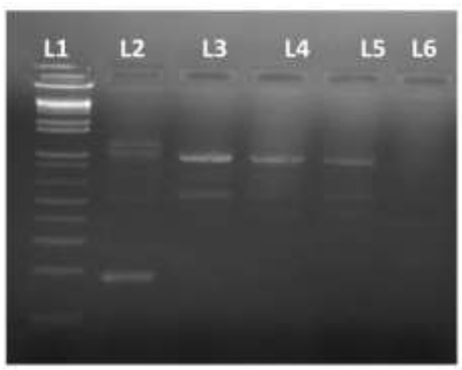
For PCR analysis, Genomic DNA was extracted (CTAB method- Doyle and Doyle, 1987) from hairy roots induced from different explants (*in vitro* and *in vivo* leaf explants and nodal segments) and control non transformed explants (*in vitro* and *in vivo* leaf explants and nodal segments). Plasmid DNA of *A.rhizogenes* was extracted using the alkaline lysis method.

Polymerase Chain reaction (PCR) amplification was done with rol B and rol C primers, which are responsible for T-DNA transfer into the explants. PCR products were run on 1.5% Agarose gel electrophoresis and the bands were scored.

PCR analysis of the transformed hairy roots showed amplification of DNA at 540 bp and 780 bp in hairy root samples and in positive control (plasmid) indicating the transformation event. But in negative control (non transformed leaf tissues), there was no amplification. This confirmed that hairy roots were developed because of integration of rol C and rol B genes of *Agrobacterium rhizogenes*. The integration has resulted in the amplification of DNA at 540 bp and 780 bp respectively in the transformed leaf explants **(Fig.1 and Fig.2)**.

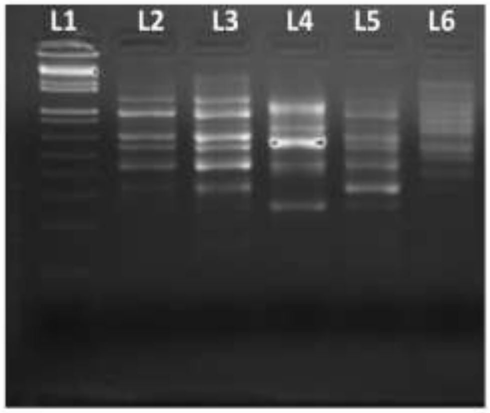
Hence, the PCR analysis has confirmed that the hairy roots were produced from the leaf explants due to the genetic transformation with *Agrobacterium rhizogenes.*

Fig 1: PCR Analysis of Hairy roots to confirm the genetic transformation with *A.rhizogenes* with rol B primers at 780 bp



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 of *A.rhizogenes* with rol C primers at 540 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)

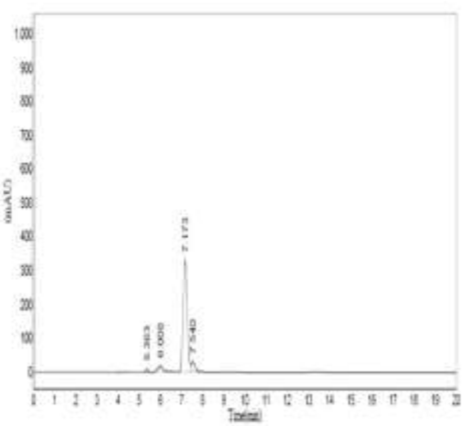
HPLC analysis of Hairy Roots

The quantification of spilanthol in hairy roots through HPLC analysis showed that the hairy root extract of *S.acmella* contained high amount of spilanthol content (0.134%) . The results of HPLC analysis and that of standard are shown in plate 4.

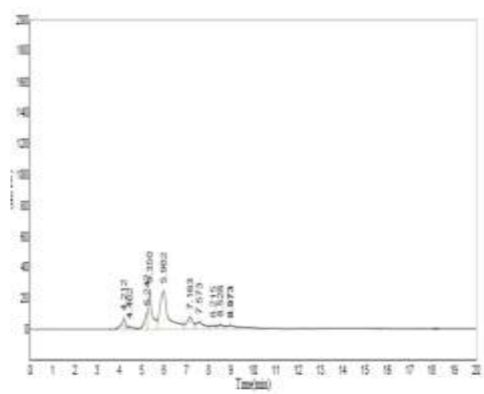
Plate 4 : Quantification of spilanthol from hairy roots of *S.acmella*

A - Spilanthol Standard

B - Spilanthol Concentration in Hairy roots (0.134%)



A



B

Discussion

Hairy root cultures offer a promise for high production of valuable secondary metabolites used as pharmaceuticals, pigments and flavors. Genetically transformed hairy roots obtained by infection of plants with *Agrobacterium rhizogenes* are suitable source for production of bioactive molecules due to their genetic stability and fast growth in culture media free of growth hormones (Shanks and Morgan, 1999). Integration of plasmid into host plant genome is stable which accounts for genetic stability of transformed root cultures (Dixit et al., 2012).

Research on hairy root production in the genus *Spilanthes* is still in its infancy. There is one report on the production of hairy roots of *Spilanthes paniculata* by infecting the cotyledons and hypocotyl segments with *A. rhizogenes* strains MTCC 2364 and MTCC 532 (Sheela et al., 2008). In this study, the nodal segments and leaf explants of *S.acmella* were infected *in vitro* with *Agrobacterium rhizogenes* strain MTCC 532 to establish cultures of transformed roots. Strain 532 is also effective in induction of hairy roots in *Plumbago rosea*, *Rubia tinctorum*, *Arachis hypogaea* and *Withania somnifera* hairy roots cultures in comparison to other strains (Yogananth and Basu, 2009; Ercan and Taskin, 1999; Karthikeyan et al., 2007; Doma et al., 2012). The present study is the first report of hairy root production from leaf and nodal segments of *S.acmella*.

Standardization of *A.rhizogenes* concentration for transformation and hairy root induction

The bacterium *Agrobacterium rhizogenes* (MTCC No. 532) was grown in Nutrient agar broth overnight and different concentrations were standardized for transformation and induction of hairy roots (0.2, 0.4, 0.6, 0.8 O.D). The percentage of transformation is 33% at 0.2 O.D, 50% at

0.4 O.D, 75% at 0.6 O.D and 55% at 0.8 O.D. Since the bacterium at the concentration of 0.6 O.D at 600 nm gave the highest transformation percentage (75%), this concentration of the bacterium was selected for all the experiments.This study is in accordance with many earlier studies such as *Arachis hypogaea*, (Liu et al., 2016), *Boerhaavia diffusa* (Sahu et al., 2013) in which maximum frequency of root induction was obtained with bacterial density of 0.6 OD at 600 nm.

Hairy root induction from nodal explants

Hairy roots were produced within 14-16 days from nodal segments, which are white, slender, with many lateral branches. 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.

For *in vitro g*rown plants, the nodal segments gave 90% hairy root induction at 72 hours of co-cultivation time.

Of the two explants, nodal segments from the *in vitro* grown plants showed good response (90%) than the field grown plants (85%). Similarly, there are earlier studies reporting hairy root induction from nodal segments in plants like *Withania somnifera* (Murthy et al., 2008), *Berberis aristata* (Brijwal and Tamta, 2015) and *Arnebia hispidissima* (Chaudhury and Pal, 2010).

Induction of Hairy roots from leaf segments

Leaf explants with and without petiole region from field grown and in *vitro* raised plants showed hairy root induction within 12-14 days of culture. For field grown plants, leaf segments with petiole, the best co cultivation time was observed to be 48 hours with 80% of hairy root induction and it is reduced to 35% with increasing the co-cultivation time to 72 hours.

For *in vitro* plants, leaf segments with petiole, the best co-cultivation time was observed to be 48 hours which gave 92% of hairy root induction and it is reduced to 55% on increasing the co-cultivation time to 72 hours. Co-cultivation period of 48 h was also found effective for *Glycyrrhiza glabra*, *Linum mucronatum* and *Artemisia annua* cultures (Mehrotra et al., 2008, Samadi et al., 2012; Giri et al., 2001).One of the important observation noticed in the present study is that the leaf explants without petiole region collected either from field grown and *in vitro* regenerated plants did not show any hairy root induction. Hairy root initiation from cut ends of petioles of leaf explants was witnessed earlier in *Pueraria phaseoloides* (Shi et al., 2003.) This may be due to difference in cell division and metabolism rate of various cells of explants to produce competent cells adjacent to wounded area for transformation and regeneration (Potrykus, 1990).

This study indicates that the type of explants and co-cultivation period influences the hairy roots production in *S.acmella.* This study is consistent with the hairy root induction study on *Berberis aristata* (Brijwal and Tamta, 2015).

Multiplication of hairy roots and fresh and dry weights of hairy roots

To multiply the hairy roots, induced roots were cultured on basal media without any hormonal concentration. They grew well in the media showed negative geotropism and good amount of lateral branching on solid MS medium. The induced hairy roots grew well in liquid MS medium when compared to solid MS medium. This is in accordance with the hairy root growth of *Pueraria phaseoloides* in which hairy roots grew more rapidly in liquid medium when compared to hairy roots in solid medium (Shi et al., 2003). The composition and type of the culture medium are known to affect the growth and proliferation of hairy roots (Huang et al., 2014).

To observe the growth and multiplication, the hairy roots induced from leaf explants were subcultured on MS and ½ MS liquid medium**.** The nature of the medium is one of the factors that will affect the root development (Suzuki et al., 1992).The hairy 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.

On MS medium, the fresh and dry weights of hairy roots were were 0.54 g and 0.021 g after 7 days of culture and increased to 2.85 g and 0.25 g after 15 days of culture. A maximum of 5.04 g and 0.8 g fresh and dry weights respectively was obtained when roots were cultured on MS medium without plant growth regulators after 30 days of culture.

On ½ MS medium, the fresh and dry weights of hairy roots were 0.75 g and 0.01 g after 7 days of culture and 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 without any plant growth regulators after 30 days of culture.

Of the two different media, more growth of the hairy roots was observed in MS half strength compared to MS basal media. These results suggested that a lower concentration of salts facilitated hairy root growth and biomasss accumulation.

Similarly, copious growth of hairy roots was observed in *Solanum xanthocarpum* on ½ MS liquid medium without hormones with doubling time of 7 days (Khatodia and Biswas, 2014). Similar results where ½ MS medium was found to be better than MS medium for hairy roots growth was observed in hairy root cultures of *Mirabilis himalaica* (Xiaozhong et al., 2015).

PCR analysis of Hairy Roots

Hairy roots were induced from different explants of *S.acmella* incubated with various concentrations of *Agrobacterium rhizogenes*.

To confirm the genetic transformation of *Agrobacterium rhizogenes*, PCR analysis of was done in the hairy roots induced from the leaf explants. For PCR analysis, Genomic DNA was extracted from non-transformed leaves, Plasmid DNA and hairy roots induced from leaves.

Polymerase Chain reaction (PCR) amplification was done with rol B and rol C primers responsible for T-DNA transfer. The rol genes (rolA, rolB, rolC and rolD) present in TL region of the Ri-plasmid have been identified as the inducer of hairy roots and biosynthesis of a variety of secondary metabolites (Palazon et al., 1998; Shkryl et al., 2008). As *A. rhizogenes* is able to transfer T-DNA of binary vectors, foreign genes can be easily inserted between TL and TR regions (Deblaere et al., 1987) and subsequently expressed in transformed roots with the aim of genetic manipulation of secondary pathways (Sharafi et al., 2013). Furthermore, the selection of transformants can visually be done through hairy root morphology, thus omitting the inclusion of antibiotic or herbicide genes as markers. Hence, *A. rhizogenes* mediated transformation enables the development of transgenic plants via marker-free selection through the use of hairy root morphology as the primary indicator of transformation (Christensen et al., 2008).

PCR products were run on Agarose gel electrophoresis and the bands were scored. PCR analysis of the transformed hairy roots showed amplification at 540 bp and 780 bp in hairy root samples and in positive control (plasmid) indicating the transformation event. but in negative control (non- transformed leaf tissues) there was no amplification. This confirmed that hairy roots developed because of rol C and rol B gene integration which resulted in their amplification at 540 bp and 780 bp respectively.

HPLC Analysis of Hairy roots

The methanolic extract of hairy roots showed significant amount of spilanthol content (0.134%).

The secondary metabolite levels of transformed root cultures are often comparable to or greater than that of intact plants (Sevon and Oksman-Caldentey 2002).

Conclusion

This study revealed that the induced hairy roots of *S.acmella* plant are rich in spilanthol content. The presence of spilanthol in hairy roots prove them to be a good alternative for the commercial production of spilanthol and can serve as a sustainable sources for spilanthol production. Therefore, switching from harvesting intact *S.acmella* plants to its hairy roots can be considered as an alternative tool for the efficient and continuous production of valuable spilanthol, which holds immense potential for pharmaceutical applications. This study is useful for scaling up of hairy roots of *S.acmella* in bioreactors for large scale production of spilanthol and can be exploited commercially. Hairy roots would be the best choice for metabolic engineering of the secondary metabolite pathways to enhance the accumulation and secretion of high value metabolites like spilanthol. Thus, they can be used as a continuous and standardized source of spilanthol in controlled conditions.

References

Birnboim H.C. and J.Doly, (1979). A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA. *Nucleic Acids Research*, 7:1513-1523.

Brijwal L. and Tamta S., (2015). *Agrobacterium rhizogenes* mediated hairy root induction in endangered *Berberis aristata* DC. *Springerplus.*4:443.

Sci., 7(2): 269-272.

Chaudhury A. and Minakshi P., (2010). Induction of Shikonin production in hairy root cultures of *Arnebia hispidissima* via *Agrobacterium rhizogenes* mediated genetic transformation. *Journal of crop science and Technology*, 13(2): 99-106.

Christensen B., Sriskandarajah S., Serek M. and Muller R., (2008). Transformation of *Kalanchoe blossfeldiana* with rol-genes is useful in molecular breeding towards compact growth. *Plant Cell Rep.* 27:1485–1495.

Deblaere R., Reynaerts A., Hofte H., Hernalsteens J.P., Leemans J. and Montagu M.V., (1987). Vectors for cloning plant cells. *Methods Enzymol.*, 153: 277–292.

Dixit A.K, Vaidya S. and Devaliya R., (2012). *Agrobacterium rhizogenes* MTCC 532 induced hairy hoot development in *Tephrosia purpurea* (L) and its effect on production of Tephrosin. *J.Sci.Res.* 4(3): 741-748.

Dobranszki J. and Silva da J.A.T., (2010). Micropropagation of apple – A review. *Biotechnol. Adv.*, 28: 462- 488.

Doma M., Abhayankar G., Reddy V.D. and Kavi Kishor P.B., (2012). Carbohydrate and elicitor enhanced withanolide (withaferin A and withanolide ,) accumulation in hairy root cultures of *Withania somnifera* (L.) *Indian J Exp Biol.* 50:484–490.

Doyle J.J. and Doyle, J.L., (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, 19:11-15.

Ercan A.G. and Taskin K.M., (1999). .*Agrobacterium rhizogenes*-mediated hairy root formation in some *Rubia tinctorum* L. population growth in Turkey. *Turk J Botany* 23:373–377.

Giri A., Giri C.C., Dhingra V. and Narasu M.L., (2001). Enhanced podophyllotoxin production from *Agrobacterium rhizogenes* transformed cultures of *Podophyllum hexandrum*. *Nat Prod Lett* ., 15:229–235.

Guillon S., Guiller J.T., Pati P.K., Rideau M. and Gantet P. (2006). Hairy root research: recent scenario and exciting prospects. *Curr Opin Biotech.*, 9:341-346.

Huang S.H., Vishwakarma R.K., Lee T.T., Chan H.S and Tsay H.S. (2014). Establishment of hairy root lines and analysis of iridoids and secoiridoids in the medicinal plant *Gentiana scabra. Bot Stud.* 55(1):17.

Karthikeyan A., Palanivel S., Parvathy S. and Bhakyaraj R., (2007). Hairy root induction from hypocotyl segments of groundnut (*Arachis hypogaea L.*) *Afr J Biotechnol.*, 6(15):1817–1820.

Khatodia S. and Biswas K., (2014). A comparative study of Hairy Root Culture induction efficiency in four medicinally important plants using *Agrobacterium rhizogenes*. *International Journal of Current Microbiology and Applied sciences.* 5(3): 625-633.

Liu S., Su L., Shuai L., Zeng X., Zheng D., Hong L., and Li L., (2016). *Agrobacterium rhizogenes*-mediated transformation of *Arachis hypogaea*: an efficient tool for functional study of genes, *Biotechnology & Biotechnological Equipment*, 30:5, 869-878.

Mehrotra S., Kukreja A.K., Khanuja S.P.S. and Mishra B.N. (2008). Genetic transformation studies and scale up of hairy root culture of *Glycyrrhiza glabra* in bioreactor. *Electron J Biotechnol.* 11(2):1–7.

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

Murthy H.N., Dijkstra C., Anthony P., White D.A., Davey M.R. and Power J.B., (2008). Establishment of *Withania somnifera* hairy root cultures for the production of withanolide *A. J. Integr.* Plant Biol. 50, 975–981.

Nelofar G.N. , Tareq A., Wani M. S. , Abid W., and Syed Naseer S. (2016). *Spilanthes acmella* an endangered medicinal plant - its Traditional, Phytochemical and Therapeutic properties – An overview . *International Journal of Advanced Research*, 4(1): 627 – 639.

Ono N.N. and Tian L., (2011). The multiplicity of hairy root cultures: prolific possibilities. *Plant Sci*.; 180: 439-446.

Palazon J., Cusido R.M., Roig C. and Pinol M.T., (1998). Expression of the rolC gene and nicotine production in transgenic roots and their regenerated plants. *Plant Cell Rep.* 17: 384–390.

Pati P.K., Rath S.P., Sharma M., Sood A. and Ahuja P.S., (2006). *In vitro* propagation of rose-a review. *Biotechnol. Adv.,* 24: 94–114.

Potrykus I., (1990). Gene transfer to plants: assessment and perspectives. *Physiol. Plant,* 19: 206–209.

Sahu L., Jena S., Swasti S., Sahoo S., and Chand P.K., (2013). *Agrobacterium rhizogenes*-mediated transformation of a multi-medicinal herb, *Boerhaavia diffusa* L.: optimization of the process and anti-microbial activity against bacterial pathogens causing urinary tract infections, *Frontiers in Life Science*, 7:3-4, 197-209.

Samadi, A.J. Carapetian R. Heidari. M. Jafari and Hassanzadeh A.G. (2012). 'Hairy Root induction in *Linum mucronatum* ssp. mucronatum, an anti-tumor lignans producing plant, *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, 40(1): 125-131.

Sevon N and Oksman-Caldentey K.M., (2002). *Agrobacterium rhizogenes*-mediated transformation: root cultures as a source of alkaloids. *Planta Med*, 68:859–868.

Shanks J.V. and Morgan M. (1999). Plant 'hairy root' culture. *Current Opinion Biotechnology*, 10: 151- 155.

Sharafi A., Hashemi Sohi H., Mousavi A., Azadi P., Razavi K.H. and Otang Ntui V., (2013). A reliable and efficient protocol for inducing hairy roots in *Papaver bracteatum. Plant Cell Tiss Organ Cult*.,113: 1-9.

Sheela C., Sharma H.P. , Ramesh C. and Jha S. (2008). *Agrobacterium rhizogenes* mediated genetic transformation of *Spilanthes paniculata* (DC.) Jansen. *International Journal of Plant Sciences*, 3(2) : 471-476.

Shi H.P., Quan H. and Spiros K. (2003). Induction of hairy roots of *Pueraria phaseoloides* and its culture in liquid and solid medium. *Sheng Wu Gong Cheng Xue Bao.*19(3): 307-11.

Shkryl Y.N., Veremeichik G.N. and Bulgakov V.P., (2008). Individual and combined effects of the *rol*A, B, and C genes on anthraquinone production in *Rubia cordifolia* transformed calli, *Biotechnol. Bioeng.*, (100): 118–125.

Suzuki S., Yoshiki T., Yamasaki N., and Yoshizaki M. (1992), Rapid propagation of *Stephania cephalantha* Hayata by tissue culture. *Jpn. J. Breed*. 42:769 – 777.

Xiaozhong L., Quan, H., Xia, X., Yin,W., (2015). Establishment of hairy root cultures and analysis of rotenoid in Tibetan medicinal plant *Mirabilis himalaica* . *Plant Omics Journal*, 8(4):335-339.

.**Yogananth N. and Basu M.J.** (2009). TLC method for the determination of plumbagin in hairy root culture of *Plumbago rosea* L. *Global J Biotech and Biochem.;* 4 (1):66–69.