Induction of hairy roots through the mediation of four strains of *Agrobacterium rhizogenes* on five host plants

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Induction of hairy roots by four strains of *Agrobacterium rhizogenes*: ATCC 15834, A4, WC and WR were studied in five plants, *Ipomoea batatas*, *Solenostemon rotundifolius*, *Vigna vexillata*, *Pachyrhizus erosus* and *Canavalia* species. Among the five plants selected for transformation and induction of hairy roots, *P. erosus* was found resistant to all the four bacterial strains. Similarly, one strain, WR also failed to induce hairy roots in all the plants. However, all the strains exhibited good growth dominated by 15834 grown in YEB medium. Hairy roots were induced from the cotyledons, hypocotyls, stem cuttings and *in vitro* plants of *I. batatas* through the transformation of 15834 and A4 strains. *S. rotundifolius* and *V. vexillata* were susceptible to the strains of A4, 15834 and WC. *Canavalia* sp. was resistant to WR and WC strains, but was susceptible to A4 and 15834. It was for the first time that hairy roots were initiated from *S. rotundifolius*, *V. vexillata* and *Canavalia* sp. The variation observed in the time of induction of hairy roots (incubation period) by a single strain (15834) in different plant species, suggests that the plant has also a definite role in determining the incubation period. Among the four strains of *A. rhizogenes*, 15834 was found to be the most efficient in transformation and initiation of hairy roots, with the shortest minimum incubation period and dominant growth in YEB medium. *A. rhizogenes* is a well known plant pathogen, which produces “hairy root disease” in susceptible plants. On modified MS medium, cotyledon explants were superior to hypocotyls. The hairy roots transformed by *A. rhizogenes* strain 15834 on *I. batatas*, *V. vexillata* and *Canavalia* sp. were also morphologically different.

**Keywords:** *Agrobacterium rhizogenes*, *Ipomoea batatas*, *Pachyrhizus erosus*, *Solenostemon rotundifolius*, *Vigna vexillata*, *Canavalia* sp., hairy roots

**Introduction**

The roots of almost all the higher plants are known to form mutualistic symbiosis with fungi of the order Glomales (*Zygomycetes*), which are known as mycorrhizas[^1] and this symbiosis is increasingly being recognized as an important and integral part of natural ecosystem throughout the world. The vesicular arbuscular mycorrhizal fungus (VAMF)-plant association is a mutualistic beneficial event, in which the plant supplies the fungus with carbon from its fixed photosynthates, while the fungus assists plants in its uptake of phosphorus, copper, zinc and other elements, which the plant cannot absorb for its growth and fitness[^3]. It is well known that mycorrhizal activity in plants, results in greater yield, nutrient accumulation and reproductive success[^4,5]. To meet the increasing demand for food globally, the crop production is to be increased. The extensive use of VAMF is one of the innovative technologies to increase crop production. The fungi themselves are good biofertilizers[^6]. However, due to the obligate symbiotic nature of the fungus, the commercial production of pure inoculum to meet the demands of the farmers is not possible[^7]. Moreover, the source of inoculum production is also limited. The hazardous effects of indiscriminate use of chemical fertilizers and other chemical agents can cause immeasurable damage to the soil and ecosystem. The relevance and importance of VAM, as an eco-friendly biofertilizer, has been well-documented and gaining momentum in India and abroad.

The present method of production of inoculum in sand or vermiculite, using proliferating plant roots is defective and has got its own limitations of high risk of contamination from the soil, sand, vermiculite and water. It is also time consuming and only small

[^1]: Zygomyces
[^2]: Glomales
[^3]: Mycorrhizas
[^4]: VAMF
[^5]: Plant association
[^6]: Fungi
[^7]: Inoculum
quantities of inoculum can be prepared. To overcome these difficulties, VAMF can be cultivated in genetically transformed hairy roots. Hairy roots are initiated by infecting host plants with *A. rhizogenes*, which is a plant pathogen, capable of inducing hairy roots in a variety of plants². Hairy root induction is the result of the integration of root inducing plasmid, (PRi) T-DNA into the plant genome and its subsequent expression⁶. This is characterized by a massive production of adventitious roots, popularly known as “hairy roots”¹⁰ in susceptible plants. Hairy roots are amenable for scale-up in large bioreactors and hence offer better advantages compared to cell cultures, and therefore, are widely preferred for the production of various chemical substances that are akin to root systems. Hairy roots can also produce recombinant proteins from transgenic roots, and thereby hold immense potential for the pharmaceutical industry¹¹,¹².

Considerable work has been done to produce pure propagules of VAMF, using hairy roots in many countries, however, work done in India to produce VAMF propagules using hairy roots is meagre. Therefore, we attempted to develop technology for the production of hairy roots from five plant species (through four strains of *A. rhizogenes*) for cultivation of VAMF-*Glomus microparum* var. *microcarpum*. In this study, attempt has been made to select the most efficient strain of *A. rhizogenes* for hairy root induction in host plants.

**Materials and Methods**

**Bacterial Cultures and Growth Medium**

The strains of *Agrobacterium rhizogenes* ATCC 15834 and A4 obtained from Dr Usha Mukundan, Head, Plant Biotechnology Laboratory, Ramniranjan Jhunjhunwala College, Ghatkopar (West), Mumbai and two wild strains WR and WC from National Chemical Laboratory, Pune, India were used in the present study.

**Bacterial Culture Medium**

The bacterial culture media selected for assessment were Nutrient Broth, Luria Bertani Broth¹³, Yeast Extract Broth¹⁴ and AB-Biotin Broth¹⁵. A loopful of bacterial cultures were inoculated into 250 mL conical flasks containing 50 mL sterilized broth of the above mentioned media. The flasks were placed in an incubated shaker at 100 rpm for 24 h at 24°C, after which the culture broths were collected and optical density was measured at 600 nm using a spectrophotometer (Genesis 2PC).

**Plant Material**

*Ipomoea batatas* (sweet potato), *Solenostemon rotundifolius* (Chinese potato), *Pachyrhizus erosus*, *Vigna vexillata* and *Canavalia* sp. (sword bean) were the five plant species selected for transformation and induction of hairy roots. The *in vitro* raised plants, stem cuttings, cotyledons and hypocotyl tissues of *I. batatas*, stem cuttings and *in vitro* plants of *S. rotundifolius*, cotyledons and hypocotyls of *P. erosus*, *V. vexillata* and *Canavalia* sp. were used as explants for transformation and induction of hairy roots. *I. batatas*, *P. erosus*, *V. vexillata* and *Canavalia* sp. were raised from quality seeds, which were surface sterilized with 0.01% mercuric chloride and 1% sodium hypochlorite solutions.

**Hairy Root Culture Medium**

Murashige & Skoog¹⁶ (MS) medium was modified to make it richer in vitamin contents and used as growth medium for induction of hairy roots from explants.

**Gene Transfer and Establishment of Hairy Root Cultures**

The plant parts, such as, stem cuttings, hypocotyls segments, *in vitro* raised plants and cotyledons were punctured with hypodermic needles attached to a syringe containing an overnight culture of *A. rhizogenes*. One hundred explants were used in each case. The explants were co-cultivated with overnight bacterial cultures and incubated at 24°C for two d in dark. Then, the explants were transferred to modified fresh MS medium containing antibiotics to eliminate bacterial growth. Successive transfers were made to make the incubating explants free from *Agrobacterium* and incubated under fluorescent light up to 25 d for hairy root induction¹⁷.

The excess of bacterium present in the hairy roots was eliminated using the antibiotics, cefotaxime and ampicillin. A combination of ampicillin and cefotaxime in the ratio 200:200 mg/L was almost effective, however, a ratio of 250:250 mg/L was very satisfactory in eliminating the bacterium.

**PCR Analysis**

Detection of the introduced gene was done by the technique described by Yu Mei et al¹⁸. The two genes, *rol B* and *vir D1* play a prominent role in transformation. DNA extraction was performed using Qiagen DNasey plant maxi kit and 200 mg of hairy roots from *I. batatas*, *V. vexillata* and *Canavalia* sp. were taken for DNA extraction. The samples were crushed in liquid nitrogen and the steps prescribed in
the Qiagen DNeasy plant maxi kit instruction manual were followed.

The rol B and vir D1 primers were supplied by Qiagen Operon, Germany. For rol B gene the 5’ primer sequence TGGATCCCAAATTGCCTTT CCTTCCACGA and 3’ primer sequence TTAGGCCT CTITTCTCAGGTTA CTGCA were used. In PCR amplification, a minimum of 780 base pair (bp) fragment could be detected. The 5’ primer sequence of vir D1 gene was ATGGTCGCAAGGACGTAAGCCCA and 3’ primer sequence was GGAGTC TTTTCAGCATGGAGC. This amplified the DNA fragment of 450 bp from the DNA samples.

For checking the presence of rol B gene, the PCR amplification was done in a final reaction volume of 30 μL containing 1× PCR buffer (Promega), 1.5 mM MgCl₂, 1 mM each of the four dNTPs, 1.25 U of Taq polymerase (Promega) and 0.5 mM each of 5’ and 3’ primers with 3 μL of the total DNA from transformed roots. After initial denaturation at 94°C for 3 min, PCR was performed for 35 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min followed by a final extension at 72°C for 7 min. The same conditions were also used for the detection of vir D1 gene.

Completed reactions were run in a 2% agarose gel along with molecular marker. Modified TAE (40 mM Tris acetate pH 8, Na₂EDTA 0.1 mM) buffer was used for electrophoresis. Ethidium bromide stained gels were analyzed in BioRad FlourS multi imager using the quantity one computer software program.

**Statistical Analysis**

Comparison of the effect of *A. rhizogenes* 15834 on five host plants was subjected to ANOVA. As the p-value is close to zero, the null hypothesis is rejected. Hence, there is a very significant difference between the five host plants on the action of *A. rhizogenes* 15834 for inducting hairy roots.

**Results**

All the strains of *A. rhizogenes* showed excellent turbidity in YEB medium and this was selected for further cultivation of this bacterium. The transformation and hairy root initiation abilities of the A4, 15834, WR and WC strains of *A. rhizogenes* are presented in Table 1. Two strains, 15834 and A4

<table>
<thead>
<tr>
<th>Host plant</th>
<th>A. rhizogenes strains</th>
<th>Hairy roots</th>
<th>Minimum time taken (d)</th>
<th>Normal% of hairy root induction</th>
</tr>
</thead>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>C</td>
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<tr>
<td>Ipomoea batatas</td>
<td>15834</td>
<td>Initiated</td>
<td>8</td>
<td>31</td>
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<td></td>
<td>A4</td>
<td>Initiated</td>
<td>12</td>
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<tr>
<td></td>
<td>WR</td>
<td>Not initiated</td>
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<tr>
<td></td>
<td>WC</td>
<td>Not initiated</td>
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<tr>
<td>Solenostemon rotundifolius</td>
<td>15834</td>
<td>Initiated</td>
<td>10</td>
<td>-</td>
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<td></td>
<td>A4</td>
<td>Initiated</td>
<td>14</td>
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<tr>
<td></td>
<td>WR</td>
<td>Not initiated</td>
<td>-</td>
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<td></td>
<td>WC</td>
<td>Initiated</td>
<td>14</td>
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<tr>
<td>Pachyrrhizus erosus</td>
<td>15834</td>
<td>Not initiated</td>
<td>-</td>
<td>Nil</td>
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<tr>
<td></td>
<td>A4</td>
<td>Not initiated</td>
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<tr>
<td></td>
<td>WR</td>
<td>Not initiated</td>
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<td></td>
<td>WC</td>
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<tr>
<td>Vigna vexillata</td>
<td>15834</td>
<td>Initiated</td>
<td>10</td>
<td>28</td>
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<td></td>
<td>A4</td>
<td>Initiated</td>
<td>16</td>
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<td></td>
<td>WR</td>
<td>Not initiated</td>
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<td></td>
<td>WC</td>
<td>Initiated</td>
<td>14</td>
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<tr>
<td>Canavalia sp.</td>
<td>15834</td>
<td>Initiated</td>
<td>12</td>
<td>27</td>
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<td>A4</td>
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<td>WR</td>
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<td>WC</td>
<td>Not initiated</td>
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Abbreviations ‘-’ Explant material not used, C-cotyledons, IP-*in vitro* plants, H-hypocotyls and SC-stem cuttings.
succeeded in initiating hairy roots in *I. batatas*. The minimum period required for initiation of hairy roots by 15834 and A4 in *I. batatas* was 8 d and 12 d, respectively. The other two strains, WC and WR failed to induce hairy roots in sweet potato. In *S. rotundifolius*, besides 15834 and A4, WC strain also induced hairy roots. These three strains of *A. rhizogenes* also induced hairy roots in *V. vexillata* (15834 needed 10 d, WC 14 d and A4 16 d for initiation of hairy roots). In *Canavalia* sp., only 15834 (in 12 d) and A4 (in 16 d) were successful in hairy root induction. *P. erosus* was not susceptible to any of the strains. WR also failed to induce hairy roots in all the five plants.

The growth medium is an important component of hairy root induction process. The MS basal medium was modified by: (i) excluding MS vitamins and including B5 vitamins (Myoinositol, 100 mg/L; Nicotinic acid, 1 mg/L; Pyridoxin HCl, 1 mg/L and Thiamine HCl, 1 mg/L); (ii) reducing sucrose-content from 30 g/L to 15 g/L; and (iii) adding either 0.5 g/L Cysteine or 0.3 g/L polyvinylpyrrolidone as an antioxidant.

The optimal medium requirements were standardized for *I. batatas*, *V. vexillata* and *Canavalia* sp. The basal MS medium or modified MS medium were not found suitable for the growth of the hairy roots of *S. rotundifolius* beyond 10-14 d. The optimum requirements for *I. batatas* were 1/5 strength of modified MS medium, 0.5 g/L cysteine HCl, pH of 5.7-6.0 and temperature of 24°C. One forth strength of modified MS medium 0.5 g/L cysteine HCl, pH of 5.7 and temperature of 25°C were ideal for *V. vexillata*. Half strength of modified MS medium 0.3 g/L polyvinylpyrrolidone, pH of 5-6 and temperature of 24°C were suitable for *Canavalia* sp.

The hairy roots were grown in liquid and solid modified MS media and sub cultured every 3 weeks. The solid medium was fortified with 0.8% phytagel (supplied by Sigma Corporation, USA). Adequate quantities of axenic cultures of hairy roots were raised by excising one root tip (1 to 3 cm long) each, from *I. batatas*, *V. vexillata* and *Canavalia* sp. and grown separately in liquid medium. The hairy roots of all the three species were morphologically different, plagiotropic, fast growing and produced lot of lateral branches (Fig. 1). In most cases, tumours were first produced from the explants of *I. batatas* and *Canavalia* sp. and then hairy roots emerged; however, *V. vexillata* produced very few tumours.

The hairy roots of *I. batatas* were white, slender, branched and brittle, which subsequently changed to light yellow. They produced several root hairs. The hairy roots of *Canavalia* sp. were thick and produced lot of lateral branches. Root tips were white and basal portion was brown. The hairy roots of *V. vexillata* were thin, soft, highly branched with several lateral branches. Newly formed hairy roots were white, which later became dark brown.

Hairy root cultures were identified both by morphological and genetic markers. The important morphological markers included profusion of rapid growth, lateral branching and plagiotropism (negatively geotropic) while the genetic markers included *vir* D1 and *rol* B, responsible for T-DNA transfer and hairy root initiation. These markers were also used to confirm bacterial gene transfer\(^9,15\). The *rol* B genes were detected from the transformed root DNA using PCR analysis. The *vir* D1 genes were not found in the hairy root DNA and this confirmed the absence of *Agrobacterium* residual genes in the transformed tissues (Fig. 2).

**Discussion**

The present investigation indicated that all the four bacterial media, in which the strains of *A. rhizogenes* were grown, supported their growth to varying
degrees. As all the strains exhibited excellent growth in YEB, it was selected as the suitable medium for maintaining cultures of *A. rhizogenes* strains.

Numerous strains or isolates of *A. rhizogenes* exist, which induce hairy roots in different plants and hence are utilized for the production of mycorrhizal inoculum, as well as for the extraction of valuable metabolites. In the hairy root induction process, the selection of *A. rhizogenes* strains, the plant species (and their explants) and the growth medium are important components. *A. rhizogenes* has four types of strains, the agropine, mikimopine, mannopine and cucumopine. The 15834 and A4 are agropine strains and they are the most often used strains owing to their strongest induction ability. Hairy roots were successfully initiated from four out of the five plants. *P. erosus* was resistant to all the four *A. rhizogenes* strains. Similarly, the other four plants were also non-responsive to the infection of the WR strain. *I. batatas* and *Canavalia* sp. were susceptible to 15834 and A4 strains and were resistant to WR and WC strains. At the same time, the Chinese potato, *S. rotundifolius* and *V. vexillata* were susceptible to three strains, A4, 15834 and WC. Except in *P. erosus*, A4 and 15834 strains succeeded in initiating hairy roots in all the other four plants. The strain 15834 was selected as the best strain for hairy root induction, because of its vigorous growth in YEB medium and lowest hairy root initiation (incubation) period (8 d) in *I. batatas*. The incubation period for hairy root initiation in *S. rotundifolius* and *V. vexillata* was 10 d and in *Canavalia* sp. 12 d after infection with 15834 strain. The strain A4 initiated hairy roots in *I. batatas*, *S. rotundifolius* and *Canavalia* sp. 12, 14 and 16 d, respectively after the infection. WC strain induced hairy roots after 14 d in *S. rotundifolius* and *V. vexillata."

Table 1 indicates that the plant has also an important role (as in the case of *A. rhizogenes* strains) in determining the hairy root induction period. Strain 15834 infected four susceptible plant species (*I. batatas*, *S. rotundifolius*, *V. vexillata* and *Canavalia* sp.) in 8 d in *I. batatas*, 10 d in *S. rotundifolius* and *V. vexillata*, and 12 d in *Canavalia* sp. It is reported that the pathogenic strain of *A. rhizogenes* carries a large plasmid, the root inducing TR-DNA, a portion of which is transferred to the plant cell, which integrates into the nuclear genome of the infected plant cell. PCR results confirmed the transfer and integration of rol B genes into the host plants. It is also known that the portion of the integrated TR-DNA (which is actually part of genetic material of the bacterium) carries with it, genetic information for coding several compounds (auxins, cytokinins, etc.), which vary from plant to plant. This variation in the compounds produced, explains why the incubation period of the hairy root initiation period varied, when the strain 15834 infected the four susceptible plant species (*I. batatas*, *V. vexillata*, *S. rotundifolius* and *Canavalia* sp.).

Expanded leaf petioles of *I. batatas*, leaves, shoot tips, cotyledons, hypocotyl tissues, *in vitro* plants, and stem cuttings of different plants were used as explants for initiating hairy roots by different workers. Hairy roots were induced from the expanded leaves of *I. batatas* by Nishiyama and Ymakawa using A13 strain after an incubation period of four weeks. At the same time, Otani et al. induced hairy roots from *I. batatas* in 7 d. Wide variations in transformation and hairy root induction percentage exist among the susceptible host plants. In sweet potato, the cotyledon explants produced 31% hairy roots, followed by *in vitro* plants 30%, hypocotyls 26% and stem cuttings 25% (Table 1). In *V. vexillata*, cotyledons and hypocotyl explants exhibited 28% and 24% of hairy root initiation frequency, respectively. *Canavalia* sp. also showed a higher hairy root initiation frequency of 27% in cotyledon and 21% in hypocotyl explants and in *S. rotundifolius* 20% each of *in vitro* plants and stem cuttings produced roots. The cotyledon explants were maximal explants in *I. batatas*, *V. vexillata* and *Canavalia* sp.

Giri et al. found that the hairy root induction efficiency of cotyledons was significantly higher than that of hypocotyls in *Artemisia annua*. Xu et al. have also observed that the cotyledon explants of *Isatis indigotica* were superior to hypocotyl explants and A4.
strain was the most suitable while comparing R1601 and 15834 in inducing hairy roots. Many investigators preferred high salt media like MS or LS for hairy root induction, often amending them suitably. In the present investigation, a few alterations were also made in the composition of MS basal medium. The sucrose content was reduced from 20 g/L to 15 g/L, excluding MS vitamin contents and including B5 vitamins to make the medium richer in vitamins. Either 0.5 g/L cysteine or 0.3 g/L polyvinylpyrrolidone was incorporated as an antioxidant to prevent oxidative browning. L-cysteine has the ability to significantly increase the transformation efficiency in cotyledonary node cells of soybean. During the investigation, we observed that the hairy roots of I. batatas excreting cysteine along with other amino acids in the media. Vanhala et al. have observed that Linsmaier and Skoog medium (LSO) supplemented with AS 50 μm [LSO+AS (acetosyringone)] was better than B50 medium supplemented with AS 50 μm (B50+AS). According to Xu et al. among MS, 1/2 MS, B5 and Whites media, MS and 1/2 MS supplemented with 30 g/L maltose were superior. The MS medium modified by the present authors was suitable for root induction and growth of hairy roots of I. batatas, V. vexillata and Canavalia sp. Even though, hairy roots were successfully initiated from S. rotundifolius, they did not grow beyond 10-14 d.

Hairy roots of V. vexillata, and Canavalia sp. initiated through the mediation of the strain 15834 were grown successfully in MS liquid medium for the first time. Hairy roots of I. batatas were induced earlier by Otani et al., Nishiyama and Yamakawa, Chandran and Potty. The hairy roots initiated by different bacterial strains exhibited different morphologies, which is caused by the different plasmids harboured by the infecting strains. The strain 15834, infection in the host plants of I. batatas, V. vexillata, S. rotundifolius and Canavalia sp., produced morphologically different types of hairy roots.

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