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First Report of *Agrobacterium rhizogenes*-induced Hairy Root Formation in *Selaginella bryopteris*: a Pteridophyte Recalcitrant to Genetic Transformation

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HIGHLIGHTS

- *A. rhizogenes* (strain LBA1334) co-cultivation with explants (root, rhizophore, stem portion near the root, and stem with intact fronds) of *S. bryopteris* led to induction of hairy roots after 6 days of infection in case of 48 h co-cultivation only.
- Out of different media like MS, SHFR (Stage Hog Fern Root) and KNOP's, SHFR showed good response in transformation as well as propagation.
- PCR with *rol* A and *vir* C gene specific primers confirmed the Ri T-DNA integration in induced hairy roots.
- Addition of TDZ (2 mg/L) and Bevistin (0.1%) in SHFR media enhanced the transformation efficiency.

Abstract: we report *A. rhizogenes*-induced hairy root formation in *S. bryopteris*, a medicinally and commercially important plant. *A. rhizogenes* strain LBA1334 co-cultivated with explants (root, rhizophore, stem portion near the root, and stem with intact fronds) for 24 and 48 h after transformation for induction of hairy roots. The induction of hairy root was observed after 6 days of infection in case of 48 h co-cultivation only. PCR with *rol* A and *vir* C gene specific primers confirmed the induced hairy roots were due to Ri T-DNA integration and not due to contaminating *A. rhizogenes*. The root network as explants showed the maximum transformation efficiency. We tested different media like MS, SHFR (Stage Hog Fern Root) and KNOP's during transformation for hairy root induction. The SHFR based media showed good response in transformation as well as propagation. Further, transformation efficiency was enhanced by addition of TDZ (2 mg/L) and Bevistin (0.1%) in SHFR media. The present work would be helpful in hairy roots-based *in vitro* production of secondary metabolites and on aspect of functional genomics of *S. bryopteris*.

Keywords:*Selaginella bryopteris;* hairy roots; *Agrobacterium rhizogenes.*

INTRODUCTION

The hairy roots of different plants are being harnessed for the *in vitro* production of valuable phyto-chemicals of medicinal and commercial importance and for wide range of uses such as pharmaceuticals, pesticides, cosmetics, food additives etc. The hairy root culture (HRC)-based *in vitro* productions were reported for bioactive molecules such as shikonins, azadirachtin, camptothecins, harpagide, ginsenosides, paclitaxel, hyoscyamine and scopolamine [1-5]. Hairy root formation is induced by *Agrobacterium rhizogenes* and it often grows comparatively as fast as or faster than plant cell cultures [6,7]. Many valuable secondary metabolites are synthesized in roots in vivo, and often synthesis is linked to root differentiation [7]. HRCs have been successfully employed also in cases where secondary metabolites accumulate only in the aerial part of an intact plant. HRC is promising for *in vitro* production of metabolites; it could be more biosynthetically efficient as compared to their mother plants [8]. Several metabolites irrespective of their origin or site of production, such as lawsone and artemisinin generally accumulates in the aerial part of the plant, but these are also produced in hairy roots [9,10]. It is reported that the plants producing high levels of secondary metabolites usually generate high-producing cell lines [11]. The preference for hairy roots is due to their genetic stability and faster growth rate, ability to produce secondary metabolites over successive generations without losing genetic or biosynthetic stability [12]. High branching and high growth rate of these roots suits for commercial up-scaling even in bioreactors [13].

Selaginella species are enriched with bioactive molecules such as flavonoids, biflavonoids, lignans, selaginellins, alkaloids, terpenoids and other phenolics [14,15]. *Selaginella bryopteris* (L.) Baker (family, Selaginellaceae) is one of the *Selaginella* species widely distributed in India and being used as folklore medicine since time immemorial [16-18]. To date no effort has been made for optimization of genetic transformation for hairy root induction in *S. bryopteris*. As the *Agrobacterium*-mediated transformation methods are not very successful in pteridophytes, deciphering functionality of genes required heterologous system such as *Arabidopsis thaliana*, *Escherichia coli* and *Saccharomyces cerevisiae*. Previously, *A. tumefaciens*-mediated stable transformation of two ferns (pteridophytes), *Pteris vittata* and *Ceratopteris thalictroides* were reported [19], with constructs containing the *P. vittata* actin promoter driving a GUS Plus reporter gene that led to recovery of transgenic sporophytes.

Earlier we initiated molecular works on *S. bryopteris* and reported RNA-Seq based transcriptome analysis and cloning and characterization of MYB transcription factors genes [20,21], and also evaluated anti-microbial properties of this important medicinal plant [22]. To the best of our knowledge, this is the first report on *A. rhizogenes*-mediated genetic transformation for induction of hairy roots in *S. bryopteris* or any other pteridophyte. In view of medicinal and commercial importance of S. bryopteris, the efficient and stable *in vitro* production method is highly desired. Therefore, there is good prospect of HRC for *in vitro* production of flavonoids/biflavonoids in *S. bryopteris* and for use in functional genomics.

MATERIALS AND METHODS

Plant material and culture

S. bryopteris plants were collected from Hills near Sheikhpura town, Bihar, India and maintained in pot containing soil, sand, compost (1:1:1) ratio in the garden of Bihar Agricultural University, Sabour, Bhagalpur, Bihar (India). Explants such as rhizophore, stem and fronds of S. bryopteris were cut properly and washed with tap water till no soil or dusts are visible. Further, explants were treated with Bevistin (0.5%) and kept on incubator shaker for 2 h, then washed with Tween-20 (0.2%) for 20 m. and with RinAlla® (5% bleach/sodium hypochloride) for a short while. After these steps, it was washed with sterile distilled water for several times under laminar Air-flow chamber. Finally, it was treated with 0.1% HgCl2 (Hi-Media, India) for 1 m. and washed with sterile water thoroughly and dried on filter paper. These dried explants were cultured on MS basal media [23].

Preparation of different media

Different media with varying compositions were tested during transformation with explants. These media were primarily MS based [23] and Stage Hog Fern Root medium (SHFR, Hi-Media, India), modified as per need with plant growth regulators (PGRs), for propagation and transformation experiments. The composition of media were as follows: (1) MS resuspension media [transformation media; in 100 mL it contained MS-Salt

= 0.220g, Glucose = 0.8g, Sucrose = 1.5g, KCl = 0.04g, MgCl2 = 0.6g; pH maintained at 5.2, 50µL acetosyringone (50 mg/ml) after autoclaving]; (2) MS co-cultivation media [in 100 mL it contained MS-salt =0.442g, Sucrose = 3g, Kinetine = 200 µl (1 mg/mL), Thidiazuron (TDZ) = 100 µL (2 mg/mL), Agar = 0.8g, pH maintained at 5.8, 200 µL cefotaxime (250 mg/mL) after autoclaving]; (3) modified Stag Horn Co-cultivation media [SHFR; in 100 mL it contained SHFR Media = 4.2 g (Hi-Media, India), TDZ* = 100 µL (2 mg/mL; * variable quantity of hormone was used), Bevistin = 0.2g, Agar = 0.4g, 200 µL cefotaxime (250 mg/mL) after autoclaving]; and (4) Root propagation media [MS-based: MS-salt = 0.442g, Agar = 0.8g, Bevistin = 0.2g, TDZ* =50 µL (2 mg/mL; * variable quantity used); and SHFR media based: SHFR = 4.4g, Agar = 0.4g, TDZ* = 50 µL (2 mg/mL; * variable quantity used)].

Protocol for co-cultivation and induction of hairy roots

Different *A. rhizogenes* strains, like MTCC 532 and MTCC 2364 (procured from CSIR- Institute of Microbial Technology, Chandigarh, India, and LBA1334 [24]; procured from procured from Indian Institute of Technology, Kharagpur, India) were used in the present study. At first two strains MTCC 532 and MTCC 2364 were used for the transformation of *S. bryopteris* in the presence of acetosyrigone for the induction of hairy roots. Later, strain LBA1334 was used for the same. In brief, the protocol was as follows, for co-cultivation, the strains were grown at 28°C in an incubator shaker with shaking (150 rpm) for 48 h in LB media (OD600=1.0) and harvested by centrifugation at 6000 rpm. The pellet was resuspended with MS-resuspension media. The explants were pierced with sterile needle and immersed in the bacterial broth culture and incubated on incubator shaker at 100 rpm for 30 m. The explants were blot dried to remove excess of bacterial inoculums. All the explants were co-cultivated on MS-transformation media with acetosyrigone (Table 1) at 25 °C in dark on incubator shaker for 24 h, and 48 h in two separate flasks. Then these explants were transferred on MS medium devoid of growth regulators and supplemented with 3% sucrose, cefotaxime (200 mg/L) and solidified with 0.8% agar (w/v). Hairy roots at the end of 15 days excised individually from the infected site were and transferred to MS medium containing 200 mg/L of cefotaxime.

Confirmation of transformation and isolation of genomic DNA

Genomic DNA was isolated from hairy roots of *S. bryopteris* using CTAB method [25]. Plasmid from *A. rhizogenes* was isolated according to the alkaline lysis plasmid isolation protocol [26]. The Polymerase Chain Reaction (PCR) was used to detect the Ri T-DNA integration in hairy roots. PCR was performed to detect the *rol* A gene using *rol* A gene specific primers, Forward: 5'-AGA ATG GAA TTA GCC GGA CTA-3' and Reverse: 5'-GTA TTA ATC CCG TAG GTT TGT TT-3' (Xcelris Genomics, India). The PCR mixture (25 µL) contained 50 ng of DNA prepared from normal and hairy roots respectively as the template, PCR was carried out on a thermal cycler (Veriti®#9902, ABI, Singapore) as follows:, 10X PCR Buffer 2.5 µL, dNTPs (10 mM) 0.5 µL, Forward Primer (10.0 µM) 0.5 µL, Reverse Primer (10.0 µM) 0.5 µL, Taq DNA Polymerase (5.0 U /µL; Xcelris Genomics, India) 0.25 µL and autoclaved distilled water 19.75 µL to make total reaction volume 25 µL. PCR for *rol* A was carried out by amplifying with initial denaturation at 94 ºC for 5 m, followed by 35 cycles of 1 m denaturation at 94 ºC, 30 s annealing at 55 ºC and 1 m extension at 72 ºC with a final extension of 72 ºC for 10 m. In control, the amplification of *vir* C gene (Forward: 5'-ATC ATT TGT AGC GAC T-3' and Reverse: 5'-AGC TCA AAC CTG CTT C-3'). The PCR conditions were same as those used for amplifying the *rol* A gene.

RESULTS AND DISCUSSION

HRC has become one of the methods of choice recently for the production of secondary metabolites in different uncultivated or endangered medicinal plants. These roots show genetic stability and can be indefinitely propagated on a synthetic medium without the use of plant growth regulators [11,27]. Further, hairy root also yields high levels of secondary metabolites in contrast to cell culture, an advantage it has due to differentiation of tissue. The hairy roots could also serve the purpose in functional genomics. Hairy root-based transformation system for functional characterization of genes/enzymes of *Selaginella* is not available. Therefore, the model plants, Arabidopsis and rice were being employed up till now for functional study of *Selaginella* genes/enzymes [14].

A. rhizogenes-mediated genetically transformed HRCs are more biosynthetically efficient and genetically stable for secondary metabolite production [28-30]. These hairy roots offer unique advantages in their genetic and biosynthetic stability [31]. Hairy roots are considered a very good system for continuous synthesis of valuable metabolic compounds in an aseptic condition in the absence of expensive growth regulators in the culture medium [12,13]. HRCs of *Brassica juncea* and *Chenopodium amaranticolor* were reported to be developed by genetic transformation using *A. rhizogenes* for removal of uranium from the solution of

concentration up to 5,000 µM [32]. HRCs of *Cichorium intybus* and *B. juncea* were used for their ability to uptake and degrade DDT (1,1,1-trichloro-2,2-bis-(4'-chlorophenyl)ethane) suggest the potential applicability and advantage of using these plant species for phytoremediation of persistent xenobiotics such as DDT in an eco-friendly and efficient manner for environmental cleanup [33].

Confirmation of Hairy root resulting from Ri T-DNA integration

A. rhizogenes induced hairy roots formation was achieved in *S. bryopteris* (Figure 1, panel a). PCR with *rol* A gene specific primer yielded a 308 bp size fragment thereby showing the Ri T-DNA integration in hairy roots (Figure 1, panel b). Further, *vir* C genes specific primers used to detect the presence of contaminating *A. rhizogenes* in the hairy roots yielded 780 bp amplicon in positive control 1 but no amplification was detected in hairy root line (Figure 1, panel b). Thus, these results confirmed that these developed hairy root lines resulted from Ri T-DNA integration and not due to contaminating *A. rhizogenes*. These hairy roots were sub-cultured on MS medium without PGR (i-iv) at day 0, 5, 10, 15, respectively (Figure 1 panel c).

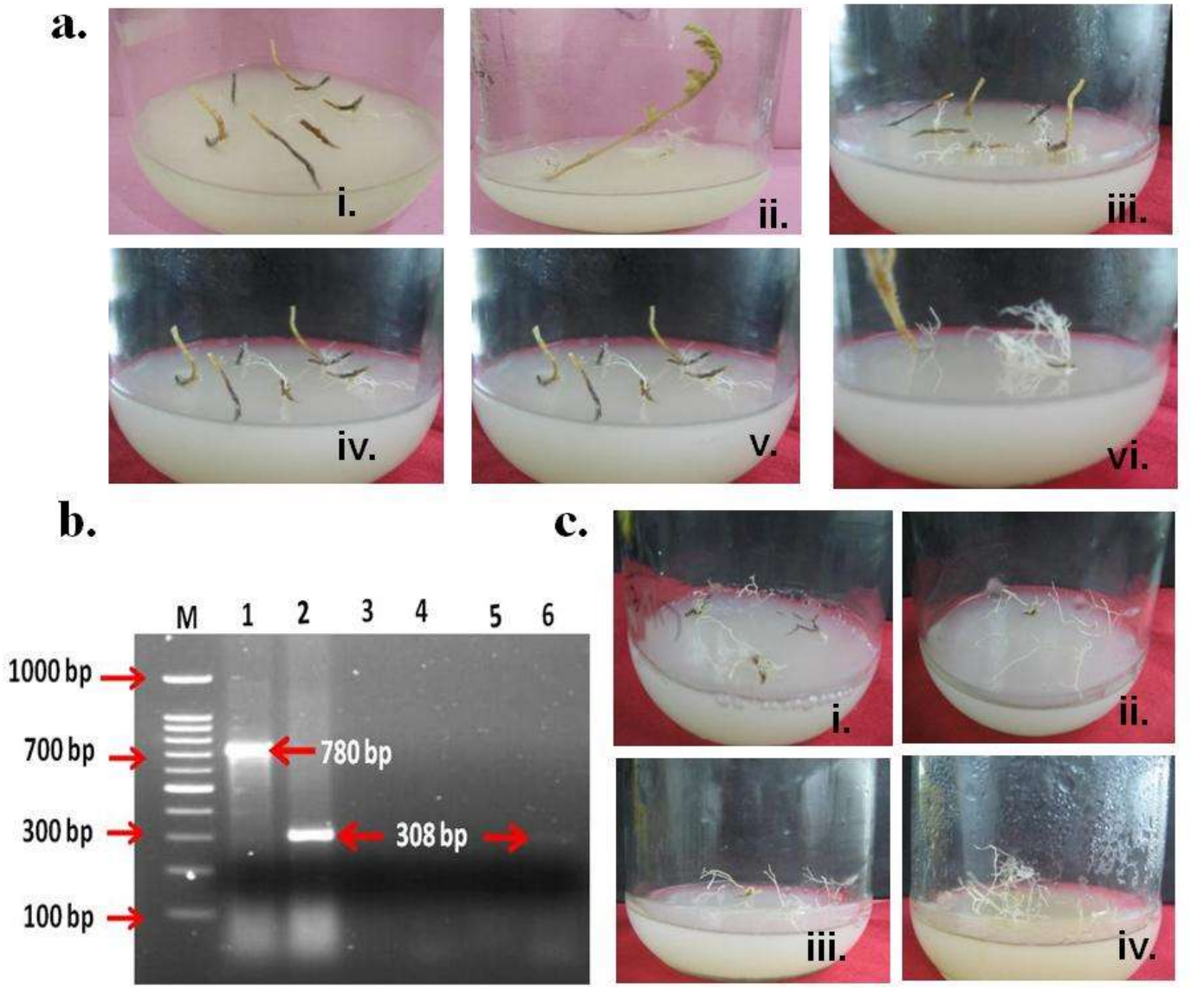

Figure 1. Hairy root induction by *A. rhizogenes* in *S. bryopteris* using root, rhizophore, stem portion near the root, and stem with intact fronds (i-vi) at day 7th, 15th and 20th, respectively on MS medium with cefotaxime (200 µg/ml) [Panel a]. PCR confirmations of hairy roots using *rol* A gene specific primers; M. 100 bp DNA marker, 1. *vir* C gene amplification with *A. rhizogenes* (positive control 1*),* 2. *rol* A gene amplification with *A. rhizogenes* (positive control 2),3*. vir* C gene amplification without any template (negative control 1), 4. *rol* A gene amplification without any template (negative control 2), 5. *vir* C gene amplification in hairy root line, and 6*. rol* A gene amplification in hairy root line [Panel b]. Subculture of hairy roots on MS medium without PGR (i-iv) at day 0, 5, 10, 15, respectively [Panel c].

Effects of *A. rhizogenes* strains, co-cultivation period, types of explants, media, and PGRs on hairy root induction

A. rhizogenes strains

Variations in virulence of bacteria, genotype of plant and type of explants play an important role in determining the frequency of hairy root induction. The virulence of the *A. rhizogenes* strains varies according to the strain and affects the frequency of the hairy root production [34]. In our experiment, two MTCC series strains (MTCC 532 and 2364) were used in the beginning but it failed to induce hairy root formation in *S. bryopteris*. Earlier in *Withania somnifera* transformation with these strains of *A. rhizogenes* (MTCC 2364, MTCC 532 including R1000), showed that strain R1000 as more effective for inducing hairy roots (50.6 %), while MTCC 2364 (29.3 %) and MTCC 532 (18.6 %) were less efficient [34]. Realizing the virulence and recalcitrance in pteridophytic plant like S. bryopteris to transformation, another strain of *A. rhizogenes*, LBA 1334 was used. This strain was found to be effective and the present study of standardization of protocol is based on this strain only.

Co-cultivation period

To see the effect of co-cultivation period the strain LBA1334 was grown along with explants for 24 and 48 h. The induction of hairy root after 6 days of infection was observed in case of 48 h co-cultivation only. The infection period along with co-cultivation period affected the hairy root induction [35,36].

Type of explants

Keeping in view the recalcitrant nature of *S. bryopteris*, different parts of plant, root, rhizophore, stem portion near the root, and stem with intact fronds were used as explants in transformation for hairy root induction (Figure 1, panel a; Table 1). The root network as explants showed the maximum transformation efficiency (70%).It was reported that the types of explants due to its meristematic activity influence the hairy roots production [37]. In *Berberis aristata*, callus was reported to be better explants in comparison to nodal segment and leaf [36].

Media

We tested different media like MS-based, SHFR and KNOP's media during transformation for hairy root induction (Table 1). The SHFR based media observed good response in transformation (80%) as well as propagation and induction time was 6 days. Liquid media of all combination have not observed good response. MS-based media did not show any response for propagation of hairy root of S. bryopteris, while KNOP showed 10% response. Different ionic strength media affects the hairy roots cultures in terms of biomass and metabolite production. In hairy root culture of *A. indica*, media varying in ionic strength such as ON, MS and B5 used yielded maximum amounts of biomass and azadirachtin in correlation with their ionic strength [1]. ON medium that contained higher ionic concentrations of inorganic salts compared to MS and B5, was found to favour the growth and azadirachtin production.

PGRs

The ability of hairy roots to grow rapidly in the absence of exogenous plant growth regulators (PGRs) and produce the same compounds found in the parental plants, makes it suitable tool for production of secondary metabolites and recombinant proteins [11]. In the present study, SHFR media was modified with plant growth regulators (PGRs), Kinetin and TDZ with or without addition of Bevistin (Table 1). SHFR media along with TDZ (2 mg/L) and Bevistin (0.1%) enhanced the transformation efficiency and propagation of hairy roots. While Kinetin (2 mg/L) and TDZ alone response was 40% and 65%, respectively.

Table1. Effect of type of explants, different media composition, and different PGRs on hairy root induction efficiency

a.Type of explants			
Type of explants	**Total number of explants**	**No. of hairy root forming explants**	**Time taken for induction of hairy root**
Root	10	7 (70%)	Early
Rhizophore	10	5 (50%)	Normal
Stem near root	10	5 (50%)	Normal
Stem with frond	10	3(30%)	Late
b. Different media composition			
Co-cultivation media	**Frequency of hairy root formation**	**Time taken in response (days)**	**Growth pattern of hairy root (propagation)**
MS-based	No Root Formation (0 in 10)	-	-
Stag Hog based	8 in 10 (80%) all type	6	Efficient
KNOP (liquid)	1 in 10 (10%) in root network only	20	Slow
c. Different PGRs			
Combination of PGRs	**Regeneration frequency of explants (%)**	**Regeneration response (in day)**	**Rate of growth (comparative)/ Visual observation (star)**
SHFR + TDZ	65	6	Good/4
SHFR + KINETIN	40	10	Slow/2
SHFR+TDZ+ KINETIN	60	6	Good/3
SHFR+TDZ+ KINETIN + Bevistin	70	6	Good/3
SHFR+TDZ+ Bevistin	75	6	Better in all/5 (best)
SHFR+KINETIN+ Bevistin	50	10	Slow/3

CONCLUSION

In *S. bryopteris* an efficient, stable and viable alternative *in vitro* method is desired for the production of bioactive molecules and HRC-based system is most suited for this. The present work would be helpful in HRC-based *in vitro* production of bioactive molecules and on aspect of functional genomics of *S. bryopteris.*

Author contributions: Conceived the idea and designed the experiment: RSS; performed the experiments: RSS, VKJ, UK; result/data interpretation: RSS, TC and VKJ; valuable suggestions and overall guidance during the experimentation: DPF and PKS; prepared and edited the manuscript: RSS and TC.

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