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Short Communication

Evaluation of influence of different strains of *Agrobacterium rhizogenes* on efficiency of hairy root induction in *Rauwolfia serpentina*

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Abstract

An improved protocol was established for hairy root induction in *Rauwolfia serpentina* using two strains of *Agrobacterium rhizogenes* MTCC 532 and 2364. Hairy root induction efficiency was standardized based on infection time, co-cultivation period, OD_{600} and acetosyringone concentration. Higher transformation efficiency was established using MTCC 532 (31%) and MTCC 2364 (24%) with 30 min infection time, 72 h co-cultivation period and 0.6 OD_{600}. Transformation efficiency was further enhanced to 55 % with 125 µM acetosyringone. MTCC 532 was proven a better strain over the MTCC 2364 in all the tested factors. Putative transgenic hairy roots were confirmed by polymerase chain reaction using *rolA* specific primers. Biomass of randomly selected 5 hairy root lines was also significantly enhanced. No significant difference of growth was recorded among the lines except line no 4. In the present study, an enhanced system for *Agrobacterium rhizogenes*-mediated hairy root culture was established which offer an effective means to attain improved transformation efficiency and ultimately beneficial for the industrial scale *in vitro* production of the secondary metabolite.

Key words: Hairy roots, *Agrobacterium rhizogenes*, *Rauwolfia serpentina*, MTCC 532, MTCC 2364

Rauwolfia serpentina (L.) Benth, commonly known as Sarpgandha is an important perennial shrub of family Apocyanaceae. The plant is a hub of more than 100 medicinally-important terpenoid indole alkaloid mainly reserpine, ajmalicine, ajmaline, yohimbine etc. Majority (85-90%) of these alkaloids are found mainly in roots (Pathania 2013). Organic synthesis of above mentioned alkaloid is expensive and tedious process due to its complex structures with regio- and stereo-specific actions. *R. serpentina* has been considered as endangered plant category by International Union for the Conservation of Nature and Natural Resources due to its over exploitation for medicinal purposes (Shetty et al. 2014). Various plant tissue culture strategies have been employed as an alternative to produce these alkaloids viz. cell suspension culture, *in vitro* clonal propagation etc. but has limited application due to the concern of genetic and biosynthetic variability of cell culture. However, hairy root culture of *R. serpentina* substantiated to be useful among all plant tissue culture techniques due to its biosynthetic steadiness, consistency in the prolonged and economic production of alkaloid (Guillon et al. 2006; Madhusudanan et al. 2008). There are several reports on *A. rhizogenes* mediated transformation of *R. serpentina* (Benjamin et al. 1993; Sarma et al. 1997; Goel et al. 2010; Mehrotra et al. 2012; Mehrotra et al. 2013a; Mehrotra. 2013b; Ray et al. 2014; Shetty 2014; Srivastava et al. 2016) but standardization of protocol was not established properly. In the present study, two *A. rhizogenes* strains, MTCC 532 and MTCC 2364 were utilized in the development of efficient protocol for hairy root induction of *R. serpentine* leaf explant.

To carry out hairy root induction, plants of *Rauwolfia serpentina* were purchased from Sushila Tiwari Herbal Garden, Rishikesh, Uttarakhand, India. Young leaves were used as explants for transformation experiments. *Agrobacterium rhizogenes* strains MTCC 532 and MTCC 2364 were purchased from Institute of Microbial Technology, Chandigarh, India. MTCC 532 and MTCC 2364 were grown in Nutrient Broth and

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Xanthomonas media, respectively at 25°C under shaking condition (200 rpm). Optical density (OD_{600}) of bacterial cultures was recorded at 600 nm. Explants were surface sterilized with 0.1% $HgCl_2$ solution and 70% ethanol. Surface sterilized explants were then cultured (Pre-culture period) on MS medium for 2d. Bacterial culture was grown up to desired OD_{600}, centrifuged at 4000 rpm for 10 min and pellet was dissolved in liquid MS medium.Pre-cultured leaves were then infected with bacterial suspension with gentle shaking, blot dried and cultured (co-cultivation period) for 2 d on MS medium.Co-cultivated explants were washed with 250 mg/l cefotaxime, blot dried andthen cultured on selection medium (½ MS medium with 250 mg/l cefotaxime) for hairy root induction. Explants which were not infected with *Agrobacterium* were utilized as control.

Various parameters such as, Optical density (OD_{600}), co-cultivation period, infection time, acetosyringone concentration were assessed for standardization of transformation efficiency. Infection time were tried from 15 to 45 min at an interval of 15 min. Co-cultivation duration (24 h, 48 h, 72 h and 84 h) were evaluated for transformation efficiency. Optical density (OD_{600}) values of the *A. rhizogenes* cell culture tried from 0.2 to 1.0 at an interval of 0.2. Finally, various concentration of acetosyringone (50-150 μM) were tested for further enhancement of transformation efficiency. Percentage of transformation efficiency (%) was calculated as using the given below formulae

$$\frac{\text{Total no. of explants inducing trangesnic hairy roots}}{\text{Total no. of explants infected with } A.\ rhizogenes} \times 100$$

Molecular analysis of transgenic roots was carried out by isolating the genomic DNA from normal roots (Non-transformed) and transgenic hairy roots using Plant DNeasy mini kit (Qiagen) according to the manufacture's instructions. Plasmid DNA were isolated from *A. rhizogenes* using the plasmid isolation kit (Qiagen) according to the manufacture's instruction and utilized as positive control. PCR was performed for the identification of the presence of 360bp of the *rolA* gene using the gene specific primers set: forward primer 5'GGAATTAGCCGGACTAAACG-3' and reverse 5'-CCGGCGTGGAAATGAATCG-3'.

PCR positive five hairy roots line has been randomly selected and evaluated for growth kinetics. 100 mg hairy roots were used were used as inoculum. Increase in fresh weight was recorded after every 7 days. All the parameters were evaluated in four independent replicates and each replicate includes 20 explants.Data were analyzed for significant differences using IBM SPSS Statistics 20 program. The different letters indicate significant difference ($P<0.05$)

Plants of *R. serpentina* were grown in earthen pots in the laboratory condition (Fig. 1A). Explants were pre-cultured and co-cultivated with *A. rhizogenes* (O_{D600} 0.6) for 15 min to induce infection.After co-cultivation for 2 d, the infected explants were washed with 250 ppm cefotaxime and cultured on ½ MS selection medium for hairy root induction (Fig. 1B). Putative transgenic white hairy roots were observed on the explants within 18 days after the transfer on to selection medium (Fig. 1C). No hairy roots were emerged from control explants. Hairy roots (100 mg) were transferred to propagation medium from 5 randomly selected PCR positive lines for growth kinetics study (Fig. 1D).

MTCC 532 induced hairy roots were thick and long as compared to MTCC 2364 induced hairy roots. MTCC 532 induces one root per explant while on the other hand more number of roots emerged from each MTCC 2364 infected explant. All the putative hairy root lines were confirmed through amplification of *rolA* gene. The 360 bp amplified product was observed on 1.2% agarose gel using gene specific primes (Fig. 2). Fresh weight of all the selected hairy root lines was significantly increased in all the tested duration (7 d, 14 d, 21 d and 28 d). No significant difference was observed among the lines except line no. 4. Transformation efficiency was significantly ($P < 0.05$) enhanced to 20% and 13% both for MTCC 532 and MTCC 2364 respectively on increasing the infection time from15 to 30 min. Transformation efficiency further reduced to 10% and 8% for MTCC 532 and MTCC 2364 respectively on enhancing the infection time to 45 minutes.

Co-cultivation period of 72 h was observed as best for higher transformation efficiency (25% and 19% for MTCC 532 and MTCC 2364 respectively), whereas the efficiencies were significantly ($P < 0.05$) decreases for both shorter and longer co-cultivation periods.

At 0.6 optical density (O_{D600}) of *A. rhizogenes* culture, significantly ($P < 0.05$) higher 31% and 24% transformation efficiency were observed for MTCC 532 and MTCC 2364 respectively. Although further increase in OD_{600} of 0.8 and 1.0 resulted in significantly ($P < 0.05$) linear decrease of transformation efficiency. Transformation efficiency linearly enhances on

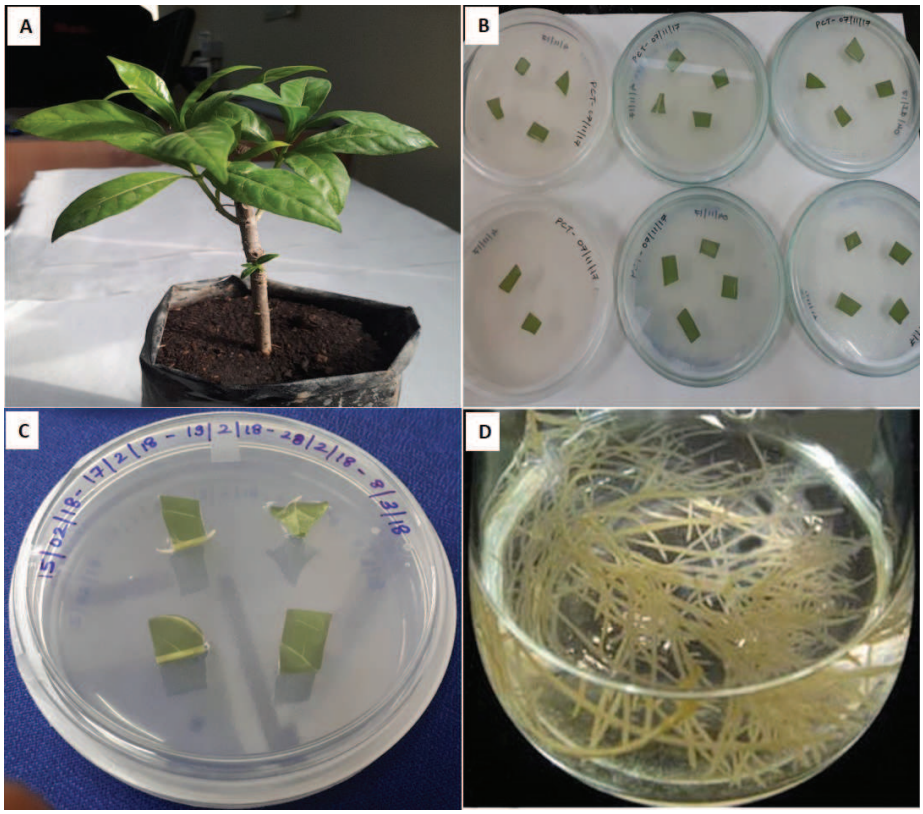

Fig. 1. ***A. rhizogenes*** **(MTCC 532 and 2364) mediated hairy root induction from leaf explants of** ***R. serpentina*** **A = *R. serpentina* plants grown in pots, B = Explants transferred to selection media after infection with *A. rhizogenes* (MTCC 532 and 2364), C = Induction of hairy roots form leaf explants and D = Proliferation of hairy roots from 100 mg hairy roots on liquid ½ MS medium**

increasing acetosyringone concentration up to 125 µM. Significantly ($P < 0.05$) higher transformation efficiency (55%) was observed at 125 µM acetosyringone concentration. Further increase in acetosyringone concentration (150 µM) significantly ($P < 0.05$) decreases the transformation efficiency to 41%.

Development of efficient hairy root culture protocol depends on various factors such as explant type, *Agrobacterium* strain, pre-culture time, co-cultivation time, optical density of *Agrobacterium*, acetosyringone concentration etc. (Barik et al. 2005; Crane et al. 2006; Khare et al. 2010). In the present study for the first time *Agrobacterium rhizogenes* strains MTCC 532 and MTCC 2364 have been evaluated for hairy root induction. Choice of explant is a very crucial step in optimizing T-DNA transfer from *A. rhizogenes* as the physiological condition of different explants vary which influences the transformation efficiency. In the present study, leaves of *Rauwolfia serpentina* were chosen as the preferred type of explant for the development of hairy roots based on previous reports (Sarma et al. 1997; Goel et al. 2010; Mehrotra et al. 2013a,b; Ray et al. 2014; Srivastava et al. 2016). Randomly selected five lines were evaluated for growth kinetics. All the lines showed significant ($P < 0.05$) growth of hairy roots at an interval of 7 d, 14 d, 21 d, 28 d. No significant ($P < 0.05$) difference of growth was observed among the lines except line no 4. Different characteristics of hairy root lines were due to the diverse insertion sites in the host plant (Gai et al. 2015). Experiments for enhancement of transformation efficiency for hairy root development were initiated with the standardization of infection time. High probability of transgene insertion into explant may be enhanced by longer infection period which leads to the adherence of maximum bacteria to the surface of wound site. Pre-culture period (2 d), $O.D._{600}$ (1.0) and co-cultivation duration (2d) was kept constant during the standardization of infection time. Significantly ($P < 0.05$) higher transformation efficiency was observed for 30 min infection time. However, enhancing the infection time to 45 min resulted in a decline in efficiency, perhaps may be due to the hypersensitive response of explants to bacteria (Orlikowska et al. 1995). Hence, we had used 30 min infection time in further standardization experiments.

Co-cultivation period was attempted after standardization of infection time because it is a crucial period which should be suitable for the explant to survive the *Agrobacterium* overgrowth after incubation and help the proper transfer of T-DNA. The longer co-cultivation time led to the reduced viability of the plant cells and thus delays the growth of transformed explants (Christoph et al. 1997). In the present study, 72 h co-cultivation period was observed significantly best for higher transformation efficiency. The *Agrobacterium* cell density used for the co-cultivation is an important

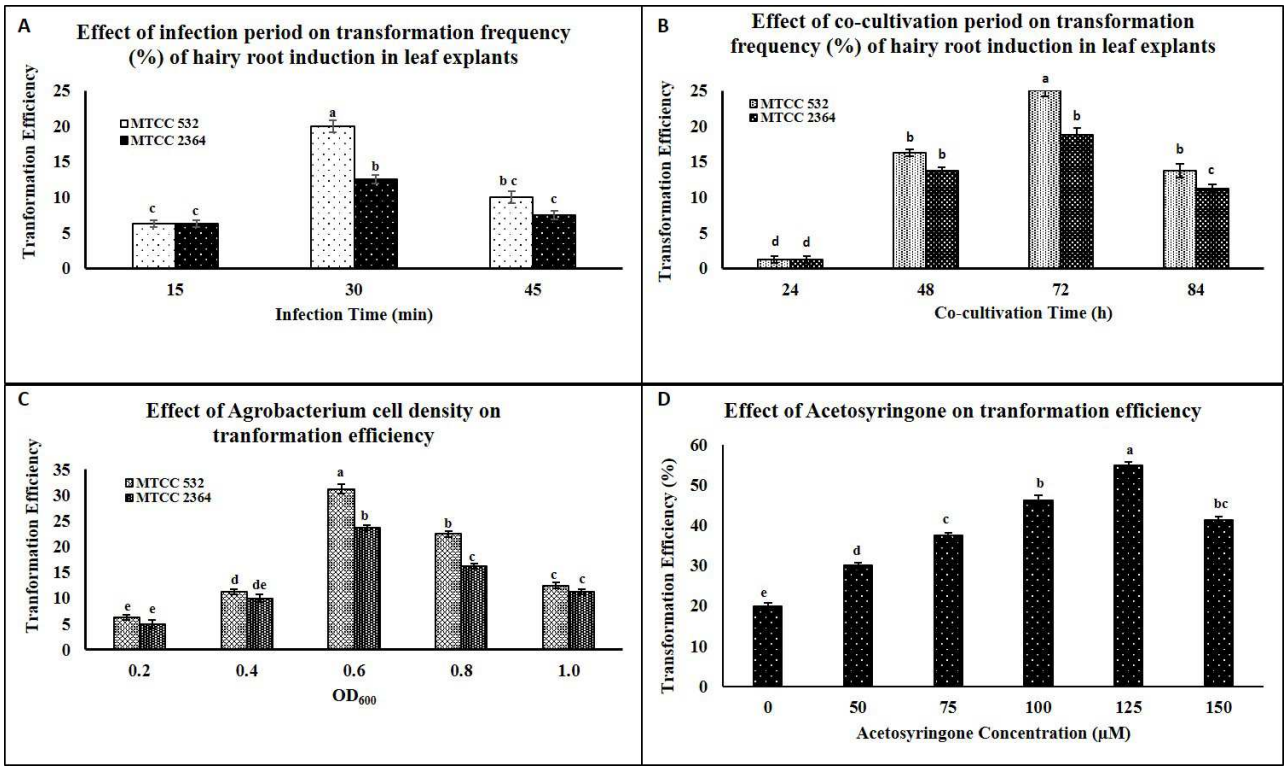

Fig. 2. Optimization of factors influencing genetic hairy root induction in leaf explants of *R. serpentina* using, *A. rhizogenes* (MTCC 532 and 2364). A = Effect of infection time on hairy root induction. The leaf explant were pre-cultured (48 h), infected in *Agrobacterium* suspension (1.0 OD600) for 15-45 minute and co-cultivated for 48 h. B = Effect of co-cultivation time on hairy root induction. The leaf explant were pre-cultured (48 h), infected in *Agrobacterium* suspension (1.0 OD600) for 30 minute and co-cultivated for 24- 84 h. C = Effect of bacterial density on hairy root induction. The leaf explants were pre-cultured (48 h), infected in *Agrobacterium* suspension (0.2-1.0 OD600) for 30 min and co-cultivated for 72 h and D = Effect of acetosyringone on hairy root induction. The leaf explants were pre-cultured (48 h), infected in *Agrobacterium* suspension (0.6 OD600) for 30 min, co-cultivated for 72 h with acetosyringone (50-150 µM)

factor for the transformation experiments as the explant used for co-cultivation differs in the strength for overcoming the effect of immersion in *Agrobacterium* culture containing medium.To evaluate the effect of O.D., pre-culture period (2 d), infection time (30 min) and co-cultivation duration (72 h) was kept constant.*A. rhizogenes* suspension at an OD_{600} of 0.2, 0.4 and 0.6 linearly increases the transformation efficiency. Higher OD_{600} (0.8 and 1.0) resulted in increase of bacterial attachment which may cause growth of *A. rhizogenes* itself within the explant (Sreeramanan et al. 2008). Acetosyringone has already been reported to improve the efficiency of hairy root development at a concentration of 50-200 µM acetosyringone. In our study higher (54%) transformation efficiency was observed at 125 µM acetosyringone; however the efficiency decreased on further increase of acetosyringone concentration (150 µM). Moreover, plant genotype and bacterial strains plays a important role in the difference of transformation efficiency at different concentration of acetosyringone (Godwin et al. 1991).

In the present study, several factors were optimized which has a pivotal role on the transformation efficiency of hairy root induction of *R. serpentina* leaf explant with strain MTCC 532 and MTCC 2364. To our knowledge, this is the first report in *R. serpentina* for detailed study of factor affecting transformation efficiency. Thus, we believe that the optimized protocol could be useful for the industrial production secondary metabolites using *R. serpentine* hairy root culture system.

Authors' contribution

Conceptualization of research (NK); Designing of the experiments (NK); Contribution of experimental materials (NK, PB, SKV); Execution of field/lab experiments and data collection (PB); Analysis of data

and interpretation (NK, PB, SKV); Preparation of manuscript (NK, AK, JSS, GKA).

Declaration

The authors declare no conflict of interest.

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