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SCIENCE

Induction and Growth Optimisation of hairy root culture of *Chlorophytum* species

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ABSTRACT

We aimed to develop a protocol for development of hairy root culture and to enhance secondary metabolite level in Chlorophytum species. Explants such as leaf and tubers of Chlorophytum species were used however only tubers pre-incubated in cytokine (1 mg/l) had positive response for the induction of hairy roots. The hairy roots emerged from the incubated tubers after 9–12 days of co–cultivation with Agrobacterium rhizogenes R1000. The Transformants showed a logarithmic increase in the number of hair like structures formation on surface of tubers. This faster growth of transformed plants was obtained by using one of super virulent strain of Agrobacterium rhizogenes R1000. Genetic markers included rolA responsible for T-DNA transfer and hairy root initiation to confirm bacterial gene transfer were detected from transformed root. Hairy roots showed significantly enhanced production of saponins. Percentage yield of transformed roots of Chlorophytum laxum was 10% while that of non-transformed tissue of same plant was only 2.5%. Our result suggests that saponin level in transformed tissue is higher than non-transformed tissue.

Keywords: Agrobacterium rhizogenes, *Chlorophytum*, hairy roots, saponins

INTRODUCTION

Agrobacterium rhizogenes, a negative soil bacterium, mediated hairy roots promises viable alternative for transgenic plant development and production of secondary metabolites and recombinant proteins.[1-5] Safed Musali (Chlorophytum species of monocotyledonous family of liliaceae) is an important ayurvedic medicinal plant having rejuvenating, aphrodisiac and effective immunomodulatory effects.[6] Therapeutic effects of safed musli are attributed to the presence of large amount of saponins. Among all species, Chlorophytum borivilianum produces the highest yield and highest saponin content.[7] Its International market demand is over 300-700 tons per year and factors such as poor seed germination and dormancy affect uniform supply of this musli.[8] This has resulted in increased commercial exploitation of most of this species has leading to its endangered plant status.[9] Although various tissue culture techniques for the Chlorophytum species are previously reported, the protocol for hairy root culture development of Chlorophytum species is not available. Chlorophytum species belongs to monocotyledonous Liliaceae family, which responds poorly to genetic transformation by Agrobacterium rhizhogenes due to lack of chemo tactic effect.[10] Hence, our aim was to find a solution to conserve this endangered plant species, enhance its saponin productivity and develop a protocol for successful genetic transformation in monocotyledonous Chlorophytum species by Agrobacterial infection by using three different Agrobacterium strains MTCC 2364, MTCC 532 and R1000.

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MATERIALS AND METHODS

Plant material, *Agrobacterium rhizogenes* strain and Chemicals

Four species of Chlorophytum were selected for comparative study i.e., Chlorophytum borivilianum, C. tuberosum, C. laxum and C. comosum. These two species were authenticated by Dr. Arvind S. Dhabe, Herbarium in-charge, Department of Botany, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad. Three strains of Agrobacterium rhizogenes i.e. MTCC 532, MTCC 2364 and R1000 were procured form MTCC Chandigarh, India. All tissue culture Medias and chemicals used are of Hi-media. Chemicals required for chromatographic analysis were purchased from Sigma-Aldrich.

Optimization of hairy roots

Hairy root of Chlorophytum species was optimised for species, explants, culture medias, Agrobacterium strains, co-cultivation method, cultivation duration, acetosyringone treatment, temperature and pH condition and cefotaxime (antibiotic) concentrations.

Three different culture media (Gamborg's B5 medium, MS media half strength and MS media), tuber and leaf explants of four different Chlorophytum species and three strains of Agrobacterium rhizogenes (MTCC 532, MTCC 2364 and R1000) were used to optimise hairy root development.

Initially, surface sterilization of different explants of Chlorophytum species was achived by treating with surface sterilants like mercuric

chloride, 70% ethanol and Sodium hypochlorite. After washing thoroughly, the explants were surface sterilized with 0.1% mercuric chloride for 5–8 minutes followed by 8–10 rinses with sterile distilled water, to remove traces of mercuric chloride.[11]

Three strains of Agrobacterium rhizogenes (MTCC 532, MTCC 2364 and R1000) were sub cultured on culture medium for bacterial growth. MTCC 532 and MTCC 2364 were subcultured on liquid YEP medium while R1000 was sub cultured on liquid YMB medium. Test tubes containing bacterial strains were incubated at 28˚C for 5 days and then stored at -80˚C in refrigerator, until time of co cultivation. At the time of co cultivation, strains were allowed to be activated and then used. Sterilised explants were further co-cultivated with all three Agrobacterium rhizhogenes strains separately and incubated at 25 ± 1˚C for varying durations from 1 day to 15 days.[12] Changes were observed and analysed.

Three methods of co-cultivation by Agrobacterium strains were employed: spreading method (direct spreading of A. rhizogenes bacteria at wounded site of explants), pricking method (pricking needle contaminated with A. rhizogenes to explants) and deeping method (deeping explants in incubated bacterial culture). [13-14]

Acetosyringone is a phenolic compound, which acts as chemotactic agent for A. rhizogenes. It has enhanced transformation frequency in several monocot and dicot plants. Acetosyringone was procured from HiMedia.[1] It was used in various concentrations (50 µm, 100 µm and 150 µm). Acetosyringone was used in following two ways and comparative results were analysed.

- Inoculation of Acetosyringone in culture medium at the time of co cultivation and incubation for 2 days at 25 ± 1˚C.
- Stepwise inoculation of acetosyringone at the time of co cultivation
- Activation of bacterial culture through addition of 100 µM AS an hour prior to co-cultivation.
- Addition of 100 µM AS in co-cultivating MS medium .
- Activation of bacterial culture a) and addition in co-cultivating medium.

The explants showing Agrobacterium rhizogenes infection were subcultured on MS media and incubated at varying temperatures. The incubation temperatures ranged from 22˚-33˚C. The response of the explants at different temperatures was analyzed after six weeks of culture.

The influence of pH on the growth and proliferation of hairy roots was evaluated. The pH of media was set over a range of 4.5–7 and the study was conducted for a period of 4 weeks.

To eliminate residual bacterial growth, explants were cultured with MS media containing antibiotic (Cefotaxime 300 µg/ ml). As the antibiotic is heat liable, it was filter sterilized before addition to media. Different concentrations of antibiotic (Cefotaxime) were evaluated for effective elimination of bacteria after co cultivation.

Evaluation of hairy roots

In morphological and microscopical evaluation, color changes, plagiotropic growth, abundant lateral root formation like characters were evaluated.[13] Including microscopy of normal root and hairy root compared for phenolic content and any histological growth changes.

In growth studies of hairy Roots, growth kinetics of transformed plants obtained from the explants of Chlorophytum species using strains of Agrobacterium rhizogenes was evaluated for a period of 6-7 weeks. Explants were transferred to fresh MS semi solid media after every 4-5 days and left in incubator in dark condition at 25˚C.

Histological studies were carried to study morphological changes by comparing thin sections of transformed tissues with non transformed tissues of Chlorophytum laxum under digital microscope. These samples were fixed in a solution (formaldehyde: glacial acetic acid: ethyl alcohol; 5: 5 90; v/v). Following the fixation, tissues were dehydrated in an ethanolic graded series and then embedded in paraffin.[1] Serial sections of 6–8 µm in thickness were cut using microtome. The sections were obtained from freshly prepared wax blocks with tissue specimen by adjusting microtome and stained with safranin.

Estimation of secondary metabolites (Saponins) from both non-transformed and transformed roots were extracted, fractionated and quantified by using gravimetric methods.[7] Initially tubers were extracted with methanol for one hour. Then filtered and methanol distilled off to obtain semi solid residue. Further residue was dissolved in n-butanol, and diethyl ether was added drop wise until complete precipitate of saponins was obtained.

In chromatographic evaluation of saponins from non-transformed and hairy roots following conditions were used

Mobile phase A	Mobile phase B
Stationary phase: silica gel G	Stationary phase: silica gel G
Mobile phase: Chloroform: glacial acetic acid: methanol: water (6.5: 3.2: 1.2: 0.8)	Mobile phase: chloroform: methanol: water (7.0: 3.0: 0.4)
Spraying agent: anisaldehyde sulphuric acid	Spraying agent: anisaldehyde sulphuric acid
Treatment after spraying: heated on hot plate at 110 ºC till color developed and intensified	Treatment after spraying: heated on hot plate at 110 ºC till color developed and intensified

Opine analysis by PCR method was outsourced to Cistron services, Mumbai, India. The bacteria free roots of normal plants (control) and transformed plants were excised for DNA extraction to perform molecular analysis. PCR was employed for detection of Ri T – DNA integration in hairy roots of Chlorophytum laxum. The roots were ground in the presence of liquid N_2 and the DNA extracted using a Nucleospin extraction kit. A set of rol A specific primer was used for the amplification of 308 bp rol A gene fragment. The sequence of primers used for amplification was forward. 1kb ladder was used as a standard reference (Qiagen). The template DNA from normal and transformed root was taken at a concentration of 50 ng acts as, 1 × PCR buffer, 25 pmoles of each primer, 2.5 Mm of dNTPs and 1 unit of Taq polymerase were present in the PCR mixture (25 µl). PCR for rol A gene was carried out by amplifying with initial denaturation at 94˚ C for 5 min followed by 35 cycles of 1 min denaturation at 94˚ C, I min annealing at 55 ˚ C and 1 min extension at 72˚ C with a final extension of 72 ˚C for 10 min using a thermal cycler (Eppendorf, Germany). The PCR products obtained were analyzed using 1% agarose gel, stained with ethidium bromide. The DNA band thus obtained was observed and documented using a transilluminator equipped with a gel documentation system (Bio–Rad, USA).

RESULTS AND DISCUSSION

We optimized an effective sterilization method to overcome the problem of fungal and bacterial contamination. Treatment of culture vessels in contact with concentrated acid for 2-3 hours; washed with distilled water, dried in hot air oven and autoclaved at 121˚C and 15 lb pressure for 15 min avoided contamination. Chamber fumigation by using potassium permanganate and 40% formaldehyde found best to overcome bacterial and fungal contamination in tissue culture cabinet.

Among the three strains, Agrobacterium strain R1000 was more effective in induction of hairy root culture in Chlorophytum. Hence, it was used throughout the experiment for transformation. While Agrobacterium strain MTCC 532 and MTCC2364 showed no hairy root growth.

Plant cells in growing region of plants are highly susceptible to infection and genetic transformation by Agrobacterium. Hence, for present research work tubers and leaves explants of Chlorophytum species were selected and studied for two weeks after infection and co-cultivation. Tubers gave good response for establishment of hairy root culture. We found that Chlorophytum laxum was highly susceptible to Agrobacterium infection as compared to other Chlorophytum species.

Of the various plant parts of Chlorophytum laxum, tubers gave good response to genetic transformation. Hairy roots were observed in them after 8-9 days of co-cultivation. Hence, we conclude that, Chlorophytum laxum serves as best choice for the establishment of hairy roots culture.

Among the three co cultivation methods used, spreading and pricking techniques failed to establish direct contact between plant cells and Agrobacterium. However, in case of deeping method, satisfactory results were observed. In this technique, bacterial culture was activated at 28˚C for two nights in incubator. Then explants were injured and deeped in bacterial culture and kept on shaking incubator at frequency 90 rpm for 15-20 min. After 15 min explants were dried on sterile filter paper and transferred to culture medium devoid of phytohormones. It was kept for 2-3 nights at 25 ± 1˚ C in dark condition. Bacterial colony was observed on the surface of explants indicating good sign of successful transformation. Infection by deeping technique gave best results. (Figure 1) Pre-incubation of explants in 1 mg/ml kinetin also gave best results for hairy root establishment.

Co-cultivation is a very important step in hairy root culture development during which bacterial DNA integrates with plant genome. [15] Range of co-cultivation duration was kept 24 to 72 hr in the present study. At 24 hr, in 10 explants degree of hairy root growth was less; at 48 hr in 10 explants degree of hairy root growth was adequate while at 72 hr in 10 explants blackening and necrosis were observed.

When co-cultivation period was kept 24 hr, no results were observed for long time after sub culturing. Changes were not observed. When kept for 48 hr period, bacterial colonies were observed along the explants and hairy root growth was also observed. It gave satisfactory results after subculture. When kept for 72 hr, overgrowth was observed with tissue necrosis. Hence we conclude that, co cultivation duration of 48 hr is ideal for successful transformation in Chlorophytum species.

From previous research work on hairy root culture, it is observed that monocot species give less response to induction of hairy roots because they lack ability to produce chaemotactic effect in order to attract agro bacterium and cause transformation. Hence, very few examples of monocot hairy root culture are reported. Plant species chosen for present research work is also a monocot species. Hence it was challenge to induce hairy roots in this species. Acetosyringone treatment was vital step. The use of acetosyringone have enhanced transformation frequency by several folds in both monocots and dicot species. Hence, we used it in various manners and its influence on transformation frequency was studied. Inoculation of acetosyringone in culture medium at the time of co-cultivation: Acetosyringone in concentration of 20 mg/l was incorporated in culture medium directly. Studied for 2-3 weeks but without any results. Stepwise inoculation of acetosyringone at the time of co–cultivation: This procedure reported previously was followed in present research work. 100 µM concentration of acetosyringone was used. It gave good results. Hairy root growth was observed at the edges of tubers of C. laxum. Hence we conclude that stepwise inoculation of acetosyringone can induce hairy roots in Chlorophytum species.

Figure 1: Explants of *Chlorophytum comosum* and *Chlorophytum laxum* after infected with Agrobacterium strain R1000 A) *Chlorophytum* comosum showing bacterial colony B) *Chlorophytum laxum* showing susceptibility to Agrobacterial strain R1000 and while *Chlorophytum comosum* is not showing infection

The transformation frequency was studied by varying the incubation temperature (22–33˚C), the optimum temperature was found to be 25˚C. Hairy root growth was observed at this temperature. (Table 1) while above 25˚C growth was stopped without any further progress in growth. Thus from this study, optimum temperature condition was found to be 25˚C. The MS medium PH was set between 4.5 and 7 to analyze growth response of hairy roots. Optimised 5.5 pH caused profound rooting in all the hairy root lines. Alteration in pH did not favor the growth of hairy roots. (Table 2) Among different concentrations of cefotaxime tried, 300 and 400 µg/ml showed 98% and 100% inhibition of bacterial growth. Though, cefotaxime at higher concentration inhibits bacterial growth completely, it has an adverse effect on the plant growth as well.[1] Hence 300 µg concentration was preferred. (Table 3) After few subculture passages, in the antibiotic containing media, the plants were transferred to antibiotic free media, and 48 h old co-cultivated explants were used for the study.

Evaluation of hairy roots

Morphological evaluations such as degree of lateral branching, plagiotropism, presence of numerous hairs confirmed transformation of Agrobacterium in Chlorophytum species. The important morphological markers included profusion of rapid growth, lateral branching and plagiotrophy (negatively geotropic).

MS media was observed to be the best media as the proliferation of hairy roots grew faster in this media. In transformed cultures, fresh roots weight from initial 0.5 g reached a maximum to 1.5 g with A. rhizogenes R1000 strain in 30 days. However in control group, fresh weight was

Table 1: Temperature optimisation

No.	Temperature	Observation
1	22	-
2	24	++
3	25	+++
4	27	+
5	29	_
6	33	-

Table 2: Optimisation of Media pH

Sr. No	MS media and its pH	Observation	Response
1	4.5	--	No Rooting
2	5.0	++	Rooting
3	5.5	+++	Profuse rooting
4	5.8	++	Rooting
5	6.0	++	Rooting
6	6.5	--	No Rooting
7	7.0	--	No Rooting

Table 3: Optimisation of Cefotaxime concentration

Concentration µg/ml	Number of explants	% of explants without infection
0	10	0
100	10	0
200	10	56
300	10	98
400	10	100
500	10	100

Figure 2: Developed hairy root culture of *Chlorophytum laxum* A) Co cultivated explants of *Chlorophytum laxum* for 3 nights B) Developed hairy roots of *Chlorophytum laxum* after 9 – 10 days of co – cultivation and subculture C) close view of developed hairy roots

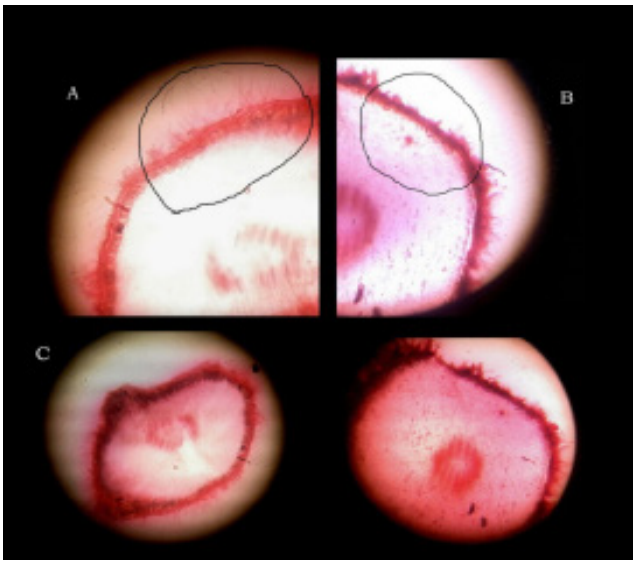

Figure 3: Histological study of transformed and non transformed tissues of *Chlorophytum laxum* A) TS of transformed tissue. Rounded portion showing significant morphological changes and emergence of hairy roots B) TS of non transformed tissue. Rounded portion with no significant morphological changes. C) images showing changes in meristematic region of transformed and non transformed tissue

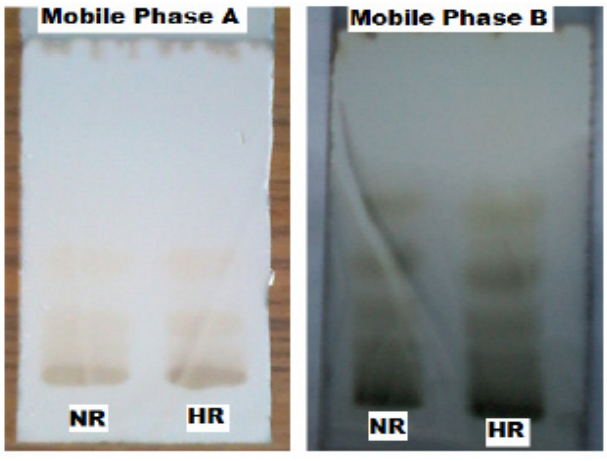

Figure 4: Thin Layer Chromatography of transformed and non transformed tissue of *Chlorophytum laxum* A) sample of non transformed tissue B) sample of transformed tissue

Figure 5: PCR analysis of hairy roots for confirmation of transgenicity where rolA is marker, CR: *Chlorophytum* roots, HR: hairy roots after infection with *A. rhizhogenes*

changed from 0.5 g to 0.7 g only. Growth of hairy roots obtained with R1000 strain was faster compared to control plants on MS basal media. Moreover control plants on basal media showed a sign of yellowing and could not be maintained after few subculture passages and an exogenous supply of phytohormones was indispensible for the maintenance of control plants for longer periods. Periodic subcultures on semisolid basal MS media and incubation under dark condition were necessary for optimum growth of transformants. Treatment of hairy roots with an antibiotic i. e. cefotaxime (300 µg/ml) for 5–6 subculture passages was a pre-requisite to eliminate the redundant bacterial growth (Figure 2)

In histological studies, darkly stained area showed meristematic activity in progress as compared to normal tissue. This is an indication of fast growth of transformed tissue. (Figure 3)

TLC analysis of saponins of normal and hairy roots showed clear band intensity difference. (Figure 4) In quantitative estimation of saponins, percentage yield of transformed roots of Chlorophytum laxum was 10% while that of non-transformed tissue of same plant was only 5.5%. Hence we concluded that saponin level in transformed tissue was higher than non-transformed tissue

Genetic markers included rolA responsible for T-DNA transfer and hairy root initiation[16-18] to confirm bacterial gene transfer were detected from transformed root DNA using PCR analysis. (Figure 5)

CONCLUSION

Our study presents a successful attempt towards the development of genetically transformed hairy roots in Chlorophytum laxum, a monocotyledonous herb that has utmost medicinal importance. We conclude that a protocol for development of hairy root culture in Chlorophytum laxum species was established successfully with significant enhancement of secondary metabolite level. Thus Hairy roots of Chlorophytum laxum initiated through the strain of Agrobacterium R1000 were grown successfully in MS solid medium for the first time.

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