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GENETIC TRANSFORMATION OF *Centella asiatica* BY *Agrobacterium rhizogenes*

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Abstract- *Centella asiatica* belonging to family Umbelliferae (Apiaceae) is an important medicinal plant. It contains pharmaceutically important bioactive compounds viz triterpenoid saponins and sapogenins, among which the asiaticoside and madecassoside are of particular interest. Asiaticoside derivatives are considered to have therapeutic importance in the treatment of Alzheimer's disease as they have shown to potentially protect cells from β-amyloid induced cell death. A soil borne gram negative bacterium, *Agrobacterium rhizogenes*, is the causative agent of hairy root disease in plants. Hairy roots are unique in being able to grow *in vitro* in the absence of exogenous phytohormones. In recent past, hairy root cultures from plants are getting considerable attention for their genetic and biosynthetic stability, rapid growth rate and ability to synthesize secondary products at levels comparable to the original plants.
Centella asiatica plants were transformed with *Agrobacterium rhizogenes* 8196 strain to induce hairy roots. Response of different explants viz root, leaf, petiole, nodal parts and incubation time was evaluated for hairy root induction and for their further growth.

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Introduction

Plants are the major source of traditional medicines culture throughout the world. In contemporary medicinal practices as well, secondary metabolites from plants constitute an important source of modern pharmaceutical drugs. They are becoming increasingly valuable products in the expanding market for herbal remedies. Various strategies have been employed to improve the production of secondary metabolites in *in vitro* systems (Bourgaud et al. 2001), including the use of genetically transformed roots.
The soil born plant pathogen *Agrobacterium rhizogenes* is responsible for hairy root formation at the site of infection. It also causes certain biochemical changes in the plant metabolism. Hairy root cultures have several properties that have promoted their use for plant biotechnological applications [14]. Hairy roots grow rapidly, show plagiotropic growth and are highly branched on phytohormone-free medium. The transformed roots are highly differentiated and can cause stable and enhanced production of secondary metabolites at levels comparable to intact plants, whereas other cell culture systems have a strong tendency to be genetically and biochemically unstable and often synthesize low levels of useful secondary metabolites [2-6].
Centella asiatica commonly known as gotu kola or brahmi is a small herbaceous annual plant of the family Apiaceae and is native to India, Sri Lanka, Indonesia, Malaysia, and other parts of Asia. It is used as a medicinal herb in Ayurvedic and traditional Chinese medicine. In common with most traditional phytotherapeutic agents, *C. asiatica* is claimed to possess a wide range of pharmacological effects, being used for human wound healing, mental disorders [15] atherosclerosis, antibacterial and antifungal [7], antioxidant [8] and anticancer purposes. *C. asiatica* has also been reported to be useful in the treatment of inflammations [9], diarrhea, asthma, tuberculosis and various skin lesions and ailments like leprosy, lupus, psoriasis and keloid. In addition, numerous clinical reports verify the ulcer-preventive and antidepressive

sedative effects of *C. asiatica* preparations, as well as their ability to improve venous insufficiency and microangiopathy [10].

Materials and methods

Plant material

Centella asiatica plants were collected from Tirupathi and aseptic cultures were established. *In vitro* established plants were used for transformation experiments. Different explants viz node, root, petiole and leaf were evaluated for genetic transformation studies.

Bacterial strain

A wild type strain of *A. rhizogenes* 8196 from MTCC, Chandigarh was used in the present study. The bacterial culture was maintained on Nutrient agar medium, and subcultured for every 15 days.

Chemicals

All the MS media components and nutrient media were procured from Himedia, India.

Establishment of *in vitro* cultures of *Centella asiatica*

Explants were treated with triton X for 2-3 mins followed by washing under running tap water for 15 mins. The explants were treated with $HgCl_2$ (0.1%) for 2-3 mins, followed by 6-7 washes with sterile distilled water in laminar air flow chamber. The explants were inoculated on Murashige and Skoog (MS) basal media and incubated in BOD incubator at 22±2 ºC under 3000 lux light, 16/8 h (light/dark) photoperiod.

Genetic transformation study

Agrobacteriun rhizogenes 8196 culture was prepared by inoculating 20 ml of Nutrient broth, with a loopful of bacteria followed by incubation on a gyratory shaker at 28º C at 200 rpm for different time period i.e. 24h, 48h, 72h.. Leaves, roots, petiole sections and nodal explants from *in vitro* grown *C. asiatica* plants were excised and evaluated for genetic transformations. Actively growing bacterial cultures of *A. rhizogenes* on nutrient broth (cell density $5x10^9$ cells/ml) were used for infecting the explants by incubating them for different time periods (10, 15, 20 min). After incubation, the explants were inoculated on MS basal media for co-cultivation, for 48 hrs. after that, the explants were transferred to MS basal media along with antibiotic cefotaxime (400 mg/L) to eliminate bacterial overgrowth.

Based on the result the best explants and the incubation period were evaluated.

Results and Discussion

Establishment of *in vitro* cultures of *Centella asiatica*

The nodal explants' gave good response when compared to other explants in establishing the aseptic cultures, after 20 days of inoculation. The *in vitro* cultures were maintained on MS basal media with a subculture period of 30 days (Fig. 1).

Genetic transformation study

The choice of explant for hairy root induction by *Agrobacterium rhizogenes* constitutes the most salient factor for the successful transformation. Plant transformation efficiency differs significantly according to the source of the explants [11]. In the present study hairy roots were produced at the base of the nodal explants after 25- 30 days of transformations (Fig. 2), followed by the leaf explants along with the petiole, showing the explants specificity.

Fig. 1- *In vitro* established *Centella asiatica*

Fig. 2- Hairy root induction at the base of the nodal explants

Only Petiole and root explants did not show any response. A 48 hrs. old *Agrobacterium rhizogene* exhibited best response for hairy root induction when compared to 24 hrs. and 72 hrs. culture (Fig. 3).

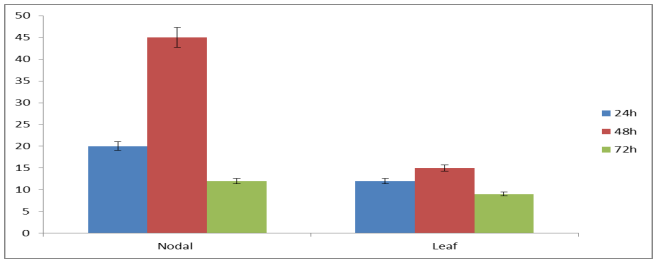

Fig. 3- Response of different explants at different growth stages of the *Agrobacterium rhizogenes*

The roots were maintained in MS basal media containing Cefotaxime for two subcultures and thereafter transferred to MS liquid media devoid of antibiotic for further proliferation. These hairy roots can be used for future experiments for enhancing the production of secondary metabolites.

The hairy root culture system is a potential approach for the production of secondary metabolites, especially pharmaceuticals. Hairy root features like rapid growth easy in maintenance and manipulation of cultures and their ability to synthesize higher amount of useful metabolites that cannot be produced in unorganized cell cultures and in some cases higher than the parent plant. The hairy root phenotype is characterized by fast hormone-independent growth, lack of geotropism, lateral branching and genetic stability [3]. The secondary metabolites produced by hairy

roots arising from the infection of plant material by *A. rhizogenes* are the same as those usually synthesized in intact parent roots, with similar or higher yields [12]. By using these hairy root cultures system it can be possible to develop a process which can be used for continuous production of triterpenoids saponins.

Agrobacterium rhizogenes mediated genetic transformation resulting in hairy root cultures is an efficient production alternative for secondary metabolites which has proved its effectiveness in the global arena. Hairy root technology has been exploited at industrial level by german company ROOTec for scale- up of hairy roots as a production alternative for important phytopahrmaceuticals [13]. The present study will be useful for large scale production of secondary metabolites with therapeutic value by using standardised process developed for multiplication of hairy root cultures.

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