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Agrobacterium rhizogenes mediated hairy root induction in two medicinally important members of family *Solanaceae*

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Withania somnifera (L) Dunal. and *Solanum surattense* Burm f., the two medicinally important members of family *Solanaceae*, were investigated for induction of hairy roots using soil borne bacterium, *Agrobacterium rhizogenes.* Explants like stem segments, hypocotyls and leaves with midrib were infected with the bacterium *in vitro*. In both plants, extensive hairy roots were induced from leaf explant containing midrib within 15 days of infection. These roots were then established on MS basal medium and their growth was observed to be independent of exogenous supply of phytohormones. The growth rate of transformed roots was 10-fold as compared to control. These roots can be grown and established in phytohormone free liquid MS medium and used as a promising source of secondary metabolites of medicinal significance.

Keywords: *Agrobacterium rhizogenes*, *Withania somnifera*, *Solanum surattense*, withanoloids, solasodine, hairy roots

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Introduction

Solanaceae family comprises a number of plants widely known for the presence of variety of natural products of medicinal significance, mainly steroidal lactones, glycosides, alkaloids and flavonoids. *Withania somnifera* (L.) Dunal. and *Solanum surattense* Burm f., the two important members of *Solanaceae*, have great medicinal importance in Indian traditional system of medicine[1].

W. somnifera(L) Dunal is a slow growing, seed propagated and evergreen shrub characterized by the presence of steroidal lactones, alkaloids and flavonoids. Over 90 different commercial preparations of this plant are available in the market[2] including root extract-based Ayurvedic preparations used for the treatment of different diseases and is considered as a general tonic. Besides roots, other plant parts are also useful in the treatment of inflammatory conditions, tuberculosis and rheumatism[3] attributed to the presence of steroids, withanoloids. Plant extract exhibits excellent antitumour activity[4].

S. surattense contains steroidal alkaloid, solasodine (SD), and other closely related glycosides[1,5]. SD serves as an important intermediate in synthesis of steroidal hormones[6] and is a potential alternative to diosgenin—a precursor in the synthesis of steroidal hormones[7].

Studies have focused on the production of commercially important secondary metabolites from *in vitro* cultures, especially callus and cell suspension cultures of various plants[8-10]. Infection of plants with a soil borne bacterium, *Agrobacterium rhizogenes*, induces the formation of proliferative and multi-branched adventitious roots at the site of infection[11], called "hairy roots". This bacterium has now been regularly used for gene transfer in many dicotyledonous plants[12].

Hairy root cultures from nearly 200 species of higher plants (at least 30 families), mostly dicots, have been reported as an established experimental system with a remarkable range of biosynthetic capabilities[13]. Subroto *et al*[14] observed *A. rhizogenes* mediated hairy root induction and subsequent regeneration of *S. nigrum* and the role of endogenous IAA on the regeneration capability of the transformed roots. Hairy root culture of this plant yielded SD comparable to that of intact plant[15]. Somaclonal variation in transformed root culture can also be obtained, which may differ in the tendency of producing secondary metabolites[16].

Though, a lot of work has been done on induction of transformed hairy roots from various plants of different genera, there are no reports on induction and establishment of hairy roots in *W. somnifera* and *S.*

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surattense. Present communication reports successful induction and establishment of hairy roots in these plants.

Materials and Methods

Plant Material

Seeds of high-yielding *W. somnifera* and *S. surattense* were obtained from Scheme for Medicinal and Aromatic Plants, Department of Botany, Mahatma Phule Agriculture University, Rahuri, MS, India and sown in plastic root trainer cups containing black cotton soil. After 15 days of seed germination, stem segments and hypocotyls were selected as explants. Leaves from 2-month-old plants were used as leaf discs. All the plant materials were first washed with detergent (5% teepol) in running tap water for 1-2 min and surface sterilized, initially in 70% ethanol followed by 0.1% $HgCl_2$ for 90 sec each and finally rinsed in sterile distilled water thrice[17].

Bacterial Strains

Two wild type strains of *A. rhizogenes*, MTCC 2364 and MTCC 532, obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India, were used for infecting the plant material. Various media, Luria Burntti (LB), Nutrient broth (NB), Tryptone broth, Yeast Mannitol broth (YMB), Tricalcium phosphate broth, and Yeast Extract Peptone (YEP) were tried for growth and maintenance of the bacterial strains. Both the strains showed excellent growth in YEP medium (*p*H, 7.0) comprising: sucrose, 5.0; peptone, 5.0; yeast extract, 5; and NaCl, 2 g ml^{-1}. Therefore, YEP medium was used in all further experiments.

Plant Tissue Culture Medium

MS basal medium[18] with 3% sucrose (w/v), at *p*H 5.5-5.7, gelled with 0.8% agar (w/v), autoclaved at 1.05 kg cm^{-2} for 18 min was used. For co-cultivation, MS medium without antibiotic and for proliferation the same medium with 250 mg/l of cefotaxime was used.

Induction and Establishment of Hairy Root Culture

For transformation studies, 48-hrs old culture of *A. rhizogenes* was first centrifuged at 3000 rpm for 5 min and the resultant cell suspension was resuspended in 5 ml of sterile MS medium. This suspension was used for infecting the plant tissues.

Co-cultivation

The surface sterilized stem segments and hypocotyl explants were placed on glass Petri dishes containing co-cultivation medium. A 10 µl aliquot of prepared bacterial suspension was applied on each explant. The same procedure was followed for leaf explants. Leaf discs (5×5 mm) were purposely wounded using surgical scalpel and placed ventral surface touching the medium. All these cultures were incubated in the incubator at 37°C for two days.

Proliferation

After two days of co-cultivation of plant tissues and bacterial cells, the explants were removed and washed thoroughly with sterile MS medium containing 1% mannitol and 250 mg/l cefotaxime. Washed explants were blot dried with sterile filter paper and placed on proliferation medium. Cultures were maintained at 25±1°C with 8 hrs photoperiod (1000 lux) on caster culture racks having photosynthetically active radiation (PAR) (Saveer Biotech, New Delhi) till induction of the hairy roots. After initiation of hairy roots the fast growing root tips were subcultured and maintained on solid MS medium in order to obtain inoculum for liquid culture. Various growth characters of established root clones were observed for six month. Sensitivity of transformants to phytohormones was tested[19].

Results and Discussion

YEP was found to be the most suitable medium for growth and maintenance of the bacterial strains (Table 1). Among the various explants, only leaf explants showed positive response to the infection of *A. rhizogenes*. Necrosis of stem segment and hypocotyl explants was observed within 24 hrs of co-cultivation. The probable reasons for this may be the delicate nature, age of the explant and the virulence of the bacterium. Both the strains of bacteria used were fast growing and dominated over the explant, resulting in death of tissues during co-cultivation.

Surface sterilized and purposely wounded leaf discs showed excellent results. During co-cultivation,

Table 1—Growth profile of *A. rhizogenes* in different media preparations

Growth medium	Optical density (600 nm)*
Luria Burntti broth (LB)	0.125
Nutrient broth (NB)	1.280
Tryptone	0.315
Yeast Mannitol broth (YMB)	0.075
Tri-calcium phosphate medium	0.009
Yeast Extract Peptone (YEP)	2.018

*O.D. taken after 24 hrs

a thin film of bacteria on explant surface and a thick growth along the edges of explant was observed within 24 hrs. In due course of co-cultivation, no signs of explants necrosis were seen; rather they remained healthy, green and swelled a bit.

After 7 days on proliferation medium, emergence of very small hairy roots was observed in both plants (Figs 1A & B), which continued to grow well (Fig. 1C). The frequency of emergence of hairy roots in both plants was found to be 20% (Table 2). During early stages of the root growth in both plants, the increase in the number of hairy root branches was almost logarithmic. The extensive branching was due to the presence of many meristems, which accounted for high growth rates of hairy roots in culture (Fig. 1D). This particular characteristic was observed to be the most common for members of *Solanaceae*[20].

These hairy root clones when maintained further on proliferation medium showed some typical features of hairy root syndrome i.e. plagiotropic growth, hormone independence and extensive lateral branching (Fig. 1C) in agreement with a previous report on *S. nigrum*[14]. Plagiotropism of hairy roots is a phenomenon common with hairy roots and is, infact, reported as one of the readily observed characteristic during *A. rhizogenes* mediated transformation[21].

Auxin sensitivity of the hairy roots showed that normally the transformed roots were hormone inde-

Table 2—Response of the various explants of *W. somnifera* and *S. surattense* to transformation by *A. rhizogenes*

Explant inoculated (No. of explants*, 60 each)		Response	Frequency (%)
W. somnifera	*S. surattense*		
Stem segments	Stem segments	-	00
Hypocotyles	Hypocotyles	-	00
Leaf	Leaf	+	20

(+)–Hairy roots induced
(-)–Hairy roots not induced
*Transformation was done in Petri plates each containing 10 explants

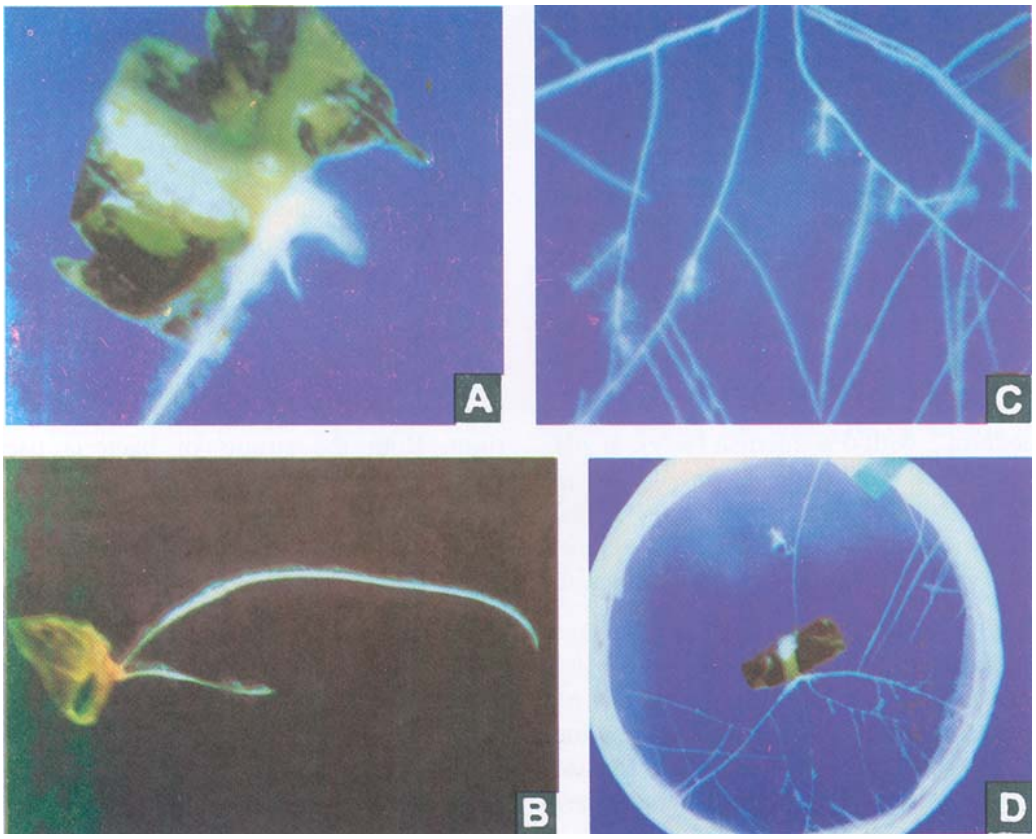

Fig 1A—Induction of hairy roots in *S. surattense* within 8 days of infection (8X); 1B: Induction of hairy roots in *W. somnifera* within 8 days of infection (4X); 1C: Proliferation of hairy roots in *S. surattense* (4X); & 1D: Birds eyeview of plate containing hairy roots of *S. surattense* (2X).

pendent and grew many folds faster than the normal roots. The growth was slightly accelerated when the growth medium was supplemented with lower concentration of IAA. As the concentration of auxin increased, swelling of the hairy root clones inclining towards formation of callus was observed.

Attempts were made for the establishments of these hairy roots on large-scale in shake flasks. For initial 48 hrs, the clones remained intact and observed to be elongated but as time passed, because of shaking perhaps, bead like structures were formed. These beads had hairs on their surface and grew well in broth resulting in increase in the biomass. The established hairy roots from both plants need to be further investigated for their potential in producing secondary metabolites of commercial importance. They may prove to be good source for the large-scale production of secondary metabolites in bioreactors from these plants.

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