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BRIEF COMMUNICATION

Hairy root culture of *Plumbago indica* as a potential source for plumbagin

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Abstract

Hairy roots of *Plumbago indica* were established at high frequency (90 %) by infecting leaf explants with *Agrobacterium rhizogene*s strain ATCC 15834. The axenic root cultures were established under darkness in hormone-free liquid Murashige and Skoog medium containing 3 % sucrose. The highest plumbagin content was found to accumulate in roots at their exponential phase of growth. A low pH (4.6) and a low concentration of sucrose (1 %) were beneficial for root growth in darkness, while pH 5.6 and 3 % sucrose under continuous irradiance enhanced plumbagin accumulation in roots up to 7.8 mg g^{-1}(d.m.). Direct shoot regeneration from hairy root culture was also achieved under continuous irradiance, thus indicated an easy way of obtaining transformed *P. indica* plants.

Additional key words: *Agrobacterium rhizogenes*, transformation, organogenesis.

Plumbagin, a naphthoquinone compound occurring mainly in *Plumbago* species was well known for its use in traditional medicines. Among all *Plumbago* species, (family *Plumbaginaceae*), *Plumbago indica* (syn. *rosea*) is the best source for harvesting plumbagin (Mallavadhani *et al.* 2002). According to several current reports (Chetia and Handique 2000) *Plumbago indica* becomes rare in several parts of India.

The objective of this work was to establish a new source for harvesting plumbagin without sacrificing the whole plant. As a result, an attempt was made to produce plumbagin *in vitro* from hairy root cultures of *P. indica*, established by genetic transformation using wild strain of *Agrobacterium rhizogenes* for the first time. The advantage of hairy root culture lies on the fact that these cultures generally grow faster and usually have higher amount of secondary metabolites in comparison to cell suspension culture and even in some cases higher than in intact plant roots (Allan *et al.* 2002). Growth of hairy roots can be scaled up by using bioreactors, hence are exploitable for commercial synthesis of plant derived natural products as evident from very recent work on anthraquinone production by hairy root culture of *Rhamnus fallax* (Rosić *et al*. 2006).

In the present study *Agrobacterium rhizogenes* strain ATCC 15834 was used to induce hairy roots of *Plumbago indica* L. The bacterial culture was maintained in solid *Agrobacterium* Broth (AB) minimal medium by routine transfer and prior to use in infecting explants, it was cultured in liquid AB medium on an orbital shaker at 28 ± 2 °C for 3 - 6 d. In 1-month-old axenic cultures of *Plumbago indica* L., bacterial infection was made on some selected sites of the stem and leaves by making wounds with a sterile needle loaded with bacterial suspension, showing absorbance (A_{600}) 0.5. Frequency of successful transformation and days taken for first appearance of hairy roots from leaf and stem explants were recorded.

Hairy roots arising from the infected sites were cut at the tips and transferred to the MS solid medium (gelled with 0.8 % agar) containing 3 % sucrose and 250 mg dm^{-3} cefotaxime (cefotaxime sodium, *ALKEM*, India) and maintained under darkness at 25 ± 2 °C. Root cultures were maintained by transferring root inocula in the same medium after every week with gradual reduction of cefotaxime concentration (200 mg dm^{-3} and 100 mg dm^{-3}, respectively). Finally, out of 10 root clones (free of bacteria), the fastest growing one was chosen for liquid

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Abbreviations: HPLC - high performance liquid chromatography; TLC - thin layer chromatography.
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culture establishment. Inocula (root tissue, *ca.* 50 mg) were transferred to 250 cm^3 Erlenmeyer flasks containing 50 cm^3 MS medium (pH 5.6) with 3 % sucrose on an orbital shaker at 70 rpm and maintained under darkness at 25 ± 2 °C. Plumbagin was detected from the plant extracts on a silica gel 60 F_{254} by thin layer chromatography (TLC) using a saturated mixture of toluene : acetic acid (99:1, v/v) as mobile phase (Panichayupakaranant *et al*. 2001) and it appeared as yellowish-red chromatographic zones on a white background. Linear ascending development was carried out in the saturated chamber up to a distance of 5 cm. The sample plumbagin was identified by comparison of R_f values with those of authentic plumbagin standard.

Effects of the duration of culture period, pH, sucrose concentration of the medium and light on growth and plumbagin production in hairy root cultures were studied. Three replicas were made for each set of experiment. For growth measurement, hairy roots were harvested periodically at 10 d intervals, weighed fresh and then dried. Experiment on effect of continuous irradiance was carried out under 41.76 µmol m^{-2} s^{-1} (white, fluorescent tubes, Philips, Mumbai, India). To study effect of pH, media pH was adjusted to 4.6, 5.6 and 6.6 before autoclaving. Sucrose in concentrations 3 (standard concentration), 2 and 1 % (m/v) was tested.

Fresh mass of hairy root tissue (*ca.* 1 g) was crushed in liquid nitrogen and then extracted with 5 cm^3 of 50 % (v/v) methanol (MeOH). The suspension was homogenized for 1 min and then centrifuged at 10 000 *g* for 20 min. The supernatant was collected and used as the source of crude plumbagin. This supernatant was then evaporated to dryness under reduced pressure and resuspended in 1 cm^3 of aqueous 50 % MeOH before injecting into HPLC.

HPLC analysis of plumbagin was carried out on a *Phenomenex*™ (Torrance, USA) C_{18} column (*Luna*, 4 µm, 250 × 4.6 mm) using a *JASCO* HPLC (Tokyo, Japan) system equipped with a *PU-2080 Plus*™ pump and a *PU-2075 Plus*™ UV-VIS detector. An isocratic linear solvent system of acetonitrile and water (20:80, v/v) with a flow rate of 1 cm^3 min^{-1} for 15 min was used to elute the plumbagin. Chromatogram was monitored at 410 nm and analyzed with *DataApex Clarity*™ software (Praha, Czech Republic). Identification of plumbagin was performed on the basis of retention time and chromatographic behaviour with those of authentic standards. Authentic standard was used as reference for quantitative analyses. HPLC elutes of plumbagin fraction was subjected to UV spectral analysis to confirm the chemical identity of plumbagin. A UV-spectral scan of the sample over the range (300 - 500 nm) was carried out in an *Analytik Jena* AG diode-array spectrophotometer (Jena, Germany) and data were analyzed with *Aspect Plus*™ software (Jena, Germany). The precise identification of the plumbagin was done by comparison of the absorption spectra of the sample with those of reference standards.

Though attempts have been made to produce plumbagin from several tissue cultures of *P. indica, e.g.* non-transformed roots (Panichayupakaranant and Tewtrakul 2002), cell suspension cultures (Komaraiah *et al.* 2001), immobilized cells (Komaraiah *et al.* 2003) and somatic embryos from suspension culture (Komaraiah et al. 2004), no report has yet been published on hairy root culture of this species.

After infecting explants with *Agrobacterium rhizogenes* strain ATCC 15834, numerous hairy roots with dense root hairs appeared from the site of infections of leaf lamina (Fig. 1*A*) and stem tissues (Fig. 1*B*) of young micropropagated *P. indica* plants. Further, these roots grew rapidly with emerging laterals in hormone-free medium showing typical hairy root phenotype (Fig. 1*C*). Higher frequency of induction (90 %) and earlier appearance (6.6 d) of hairy roots in leaf than that in stem (70 % in 18 d) is suggestive of the superiority of leaf over stem explants. It has been suggested that larger area of explants may cause more attachment of bacteria resulting in higher frequency of transformation (Mei *et al.* 2001).

In a selected axenic hairy root clone cultured for 50 d in auxin-free MS medium containing 3 % sucrose, an enhanced biomass as well as plumbagin accumulation were observed. Initially up to 10 d, root growth was slow; afterwards roots started developing into a large number of laterals that continued almost up to 30 d of culture. Afterwards, emergence of laterals was almost stopped and roots became thicker and showed tendency of callusing. Plumbagin was detected in the soluble fraction of hairy roots as evidenced from TLC analysis (Fig. 2*A*) followed by HPLC (Fig. 2*B*) and finally UV-spectral (Fig. 2*C*) analysis of the soluble fraction.

A time-course analysis of hairy root tissue for growth and plumbagin production (estimated in roots as well as in the medium) revealed that 20-d-old root culture showed highest plumbagin accumulation [5.32 mg g^{-1} (d.m.)]. Thereafter, a rise in biomass but a gradual decline in plumbagin content was noticed in cultures grown for 30 d [2.5 mg g^{-1} (d.m.)], 40 d [1.1 mg g^{-1} (d.m.)] and 50 d [0.5 mg g^{-1} (d.m.)]. Hairy root cultures of other species such as *Plumbago zeylanica* (Verma *et al.* 2002), *Tagetes patula* (Mukundan and Hjortsa 1991), and *Gmelina arborea* (Dhakulkar *et al.* 2005) also showed a similar growth pattern. In our study, it was observed that the growth of *P. indica* normal roots was slower than growth of the hairy roots. A more or less similar observation was recorded by Panichayupakarant and Tewtrakul (2002) in root cultures of *Plumbago rosea*. In hairy roots, faster growth rate was observed because of high frequency lateral root formation and consequent increase in the number of elongating tips (Mano *et al.* 1989). In the early stage of growth, there was a logarithmic increase in the number of branches (Flores and Filner 1985) and in the presence of numerous meristems which account for high growth rate for hairy root in culture, exceeds over most non-transformed root culture (Quattrocchio *et al.* 1986).

Also plumbagin content in non-transformed roots was much lower than that in the hairy roots [2.1 mg g^{-1}(d.m.)]. Further, a detectable amount of plumbagin was always

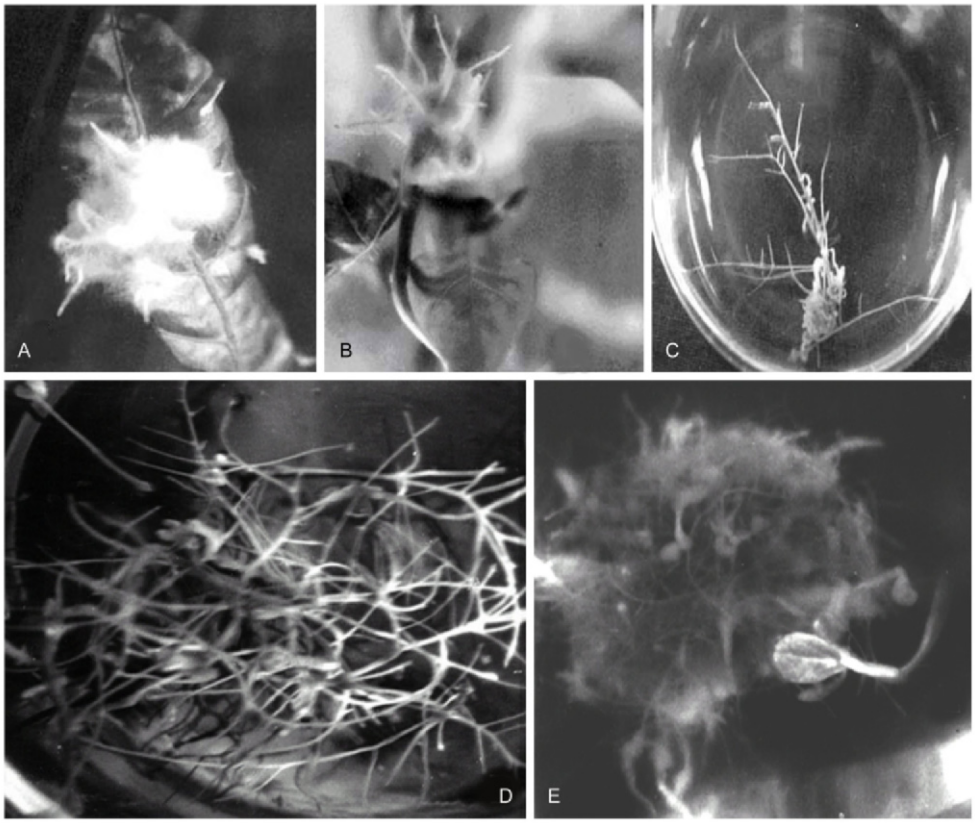

Fig. 1. Induction of hairy roots from different explants of *Plumbago indica* and light induced organogenesis in hairy root cultures: *A* - hairy root emerging from leaf lamina, *B* - hairy root emerging from stem tissue of internodes, *C* - hairy roots growing on auxin-free medium, *D* - initiation of greenish shoot bud formation (indicated by *black arrow*), *E* - growth of single shoot bud in liquid MS-medium.

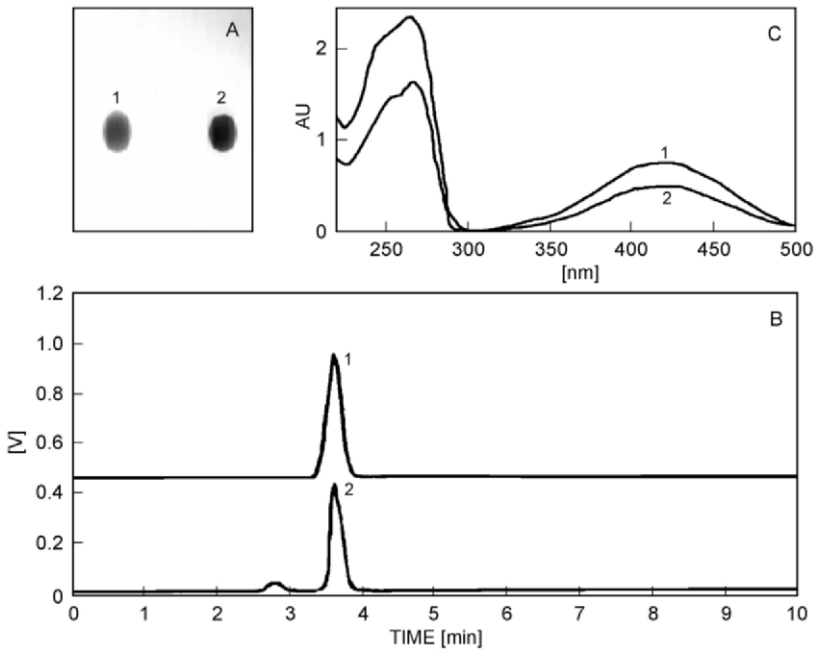

Fig. 2. Identification of plumbagin in hairy root cultures of *P. indica*: *A* - detection of plumbagin by TLC (*lane 1* - plumbagin standard, *lane 2* - plumbagin from hairy roots tissue); *B* - HPLC chromatogram showing plumbagin from soluble fraction (1 - plumbagin standard, 2 - plumbagin from hairy roots tissue, chromatograms were monitored at 410 nm); *C* - confirmation of chemical identity of plumbagin by UV-spectroscopy (1 - plumbagin standard, 2 - plumbagin from hairy roots tissue).

determined in the spent medium indicating a leaching of metabolite in the medium during culture.

Neither of the used pH (4.6, 5.6, and 6.6) showed adverse effect on hairy root growth of *P. indica*, and the pH 4.6 was found most suitable in respect to growth (0.34 g) and plumbagin contentt [7.23 mg g^{-1}(d.m.)] as obtained from 20-d-old root culture. Similar to the present results, better growth and higher diosgenin accumulation in *Trigonella foenum-graecum* hairy roots (Merkli *et al*. 1997) was observed in lower pH (5.0 and 4.8 before and after autoclaving, respectively) in comparison with that grown in higher pH such as 5.5 and 5.9. Although several investigations have been made on influence of pH on growth and secondary metabolite formation in hairy roots of different plant species (Mukundan and Hjorsto 1991), the results regarding this aspect are not clear.

In order to investigate the effect of sucrose in growth and plumbagin production, different sucrose concentrations (1, 2 and 3 %; m/v) were tested. It was observed that supplementation of 2 % sucrose yields maximum plumbagin accumulation [biomass (0.13 g), plumbagin content 6.18 mg g^{-1}(d.m.)], whereas, maximum biomass was observed at 1 % sucrose [biomass (0.14 g), plumbagin content 5.43 mg g^{-1}(d.m.)] followed by 3 % sucrose concentration [biomass (0.12 g), plumbagin content 5.2 mg g^{-1}(d.m.)]. A similar situation to the present one was observed in *Plumbago rosea* non-transformed root culture, where supplementation of 1 % sucrose to the culture medium showed enhanced accumulation of plumbagin without affecting the biomass growth (Panichayupakaranant and Tewtrakul 2002). Also in *Solanum khasianum* (Jacob and Malpathak 2004) supplementation of 1 % sucrose caused an increase in solasodine production without any increase in biomass growth.

Inhibition of growth of hairy root lines of *P. indica* was observed when incubated under continuous irradiance. The root mass obtained from culture grown under irradiance was relatively less (0.12 g) than that dark grown under dark (0.16 g). However, induction of morphogenesis and enhancement of plumbagin content in hairy roots were found to be associated with irradiance. Prominent greenish shoot buds appeared on hairy roots. Eventually, from 3rd week of the culture and onwards, these buds sprouted to small leafy green shoots attached firmly with root epidermis by very small stalks (Fig. 1*D*,*E*). The light grown root showed better plumbagin yield [7.8 mg g^{-1}(d.m.)] than dark-grown roots [4.9 mg g^{-1}(d.m.)]. Light-induced growth inhibition (Mukundan and Hjortsa 1991) with concomitant increase in the synthesis of secondary metabolites as compared to dark-grown hairy root cultures was reported earlier (Hagimori *et al*. 1982, Ohlsson *et al*. 1983)

P. indica hairy root culture thus provides an attractive system for the production of plumbagin; a secondary metabolite with high economical and medicinal relevance. In this communication we report for the first time, the induction of hairy root cultures of *P. indica* and optimization of growth condition for plumbagin production. It is worthwhile to mention that the work is currently in progress to study the enzymatic route of plumbagin synthesis as well as to explore the possibility of plumbagin production at industrial scale using hairy root culture of the species.

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