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Rapid *in vitro* shoot multiplication and analysis of plumbagin in *Agrobacterium rhizogenes* mediated hairy root culture of *Plumbago zeylanica* L.

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ABSTRACT

Plumbago zeylanica L. is an important medicinal plant and roots are used for dyspepsia, piles, diarrhoea, skin diseases, leprosy and rheumatism. A protocol for rapid *in vitro*-multiplication through axillary bud proliferation was developed. Approximately 7 shoots were produced from a single nodal segment of a four year old field grown plant after 4 weeks of culture on Murashige and Skoog's (MS) basal medium supplemented with 2 mg/l BAP + 100mg/l adenine sulphate. Optimum number of roots was induced (~15/shoot) upon transferring the individual regenerant to half strength MS medium supplemented with 0.5mg/l IBA. Hairy roots were initiated with the A4 strain of *Agrobacterium rhizogenes* which exhibited optimum growth in half strength MS medium containing 4% sucrose. Growth kinetic studies demonstrated a maximum 11 fold increase in root biomass yield after 6 weeks of culture. The fresh hairy roots produced 0.61% higher amounts of plumbagin over the untransformed control roots. The present research findings revealed for the first time the potentialities of the hairy root cultures of *P. zeylanica* for the production of the important secondary metabolite, plumbagin.

1. Introduction

Plants have been an important source of medicine for thousands of years. Even today, the World Health Organization estimates that up to 80% of people still rely mainly on traditional medicines for remedies. *Plumbago* species L. (Plumbaginaceae) is a medicinal shrub distributed throughout India. The plant is commonly known as Ceylon leadwort (English), Chita or Chitra (Hindi) and Chitramoolam (Tamil). The tuberous roots of *Plumbago* species are used as an important indigenous ayurvedic drug. The root contains plumbagin, 3-chloroplumbagin, 2,3-biplumbagin, 6,6-biplumbagin, zeylinone, isozeylinone, chitranone, droserone, plumbagic acid and plumbazeylanone (Chinnamadasamy *et al.,* 2010). The leaves and stem contains little or no plumbagin. Roots of *Plumbago zeylanica* L. are used for the treatment of various ailments, such as dyspepsia, piles, diarrhoea, skin diseases, leprosy and rheumatism (Rout *et al.,* 1999). Roots are also reported as antibacterial, antifungal, abortifacient (Uma Devi *et al.,* 1999) and reported to be substitute for cantharides (Chetia and Handique, 2000). The pharmacological importance of this perennial shrub lies in its ability to produce a napthoquinone, called plumbagin (Modi, 1961), mainly found in its roots. Discoveries of the tumor inhibitory substance (Krishnaswami and Puroshothaman, 1980) and radiomodifying effects (Uma Devi *et al.,* 1999) of plumbagin have enhanced the demand of this medicinal plant for its roots. Conventional propagation of the plant is rather difficult and insufficient to meet the growing demand owing to the poor germination of seeds and death of young seedlings under natural conditions (Anonymous, 1989). Moreover, indiscriminate collection of the roots from the natural habitat to meet the growing demands of the pharmaceutical companies and various adverse biotic factors affecting the wild population coupled with inadequate attempts for its replenishment have led to

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acute depletion of the natural population. Hence, rapid *in vitro* micropropagation approaches are highly important for steady supply of plants for commercial use. Reports on plant regeneration from callus cultures of *P. zeylanica* (Debata and Susmita, 1998, Rout *et al*., 1999, Verma *et al*., 2002, Mallikadevi *et al*., 2008) and clonal multiplication of *P. indica* (Chetia and Handique 2000) and *P. rosea* (Kumar and Bhavanandan, 1988, Jose *et al*., 2007) through axillary bud proliferation was reported earlier. Establishment of an efficient and reproducible organogenic system in such important medicinal plant species is a pre-requisite not only for basic research but also for commercial exploitation of plumbagin. *In vitro* plant also have greater significance in genetic transformation studies through *Agrobacterium rhizogenes* for hairy root culture. Selvakumar *et al.* (2001) reported shoot multiplication in *P. zeylanica* earlier with very less number of shoot regeneration which need further refinement in rate of *in vitro* shoot multiplication.

Furthermore, as the medicinally active constituents of *P. zeylanica* are mainly obtained from the root tissues, standardization of *Agrobacterium rhizogenes* mediated genetic transformation for hairy root cultures and evaluation of plumbagin production by transformed culture have greater importance. In a wide range of medicinal plants, hairy root cultures have already proven to be an efficient alternative production system for root derived phytochemicals of consistent quality within much shorter time than is commonly expected from *in vivo* grown plants (Tepfer, 1990, Wysokinska and Chimel, 1997, Canto-Canche and Loyola-Vargas, 1999, Banerjee *et al.,* 2002). The present study dealt with *in vitro* multiplication of *P. zeylanica* for large number of micropropagated plant in one hand and standardization of hairy root culture in regenerated elite clones through *A. rhizogenes* and evaluation of plumbagin in hair roots on the other hand.

2. Materials and methods

2.1 Nodal multiplication, rooting and hardening

Plants of *Plumbago zeylanica* are collected from medicinal garden of College of Agriculture, Orissa University of Agriculture and Technology, Bhubaneswar. Young, nodes from newly sprouted young stems (2-3 weeks old) of *P. zeylanica* were cut and washed in distilled water. They were washed with 2% Tween-20 solution for 8-10 min and washed with 70% ethanol followed by 0.1% (w/v) mercuric chloride for a 5 min and thoroughly rinsed three-four times in sterilized distilled water. About 1.0 cm long sprout segments cultured on agar solidified MS (Murashige and Skoog 1962) medium (pH 5.8) supplemented with various concentration of 0.5-5.0 mg/l BAP along with 25-100 mg /l adenine sulphate for induction and proliferation of multiple shoots. Cultures were maintained under cool-white fluorescent light (3,000 lux) at 25±2°C with 16 h photoperiod. Sub-culture was done after 3-4 weeks interval in MS medium with same concentration of hormones. After induction and proliferation of multiple shoots, these are transferred into rooting medium i.e. MS supplemented with 0.1 – 1.0 mg/l IBA. The regenerated rooted plantlets were first pre-hardened with distilled water in culture room for 48 h so as to acclimatize them. Then these plants were transferred to polybags containing sand, soil and vermiculite (1:1:1) under green house condition for 7 d for hardening.

2.2 Hairy root culture through Agrobacterium rhizogenes

The aseptically grown nodal explants and leaves were infected with 48h old suspension of the *A. rhizogenes* both A4 and MTCC532 strain (OD value 0.8 at absorbance 620 nm) grown in liquid YMB medium (Hooykaas *et al.,* 1977) having 200 mM acetosyringone. After co-cultivation with bacteria for 24h to 96 h on semi-solid, hormone-free MS medium, the explants were transferred onto the same medium containing 500mg/l of cephalaxin (Ranbaxy, India) under dark conditions in order to get bacteria free culture. Hairy roots were induced after 7-10 days after transferring of bacteria free culture on hormone free MS medium. The emerging hairy roots were subsequently transferred to half or full strength of liquid MS medium with 3% or 4% sucrose under dark condition for further growth.

2.3 Plumbagin content in transformed and non-transformed roots

Dry hairy roots (1g weight) were consequently chemically extracted (in triplicate) according to the earlier reported protocol (Gupta *et al.,* 1999, Basu and Yogananth 2009) and the plumbagin content was quantified through a HPTLC. Chemical extraction and quantification of plumbagin were also carried out in the fresh, non-transformed control roots as well as in the dry hairy roots at the optimum growth phase for comparative analysis. The soxhlet extraction was done from transformed and non-transformed dried roots using methanol. Plumbagin was identified by TLC method using silica-gelG-60 powder pre-coated TLC plates (E-mark, Germany) for standard and extracted samples. Solvent system used for TLC plates was toluene:glacial acetic acid (99:1) and the light red spot of authentic sample was identified as plumbagin in UV light and after derivatization in anisaldehyde sulphuric acid reagent followed by heating at 110^{0}C for 10 min. Quantification of plumbagin from transformed and non-transformed dried roots was carried out through HPTLC method using Camag TLC applicator. The HPTLC finger prints profile was snapped

by Cammag Reprostar III, before derivatization under UV light 254 nm, 366 nm and after derivatization. The isolated plumbagin peak was confirmed by comparing the spectrum obtained by TLC scanner, which was completely in agreement with the reference standard.

3. Results and discussion

3.1 Nodal shoot multiplication, rooting and hardening

The best multiple shoot initiation response was noted in MS medium supplemented with 2mg/l BAP along with 100mg/l adenine sulphate. Approximately 7 numbers of shoots per node were recorded after three to four weeks of culture (Table 1; Figs. 1 and 2). Root induction was observed with all media combinations tried with IBA (Table 1). The average number of roots (~15) with root length (~2-3 cm) were induced on half strength MS medium containing 0.5 mg/l IBA and well developed plants with good root systems were produced within two weeks (Table 1, Fig. 3). Well developed plants with good root systems were produced within two weeks of transfer to this medium. Sub-culturing was done after 3-4 weeks intervals in MS medium in same concentrations of growth hormones to provide proper nutrients for growth of the *in vitro* regenerated plants. The reported direct shoot regeneration protocol can be exploited commercially to multiply elite clones more rapidly and within a shorter time period, and also can be used for developing *in vitro* strategies for the conservation of this useful medicinal plant. Adenine sulphate with BAP in the present experiment showed very exciting result in shoot multiplication in *P. zeylanica* with average of 7 shoots as compared to only one shoot per explants with IBA and adenine sulphate combination as reported by earlier (Selvakumar *et al.,* 2001). Lowering the concentrations of both the plant growth regulators to half of their original strengths improved the growth, and elongated shoots with 8–10 inter-nodes could be obtained within the next 3 weeks of culture. Lowering the concentration of BAP improved differentiation of shoot buds has also been reported in case of *P. indica* (Anonymous, 1989). Hence, the multiplication rate as revealed in the present study significantly exceeds that of the report of Selvakumar *et al.* (2001) within a relatively shorter time period. More than 90 % of plantlets survival was observed on hardening for one week. However, the rate of survival decreased to 55% after two three weeks of acclimatization. It was observed that gradual acclimatization of *in vitro* grown plants to external environment is most essential for *P. zeylanica.* More than 85 % of the plants transferred to pots survived under field conditions (Fig. 4).

Table 1

Effect of various concentrations of plant growth regulators on shoot and root formation in *P. zeylanica* culture.

	Shoot multiplication			Rooting of micropropagated shoot	
BAP (mg/l)	ADS (mg/l)	No. of shoots/node	IBA(mg/l)	No. of roots/shoot	Rooting response
0.5	0	1.0±0.2	0.1	0	No rooting
1.0	25	2.2±0.6	0.2	1.0±0.25	No rooting
1.5	50	4.6±0.5	0.3	2.3±0.55	Slow rooting
2.0	100	7.5±0.4	0.4	7.2±0.75	Profuse rooting
2.5	100	4.2±0.5	0.5	15.1±0.45	Profuse rooting
3.0	100	3.7±0.9	0.6	9.5±0.33	Profuse rooting
3.5	100	2.5±0.24	0.7	5.2±0.65	Slow rooting
4.0	100	2.2±0.54	0.8	4.8±0.44	Slow rooting
4.5	100	1.7±0.46	0.9	3.2±0.05	Slow rooting
5.0	100	1.5±0.87	1.0	3.0±0.15	Slow rooting

3.2 Hairy root culture and kanamycin based selection

Nodal and leave explants from such *in vitro* raised plantlets served as explants for genetic transformation studies. The relative transformation frequency in A4 strain of *A. rhizogenes* was recorded to be 78.29% and 50.35% in shoot and leaf explants respectively in 150 min of co-culture after 3 weeks of bacterial infection (Table 2). No transformation was recorded in and MTCC532 strain. Excision and sub-culturing of the emerging hairy root clones (20-30 in number) on full and half strength MS medium with 3% sucrose exhibited a very thin and fragile appearance with limited growth characteristic. However, half strength liquid MS medium containing 4% sucrose supported active proliferation of one healthy looking root line (Fig. 5) as has also been reported earlier by Banerjee *et al.* (1998) in case of *Valeriana wallichii*. A gradual increase in the hairy root induction was

noticed with an increase in co-cultivation from 1 to 3 days. The developed roots exhibited fast growth and high lateral branching on growth regulator free MS medium and plagiotropism. The biomass in hairy root culture was higher than in non transformed root culture. The survival percent of regenerated transformed plants from callus (20%) and multiple shoots (30%) by *A. rhizogenes* (A4) strain in 120 min infection time and 2d co-cultivation periods. No regeneration was found from leaf and multiple shoots in *A. rhizogenes* (MTCC532) strain. The growth inhibiting dose of kanamycin was determined by transfering the transformed and non transformed lines on MS medium supplemented with various concentrations of kanamycin (0-60 mg l^{-1}). It was observed that lethal dose for control plant 60 mg l^{-1} kanamycin used for primary screening of putative transformants. The optimized selection method eliminates the regeneration of non-tranformed plants.

3.3 *Plumbagin content transformed and non-transformed roots*

Growth index analysis of this hairy root clone revealed a gradual increase in fresh weight up-to 6 weeks of culture at which a maximum 11 fold increase in biomass could be recorded (Fig. 5). The plumbagin production potentials of the fresh hairy root cultures paralleled with the different growth phases and reached its maximum during the optimum growth period, i.e. 6 weeks of culture. Plumbagin content of the fresh hairy roots with that of the fresh, non-transformed control roots and the dry hairy roots at the optimum production phase (i.e. 6 weeks of culture), the fresh hairy roots were found to possess 8.13% plumbagin than non-transformant roots (7.52%) (Figs. 6 & 7). Besides this, the fresh weight : dry weight ratio of the hairy roots being 20:1, the fresh hairy roots were found to possess 49.4 times higher amount of plumbagin than the dry *in vitro* roots in *Plumbago zeylanica.* The present study demonstrates for the first time, successful induction and establishment of *A. rhizogenes* mediated hairy root cultures in *P. zeylanica* with higher potential for the production of the active compound – plumbagin. The production of this compound from hairy root cultures can further be optimized for commercial production.

Table 2

Effect of co-culture period on mean percentage of root induction in shoot tips and leaves (A4 strain)

Co-culture period	Response of shoot tips			Response of leaves		
	7 d	15 d	21d	7 d	15 d	21d
30 min	22.27±0.25	38.89±0.35	44.44±0.36	0	0	0
60 min	33.33±0.79	45.23±0.44	50.30±0.87	0	0	0
90 min	38.78±1.01	51.25±0.26	55.29±0.96	11.10±0.03	16.66±0.34	27.89±0.12
120 min	52.23±0.96	56.26±1.04	62.15±0.16	22.21±0.33	28.24±0.24	35.25±0.15
150 min	55.15±0.39	63.67±0.65	78.29±0.97	23.26±0.43	38.89±0.45	50.35±0.67
180 min	35.25±1.12	42.30±0.26	50.29±0.88	5.52±0.24	12.14±0.33	16.34±0.45

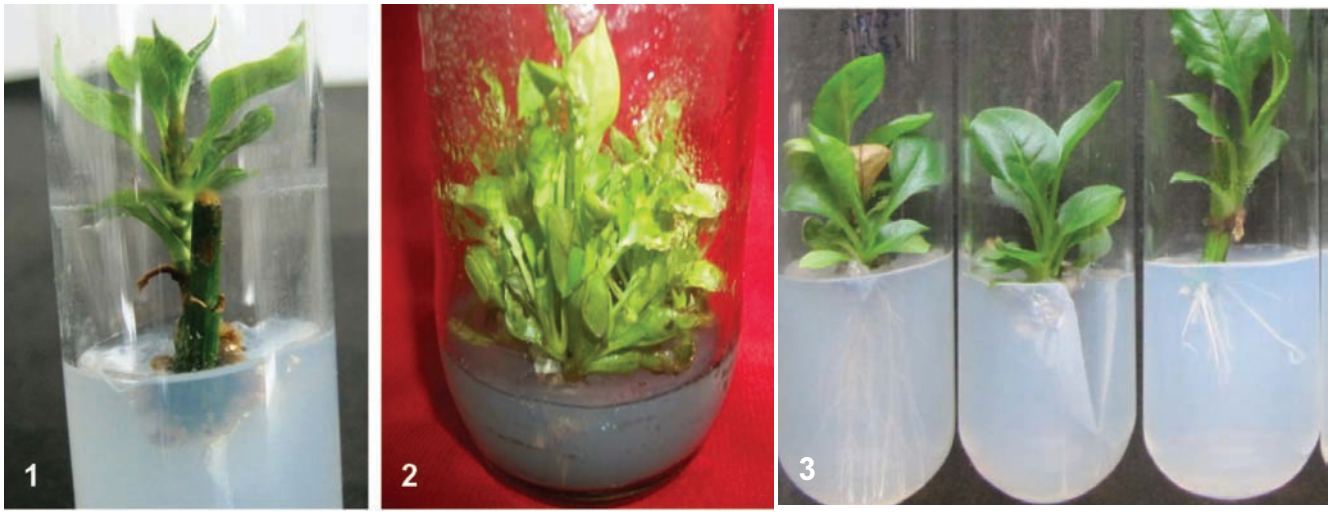

Fig. 1. Aseptic nodal culture establishment of *P. zelinica* in MS medium supplement with 1.5 mg/l BAP. Fig. 2. Nodal shoot multiplication of *P. zelinica* in MS medium supplement with 2mg/l BAP + 100 mg/l adenosine sulphate. Fig. 3. *In vitro* rooting of multiple shoots of *P. zelinica* in MS medium supplemented with 0.5 mg/l IBA.

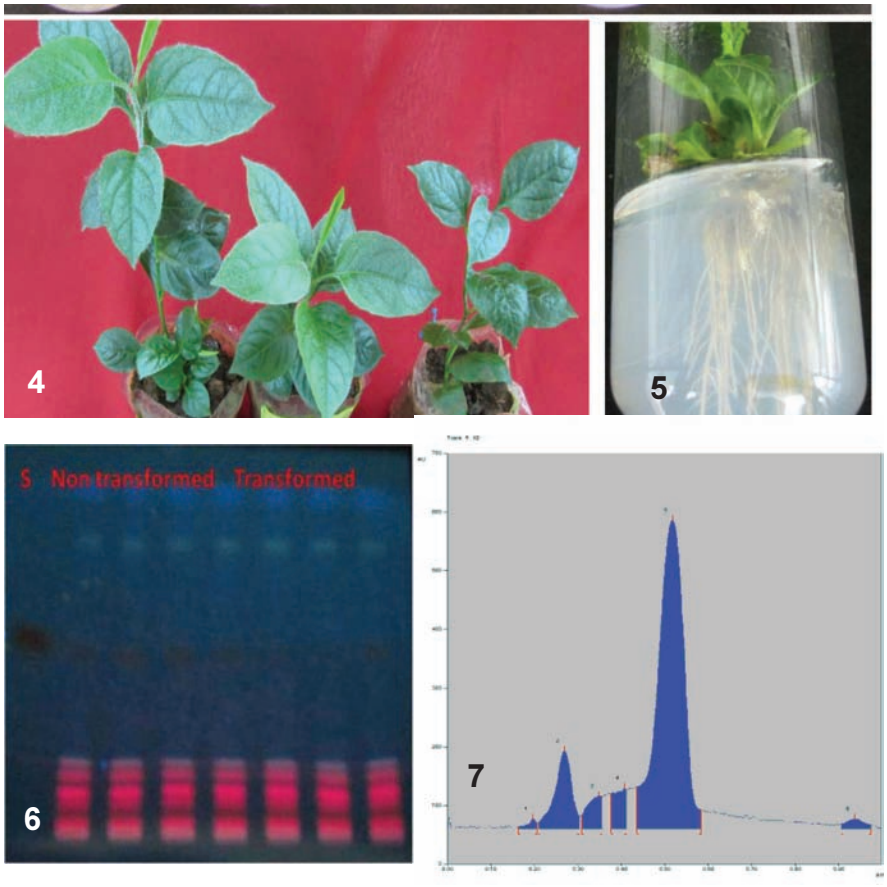

Fig. 4. Hardening of rooted plants of *P. zelinica* in net house. Fig. 5. Hairy root initiation of *P. zelinica* aseptically grown nodal shoot tip transformed with *Agrobacterium rhizogenes.* Fig. 6. HPTLC plats showing plumbagin in transformed and non transformed roots. Fig. 7. HPTLC picks showing plumbagin content in transformed and non-transformed root samples.

4. Conclusion

In vitro nodal multiplication, rooting and hardening of *Plumbago zeylanica,* a medicinal plant having potential active principle napthoquinone - plumbagin, is standerized. Hairy root culture through *Agrobacterium rhizogenes* mediated genetic transformation is also successful with A4-strain. Pricking method in aseptically grown *in vitro* generated multiple shoots and leaves were found more effective in shoots as compared to leaf explants. The establishment of hairy root cultures through *Agrobacterium rhizogenes* mediated genetic transformation could produce more roots as compared to normal root produced with a higher production of a bioactive compound, plumbagin, in transformed root. Plumbagin content was found to be more in transformed roots (8.13%) than non- transformed roots (7.52%) as detected by HPTLC method.

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