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Genetic Transformation of *Plumbago zeylanica* with *Agrobacterium rhizogenes* Strain LBA 9402 and Characterization of Transformed Root Lines

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Abstract

High frequency transformation (73.80 ± 2.24%) has been obtained in *Plumbago zeylanica* using nodes and internodes of axenic whole plants infected with *Agrobacterium rhizogenes* strain LBA 9402. The root lines could be distinguished morphologically into two types **:** Root lines of morphotypes I and II. While morphotype I showed profuse branching with short (< 1 cm), highly dense hairy laterals, the roots of morphotype II roots were characterized also by profuse branching with long hairy laterals (> 3 - 4 cm). Only four of the ten root lines showed integration of four *rol* genes (*rolA*, *rolB*, *rolC* and *rolD*) of TL-DNA. None of the root lines showed presence of any of the five genes of TR-DNA. It is noteworthy that the root morphotypes (I and II) showed a clear distinction in the nature of integration and expression of *rol* genes. The transformed root lines varied significantly ($p \leq 0.05$) with respect to DW (GI DW basis, 2.19 ± 0.24 - 5.31 ± 0.6) after 4 weeks of culture on solid modified MS; and plumbagin contents in root lines (4.81 ± 0.16 - 6.69 ± 0.34 mg/g DW) were higher than that reported earlier. Transformed root lines of *P. zeylanica* maintained *in vitro* on phyto-hormone devoid medium for over 2 years can be used for scale up studies for the production of plumbagin in bioreactors.

Introduction

Plumbago zeylanica L. is an important medicinal plant indigenous to South-east Asia and a recognized natural resource of anapthoquinone compound called plumbagin isolated mainly from its roots (Mallavadhani et al. 2002). Plumbagin

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has been reported to have antitumor, antimicrobial, (Hazra et al. 2008, Didry et al. 1994) properties. It is also the principal active component of a microfilaricidal drug (Mathew et al. 2002). Wide medicinal properties of plumbagin led to the indiscriminate collection of the roots from the natural habitat (Chetia and Handique 2000). Moreover, the low germination of seeds and death of young plantlets under natural environment makes conventional propagation of the plant challenging and inadequate to meet the growing demand of the pharmaceutical companies. Various *in vitro* protocols have been used in an effort to boost plumbagin production in *Plumbago* species including the induction of adventitious roots in *P. indica* (Panichayupakaranant and Tewtrakul 2002) and *P. zeylanica* (Sivanesan and Jeong 2009).

Hairy root cultures have been reported to be a potent substitute for secondary metabolite production system for root-derived phytochemicals in a wide range of medicinal plants owing to high productivity and genetic stability (Roychowdhury et al. 2013, Chandra 2012). There are a few reports on genetic transformation of *P. zeylanica* for production of plumbagin. Verma et al. (2002) reported a very low transformation frequency by *A. rhizogenes* strain A4 and low accumulation of plumbagin (0.017 %). *P. zeylanica* transformed with recombinant *A. rhizogenes* strain ATCC 15834 containing the PL-scFv gene exhibited 2.2 times higher plumbagin content than those obtained from the wild-type (Sakamoto et al. 2012).

In the present study, authors report genetic transformation of *P. zeylanica* using *A. rhizogenes* strain LBA 9402.Morphological and molecular characterization of one year old transformed root lines has been done in detail for identification of high plumbagin yielding rhizoclones.

Materials and Methods

Plumbagozeylanica shoots were collected during May-June of 2011 from 10-year-old plants growing in the Experimental Garden, Department of Botany, University of Calcutta. Shoot cultures of *Plumbago zeylanica* L. were sterilized and established following the method described by Verma et al. (2002). The shoot cultures were maintained on the solid MS containing 3% (w/v) sucrose, 0.75% (w/v) agar and supplemented with 4.4 μM BA, maintained at 24 ± 1°C and 50 - 60% relative humidity in the presence of light (with a 16/8 hrs light/dark photoperiod. Roots were initiated in axenic microshoots cultured on solid MS for 7 - 10 weeks.

Agrobacterium rhizogenes strain LBA 9402 (pRi 1855) (Petit *et al*. 1983) was used in the present study. For **a** transformation experiment, bacteria from a single colony was grown for 24 hrs. in 10 ml of liquid YMB medium, pH 7.0, on a gyratory shaker at 26°C at 180 rpm. Acetosyringone (200 μM) was added to the bacterial suspensions ($O.D._{600nm}$ > 1) 2 hrs before infection.

Axenic *P. zeylanica* plants (3 - 4 cm in length, 6 - 8 weeks old) and leaves excised from such plants cultured on solid MS were used for infection. Excised leaf explants were wounded at midrib and whole plants were wounded at nodes and internodes with a hypodermic needle loaded with the bacterial suspension as reported for *Tylophoraindica*(Chaudhuriet al. 2005). Liquid bacterial media that had not been inoculated with bacteria was used as a control.

The infected, excised leaves were kept on a filter paper soaked with liquid MS in Petri plates (9 cm diam) for cocultivation in the dark for 72 hrs. After 72 hrs of culture, the explants were washed in ampicillin (1,000 mg/l) for 30 min and rinsed four to five times in sterile distilled water. The explants were then cultured in Petri plates containing 25 ml solid MS supplemented with 500 mg/l ampicillin, in the dark for 8 weeks. Inoculated whole plants were cultured on solid MS for 8 weeks in culture tubes under a 16/8 hrs (light/dark) photoperiod.

For each experiment, 30 leaf explants and 20 plants were infected and all of the experiments were repeated thrice. Data for wound callus formation and transformed root induction were recorded every week up to 7 weeks after initial infection.

Roots initiated at the wound sites of the infected excised leaf explants or at wound sites of the whole plants were excised and cultured in the dark in Petri plates (9 cm diam) containing 25 ml of solid MS or modified MS (Tepfer 1995) supplemented with ampicillin (500 mg/l). Each excised primary root was propagated as a separate line and sub-cultured at 4 week intervals. Each line was screened for the presence of bacteria by culturing the root segments in YMB medium. Fast growing axenic lines were subcultured at a 4-week interval and maintained on ampicillin free solid modified MS after 1 year of infection.

The growth and morphology of one year old transformed root lines maintained on solid modified MS medium by subculturing at 4-week intervals were studied. Roots excised from 8-week old intact non-transformed *in vitro* plantlets grown on solid MS medium were used controls.

Five root tips [~ 0.1 g fresh weight (FW)] of each root line, were subcultured on solid modified MS (25 ml) in 9-cm Petri plates and cultured for 28 days in the dark. Twenty-five root tips were used as explants for each root line and the experiments repeated thrice. The roots derived from each explant, were washed with deionized water, blotted dry and the FW determined. The roots were then

oven-dried (55°C) to a constant weight to obtain the dry weight (DW). Growth was measured as an index of DW [growth index, GI = (harvest DW-/inoculum DW)].

Confirmation of the transgenic nature of the roots and molecular characterization of transformed root cultures were performed on the basis of the presence and/or absence of 18 ORFs from TL-DNA including four *rol* genes and 5 ORFs from TR-DNA (Slightom et al. 1986). PCR was performed using genomic DNA isolated from transformed root lines after 12 months of maintenance *in vitro* and non-transformed roots from *in vitro* grown plants by cetyl trimethyl ammonium bromide (CTAB) based on the method developed originally by Doyle and Doyle (1987) with modifications, PCR programs and primers spanning *rolA* (Diouf et al. 1995), *rolB* (Wang et al. 2001), *rolC* (Sevón et al. 1997), *rolD* (Christensen et al. 2008) and rest of 14 ORFs from TL-DNA genes and 5 ORFs from TR-DNA genes (Taneja et al. 2010). Plasmid DNA of pLJ1 for TL-DNA (Jouanin 1984) was used as positive control for *rol* genes and for other ORFs plasmid DNA from *A. rhizogenes* strain LBA 9402 was used as positive control. Genomic DNA of non-transformed roots excised from *in vitro* grown plants was used as negative control.

PCR reactions were carried out in a programmed thermal cycler (Gene Amp® PCR system 2400; Perkin Elmer, Foster City, Calif). All PCR products were separated by electrophoresis on 1.2% agarose gel, stained by ethidium bromide (0.5 μg/ml) and visualized under UV light. The images were captured using BioRad Gel Doc™ XR Imager.

As *virD1* gene resides outside the T-DNA of bacterial Ri plasmid and not transferred to the host plant genome, *virD1* specific primers were used to eliminate the chances of false positive PCR products resulting from to bacterial contamination.

Expression of four *rol* genes at the transcription level was analyzed by reverse transcriptase polymerase chain reaction. Total RNA was isolated from non transformed, 18 months old transformed root lines according to the method of Salzman et al. (1999) and Wang and Stegemann (2010) with modifications. The RNA was quantified spectrophotometrically (Biospectrometer, Eppendorf). For cDNA synthesis 1 μg total RNA was reverse transcribed using transcriptor first strand cDNA synthesis kit (Bio Bharati Life Science, India) following the manufacturer's protocol in a Nexus master cycler (Eppendorf, Germany). The primers and amplification conditions for PCR analysis with cDNAs were same as that were used for PCR analysis with genomic DNA as described above.

For analysis of plumbagin, 100 mg dried and powdered tissue of each root line, harvested from 4 week old culture on solid modified MS, was extracted and

analyzed by HPTLC following methods reported earlier (Martin et al. 2011; Sasikumar et al. 2010). A CAMAG HPTLC system (Muttenz, Switzerland) comprising a Linomat-5 automated sample applicator equipped with a 100µl syringe, CAMAG TLC scanner along with winCATS software (version: 1.4.6) and a twin-trough glass tank was used for the analysis. Plumbagin was detected on the basis of R_f (0.49). For quantitative analysis peak areas were used to calculate the amount of the plumbagin present in the cultured tissue and these were compared to the standard compound. The standard samples were used to construct a calibrated graph by plotting peak areas versus amount of plumbagin injected over a range of 100 - 500 ng and the calibration curve of plumbagin was linear in the range of 100 - 500 ng. The samples were extracted and analyzed in triplicate.The plumbagin content was expressed as mg g/DW.

All the experiments were set up in a randomized design. All experiments were repeated at least three times. Data were examined by a ANOVA to detect significant differences ($p \leq 0.05$) between the mean (Sokal and Rohlf 1987). A post hoc mean separation was performed by the Tukey's multiple comparison test at the same 5% probability level using SPSS software (version 16.0). Variability in the data was expressed as the mean ± standard deviation (Sd).

Results and Discussion

Excised leaf explants (2%) showed root induction within 5 - 6 weeks of infection with *Agrobacterium rhizogenes* strain LBA 9402. The roots never appeared on the leaf explants and whole plants not infected with *A. rhizogenes* (Fig. 1A). Root induction following inoculation of intact shoots occurred after 3 - 4 weeks of infection either directly or indirectly via reddish-white callus formation. 73.80 ± 2.24% of intact shoots showed root initiation when infected with LBA 9402.

Root induction frequency was (65.02 ± 1.16%) via wound callus formation and 71.91 ± 1.87% directly from wound site in internodes of whole plants as compared to 32.41 ± 1.35% via wound callus formation and 35.74 ± 0.96% directly from wound site in nodes of whole plants (Fig. 1 B, C, D).

The results demonstrated that the nodes and internodes of axenic plants of *P. zeylanica* were the best target sites for transformed root induction (Fig. 1 E) than excised leaf explants using *Agrobacterium rhizogenes* strain LBA 9402. Earlier reports on root induction with *Agrobacterium rhizogenes* strains MTCC 532 and A4 have used leaf explants of *P. zeylanica* (Sivanesan and Jeong 2009, Verma et al. 2002). In the present study, improved infectivity of *A. rhizogenes* strain LBA 9402 was obtained in *P. zeylanica* using whole plants instead of excised leaf explants. The results demonstrated that the nodes and internodes of axenic plants of *P. zeylanica* were the best target sites for transformed root induction (Fig. 1 E)

than excised leaf explants using *Agrobacterium rhizogenes* strain LBA 9402. In the earlier reports on root induction with *Agrobacterium rhizogenes* strains MTCC 532 and A4, leaf explants of *P. zeylanica* (Sivanesan and Jeong 2009, Verma et al. 2002) were used. In the present study, the whole plants of *P. zeylanica* instead of excised leaf explants were used to obtain improved infectivity of *A. rhizogenes* strain LBA 9402.

Fig. 1. Induction of transformed roots following infection with *Agrobacterium rhizogenes*strain LBA 9402 at nodes and internodes of axenic whole plants of *Plumbago zeylanica*. A. No root induction in control plants. Bar = 0.6 mm**.** B. Rhizogenesis in wound callus (arrow) at internode. Bar = 0.6 mm**.** C. Rhizogenesis in wound callus (arrow) at node. Bar = 0.6 mm. D. Direct induction of root (arrow) at internode. Bar = 0.6 mm. E. Roots induced at infection site (arrow) after 5 weeks in rooted plants. Bar = 1 mm.

Root lines were established from a single primary root (~3 - 4 cm long with 1 - 2 laterals) excised from each infection site. Roots when cultured on MS supplemented with antibiotic, showed poor growth and turned brown after 4 - 6 weeks and none of the root lines survived.

Root lines cultured on modified MS supplemented with ampicillin (500 mg/l) showed rapid growth**,** and developed laterals. After six months of infection, 10

fast-growing, putatively transformed axenic root lines were selected and maintained on solid modified MS without ampicillin.

Non-transformed roots did not survive in MS or modified MS medium with or without ampicillin (500 mg/l). Hence roots from 4 - 6 week**s** old *in vitro* grown plants on solid MS were used as control.

Root lines transformed with *A. rhizogenes* showed morphological characteristics of typical Ri transformed roots. They were highly branched with profuse hair, and showed plagiotropic growth on modified MS. The root lines could be morphologically distinguished into two types: morphotype I (Fig. 2A) - profuse branching with short (< 1 cm), highly dense hairy laterals all along both sides of theprimary roots (root lines PzIX15, PzIX28, PzIX25 and PzIX40) and morphotype II (Fig. 2B) - profuse branching with long hairy laterals (> 3 - 4 cm) all along the surface of the primary root on both sides (root lines PzIX11, PzIX26, PzIX32, PzIX33, PzIX34 and PzIX47). None of the transformed root lines showed spontaneous callusing.

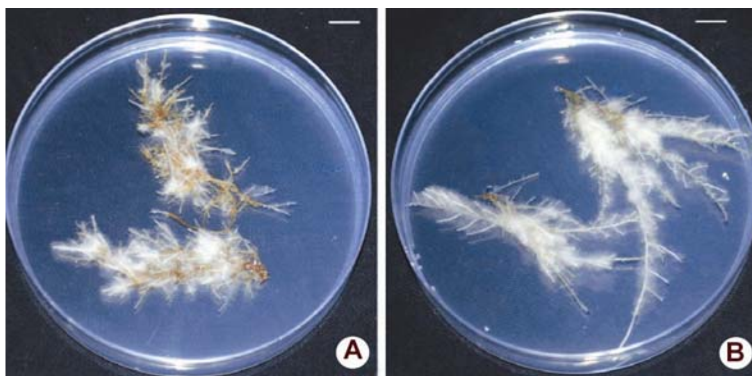

Fig. 2. Two morphotypes of *P. zeylanica* hairy root lines.A. Root line PzIX15 showing Morphotype I. Bar = 7.5 mm. B. Root line PzIX33 showing morphotype II. Bar = 7.5 mm.

PCR analysis of 10 transformed root lines was done after 12 months of maintenance *in vitro*, for the presence of four TL-DNA *rol* genes (*rolA*, *rolB*, *rolC* and *rolD*) and five TR-DNA genes (*ags*, *mas1*, *mas2*, *aux1*, *aux2*). It is worth mentioning that only four of the ten root lines showed integration of four *rol* genes (*rolA*, *rolB*, *rolC* and *rolD*) of TL-DNA (Table 1). Five root lines showed thepresence of *rolA*, *B* and *C* genes and absence of *rolA* gene,while one root line showed the presence of only two *rol* genes (*rolB* and *rolC*). None of the root lines showed the presence of any of the five genes of TR-DNA.

RT-PCR analysis of 18 month old root lines revealed the presence and expression of the four *rol* genes, indicating that the *rolA*, *rolB*, *rolC* and *rolD* genes were stably integrated, retained and expressed at the transcription level in root

lines PzIX15, PzIX28, PzIX25 and PzIX40. The PCR products were of the expected size and identical with those of the corresponding positive controls. In 18-month-old root lines PzIX11, PzIX33, PzIX34, PzIX47 and PzIX26, integration and expression of *rolB*, *rolC* and *rolD* genes was confirmed, while expression of *rolA* gene was not detected, as expected. On the other hand, integration and expression of only two *rol*genes, *rolB* and *rolC*, were detected in 18-month-root-line PzIX32, where as the other two *rol* genes, *rolA* and *rolD* were not integrated and expressed (Fig. 3).

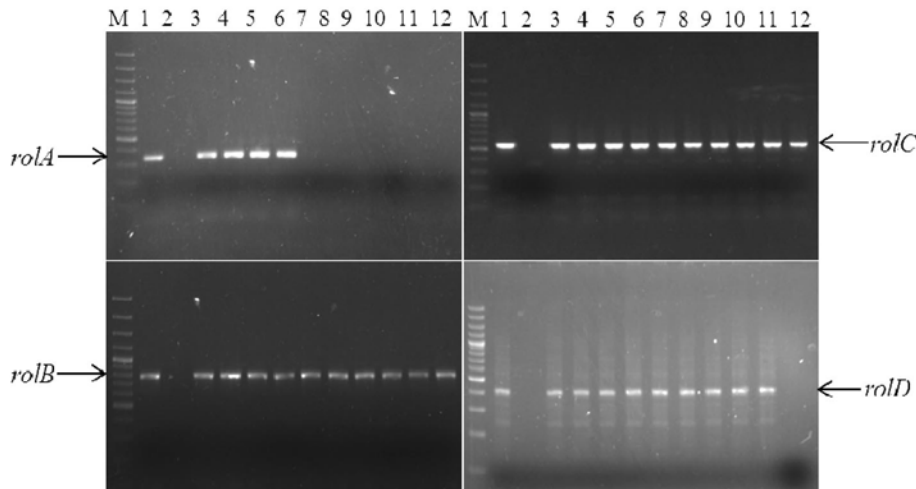

Fig. 3. Expression of *rol* genes at the transcription level in LBA 9402-transformed root lines using *rolA*, *rolB*, *rolC* and *rolD*-specific primers. Lane M: Molecular marker (100 bp plus DNA ladder); Lane 1: Positive control (pLJ1); Lane 2: Negative control (non-transformed root); Lanes 3 - 12: LBA 9402-transformed root lines (PzIX15, PzIX28, PzIX25, PzIX40, PzIX11, PzIX26, PzIX33, PzIX34, PzIX47 and PzIX32, respectively).

Table 1. Morphological analysis and *rol* gene integration in 10 transformed root lines of *P. zeylanica* (n=15, each experiment repeated three times).

Hairy root lines	Morpho-type	Description	TL-DNA			
			rolA	*rolB*	*rolC*	*rolD*
Pz IX15	I	Profuse branching with	+	+	+	+
Pz IX28	I	short (<1 cm), highly dense	+	+	+	+
Pz IX25	I	hairy laterals all along the	+	+	+	+
Pz IX40	I	both sides of primary roots	+	+	+	+
Pz IX11	II	Profuse branching with long	-	+	+	+
Pz IX33	II	hairy laterals (>3-4 cm) all	-	+	+	+
Pz IX34	II	along the both sides of	-	+	+	+
Pz IX26	II	primary roots	-	+	+	+
Pz IX47	II		-	+	+	+
Pz IX32	II		-	+	+	-

'+' and '-' represents the presence and absence of the corresponding genes, respectively.

Root lines of morphotype I thus showed presence and expression of all four *rol* genes of TL-DNA, while the root lines of morphotype II showed absence of *rolA* gene; although *rolD* was also absent in one root line of morphotype II. It is noteworthy that the root morphotypes (I and II) showed a clear distinction in the nature of integration and expression of *rol* genes.

The integration of *rolB* and *rolC* gene in all root lines verifies the absolute requirement of these genes in root initiation and further growth (Nilsson and Olsson 1997, Sinkar et al. 1988). As in several other species (Altamura 2004, Alpizar et al. 2008), none of the transformed root lines showed integration of TR-DNA genes in *P. zeylanica* confirming that the *aux* genes do not play a key role in the hairy root induction.

Transformed root lines were analyzed for integration of 18 ORFs from TL-DNA (including four *rol* genes). Five ORFs of TL-DNA *viz.*, ORF 1, 3, 7, 8 and 14 were not detected in any of the ten root lines (Table 2). On the basis of the presence or absence of the remainder of the 13 ORFs from TL-DNA, the root lines could be grouped into four types: molecular phenotypes (MPhe)-type I includes four root lines of morphotype I (lines PzIX15, PzIX28, PzIX25 and PzIX40) showing integration of 12 ORFs including four *rol* genes, *viz.*, ORFs 2, 4, 5, 9, 10 (*rolA*), 11 (*rolB*), 12 (*rolC*), 13, 15 (*rolD*), 16, 17, 18 (Fig. 4).

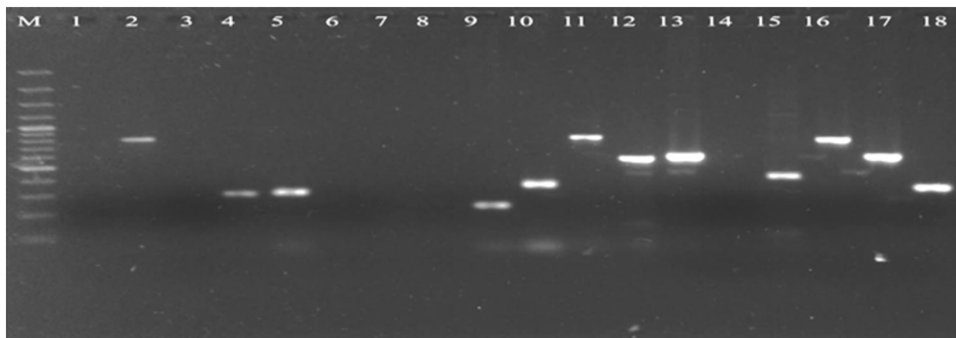

Fig. 4. Integration of T-DNA ORFs in Ri-transformed root line PzIX15 (MPhe-type I) by PCR analysis with ORF specific primers. Lane M: Molecular marker (100 bp plus DNA ladder); Lane 1-18: PCR amplicons of ORF 1-18, respectively, with genomic DNA of LBA 9402-transformed root line PzIX15.

Thus the morphological and molecular characterization of the transformed root lines of morphotype I revealed that they are distinctly different from other transformed root lines of *P. zeylanica*. MPhe-type II comprises four root lines (PzIX11, PzIX33, PzIX34, PzIX47) showing integration of 11 ORFs and showing absence of *rolA* gene (ORF 10). Root line PzIX26 (MPhe-type III) showed

Table 2. Analysis of T-DNA ORFs in *A. rhizogenes* transformed root lines of *P. zeylanica*

Molecular /morphological phenotype	Ri transformed root lines	ORF1	ORF2	ORF3	ORF4	ORF5	ORF6	ORF7	ORF8	ORF9	ORF10 *rolA*	ORF11 *rolB*	ORF12 *rolC*	ORF13	ORF14	ORF15 *rolD*	ORF16	ORF17	ORF18
MPhe-type I/ morphotype 1	PzIX15	-	+	-	+	+	-	-	-	+	+	+	+	+	-	+	+	+	+
	PzIX28	-	+	-	+	+	-	-	-	+	+	+	+	+	-	+	+	+	+
	PzIX25	-	+	-	+	+	-	-	-	+	+	+	+	+	-	+	+	+	+
	PzIX40	-	+	-	+	+	-	-	-	+	+	+	+	+	-	+	+	+	+
MPhe-type II/ morphotype II	PzIX11	-	+	-	+	+	-	-	-	+	-	+	+	+	-	+	+	+	+
	PzIX33	-	+	-	+	+	-	-	-	+	-	+	+	+	-	+	+	+	+
	PzIX34	-	+	-	+	+	-	-	-	+	-	+	+	+	-	+	+	+	+
	PzIX47	-	+	-	+	+	-	-	-	+	-	+	+	+	-	+	+	+	+
MPhe-type III/ morphotype II	PzIX26	-	+	-	+	+	+	-	-	+	-	+	+	+	-	+	+	+	+
MPhe-type IV/ morphotype II	PzIX32	-	+	-	+	+	-	-	-	+	-	+	+	+	-	-	+	+	+

'+' and '-' represent the presence and absence of corresponding ORFs, respectively.

Table 3. Comparison of growth index (GI, DW basis), plumbagin content (mg/gDW) and productivity (mg per Petri plate) in ten transformed root lines cultured on modified MS for 28 days in the dark (n = 15, each experiment repeated three times).

Morphotype	Molecular phenotype	Root line	Growth index (DW basis) ± Sd	Plumbagin content (mg/gDW) ± Sd	Plumbagin production (mg) per Petri plate
I	MPhe-type I	PzIX15	3.8 ± 0.60 bc	4.47 ± 0.14 a	0.16 ± 0.03 ab
		PzIX28	2.19 ± 0.24 a	6.69 ± 0.34 c	0.18 ± 0.02 ab
		PzIX25	2.83 ± 0.16 ab	4.37 ± 0.2 a	0.11 ± 0.02 a
		PzIX40	3.63 ± 0.18 b	4.41 ± 0.40 a	0.17 ± 0.040 ab
		PzIX11	4.00 ± 0.17 bc	5.62 ± 0.38 b	0.22 ± 0.01 b
II	MPhe-type II	PzIX33	5.31± 0.60 d	6.69 ± 0.27 c	0.48 ± 0.07 c
		PzIX34	2.58±0.45 a	5.75±0.34 b	0.14±0.03 a
		PzIX47	2.66±0.21 a	4.81±0.16 a	0.13±0.01 a
	MPhe-type III	PzIX26	3.0 ± 0.4 ab	4.44 ± 0.18 a	0.12 ± 0.02 a
	MPhe-type IV	PzIX32	4.1 ± 0.44 c	5.59 ± 0.24 b	0.16 ± 0.02 ab

Values represent mean ± Sd, mean values with the same letter in a column were not significantly different at $p \leq 0.05$ according to ANOVA and Tukey's multiple comparison test.

integration of 12 ORFs *viz*., ORFs 2, 4, 5, 6, 9, 11 (*rolB*), 12 (*rolC*), 13, 15 (*rolD*), 16, 17, 18 and absence of *rolA* gene while root line PzIX32 (MPhe-type IV) showed presence of 10 ORFs and absence of *rolA* (ORF 10), *rolD* (ORF 15) and ORF 6. Such variations in integration of ORFs of T-DNA have been reported in hairy roots of *Catharanthus roseus* (Taneja et al. 2010) and *Rauvolfia serpentina* (Ray et al. 2014). Thus it may be suggested that the morphological difference between morphotypes I and II root lines may be due to presence or absence of *rolA* gene in *P. zeylanica* hairy root cultures. Variability in growth and plumbagin content in transformed root lines.

The ten transformed root lines varied significantly ($p \leq 0.05$) with respect to DW (GI DW basis, 2.19 ± 0.24 - 5.31 ± 0.6) after 4 weeks of culture on solid modified MS irrespective of the morphotype of roots. Plumbagin content in root lines varied from 4.81 ± 0.16 - 6.69 ± 0.34 mg/gDW (Table 3).

Plumbagin accumulation in all ten transformed root lines of *P. zeylanica* was higher than reported earlier (Verma et al. 2002, Sakamoto et al. 2012). Transformed root line PzIX33 showed an optimum GI with highest plumbagin content (0.669 ± 0.014 %) and productivity (0.48 ± 0.07 mg per Petri plate).

Our result showed a significant variation in growth and plumage content in between the root lines studied; this variation in growth, secondary metabolite content in between transformed root lines have been reported earlier in *Tylophora indica*, *Atropa belladonna* and *Catharanthus roseus* (Chaudhuri et al. 2005,

Aoki et al. 1997, Batra et al. 2004) and has been attributed to the nature of the T-DNA integration into the host genome, copy number of T-DNA inserted and differential expression of *rol* genes etc. (Jouanin et al. 1987, Ambros et al. 1986). Hence, in the present study the insertion of Ri T-DNA in transformed root lines ultimately has produced a stimulatory effect on plumbagin content irrespective of the total or partial integration of T-DNA genes. The variability of growth and plumbagin content in the transformed root lines in the present study provide (gives) an opportunity for screening and identification of high plumbagin producing transformed root lines of *P. zeylanica*. Transformed root lines of *P. zeylanica* maintained *in vitro* in the medium without any phytohormone for over 2 years can be used for scale up studies for the production of plumbagin in bioreactors.

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References

Alpizar E, Dechamp E, Lapeyre-Montes F, Guilhaumon C, Bertrand B, Jourdan C, Lashermes P, Etienne H (2008) *Agrobacterium rhizogenes*-transformed roots of coffee (*Coffeaarabica*): Conditions for long-term proliferation and morphological and molecular characterization. Ann**.** Bot. **101**: 929-940.

Altamura MM (2004) *Agrobacterium rhizogenes rolB* and *rolD* genes: regulation and involvement in plant development. Plant Cell Tissue & Org. Cult. **77**: 89-101.

Ambros PF, Matzke MA, Matzke JM (1986) Detection of 17 kb unique sequence (T-DNA) in plant chromosomes by *in situ* hybridization. Chromosoma **94**: 11-18.

Aoki T, Matsumoto H, Asako Y, Matsunaga Y, Shimomura K (1997) Variation of alkaloid productivity among several clones of hairy roots and regenerated plants of *Atropa belladonna* transformed with *Agrobacterium rhizogenes* 15834. Plant Cell Rep **16**: 282-286.

Batra J, Dutta A, Singh D, Kumar S and **Sen J** (2004) Growth and terpenoid indole alkaloid production in *Catharanthus roseus* hairy root clones in relation to left- and right-termini-linked Ri T-DNA gene integration. Plant Cell Rep. **23**: 148-154.

Chandra S (2012) Natural plant genetic engineer *Agrobacterium rhizogenes*: Role of T-DNA in plant secondary metabolism. Biotechnol. Lett. **34**: 407-415.

Chaudhuri KN, Ghosh B, Tepfer D and **Jha S** (2005) Genetic transformation of *Tylophora indica* with *Agrobacterium rhizogenes* A4: Growth and tylophorine productivity in different transformed root clones. Plant Cell Rep. **24**: 25-35.

Chetia S and **Handique PJ** (2000) High frequency *in vitro* shoot multiplication of *Plumbago indica*, a rare medicinal plant. Curr. Sci. **78**(10): 1187-1188.

Christensen B, Sriskandarajah S, Serek M and **Müller R** (2008) Transformation of *Kalanchoeblos sfeldiana* with *rol*-genes is useful in molecular breeding towards compact growth. Plant Cell Rep. **27**: 1485-1495.

Didry N, Dubrevil L and **Pinkas M** (1994) Activity of anthraquinonic and naphthoquinonic compounds on oral bacteria. Die Pharmazie **49**: 681-683.

Diouf D, Gherbi H, Prin Y, Franche C, Duhoux E and **Bogusz D** (1995). Hairy root nodulation of *Casuarinaglauca*: A system for the study of symbiotic gene expression in an actinorhizal tree. Mol. Plant Microbe. Interact. **8**(4): 532-537.

Doyle JJ and **Doyle JL** (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. **19**: 11-15.

Hazra B, Sarkar R, Bhattacharya S, Ghosh PK, Chel G and **Dinda B** (2008) Synthesis of plumbagin derivatives and their inhibitory activities against Ehrlich ascites carcinoma and *Leishmania denovani* promastigotes *in vitro*. Phytother Res. **16**:133-137.

Jouanin L (1984) Restriction map of an agropineRi plasmid and its homologies with Ti plasmids. Plasmid **12**:91–102

Jouanin L, Guerche D, Pamboukdjian N, Tourneur C, Casse-Delbart F and **Tourneur J** (1987) Structure of T-DNA in plants regenerated from roots transformed by *Agrobacterium rhizogenes* strain A4. Mol. Gen. Genet. **206:** 387-392.

Mallavadhani UV, Sahu G and **Muralidhar J** (2002) Screening of Plumbago species for the bio-active marker plumbagin. Pharm. Biol. **40**(7): 508-511.

Martin K.P, Sabovljevic A and **Madassery J** (2011) High-frequency transgenic plant regeneration and plumbagin production through methyl jasmonate elicitation from hairy roots of *Plumbago indica* L. J. Crop Sci. Biotech. **14**(3): 205-212.

Mathew N, Paily KP, Vanamil AP and **Balaraman KK** (2002) Macrofilaricidal activity of the plant *Plumbago indica/rosea in vitro*. Drug Dev. Res. **56**:33-39.

Nilsson O and **Olsson O** (1997) Getting to the root: the role of the *Agrobacterium rhizogenesrol*-genes in the formation of hairy roots. Physiol. Plant **100**:463-473.

Panichayupakaranant P and **Tewtrakul S** (2002) Plumbagin production by root culture of *Plumbagorosea*. Electron. J. Biotechnol. **5(3)**: 11-12.

Petit A, David C, Dahl G, Ellis J, Guyon P, Casse-Delbart F and **Tempe´ J** (1983) Further extension of the opine concepts: Plasmids in *Agrobacterium rhizogenes* co-operate for opine degradation. Mol. Gen. Genet. **207**: 245-250.

Ray S, Samanta T, Majumder A, Bandyopadhyay M and **Jha S** (2014) Cytogenetic characterization of *Agrobacterium rhizogenes* transformed root lines of *Rauvolfia serpentina*. Nucleus **57**(2): 105-112.

Roychowdhury D, Majumder A and **Jha S** (2013) *Agrobacterium rhizogenes* mediated transformation in medicinal plants: prospects and challenges. *In:* Chandra S et al. (eds) Biotechnology for medicinal plants. Berlin, Heidelberg: Springer pp. 29-68.

Sakamoto S, Putalun W, Pongkitwitoon B, Juengwatanatrakul T, Shoyama Y, Tanaka H and **Morimoto S** (2012) Modulation of plumbagin production in *Plumbago zeylanica* using a single-chain variable fragment antibody against plumbagin. Plant Cell Rep. **31**: 103-110.

Salzman RA, Fujita T, Zhu-Salzman K, Hasegawa PM and **Bressan RA** (1999) An improved RNA isolation method for plant tissues containing high levels of phenolic compounds or carbohydrates. Plant Mol. Biol. Reporter. **17**: 11-17.

Sasikumar, Meenaa and **S kavitha, Sriram** (2010) Hptlc analysis of various market samples of a traditional drug source - Kodiveli *(plumbago zeylanica* Linn) Intl. J. Pharm. and Pharmaceut. Sci. **4**: 130-132.

Sevón N, Dräger B, Hiltunen R and **Oksman-Caldentey KM** (1997) Characterization of transgenic plants derived from hairy roots of *Hyoscyamu smuticus.* Plant Cell Rep. **16**: 605-611.

Sinkar VP, Pyihoud R, White FF, Nester FW and **Gordon MP** (1988) *rolA* locus of the Ri plasmid directs developmental abnormalities in transgenic tobacco plants. Genes Dev. **2**: 688-697.

Sivanesan and Jeong (2009) Induction and establishment of adventitious and hairy root cultures of *Plumbago zeylanica* L. Afr. J. Biotechnol. **8**(20): 5294-5300.

Slightom JL, Durand-Tardif M, Jouanin L and **Tepfer D** (1986) Nucleotide sequence analysis of TL-DNA of *Agrobacterium rhizogenes* agropine type plasmid. Identification of open reading frames. J. Biol. Chem. **261**: 108-121.

Sokal RR and **Rohlf FJ** (1987) Introduction to biostatistics. WH Freeman, New York.

Taneja J, Jaggi M, Wankhede DP and **SinhaAK** (2010) Effect of loss of T-DNA genes on MIA biosynthetic pathway gene regulation and alkaloid accumulation in *Catharanthus roseus* hairy roots. Plant Cell Rep. **29**: 1119-1129.

Tepfer D (1995) *Agrobacterium rhizogenes* mediated transformation: transformed roots to transformed plants. *In:* Potryukus I (ed) Gene transfer to plants, Springer Velrag, Berlin, Hiedelberg, pp. 45-52.

Verma PC, Singh D, Rahman L, Gupta MM and **Banerjee S** (2002) *In vitro* studies in *Plumbago zeylanica*: Rapid micropropagation and establishment of higher plumbagin yielding hairy root cultures. J. Plant Physiol. **159**: 547-552.

Wang YM, Wang JB, Luo D and **Jia JF** (2001) Regeneration of plants from callus cultures of roots induced by *Agrobacterium rhizogenes* on *Alhagi pseudoalhagi*. Cell Res. **11**(4): 279-284.

Wang L and **Stegemann JP**(2010) Extraction of high quality RNA from polysaccharide matrices using Cetlytrimethyl ammonium Bromide. Biomaterials **31**(7): 1612. doi:10.1016/j.biomaterials. 2009.11.024.