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Agrobacterium rhizogenes-Mediated Transformed Roots of *Rauwolfia serpentina* for Reserpine Biosynthesis

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ABSTRACT

Root extracts of *Rauwolfia serpentina* have been used for centuries in Ayurvedic medicine as a panacea for a wide variety of physical as well as mental disorders. The potential of *Agrobacterium rhizogenes*-mediated genetic transformation for the synthesis of phytomolecules of high pharmaceutical value is now well established and documented. Transgenic roots were induced from *R. serpentina* leaf explants in response to *A. rhizogenes* A4 strain on semi-solid ½-strength MS medium. Amongst 200 hairy root clones developed, 27 showing persistent and incessant growth over several generations were selected. Transformed roots grew vigorously and branched profusely on hormone-free liquid B_5 medium with 3% sucrose with higher biomass yields compared to the control and showed two stable and distinct morphotypes. Medium devoid of any carbon source served as the control. The transformed nature of the roots was confirmed by PCR amplification with *rol*A primers. Growth kinetic studies exhibited the highest growth index (58.57 ± 1.92) at the 10^{th} week followed by slow growth in the subsequent period up to 14 weeks. Reserpine content increased with root growth and was highest in 10-weeks-old cultures. Hairy root clones showed a wide array of variation in relative reserpine content, varying from 0.0064 to 0.0858% dry weight (DW). On the basis of relative reserpine content, these hairy root clones were classified into 5 different groups. SM_{12} clone had the highest reserpine level (0.0858% DW) producing 2-3 times more than the content of field-grown roots harvested after 18-24 months. A distinct relationship between root morphology and reserpine content was observed. The present study is the first report of reserpine production in quantifiable amounts from the hairy roots of any *Rauwolfia* species.

Keywords: genetic transformation, hairy roots, HPLC

INTRODUCTION

Roots of *Rauwolfia serpentina* are the principal natural source of the alkaloid 'reserpine' known for various pharmacological activities (Muller *et al.* 1952). The extracts of roots and total alkaloids *R. serpentina* are highly effective in hypertension, insomnia, giddiness, anxiety states, maniacal behavior, psychosis, schizophrenia and hyperglycemia (Duke 1985; Trivedi 1995; Bhattacharjee 1998). Reserpine depletes catecholamines (epinephrine and norepinephrine) and serotonin (5-hydroxytryptamine) from central and peripheral neurons by interfering with the uptake of these amines from the cytosol into vesicles and granules. The domestic demand for *R. serpentina* roots is continuously increasing. Long duration required for root harvesting (18-24 months) and poor seed germination in the crop has restricted its commercial cultivation. Plant tissue culture techniques could be helpful in circumventing these problems. *In vitro* clonal propagation and indole alkaloids from multiple shoots of *R. serpentina* (Roja *et al.* 1985; Mathur *et al.* 1987; Roja and Heble 1996; Goel 2007) and isolation of alkaloids along with enzymes involved in their biosynthesis in cell suspension cultures (Ohta and Yatazawa 1979; Stockigt *et al.* 1981, 1983; Schubel and Stockigt 1984; Shimolina *et al.* 1986; Yamamato and Yamada 1986; Roja *et al.* 1987; Yamamato and Yamada 1987; Molokhova *et al.* 1988; Kunakh and Alkhimova 1989; Schuebel *et al.* 1989; Obitz *et al.* 1995; Kirilova *et al.* 2001) have been reported. However, the problem of genetic and biosynthetic instability of cell cultures and resurgence of interest in the potential of *Agrobacterium rhizogenes*-mediated hairy roots has opened up a new area for enhanced secondary metabolite production (Benjamin *et al.* 1993; Falkenhagen *et al.* 1993; Sheludko and Kostenyuk, 1994; Klushychenko *et al.* 1995; Sheludko *et al.* 2002). However, commercial production of reserpine through hairy root cultures in *R. serpentina* has not been achieved so far. Therefore, the present study was aimed to enhance *A. rhizogenes*-mediated hairy root biomass and select high reserpine-yielding hairy root clones of *R. serpentina* as a potential alternative source.

MATERIALS AND METHODS

Hairy root induction

In vitro cultures of *R. serpentina* maintained on MS (Murashige and Skoog 1962) medium with 1.0 mgl^{-1} BAP (6-benzylaminopurine, Sigma-Aldrich, India) and 0.1 mgl^{-1} NAA (α-naphthelene acetic acid, Sigma-Aldrich) served as explant source for hairy root induction (Goel *et al.* 2007). Two wild type strains *of A. rhizogenes* viz. A4 (pRiA4) and LBA 9402 were used for transformation events and were grown at 28°C for 48 h in YMB (yeast mannitol broth, Hi-Media, India) medium. Fresh suspension was prepared by inoculating a single bacterial colony in 10 ml YMB medium and incubating for 48 h at 28°C at 100 rpm. Bacterial growth was estimated by optical density at 660 nm using a Nanodrop (ND-1000) spectrophotometer.

Bacterial inoculation in the explants and co-cultivation

In vitro leaf explants were pricked with a sterile needle using the

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bacterial suspension for induction of hairy roots. Leaf explants pricked using sterile distilled water served as the control. Pricks were made on the upper surface of the leaves so as to only cause sub-lethal injury. Explants were co-cultured on hormone-free MS basal medium with 3% sucrose and 0.8% agar at 25 ± 2°C and 40 µmol m^{-2} s^{-1} light intensity. After 2-3 days the explants were transferred to MS medium supplemented with 1 µg/µl of antibiotic "Sporidex" (Ranbaxy) to eliminate vestigial bacteria. The explants were repeatedly cultured on antibiotic supplemented medium until bacterium completely disappeared.

Effect of co-cultivation medium on hairy root induction

After one week of incubation on MS medium, half of the disinfected explants were transferred to hormone-free ½-strength MS antibiotic medium and the remaining half were left on the same medium. Transformation frequency (TF %) was recorded up to the 6th week after root induction by the following formula

$$\text{Transformation Frequency (TF \%)} = \frac{\text{No. of explants showing hairy root emergence}}{\text{Total No. of explants infected}} \times 100$$

Disinfection and maintenance of transformed hairy roots

Putatively transformed roots 1.0-1.5 cm in length were excised from the explants and were transferred to hormone-free liquid MS medium containing antibiotic. Normal (non-transformed) roots obtained from *in vitro* shoot cultures were also maintained under same culture conditions.

Confirmation of transformed nature of hairy roots

In order to confirm the transformed nature of the hairy roots the putatively transformed and non-transformed roots were subjected to polymerase chain reaction (PCR) with primers for universal wild type *A. rhizogenes* A_4 strain specific *rol*A gene harbored within the T-DNA. Forward (5′-GGAATTAGCCGGACTAAACG-3′) and reverse (3′-CCGGCGTGGAAATGAATCG-5′) primers for *rol*A were procured from Genie Bangalore (India). Primers for the *VirD1* gene (forward 5′-ATGTCGCAAGGCAGTAAGC-3′ and reverse 3′-CGACGGTTGCTCCTGCTGA-5′), coding for DNA outside the T-DNA of the Ri plasmid, were also used to rule out the possibility of *A. rhizogenes* contamination in hairy roots. This involved isolation of DNA (Khanuja *et al.* 1999) from roots and *A. rhizogenes* (Sambrook *et al.* 1989) followed by PCR amplification, which was carried out in a total volume of 25 µl in a Bio-Rad i-cycler version 4.006. The reaction comprised of 25-30 ng of template DNA, 0.3 U of *Taq* DNA polymerase, 0.25 µl of each dNTP, 1.5 mM $MgCl_2$ buffer and 5 pmol of each primer. After initial denaturation at 94°C (5 min), the program was run for 35 cycles consisting of 94°C denaturation step (1 min), 60°C primer annealing step (1 min) and 72°C amplification step (1 min), at the end of the run a final amplification period (5 min; 72°C) was appended. Amplified DNA was loaded onto 1.2% agarose gel in TAE buffer stained with 0.5 µg/ml ethidium bromide and photographed on a polaroid gel documentation system.

Media optimization for hairy root growth at shake flask level

MS, LS (Linsmaier and Skoog 1965), B_5 (Gamborg *et al.* 1968), and NB (Nistch and Nistch 1969) basal culture media at ½ and ¼ strengths with 3% sucrose were examined to try and obtain higher biomass yield. The pH of the medium was adjusted to 5.86 ± 0.02 prior to autoclaving. A single root with lateral branches weighing approx. 20 mg was inoculated in 20 ml medium and growth was recorded after 6 weeks of inoculation. In another experiment, different pH levels (3.86, 4.86, 5.86, 6.86 and 7.86) and different levels of sucrose (0, 1.5, 3.0, 4.5 and 6.0%) were tested in the optimized hormone-free liquid basal medium. A minimum of three replicates were maintained for each treatment.

Qualitative analysis of the selected fast-growing hairy root clones

Dried hairy roots of *R. serpentina* were extracted as per the protocol described earlier (Goel *et al*. 2009). The vacuum-dried extracts were checked on a TLC plate 20 × 20 cm silica gel 60 F_{254} (Merck Darmstadt, Germany). The plate was run in chloroform: methanol (95: 5) and visualized under UV light at 254 nm. In order to isolate reserpine, preparative TLC was carried out and the spot corresponding to authentic reserpine (Sigma-Aldrich) was eluted and redissolved in chloroform: methanol (3: 1). It was filtered and concentrated and run three times in the same mobile phase followed by an ethyl acetate: hexane: methanol (65: 25: 10) mixture. Finally, the plate was developed with Dragondorff's reagent.

Growth kinetic studies in *R. serpentina* hairy roots and reserpine biosynthesis

Growth kinetic studies were carried out to assess the optimum growth period, higher biomass and reserpine production in five (SM_{14}, SM_{19}, SM_{21}, SM_{28}, and SM_{30}) randomly selected fast-growing hairy root clones. Initially, about 150 mg of roots were inoculated in 50 ml medium. A minimum of three replicates were harvested at 2-week intervals from the 4th week onwards up to the 12th week. Different parameters i.e. dry matter (DM) %, growth index (GI) and reserpine content (% DW) were recorded using the following formula:

$$\text{Dry matter (\%)} = \frac{\text{Biomass dry weight}}{\text{Biomass fresh weight}} \times 100$$

Quantitation of reserpine through HPLC

Quantitative estimation of reserpine in hairy root clones was carried out by reverse-phase high-performance liquid chromatography (RP-HPLC) using photodiode array (PDA) detection (Srivastava *et al.* 2006). An analytical HPLC system consisted of LC-20AD solvent delivery pumps, a DGU-20A5 degasser, a CTO-20A column oven, 10AF auto-sampler and a SPD-M 20A photodiode array detector was used. Data acquisition was performed on lab Solution 3.21. Separation was achieved with a binary gradient program for pump A (acetonitile), and pump B (0.01 M phosphate buffer (NaH_2PO_4)) containing 0.5% glacial acetic acid at pH 3.5. A chromolith RP-18e HPLC column, 4.6 × 100 mm ID was used for all analyses. Column temperature was maintained at 26 ± 2°C and analysis was performed at a flow rate of 0.1 ml/min throughout the gradient run and data acquisition was performed at λ = 254 nm. Solvents were of HPLC grade (Merck, Darmstadt, Germany). Dried extracts from 10-week-old hairy root samples were sonicated and dissolved in methanol (methanol-HCl 98: 2, v/v) at 1 mg/µl on a dry weight (dw) basis. The reserpine (Sigma) standard was prepared in methanol (1 mg/ml). Reserpine content (0.030-0.034% dw) in var. 'CIM-Sheel' developed at CIMAP (Gupta *et al.* 2005) was used as the benchmark to categorize the hairy roots.

Statistical analysis

The results from growth kinetics experiments were analyzed by two-way ANOVA. The results were interpreted as statistically significant at $P > 0.01$. This was computed as the ratio of mean square corresponding to the treatment to the mean square value representing the error variability from entire samples as opposed to using the value corresponding to the error variability computed by two-way ANOVA in the denominator of the ratio used to calculate the *F*-value.

RESULTS

Hairy root induction

Agrobacterium A4 strain was capable of inducing transgenic roots in *R. serpentina* leaf explants after 6 weeks with

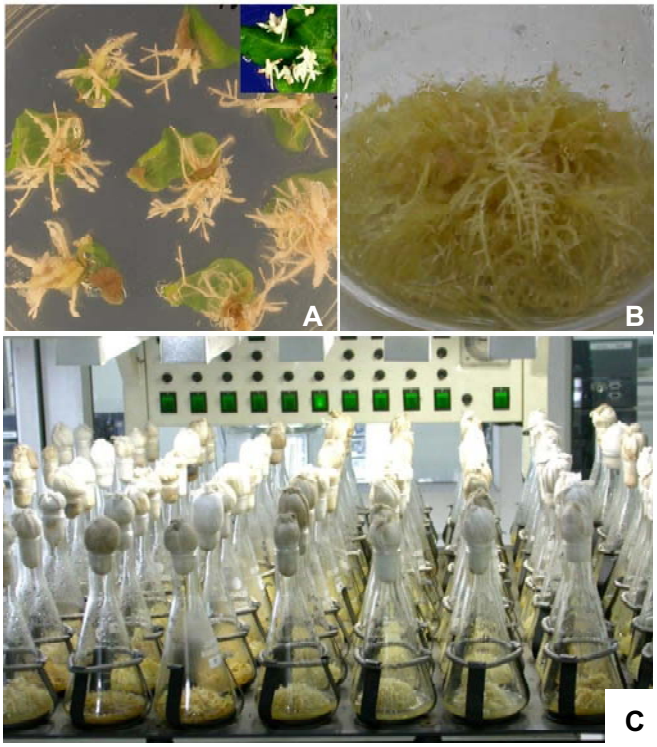

Fig. 1 *A. rhizogenes* mediated genetic transformation in *R. serpentina*. Hairy root induction in leaf explant (**A**) emergence of root bunches (inset); profuse hairy root growth in liquid B_5 medium (**B**); maintenance of various hairy root clones at shake flask level (**C**).

70% transformation frequency (TF) vs. 45% in LBA 9402. The average number of roots produced by A4 and LBA 9402 strains were 6 and 4, respectively. LBA 9402 induced callus before the onset of root emergence. Upon transfer of agro-infected leaf explants from MS to ½-MS medium, roots emerged from leaf explants on the 19th day of co-cultivation compared to hormone-free MS medium where roots were visible on the 27th day. Relative TF% on ½- and full-MS medium was 85.93 and 70.27, respectively after 6 weeks of culture. The transformed roots exhibited typical features of fast growth, profuse branching and negative geotropism (**Fig. 1A, 1B**).

Maintenance of transformed hairy roots cultures

Emergence of root(s) from each needle prick on the *in vitro* leaf explants was considered as a distinct transformation event and was maintained as an individual root clone. More than 200 different hairy root clones were initially induced and the majority of these root clones formed callus and slow growth in hormone-free medium and therefore were discarded leaving behind only 40 fast-growing hairy root clones. These clones were named SM_1, SM_2, and SM_3, etc. Some of the clones lost their growth potential during the course of subculture. Finally, 27 hairy root clones which grew vigorously with profuse branching on hormone- and antibiotic-free medium and showed persistent and incessant growth even after three years were selected (**Fig. 1C**). These roots showed two distinct morphological phenotypes that remained stable over subsequent generations. Of the 27 clones, four were morphologically different. These four root clones were thin, up to 15 cm long, less branched, soft, flexible, greenish white in color and were able to survive up to 7-8 months without further sub-culture, whereas remaining clones were highly branched, only 5-6 cm long, creamish in color, brittle and turned reddish on maturity and could survive up to 16-20 weeks without sub-culture. Non-transformed roots exhibited very slow growth in hormone-free medium.

Confirmation of transformed nature of hairy roots

PCR analysis of the DNA with *rol*A primers exhibited the amplification of the TL-DNA fragment (600 bp) in transformed roots (**Fig. 2**). Non-amplification of DNA from transformed root with the *vir*D1 primers (**Fig. 3**) confirmed the lack of *Agrobacterium* contamination in hairy root clones. The expected amplification (650 bp) was obtained with *A. rhizogenes* A4 DNA (positive control). Non-transformed roots did not show any amplification either with *rol* A or *vir* D1 primers.

Effect of nutrient medium composition, pH and sucrose concentration

Amongst various media (MS, LS, B5 and N6) tested, liquid basal B_5 medium at pH 5.86 with 3% sucrose supported fast growth and highest biomass production of hairy root clones. Hairy root growth decreased as the strength of the culture medium decreased. There was a consistent increase in the growth of hairy roots with an increase in media pH from 3.86 to 5.86 followed by a decrease at higher pH levels i.e. 6.86 and 7.86, respectively. Highest root biomass (3.26 ±

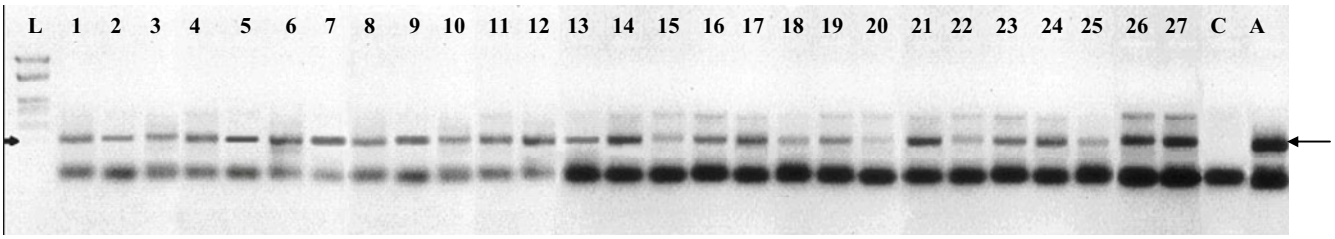

Fig. 2 PCR with *rol* A primers and hairy root clones. M = marker DNA; 1-27 = hairy root clones; C = control (non-transformed root); A = DNA from *A. rhizogenes* A4 strain. Arrow indicated 600-bp fragment.

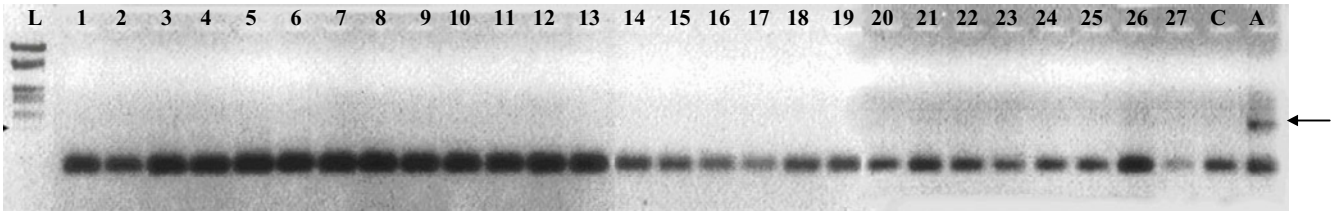

Fig. 3 PCR with *vir* D1 primers and hairy root clones. M = marker DNA; 1-27 = hairy root clones; C = control (non-transformed root); A = DNA from *A. rhizogenes* A4 strain. Arrow indicates 650-bp fragment.

Table 1 Growth kinetics and reserpine content of hairy root clone(s) at different growth periods.

Growth (weeks)	Growth parameters	Hairy root clones				
		SM_{14}	SM_{19}	SM_{21}	SM_{28}	SM_{30}
4	Dry wt. (g)	0.26 ± 0.033	0.44 ± 0.082	0.43 ± 0.025	0.24 ± 0.058	0.47 ± 0.036
	Dry matter %	10.92	8.99	8.5	11.61	7.3
	Growth index	15.18 ± 1.77	31.62 ± 5.55	32.44 ± 0.81	12.78 ± 3.53	41.99 ± 4.2
	Reserpine (% dw)	0.0131	0.0213	0.0332	0.0293	0.0411
6	Dry wt. (g)	0.50 ± 0.057	0.73 ± 0.023	0.66 ± 0.12	0.63 ± 0.075	0.73 ± 0.053
	Dry matter %	9.14	11.93	10.05	10.84	9.27
	Growth index	35.8 ± 2.23	39.95 ± 8.0	42.75 ± 1.81	37.98 ± 2.29	51.82 ± 4.56
	Reserpine (% dw)	0.0162	0.0263	0.0388	0.0323	0.0345
8	Dry wt. (g)	0.62 ± 0.01	0.67 ± 0.023	0.68 ± 0.026	0.44 ± 0.031	0.67 ± 0.026
	Dry matter %	8.86	9.49	8.92	8.40	8.29
	Growth index	45.74 ± 2.06	46.06 ± 7.02	49.78 ± 2.09	34.11 ± 2.08	52.86 ± 3.64
	Reserpine (% dw)	0.0201	0.0339	0.0320	0.0458	0.0506
10	Dry wt. (g)	0.68 ± 0.006	0.73 ± 0.03	0.73 ± 0.046	0.68 ± 0.012	0.66 ± 0.006
	Dry matter %	7.55	7.77	8.34	7.91	7.45
	Growth index	58.97 ± 0.26	61.62 ± 2.13	57.31 ± 4.11	56.67 ± 0.63	58.29 ± 2.05
	Reserpine (% dw)	0.0230	0.0426	0.0422	0.0546	0.0562
12	Dry wt. (g)	0.65 ± 0.031	0.66 ± 0.038	0.78 ± 0.006	0.66 ± 0.014	0.68 ± 0.039
	Dry matter %	7.07	7.26	9.1	7.96	7.38
	Growth index	60.7 ± 1.1	59.93 ± 2.62	56.2 ± 3.67	54.00 ± 0.30	60.6 ± 3.2
	Reserpine (% dw)	0.0139	0.0414	0.0365	0.0472	0.0528

Table 2 ANOVA table for the study of effect of hairy root lines and culture period on the growth index of *R. serpentina.*

Source of variation	Df	S.S	M.S = S.S/ df	F_{cal}	F_{tab}
Hairy root lines (L)	4	685983.3 (±2643.76)	MSL= 171495.8 (±660.94)	MSL/MSE= 3.2113 (±3.1799)	$F_{4,16} = 4.7726$
Culture period (T)	4	683644.3 (±2666.98)	MST= 170911.1 (±666.74)	MST/MSE= 3.2003 (±3.2078)	$F_{4,16} = 4.7726$
L*T	16	854466.4 (±3325.56)	MSE= 53404.15 (±207.85)	-	-
Total	24	2224094 (±8636.3)	-	-	-

Since F values for hairy root lines (L) and growth period (T) are less than the tabulated value, therefore H0 is accepted at 1% level of significance. Values in bracket are the ANOVA of respective standard deviation

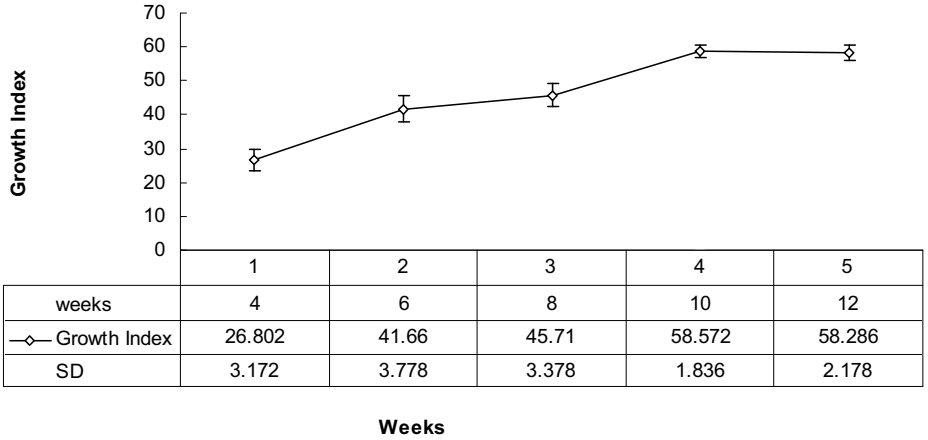

Fig. 4 Average growth indices (AGI) of five hairy root clones at different growth periods.

0.15) was obtained at 3% sucrose. Sucrose concentration beyond 3% inhibited growth. Roots exhibited mortality within 5-6 weeks in medium devoid of a carbon source (data not shown).

Growth kinetics studies in *R. serpentina* hairy root clones and reserpine analysis

Growth kinetic studies firmly revealed a continued increase in root growth in clones SM_{19}, SM_{21} and SM_{28} until the 10th week of culture followed by a marginal decline. Although clones SM_{14} and SM_{30} exhibited a continuous increase in biomass up to the 12th week of culture, a subsequent increase was not significant during the 10-12th week culture period (**Table 1**). Growth index (GI) of each of the 5 hairy root clones exhibited a definite sigmoid growth pattern. A continuous increase in root biomass was recorded during first 10 weeks. Highest GI (58.57 ± 1.92) was recorded at the 10th after which there was no significant increase in biomass (GI = 58.29 ± 3.02) up to the 12th week of culture (**Fig. 4**). In all 5 hairy root clones, reserpine content also reached the highest level after 10 weeks of growth followed by a decline in subsequent weeks, irrespective of their relative reserpine content (**Table 1, Fig. 5**). As is evident from the ANOVA (**Table 2**), since *F* values for hairy root lines (L) and growth period (T) are less than the tabulated value therefore the observations were accepted at $P = 0.01$.

Qualitative analysis of the selected fast growing hairy root clones

Reserpine was detected at $R_f = 0.5$ in chloroform and methanol (95: 5) and at $R_f = 0.75$ when the same TLC plate was run in ethyl acetate: hexane: methanol (65: 25: 10). A single spot was detected at $R_f = 0.5$ when the same fraction was run anew in ethyl acetate: hexane: methanol (65: 25: 10).

Selection of high reserpine-producing hairy root clones in *R. serpentina*

Hairy root clones exhibited a wide range (0.0064-0.0858% dw) of reserpine content (**Fig. 6**). Compared to the reserpine content (0.03-0.034) of var. 'CIM-Sheel', all root clones

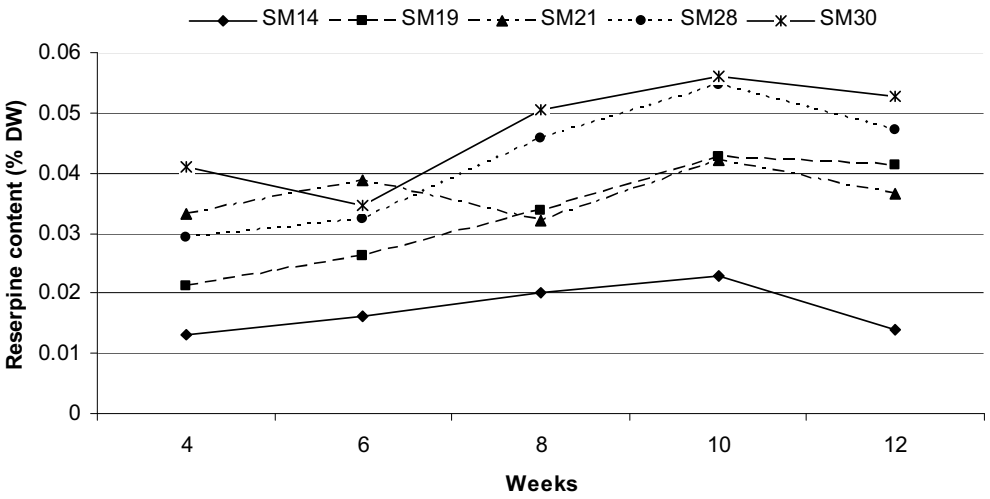

Fig. 5 Reserpine content in hairy root clones of *R. serpentina* at different growth periods.

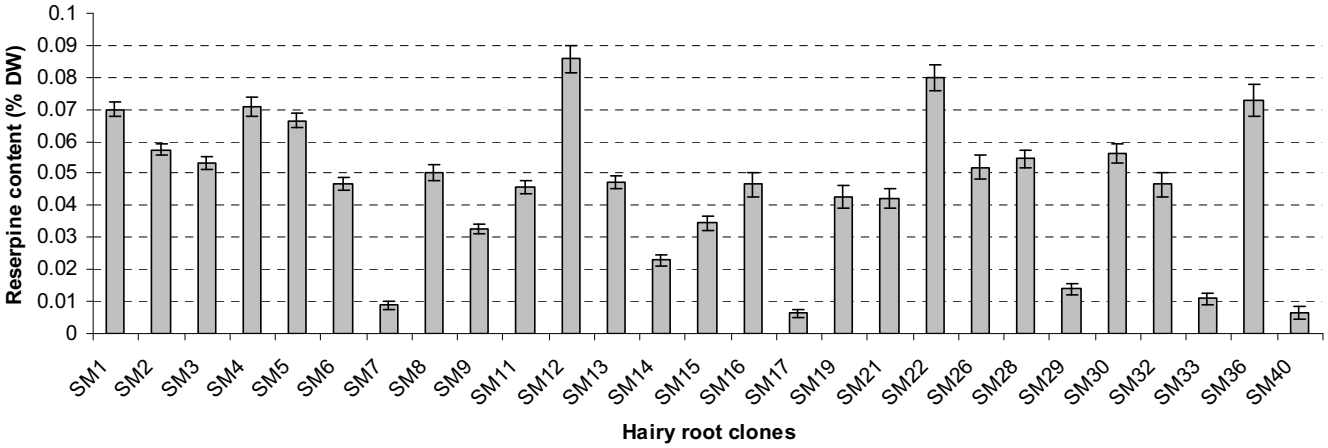

Fig. 6 Variation in reserpine content in different hairy root clones after 10 weeks of growth.

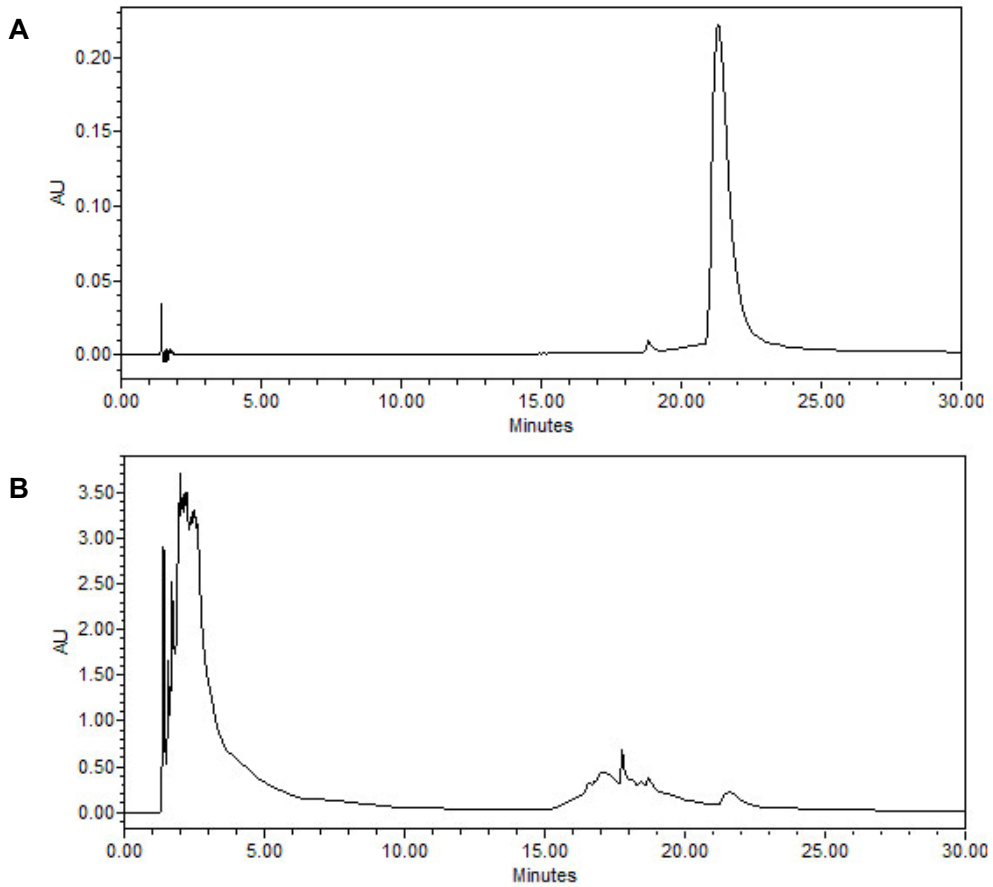

Fig. 7 HPLC chromatogram of reserpine standard (**A**) and hairy root clone SM_{12} (**B**).

Table 3 Reserpine content in 10-weeks-old *R. serpentina* hairy root clones.

Category	Hairy root clone	Tissue DW (g)	Extract wt. (mg)	Reserpine (% DW)
Group 1	SM_{12}	0.66	92.1	0.0858
	SM_{22}	1.33	192.4	0.0799
	SM_{36}	1.37	250.6	0.0729
	SM_{4}	1.26	175.4	0.0709
	SM_{1}	1.41	207.0	0.0701
Group 2	SM_{5}	1.54	218.2	0.0665
	SM_{2}	1.17	178.2	0.0574
	SM_{30}	1.52	250.6	0.0562
	SM_{28}	1.30	231.2	0.0546
	SM_{3}	1.96	243.1	0.0533
	SM_{26}	1.35	225.3	0.0519
	SM_{8}	1.31	170.4	0.0502
Group 3	SM_{13}	1.07	173.3	0.0473
	SM_{6}	1.35	195.7	0.0467
	SM_{16}	1.25	149.6	0.0466
	SM_{32}	1.04	163.6	0.0465
	SM_{11}	1.38	208.3	0.0458
	SM_{19}	1.36	110.1	0.0426
	SM_{21}	0.94	152.9	0.0422
Group 4	SM_{15}	1.39	97.2	0.0345
	SM_{9}	1.43	171.5	0.0326
Group 5	SM_{14}	1.55	186.4	0.0230
	SM_{29}	1.49	114.8	0.0139
	SM_{33}	1.50	114.8	0.0109
	SM_{7}	1.06	51.4	0.0089
	SM_{40}	2.08	117.1	0.0065
	SM_{17}	3.07	354.5	0.0064

were grouped into 5 different categories (**Table 3**). All 5 clones SM1, SM_4, SM_{12}, SM_{22} and SM_{36} synthesized most reserpine, with SM_{12} containing the highest reserpine content (0.0858% dw) (**Fig. 7B, Table 3**). Most of the hairy root clones exhibited a relatively higher reserpine content (0.0422-0.0665% dw). Reserpine content (0.0326-0.0345% dw) in clones SM_9 and SM_{15} was almost equivalent to 'CIM-Sheel' and 6 clones recorded lower reserpine content than the control. Reserpine content in SM_{12} was about 14 times higher than that produced by SM_{17}, which revealed the variable nature of hairy roots for alkaloid production. A fair relationship between root morphology and reserpine content was also observed. As already mentioned, four clones were morphologically distinct from others: they produced a very low amount (0.0064-0.0139% dw) of reserpine. On the other hand, other clones produced a higher amount of reserpine.

DISCUSSION

The differences in virulence and morphology may be attributed to the different Ri-plasmids harbored by the strains (Nguyen *et al.* 1992; Akramian *et al.* 2008). The plagiotropic characteristic of hairy roots is advantageous as it increases aeration in liquid medium and roots grown in air have an elevated accumulation of biomass. The faster growth of transformed roots may be attributed to their extensive branching, resulting in many meristems (Flores *et al.* 1999; Giri and Narasu 2000; Srivastava and Srivastava 2007). The hairy roots are usually non-chimeric because they originate from single cells and each hairy root clone consists of uniformly transformed cells (Ohara *et al*. 2000). Hormone autonomy is due to the capability of endogenous synthesis of auxin in hairy roots. Genes present on *A. rhizogenes* T-DNA are involved in overproduction of plant hormones at the infection site, causing the hairy root diseases (Machado *et al*. 1997; Bulgakov 2008). The difference in morphology and branching and growth pattern may be due to the difference in endogenous hormone level of target cells because *rol* expression is largely an auxin-inducible system and the fate of *rol*-induced meristem depends upon the local hormonal balance of a cell/tissue (Arroo *et al*. 1995; Baumann *et al*. 1999). Putatively transformed roots of *R. serpentina* demonstrated amplification of *rol*A. In earlier reports the transformed nature of hairy roots in *R. serpentina* was confirmed by opine analysis (Falkenhaegen *et al*. 1993; Benjamin *et al*. 1994). The literature so far does not support any evidence of genetic transformation at the molecular level in this species; this is the first report of molecular evidence of genetic transformation in *R. serpentina*. Stable integration of Ri T-DNA into the host plant genome accounts for the genetic stability of transformed root cultures. Their biochemical stability leads to a high growth rate with a stable and high level of production of secondary metabolites (Kamada *et al.* 1986). Secondary metabolite biosynthesis in transformed roots is genetically controlled (Hamill and Rhodes 1988) but it is also strongly influenced by nutritional and environmental factors (De-Eknamkul and Ellis 1984; Hilton and Rhodes 1993). These genetically transformed root cultures can produce levels of secondary metabolites comparable to that of intact plants. The *rol* genes in Ri T-DNA induce changes in sensitivity to plant hormones and/or in the metabolism of plant hormones (Akutsu *et al*. 2004). Owing to the random integration of T-DNA into the host plant genome, the resulting hairy roots often show variable patterns of secondary metabolite accumulation. Due to a certain amount of heterogeneity, repeated selection seems to be an important approach to obtain high-yielding hairy root lines (Yukimune *et al.* 1994). To the best of our knowledge this is the first report of reserpine synthesis in the hairy roots of *R. serpentina*.

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