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Genomic and metabolomic fingerprinting of *Phyllanthus amarus* (Schumm & Thonn) hairy root clones

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Abstract

This study deals with the establishment of hairy root cultures of *Phyllanthus amarus*, using *Agrobacterium rhizogenes* strain ATCC 15834. Genomic and metabolomic profiles of hairy root clones were determined by employing amplified fragment length polymorphism (AFLP) and gas chromatography-mass spectrometry (GC/MS) techniques. Hairy root clones and control showed ample differences in their AFLP profiles. AFLPs revealed 15-29 plasmid-specific bands and variable number of additional and/or missing bands of varied size in different clones, suggesting random and multiple integrations of Ri T-DNA. GC/MS analysis revealed marked differences in the profiles of compounds in various hairy root clones, indicating that the clones are distinct and dissimilar in their secondary metabolite production. These differences in the metabolite profiles of various clones may be attributed to the random T-DNA integrations resulting in missing and/or additional bands as well as changes in the gene expression pattern of recipient genomes. The hairy root clone Ph19 was found to produce maximum amount (27 g/100g dry weight) of a novel compound, Amarone. The overall results amply indicate the genome-wide changes caused by Ri T-DNA integrations leading to altered gene expression and wide variability in secondary metabolite profiles of different hairy root clones.

Key words: *Phyllanthus amarus*, Hairy roots, AFLP, metabolomic profiles

Introduction

Globally, plants serve as major sources of pharmaceuticals and other fine chemicals. Although plant cell culture is considered as an alternative to plant tissues for extraction of secondary metabolites, the major constraint with cell cultures is their genetic instability, resulting in low yields of secondary metabolites. Hairy root cultures induced by *Agrobacterium rhizogenes* are being widely employed as sources of useful compounds due to their rapid growth in culture media without phytohormones and relatively higher productivity of secondary metabolites compared to cell or callus cultures, or in some cases even the roots of the mother plant (Hamill *et al*., 1987). Hairy roots are genetically stable as they originate from single-plant-cell-infection by *Agrobacterium rhizogenes* (Bourgaud *et al*., 1999), and are capable of synthesizing metabolites that are normally found in roots (Li *et al.*, 2000). The productivity of secondary metabolites in hairy roots depends on the genetic character of the hairy roots, but is also influenced by the nutritional and environmental factors (Aoki *et al.*, 1997). In addition to enhanced production of secondary metabolites, hairy root cultures also were shown to synthesize new compounds not normally associated with the parent plant (Wysokinska and Chmiel, 1997). Hairy root cultures have been developed and employed for production of different useful compounds in various plants such as *Salvia* (Kuzma *et al.*, 2009), *Papaver* (Le Flem-Bonhomme *et al.*, 2004), *Azadirachta* (Allan *et al.*, 2002), *Coleus forskohlii* (Asada *et al*., 2012), *etc*. Also hairy root cultures were used to study biotransformation capabilities in *Anethum graveolens* (Faria *et al.*, 2009).

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The genus *Phyllanthus* (Euphorbiaceae) comprises of more than 600 species widely distributed throughout the tropical and subtropical regions. *Phyllanthus amarus*, previously known as *Phyllanthus niruri*, is an important herb, often used in the treatment of liver disorders and jaundice in the traditional Indian medicine (*Thygarajan et al.*, 1990). It is also used extensively for mitigating diseases, such as asthma, malaria, dysentery, influenza, dyspepsia, colic, dropsy, epilepsy and urinogenital disorders (Calixto *et al.*, 1998; Chopra *et al*., 1986; Mehrotra *et al.*, 1990). Its antiviral activity extends to human immunodeficiency virus-1 reverse transcriptase (Notka *et al.*, 2004). The crude extracts of *P. amarus* were found to show hypoglycemic (Moshi *et al.*, 1997), hypotensive, diuretic (Nadkarni, 1993), antiplasmodial, antibabesial (Matsuura *et al.*, 2005) and antioxidant properties (Karuna *et al.*, 2009). The plant extract also caused inhibition of calcium oxalate internalization in urolithiasis (Campos and Schor, 1999). The plant as well as various cultures of *Phyllanthus* have been well studied phytochemically and are known to contain lignans, alkaloids, flavonoids, phenols, tannins and terpenoids (Elfahmi *et al.*, 2006; Ishimaru *et al.*, 1992; Singh *et al.*, 1989). Among these, lignans have been evaluated intensively and were found to possess antitumor activity (Satyanarayana and Venkateswarlu, 1991). Furthermore, the crude extract of plant revealed anticancer activity with respect to hepatocellular carcinoma (Rajesh Kumar and Kuttan, 2000). Limited published reports, dealing with the establishment of hairy root cultures from *P. amarus* for production of phenolic compounds (Ishimaru *et al*., 1992) and their bioactivity against hepatitis B surface antigen (Bhattachrya and Bhattachrya, 2004), are available.

Amplified fragment length polymorphism (AFLP) is a novel polymerase chain reaction (PCR)-based method for plant DNA fingerprinting which reveals significant levels of DNA polymorphism (Vos *et al.*, 1995). This method is highly reproducible and does not require prior knowledge about the genome under study. AFLP analysis has been used extensively to assess genetic relationships of wide range of species such as *Hibiscus* (Cheng *et al.*, 2004), *Brassica* (Zhao *et al.*, 2005), *Uniola* (Subudhi *et al.*, 2005), *Aegiceras corniculatum* (Deng *et al.*, 2009), besides somaclonal variants of *Syngonium* (Chen *et al*., 2006), *Arabidopsis* (Polenco and Ruiz, 2002) and tissue culture-derived date palms (Saker *et al*., 2006). AFLP has also been employed for studying the biodiversity of *Streptococcus thermophilus* (Lazzi *et al*., 2009).

Metabolomics aim at unbiased identification and quantification of all metabolites present in a sample from an organism grown under defined conditions (Bhalla *et al*., 2005). The metabolome is closely related to phenotypes and is more context-dependent. Multivariate spectrometric detection methods like gas chromatography coupled with mass spectrometry (GC/MS) or high performance liquid chromatography coupled with mass spectrometry methods (HPLC/MS) are advantageous for identifying and quantifying number of compounds from a mixture (Abhyankar *et al*., 2005). GC/MS is still a popular method of metabolite profiling due to its ease in handling and availability of large databases to identify the various compounds. GC/MS analysis was used for metabolite profiling in *Ailanthus ultissima* (Mastelic and Jerkovic, 2002), *Phanerochaete* (Miura *et al.*, 2004) and loblolly pine (Morris *et al.*, 2004) and for analysis of essential oils in *Anethum graveolens* (Santos *et al.*, 2002). Also methods like solid phase micro extraction (SPME) coupled with GC/MS were used to analyze the regulation of floral scent production in *Petunia* (Verdonk *et al*., 2003). ^{1}H NMR fingerprinting was used to study the metabolic changes, caused in *Lemna minor* by various phytotoxic substances (Aliferis *et al.*, 2009) besides metabolomics coupled with other functional genomics approaches showed a promise for discovery of biomarkers of human disease (Lewis *et al*., 2008). ^{1}H NMR studies with transgenic and non-transgenic *Arabidopsis* could potentially be applied in the biosafety assessment of transgenic plants, as it was possible to distinguish wild type plants from transgenic plants based on their metabolic profiles (Ren *et al.*, 2009).

In this investigation, we dealt with the establishment of hairy root clones of *P. amarus* and their genomic and metabolomic profiling, using AFLP and GC/MS analyses. Furthermore, an attempt has also been made to correlate the various changes observed in the metabolite profiles with the alterations that occurred in the genomes of different hairy root clones. An overview of the results points to the role of *P. amarus* hairy root extract as a potential source of secondary metabolites.

Materials and Methods

Establishment of hairy root cultures of P. amarus

Seeds from a single plant of *P. amarus*, growing in the Institute's (Centre for Plant Molecular Biology, Osmania University) garden were collected and sown in the net house. These plants were employed in the present investigation. Multiple shoots were induced by culturing nodal sectors from a single plant of *P. amarus* on Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962), supplemented with 2.0 mg/l benzyl amino purine (BAP), sucrose (30 g/l) and solidified with 0.8% agar. Cultures were maintained at 25 $\pm$ 2°C in light by routine subculturing for every 4 weeks. Stem pieces (7.0-8.0 mm) and leaves from the multiple shoots were used for transformation. The leaves were cut from apical and distal ends. The stem pieces and the leaves were placed on MS basal medium, containing 30 g/l sucrose solidified with 0.8% agar along with 100mM acetosyringone and incubated for 2 days at 25 $\pm$ 2°C in light for preculture. Transformation experiments were carried out with *Agrobacterium rhizogenes* strain ATCC 15834 as described earlier (Abhyankar *et al.*, 2005).

Hairy root clones were separated based on their origin and growth pattern. Hairy roots originating from different explants were deemed as separate clones. Ten hairy root clones, *viz.*; Ph1, Ph2, Ph8, Ph10, Ph13, Ph15, Ph16, Ph17, Ph18 and Ph19, were selected for this study. These clones were maintained on MS basal medium, containing cefotaxime (250 mg/l) for 3 months, followed by atleast 18 months on MS basal medium sans antibiotics in dark with subculturing after every 6 weeks.

Seeds were also germinated *in vitro* on MS basal medium and the resulting seedlings were grown for 6 weeks and shoots and roots collected from these seedlings were used for GC/MS analyses.

Growth analysis

An inoculum (500 mg) of hairy roots was cultured on 50 ml MS basal medium with 0.8% agar for growth analysis. Growth patterns were analyzed by recording fresh and dry weights of the hairy roots for 5 weeks at weekly intervals. Four replicates were used for fresh and dry weight analysis. The fresh and dry growth indices were calculated using the following formulae: Fresh growth index (FGI) = final fresh weight – initial fresh weight / initial fresh weight; Dry growth index (DGI) = final dry weight – initial dry weight (calculated) / initial dry weight (calculated).

AFLP *analysis*

Genomic DNA of each hairy root clone and untransformed control was isolated using 200 mg of root tissue by the method of Zhang and Stewart (2000). Plasmid DNA was isolated from *Agrobacteriun rhizogenes* strain ATCC 15834 by standard methods (Sambrook and Russell, 2001). About 300ng of DNA from each of the clones, untransformed control and plasmid was used for AFLP reactions. AFLP was carried out mainly using the method of Vos *et al.*, (1995) with appropriate modifications. Amplification reactions were carried out using *EcoR*I and *Mse* I (E/M) enzyme combinations. Preamplification was done using *EcoR*I primers with an addition of a single base (A) and *Mse* I primers with the addition of cytosine (C) at 3' end. PCR conditions for preamplification were: 94^{0}C for 20 seconds, 56^{0}C for 30 seconds and 72^{0}C for 2 minutes (21 cycles). For selective amplification, 10 combinations of three nucleotides were used with *EcoR*I / *Mse*I primers. Out of these, only 6 combinations were presented in this paper. These are: I) E-ACC / M-CTA; II) E-AAC / M-CTC; III) E-AAC / M-CAC; IV) E-AGG / M-CTC; V) E-ACC / M-CTG and VI) E-ACG / M-CAA. Radioactive labeling was done using the ^{33}P á dATP. The PCR conditions were: 94^{0}C for 20 seconds, followed by 30 seconds annealing step (annealing temperature in first cycle was 65^{0}C, subsequently it was reduced by 1^{0}C in each cycle for the next 10 cycles till it reached 56^{0}C), followed by extension step at 72^{0}C for 2 minutes. Once the annealing temperature reached 56^{0}C, 21 more cycles were performed. The PCR products were separated on a 5% denaturing polyacrylamide gel along with the 50 base pairs (bp) ladder to determine the band size of different samples. The gel was then dried for one hour at 80^{0}C and autoradiographed for 4 days on Kodak X-ray film at -70^{0}C. The fingerprinting pattern was analyzed using the software SynGene Gene Tools, version 3.00.22 (SynGene Laboratories).

Metabolite profiling

Metabolite profiles were analyzed by GC/MS method for hairy root clones and also for untransformed roots and shoots as control. All the solvents used were of analytical grade. For extraction, hairy roots, control roots and shoots were dried at room temperature for 2 - 3 days and then kept at 50^{0}C for 2 hours. The dried roots and shoots were powdered finely and about 750 mg of each powder was extracted separately with 30 ml of n-hexane at room temperature for 2 days. The extracts were filtered through Whatman filter paper no. 1 and concentrated to 4.5 ml. The extracted sample powders were dried thoroughly to remove all traces of hexane and were re-extracted with 30 ml of methanol at room temperature for 2 days. The extracts were filtered through Whatman filter paper no. 1 and concentrated to 4.5 ml. The GC/MS analyses for hexane and methanol extracts were carried out, using Agilent 6890 series gas chromatograph equipped with 5973 N mass selective detector (MSD).

GC *conditions*: Column HP - 5 (30 M, 0.25 mm ID and 0.25 µ film thickness), injection temp.250^{0}C; interface temp. 280^{0}C; oven temp. was programmed from 50^{0}C, hold for 2 minutes, then increased by 10^{0}C/min till it reached 280^{0}C, and then hold for 5 minutes. Helium was used as a carrier gas at a flow rate of 1.0 ml/min. Injection was done in split mode (10:1) with volume of 1.0 µl.

MS *conditions*: Source temp. 230^{0}C, quadrupole temp.150^{0}C, MS was scanned from m/z 30 to 600 at a scan rate of 1.0 scan/ sec. The experiment was repeated thrice with three replicates and all the compounds were identified by library match (>95%).

Extraction and isolation of Amarone

Agar was completely removed from the hairy roots and then they were dried at room temperature for 2 days followed by drying at 50°C for 2h. Dried roots were powdered finely and ten grams of root powder was extracted (3x) at room temperature with 200 ml of methanol each time. The extract was concentrated and the concentrate was chromatographed over a column of neutral alumina (50 x 0.5 cm). Fractions were eluted by subsequent use of the following eluents: chloroform (500 ml), chloroform/ethyl acetate (1:1, 500 ml), ethyl acetate (500 ml), ethyl acetate/methanol (1:1, 750 ml), methanol (500 ml), methanol/acetone (1:1, 750 ml) and acetone (2000 ml). Fractions of 50 ml each were collected and were profiled by TLC. Fractions with corresponding profiles were combined and concentrated.

Results

Establishment and growth of hairy root clones

White friable callus developed at the cut ends of stem pieces in 10-12 days and hairy roots emerged from this callus mass in 15-20 days. Whereas, leaves neither showed callus formation nor hairy root induction, but turned yellow and were dead within 2 weeks after infection. Further, control explants except minor swelling at the cut ends, did not produce any roots even after 4 weeks of culture. The hairy roots, produced by stem pieces, were delicate, thin, thread-like and white in appearance. Each callus mass along with the roots was separated from the explant and were cultured on MS basal medium supplemented with cefotaxime (250 mg/l). Single hairy root originating from specific explant was considered as distinct clone. The various hairy root clones, upon subculture, put forth a large number of primary and secondary laterals and grew into a compact-thick mat on the medium (Figure 1a). All the clones were morphologically similar in appearance and growth, but showed clear variations in lateral branching. Clones Ph1, Ph10, Ph13, Ph15 and Ph19 showed normal branching pattern, with short laterals developing from both sides of the main root (Figure 1b). Two clones, Ph2 and Ph8, disclosed longer laterals which could not be distinguished from the main roots (Figure 1b). Whereas, clones Ph16 and Ph18 showed development of laterals only from one side of the main root (Figure 1b). In clone Ph17, the laterals were observed emerging after considerable growth of the main root (Figure 1b). Although different clones exhibited varied branching pattern, the growth profiles of all the clones showed a typical sigmoid growth pattern with a doubling time of 5-7 days. Wide variations were observed among clones for FGI and DGI values (Figure 2). However, DGI was found to be greater than that of FGI. After 5 weeks of culture, Ph2 and Ph19 showed maximum FGI of 11.74, while Ph10 showed a minimum FGI of 7.00.

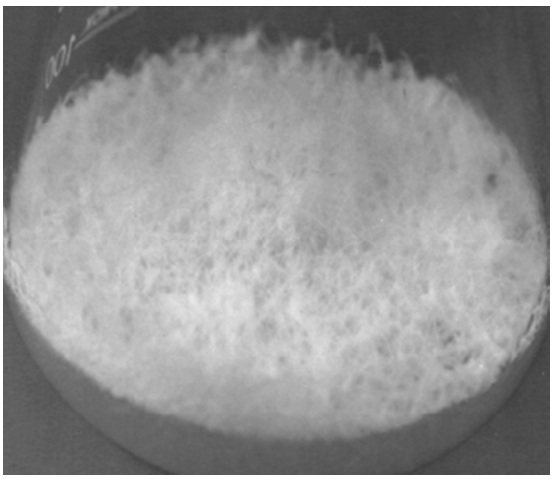
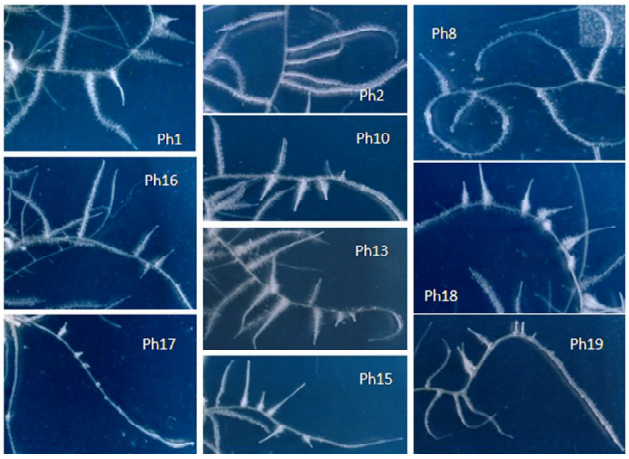


Figure 1: (a) Hairy root culture of *Phyllanthus amarus* after 4 weeks of subculture grown in dark on MS basal medium with 0.8% agar



(b) *P. amarus* hairy root clones after 10 days of subculture showing different lateral branching patterns.

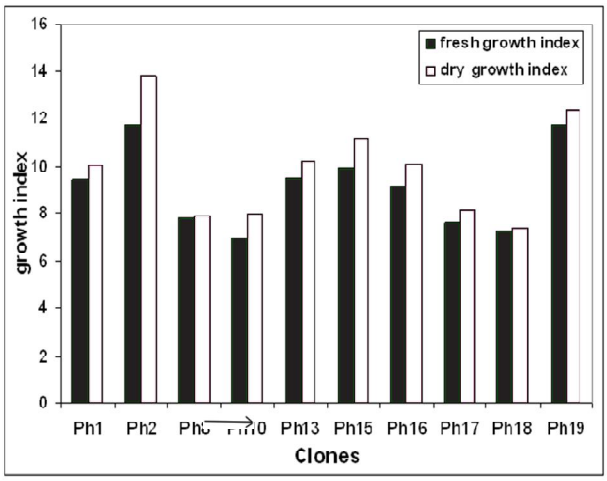


Figure 2: Growth indices of *P. amarus* hairy root clones after 5 weeks of subculture on MS basal medium with 0.8% agar. FGI: fresh weight growth index; DGI: dry weight growth index. Each bar represents mean of four replicates.

AFLP *analysis*

AFLPs were carried out using six combinations of *EcoRI* and *MseI* primers with three selective nucleotides. Each combination yielded a different set of amplified fragments (Figure 3). Various clones showed marked differences in the profile of amplified fragments in each combination. Clone Ph1 revealed a total of 295 amplified fragments from six combinations, out of which 225 bands were found common with the untransformed control. Also, a total of 17 plasmid-specific bands were observed with variable number of bands in each combination. Twenty eight bands, present in the control, were absent in clone Ph1; whereas, 53 additional bands were present in this clone which were absent in the control as well as in the plasmid. Out of these 53 additional bands, 11 bands were clone-specific for Ph1.

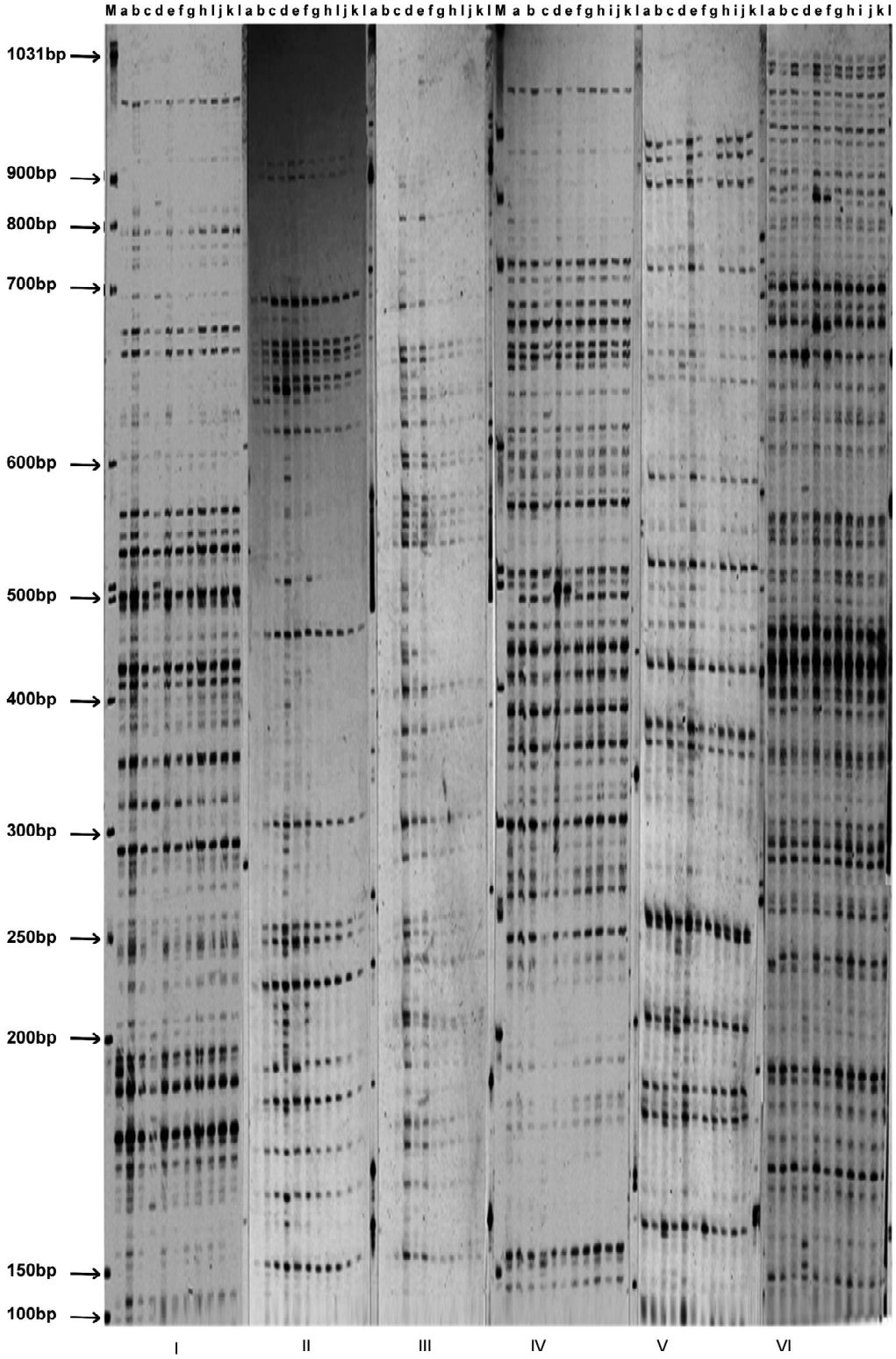


Figure 3: AFLP profiles of *P. amarus* hairy root clones, untransformed control and plasmid. 50bp ladder was run along with the samples. E) *Eco RI* primer, M) *Mse I* primer, I) E-ACC / M-CTA, II) E-AAC / M-CTC, III) E-AAC / M-CAC, IV) E-AGG / M-CTC, V) E-ACC / M-CTG and VI) E-ACG / M-CAA primers with selective nucleotide combinations used for amplification. In each combination lanes represent: M) marker, a) untransformed control, b) clone Ph1, c) Ph2, d) Ph8, e) Ph10, f) Ph13, g) Ph15, h) Ph16, i) Ph17, j) Ph18, k) Ph19 and l) plasmid.

Ph2 showed 203 common bands with that of control. In this clone, plasmid-specific bands ranged from 1-8 in each combination, totaling to 21 from 6 combinations. As compared to control, 48 bands were missing in the clone while 70 additional amplified fragments were observed in the clone. However, out of these bands, only 4 bands were found clone-specific.

Out of 303 total bands disclosed by Ph8, 189 bands were found common with the control. Also, 29 plasmid-specific bands were observed in 6 combinations ranging from 1-9 bands in each combination. When compared to the control, 61 bands were missing in the clone, while 85 additional bands were noticed in the clone. Seventeen out of these 85 bands were found clone-specific.

Clone Ph10 showed the maximum number (312) of bands in all 6 combinations. Out of 312 amplified fragments, 210 bands were found common with the untransformed control. The clone also disclosed 28 plasmid-specific bands ranging from 1-10 in each combination. As compared to the control, 43 bands were missing while 74 additional bands were observed in the clone. Out of these 74 bands, only 8 bands were found clone-specific.

The clone, Ph13, revealed 277 amplified fragments, of which 196 bands were found common with the control. In this clone, 22 plasmid-specific bands were observed in 6 combinations. As compared to the control, 57 bands were absent in Ph13, while 59 additional bands were found to be present. However, 3 out of these 59 additional bands were found clone-specific.

Out of 259 total bands observed in Ph15, 193 bands were found common with the control. Also, 15 plasmid-specific bands were noticed in the clone in 6 combinations. As compared to the control, 59 bands were missing in the clone, while 51 additional bands were visualized. Three out of these 51 bands were found clone-specific.

From a total of 270 amplified fragments shown by Ph16, 202 bands were found common with that of the control. In this clone, a total of 15 plasmid-specific bands were also observed in all the 6 combinations. In Ph16, as compared to the control, 51 bands were missing, while 53 additional amplified fragments were observed. Out of these bands, only 2 were found clone-specific.

In clone Ph17, a total of 255 amplified fragments were observed, out of which 194 bands were found common with the untransformed control. Further, the clone exhibited a total of 17 plasmid-specific bands from 6 combinations. As compared to the control, 59 bands were missing, while 44 additional bands were observed in this clone. Out of 44 additional bands only 2 bands were found clone-specific.

Clone Ph18 divulged a total of 253 amplified fragments, of which 192 bands were common with the control. Also, a total of 19 plasmid-specific bands were observed in this clone from 6 combinations. When compared to the control 60 bands were absent, while 42 additional amplified fragments were observed in the clone. Out of these additional bands, only 1 band was found to be clone-specific.

The clone, Ph19, demonstrated a total of 244 amplified fragments, of which 175 bands were found common with that of control. A total of 23 plasmid-specific bands were also observed in the clone from all 6 combinations. In this clone, compared to the control, 77 bands were missing, while 46 additional bands were present. However, 5 out of these 46 bands were found clone-specific in Ph19.

The various hairy root clones revealed more number of missing bands than plasmid-specific bands. Based on AFLP banding pattern, the hairy root clones, compared to the control, could be classified into 2 broad groups, *viz.*; i) Ph1, Ph2, Ph8, Ph10, Ph13 and Ph16 which showed less number of missing bands than the number of additional bands, and ii) Ph15, Ph17, Ph18 and Ph19 which showed more number of missing bands than the additional bands. Clone Ph8 showed maximum number of 85 additional bands and 28 plasmid-specific bands, while clone Ph19 showed maximum number of 74 missing bands. However, least number of additional (42) and missing (28) bands were disclosed by clones Ph18 and Ph1, respectively.

Metabolite profiling by* GC/MS *and amarone production

Solvent extracts of hairy root clones along with untransformed control roots and shoots were subjected to qualitative analyses by GC/MS. The various compounds identified in hexane and methanol extracts of hairy root clones and that of controls are presented in Table 1. Striking differences were observed in the compound profiles of controls and hairy root clones (Figure 4). Control roots and shoots showed a total of 9 and 17 compounds, respectively, out of which only 2 compounds were found common between control roots and shoots. The hairy root clones showed 6-14 compounds as revealed by GC/MS analysis. Only one compound (octane) was produced in all hairy root clones and controls, while 1-octadecene was found only in hairy root clones but was absent in control roots and shoots. Three and 6 compounds were exclusively present in control roots and shoots, respectively. The various clones, except Ph1 and Ph18, revealed 1-3 clone-specific compounds. Octadecane and β-sitosterol were the two clone-specific compounds observed in Ph2, while Ph10 showed two different clone-specific compounds (cyclohexane 1,3-dimethyl and pentadecane). Three clone-specific compounds, *viz.*; eicosane, heptacosane and morphine, 3-O (4-trifluoromethylphenyl) were exclusively present in Ph13. Five clones, *viz.*; Ph8, Ph15, Ph16, Ph17 and Ph19 demonstrated one clone-specific compound each.

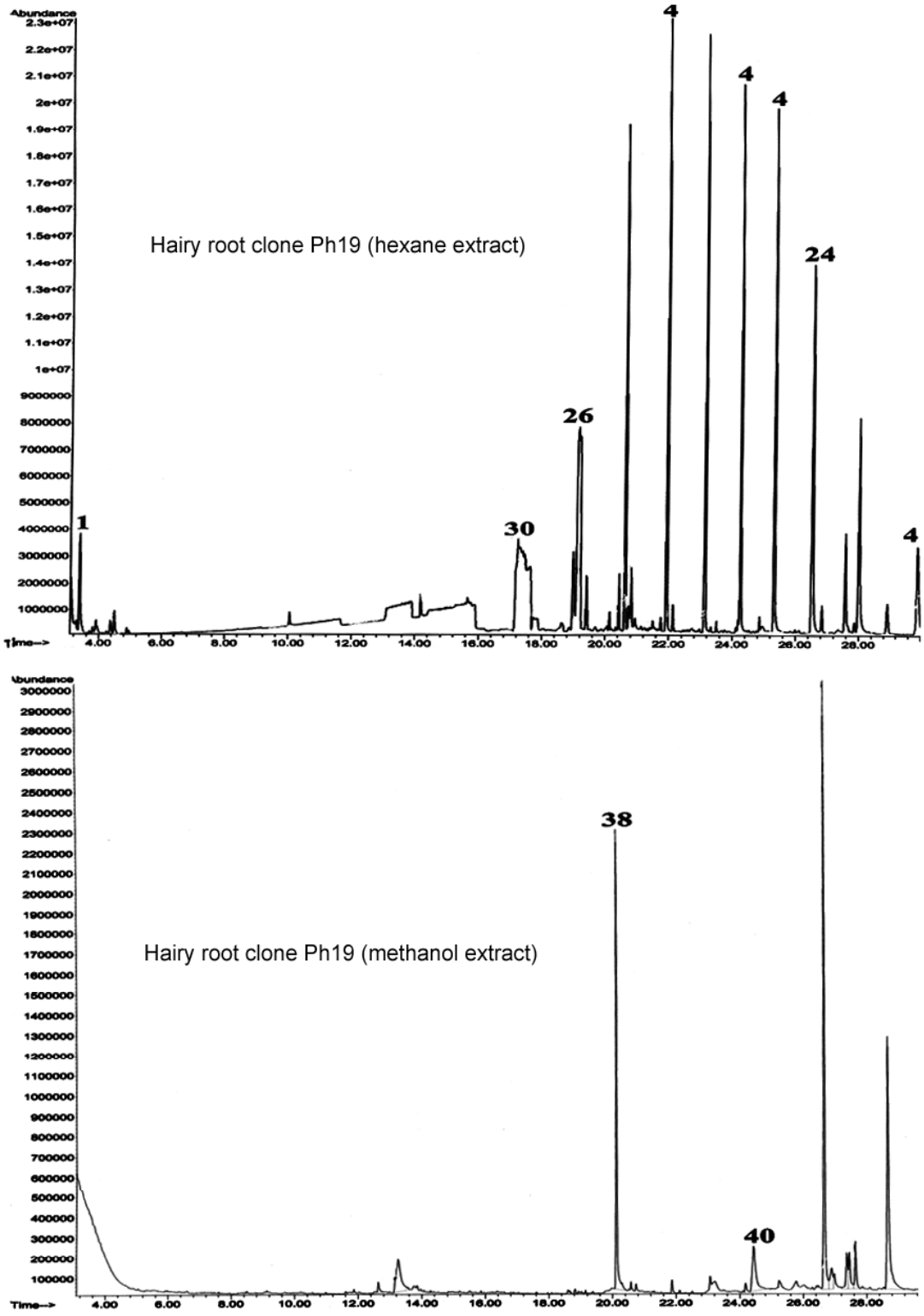


Figure 4: GC chromatograms of hexane and methanol extracts of *P. amarus* transformed hairy root clone 19. The peaks are numbered as they appear in Table 1.

GC/MS and AFLP analyses depicted marked differences in their profiles of secondary metabolites and amplified fragments (Table 2). Three compounds, *viz.*; phenol, 2,6-dibromo-4-nitro; 2-methoxy-3,8-dioxocephal and 1,2-benzenedicarboxylic acid were observed only in control roots which were absent in control shoots and hairy root clones. The presence of these 3 compounds may be associated with 4 control-specific bands (I-575, IV-622, V-237 and VI-977) which were missing in all the clones. Phenol, 2,4-bis(1,1-dimethylethyl) was present only in clone Ph2 and control roots. Also, untransformed control and clone Ph2 showed 2 specific bands (IV-250 and VI-622) which were absent in other clones. Benzoic acid was found only in clone Ph1 and control shoots, while it was absent in control roots and all other hairy root clones. Clone Ph1 and control showed five amplified fragments (I-192, I-187, II-751, IV-435 and VI-363) which were absent in all other clones. Also, stearic acid was produced in clone Ph15 and control shoots which was missing in control roots and various other clones. Further, clone Ph15 and control showed two common bands (VI-654 and 632) that were missing in other hairy root clones.

Table 1: GC/MS profiles of *P. amarus* untransformed roots, shoots and hairy root clones

S. No	Compound	Cont roots		Cont shoots		Ph1		Ph2		Ph8		Ph10		Ph13		Ph15		Ph16		Ph17		Ph18		Ph19	
		H	M	H	M	H	M	H	M	H	M	H	M	H	M	H	M	H	M	H	M	H	M	H	M
1.	Octane	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
2.	p-xylene	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	-	-
3.	Phenol,2,4-bis (1,1-dimethylethyl	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4.	4-(3,4-dimethoxy benzylidene)	+	+	-	-	-	+	-	+	-	+	-	+	-	+	+	+	-	-	+	+	-	+	+	-
5.	Cyclopentane, 1-ethyl-2-methyl	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-
6.	Heptane, 2,6-dimethyl	-	-	+	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-
7.	Thiophene, tetra-hydro-,1,1-dioxide	-	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	-	-
8.	Hexadecenoic acid, z-11	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9.	Palmitic acid	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
10.	9-octadecenoic acid(z)	-	-	+	-	+	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-
11.	Stearic acid	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
12.	9-octadecenal(z)	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13.	9,12-octadecadienoic acid	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14.	Nonacosane	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
15.	Dimethyl2,3-bis(1,3 -dimethylin)	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16.	Nonane	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
17.	Octadecane	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18.	Trans-1-butyl-2 -methyl cycloprop	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19.	Cyclohexane,1,3 -dimethyl trans	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
20.	Heptane,2,4-dimethyl	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-
21.	Eicosane	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-

22.	Heptacosane	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
23.	Hexadecanoic acid, methyl ester	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-
24.	3-(4-N,N-dimethyl aminophenyl) propenoic acid	+	+	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	+	+	-	-	+	-
25.	Methyl oleate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
26.	Cyclodecasiloxane, eicosamethyl	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	-	+	-
27.	Eseroline, 7-bromo - methyl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
28.	Cyclohaptasiloxane, tetradecamethyl	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	+	+	-	-	-	-
29.	Cyclooctasiloxane, hexadecamethyl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
30.	Silane,(3β, 11 β, 20S pregnane	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
31.	Benzoic acid, 2,5-bis (trimethyl siloxy)-, methyl ester	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
32.	2-methoxy-3,8-dioxo	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
33.	1,2-benzenedic arboxylic acid	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
34.	Benzoic acid	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
35.	1,3-isobenzofuran-dione	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
36.	Neophytadiene	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
37.	Dimethyl 2,3-bis(1,3 - dimethylindol-2yl) fumarate	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
38.	1-octadecene	-	-	-	-	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
39.	Erucylamide	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
40.	Stigmasterol	-	-	-	-	-	-	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	-	-	+
41.	B sitosterol	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
42.	Pentadecane	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
43.	Morphine,3-O (4-trifluoromethyl phenyl)	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-

+ compound present; - compound absent; H – hexane extract; M – methanol extract

Clone Ph2 alone showed the presence of octadecane and β-sitosterol which may be associated with the four missing bands (I-453, I-163, VI-333, VI-261) and four clone-specific bands (III-829, III-641, III-637 and III-583). Nonane was produced exclusively by Ph2 and Ph10, while it was missing in the control and other clones. Further, six additional amplified fragments (III-693, III-625, III-271, III-112, IV-900 and V-652) were observed only in Ph2 and Ph10, which were missing in the remaining clones and control. Clones Ph2 and Ph15 showed presence of methyl ester of hexadecanoic acid and the clone-specific band VI-920 may be associated with this compound.

Clone Ph8, containing 9 missing bands (I-765, I-736, IV-642, IV-341, IV-184, VI-640, VI-483, VI-433 and VI-368) and 17 clone-specific bands (I-462, II-613, II-549, II-522, II-491, II-462, II-458, 202, III-386, IV-924, IV-145, V-364, V-215, VI-1014, VI-196, VI-174 and VI-153) showed the exclusive presence of trans-

Table 2: GC/MS and AFLP analyses of hairy root clones showing correlation between secondary metabolites and amplified fragments

Presence of compound in	Compound	Presence of bands in	Presence of control / clone-specific band(s)	Absence of control / clone-specific band(s)
Control roots	phenol, 2,6-dibromo -4-nitro; 2-methoxy-3, 8-dioxocephal; 1, 2-benzenedicarboxylic acid	Control roots	I-575, IV-622, V-237 and VI-977	–
Control roots and Ph2	Phenol, 2,4-bis(1,1-dimethylethyl)	Control roots and Ph2	IV-250 and VI-622	–
Control shoots and Ph1	Benzoic acid	Control shoots and Ph1	I-192, I-187, II-751, IV-435 and VI-363	–
Control shoots and Ph15	Stearic acid	Control shoots and Ph15	VI-654 and VI-632	–
Ph2	Octadecane; β-sitosterol	Ph2	III-829, III-641, III-637 and III-583	I-453, I-163, VI-333, VI-261
Ph2 and Ph10	Nonane	Ph2 and Ph10	III-693, III-625, III-271, III-112, IV-900 and V-652	–
Ph2 and Ph15	methyl ester of hexadecanoic acid	Ph2 and Ph15	VI-920	–
Ph8	trans-1-butyl-2-methyl cyclopropane	Ph8	I-462, II-613, II-549, II-522, II-491, II-462, II-458, 202, III-386, IV-924, IV-145, V-364, V-215, VI-1014, VI-196, VI-174 and VI-153	I-765, I-736, IV-642, IV-341, IV-184, VI-640, VI-483, VI-433 and VI-368
Ph10	cyclohexane, 1,3-dimethyl; pentadecane	Ph10	II-863, III-881, IV-513, V-836, V-758, V-749, V-209 and VI-417	IV-475
Ph13	Eicosane; heptacosane; morphine, 3-O (4-trifluoromethylphenyl)	Ph13	II-556, III-223 and V-201	V-729 and VI-611
Ph15	eseroline, 7-bromo-methyl	Ph15	II-741, III-199 and VI-521	IV-609, IV-381, IV-324, V-715, V-646, V-600, V-285 and VI-665
Ph16	methyl oleate	Ph16	II-227 and VI-516	–
Ph17	cyclooctasiloxane hexadecamethyl	Ph17	IV-173 and V-396	–
Ph19	silane (3β, 11 β, 20S pregnane)	Ph19	II-787, II-196, VI-693, VI-163 and VI-143	I-175, II-680, VI-385 and VI-320
All hairy root clones, but absent in control	1-octadecene	ControlAll hairy root clones	I-575, IV-622, V-232 and VI-977VI-218	Bands present in control, were missing in hairy root clonesBand absent in control

1-butyl-2-methyl cyclopropane. The clone, Ph10, which specifically showed one missing band (IV-475) and eight clone-specific bands (II-863, III-881, IV-513, V-836, V-758, V-749, V-209 and VI-417) could produce the compounds, cyclohexane, 1,3-dimethyl and pentadecane. Clone Ph13 having three clone-specific bands (II-556, III-223 and V-201) and two missing bands (V-729 and VI-611), could exclusively produce three compounds, *viz.*; eicosane, heptacosane and morphine, 3-O (4-trifluoromethylphenyl). Clone Ph15 with three clone-specific bands (II-741, III-199 and VI-521) and eight missing bands (IV-609, IV-381, IV-324, V-715, V-646, V-600, V-285 and VI-665) could specifically produce the compound, eseroline, 7-bromo-methyl. Clone Ph16 with two clone-specific bands (II-227 and VI-516) could produce the clone-specific compound, methyl oleate. However, Ph16 could not produce 4(3,4-dimethoxybenzylidene) which was present in all other clones as well as the control. Clone Ph17 with two clone-specific bands (IV-173 and V-396) produced one clone-specific compound, *viz.*; cyclooctasiloxane hexadecamethyl. Ph19 having five clone-specific bands (II-787, II-196, VI-693, VI-163 and VI-143) and four missing bands (I-175, II-680, VI-385 and VI-320) produced one clone-specific compound, *viz.*; silane (3β, 11 β, 20S pregnane), while two compounds (p-xylene and thiophene tetrahydro-1,1-dioxide) present in all other clones were missing in this clone. Compound, 1-octadecene, was produced in all the hairy root clones, but was absent in the control. Four bands (I-575, IV-622, V-232 and VI-977) which were present in the control were missing in all the hairy root clones. Also, an additional band (VI-218) that was observed in all the hairy root clones was absent in the control.

Methanolic extracts from all the hairy root clones maintained stably for a period of about 4 years, were subjected to fractionation on the neutral alumina column. Fractions eluted with acetone (fraction numbers 40-51) yielded a bright yellow liquid compound (TLC pure), amarone, with a characteristic pleasant odour. Amarone was produced in all the clones which could not be detected by GC/MS. However, this compound was missing in the untransformed roots and shoots. On dry weight basis, clone Ph19 produced highest amount (27.0%) of this compound followed by Ph2 (16.07%), Ph16 (12.76%), Ph18 (12.58%), Ph15 (10.2%), Ph 10 (10.18%), Ph17 (8.62%), Ph13 (8.44%), Ph8 (4.66%) and Ph1 (3.04%).

Discussion

Hairy root cultures established by infection with *Agrobacterium rhizogenes* are widely used as sources of useful compounds due to their rapid growth and high production of secondary metabolites (Kim *et al.*, 2009; Babaoglu *et al.*, 2004; Bhagyalakshmi *et al.*, 2004). Using stem pieces from *in vitro* maintained shoots of *P. amarus*, we have developed an efficient transformation protocol employing *Agrobacterium rhizogenes* strain ATCC 15834 with 13% frequency of hairy root induction. In the present study, stem pieces alone from *in vitro* maintained shoots demonstrated hairy root induction, while leaves failed to induce any hairy root, suggesting the importance of explant source for hairy root induction. Among the different explants tested for hairy root induction in *Pueraria phaseoloides*, leaf petioles were found more competent than cotyledons and leaves (Shi and Kintzios, 2003). Such explant specificity was also observed in *Plumbago* (Gangopadhyay *et al.*, 2008) and coffee (Alpizar *et al.*, 2006). In the present investigation, callus formation preceded hairy root induction presumably because of high level transient expression of auxin promoting genes. Hairy root induction *via* callus was also noticed in different plants like potato (Ottani *et al.*, 1990), *Tylophora indica* (Chaudhuri *et al.*, 2005) and *Mammillaria plumosa* (Gonzalez-Diaz *et al.*, 2006). On the other hand, Bhattacharya and Bhattacharya (2004) reported hairy root induction in *P. amarus* without any callus formation. These differences may be attributed to the genotypic and environmental variations.

Distinct differences observed by us in the lateral branching pattern in various hairy root clones point to the fact that each hairy root clone arose from a separate transformation event. Hairy root clones of *Gentiana* sp. (Momcilovic *et al.*, 1997), *Catharanthus roseus* (Batra *et al.*, 2004) and *Rhamnus fallax* (Rosic *et al.*, 2006) also showed wide variability in morphology and growth patterns. The doubling time of hairy root clones observed in this investigation was 5-7 days, which was within the range of transformed roots obtained from various other plant species (Wilson *et al.*, 1987). Furthermore, no decline in hairy root growth was observed in *P. amarus* even after 5 weeks of subculture. These observations are comparable to the hairy root clones of *Psoralea corylifolia* (Abhyankar *et al.*, 2005) and *Oxalis tuberosa* (Bais *et al.*, 2003). Whereas, hairy root clones in *Hyoscyamus muticus* reached stationary phase within 14 days (Vanhala *et al.*, 1995) and within 28 days in *Saussurea involucrata* (Fu *et al.*, 2006). In this study we observed an approximate 8-13 fold increase in fresh and dry weights of hairy roots after 5 weeks of subculture. These hairy roots were maintained by regular subculturing on MS basal medium for over 4 years without any callus formation or deceleration of growth.

AFLP is one of the recent innovations in genetic marker technologies having considerable potential for generating a large number of reproducible loci with genome-wide distribution (Vos *et al.*, 1995). In 6 combinations of AFLP amplifications, a total of 244-312 amplified fragments were observed in different clones, of which 175-225 bands were found common with the untransformed control, implicating that the major portion of the genomes of hairy root clones remained unaltered. A total of 15-29 plasmid-specific bands were observed in 6 combinations used for AFLPs in different clones. Out of these, each hairy root clone revealed 1-10 plasmid-specific bands of varied size in each combination,

indicating multiple and random integrations of Ri T-DNA into the genomes of these clones. Also, varied profiles of plasmid-specific bands observed in each clone suggest that different regions of Ri T-DNA have stably integrated into the genomes of different hairy root clones. Studies carried out in tobacco (Tempe and Casse-Delbart, 1989) and *Lotus corniculatus* (Petit *et al*., 1986) also demonstrated random and multiple integrations of Ri T-DNA. All the hairy root clones employed in this investigation, exhibited more number of missing bands (28-74) than plasmid-specific bands (15-29). These missing bands which were present in the control, might not have amplified in the clones due to Ri T-DNA integrations in the corresponding genomic regions. More number of missing bands, in comparison with plasmid-specific bands, observed in the clones might have resulted from integrations of Ri T-DNA corresponding to partial amplicon(s) or regions not corresponding to plasmid-specific amplicons. Also, a total of 42-85 additional bands of varied size were observed in different clones that were missing in the control. Clones Ph15, Ph17, Ph18 and Ph19 showed more number of missing bands than additional bands, which may be attributed to the integration of long T-DNA fragments that failed to act as amplicons or integration(s) that might have abolished the restriction site(s) corresponding to the amplicon(s). On the other hand, more number of additional bands than the missing bands were found in 6 other clones, viz.; Ph1, Ph2, Ph8, Ph10, Ph13 and Ph16, suggesting generation of novel amplicons resulting from interaction(s) between Ri T-DNA regions with genomic sequences at the sites of integration. Clone Ph8 showed maximum number (85) of additional bands as well as plasmid-specific (29) bands, implicating maximum integrations comprising both long and short stretches of T-DNA for generation of novel and plasmid-specific amplicons. An overview of AFLP profiles with wide variations amply suggests that different clones arose from independent transformation events with Ri T-DNA integrations at diverse locations in the genome.

GC/MS, a current popular method adopted for metabolite profiling, was employed for qualitative analyses of different compounds present in hairy root clones and controls, wherein striking differences were observed in the compound profiles. A total of 6-14 compounds were produced in different hairy root clones, while control roots and shoots disclosed 9 and 17 compounds respectively (Table 1). The overall metabolite profiles revealed the presence of 3 compounds specifically in control roots. Also, 4 control-specific bands which were missing in all the clones, were observed in the AFLP analysis. Absence of control-specific compounds in all the hairy root clones may be attributed to disruptions caused in the genomic DNA by Ri T-DNA integrations. Benzoic acid was produced only in clone Ph1 and untransformed shoots while it was absent in control roots and all other clones. Similarly, clone Ph15 and untransformed shoots exhibited the exclusive presence of stearic acid (Table 1). The presence of benzoic acid and stearic acid in hairy root clones Ph1 and Ph15, suggests that the synthetic pathways activated in the differentiated stem explants, used for transformation, continued even after transformation and differentiation in the respective hairy root clones. Further, 5 amplified fragments (I-192, I-187, II-751, IV-435 and VI-363) were specifically observed in Ph1 and control, while 2 other bands (VI-654 and VI-632) were exclusively exhibited by Ph15 and control, suggesting the plausible role of these genomic regions in the synthesis of benzoic acid and stearic acid, respectively. However, in the remaining hairy root clones these compounds were not produced owing to the absence of corresponding amplicons. The compound, phenol,2,4-bis(1,1-dimethylethyl), which was found exclusively in Ph2 and control roots with 2 specific bands (IV-250 and VI-622) indicate that the corresponding regions might be responsible for synthesis of the compound. Presence of this compound in control roots and its absence in control shoots clearly establish its *in planta* synthesis in roots. Compounds, β-sitosterol and octadecane, were exclusively produced in clone Ph2, which also showed 4 missing bands and 4 additional bands, implicating genomic alterations in the corresponding regions leading to switching on of the pathways for production of these compounds or generated a block in the metabolic pathways for utilization of these compounds. Also, clones Ph8, Ph10, Ph13 and Ph15 produced different clone-specific compounds with several additional (3-17) and some missing (1-9) bands. Clone Ph17 showed the presence of 2 clone-specific (IV-173 and V-396) additional bands and the production of the clone-specific compound, Cyclooctasiloxane hexadecamethyl, probably because of the insertional inactivation of gene(s) involved in the downstream pathway leading to accumulation of the compound. Clone Ph19 alone, having 5 additional bands and 4 missing bands, produced a clone-specific compound, silane, 3â, 11â, 20S pregnane, while 2 other compounds *viz.*; p-xylene and thiophene tetrahydro-1,1-dioxide, were missing only in this clone. It may be predicted that T-DNA integration(s) in this clone, have switched on the pathway for synthesis of the clone-specific compound; at the same time, these integrations also might have disrupted the existing pathways responsible for the missing compounds. Wide range of variation (3-27%) observed among various hairy root clones for production of the novel compound, amarone, may be attributed to the differential interactions among diverse gene products of Ri T-DNA and that of plant genome. The well-defined AFLP and metabolite profiles observed in different hairy root clones unequivocally establish the random nature and varied integration(s) of T-DNA into the genomes of different clones. In *C. roseus* hairy root clones (Batra *et al*., 2004) and transformed potato tubers (Roessner *et al*., 2001) similar variations were found in their secondary metabolite profiles.

In this investigation, a reproducible transformation protocol has been optimized for induction of hairy root cultures from shoots of *P. amarus,* using *Agrobacterium rhizogenes* strain ATCC 15834. AFLP analysis disclosed diverse alterations in the genomes of hairy root clones owing to random and multiple integrations of Ri T-DNA. Marked differences observed in the GC/MS profiles indicate that the clones are distinct and dissimilar in their secondary metabolite production. Further, the presence and absence of various AFLP bands vis-a-vis metabolite profiles, observed in different clones, implicate the role of specific amplicons in the production of specific compounds. The identified polymorphic bands, tagged to various secondary metabolites, offer scope for isolation of gene(s) involved in the biosynthesis of specific compounds. The clone Ph19, endowed with 27.0% amarone on dry weight basis, might serve as a source for production of this compound on commercial scale.

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Conflict of interest

The authors declare no conflict of interest.

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