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Amplified fragment length polymorphism and metabolomic profiles of hairy roots of *Psoralea corylifolia* L.

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Abstract

A reproducible protocol for establishment of hairy root cultures of *Psoralea corylifolia* L. was developed using *Agrobacterium rhizogenes* strain ATCC 15834. The hairy root clones exhibited typical sigmoid growth curves. Genomic and metabolomic profiles of hairy root clones along with that of untransformed control were analysed. Hairy root clones, Ps I and Ps II, showed significant differences in their amplified fragment length polymorphism (AFLP) profiles as compared to that of control, besides exhibiting Ri T-DNA-specific bands. These results amply indicate the stable integration of Ri T-DNA into the genomes of these clones. Further, the variations observed between clones in the AFLP profiles suggest the variable lengths and independent nature of Ri T-DNA integrations into their genomes. An isoflavonoid, formononetin, and its glycoside were present only in the hairy root clones while they were absent in the untransformed control. Variations observed in the metabolite profiles of these clones may be attributed to the random T-DNA integrations and associated changes caused by them in the recipient genomes. GC/MS analyses revealed the production of three and six clone-specific compounds in Ps I and Ps II, respectively, suggesting that the clones are dissimilar in their secondary metabolism. HPLC/UV–MS analyses disclosed substantial increases in the total isoflavonoids produced in Ps I (184%) and Ps II (94%) compared to untransformed control.

Keywords: *Psoralea corylifolia*; Papilionaceae; Hairy roots; Metabolomics; AFLP; Isoflavonoids; Formononetin

1. Introduction

Psoralea corylifolia L. is an important medicinal plant found in the tropical and subtropical regions of the world. It was found to synthesize diverse phenylpropanoids such as furanocoumarins, isoflavonoids, etc. (Boardley et al., 1986). These compounds are mainly used to cure leucoderma, leprosy, psoriasis and inflammatory diseases of skin (Chadha, 1985). Flavonoids, in general, were found to confer protection against several cancers (Herman et al., 1995). Flavonoid, daidzein (4′,7-dihydroxy isoflavone), was found to reduce the incidence of breast cancer (Adlercreutz et al., 1991), and could play a key role in conferring protection against colon cancer (Setchell et al., 1981). Recently, it was reported that daidzein could prevent osteoporosis by enhancing osteoblast viability besides its anabolic effect on the bone metabolism (Jia et al., 2003). Also, daidzein was found in whole plants as well as in different callus cultures of *Psoralea* (Bourgaud et al., 1995). Further, it was demonstrated that certain cell lines, obtained from callus cultures of different *Psoralea* species,

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produce high levels of daidzein as compared to whole plants (Bouque et al., 1998). Hairy root cultures induced by *Agrobacterium rhizogenes* represent an attractive alternative to callus and cell cultures for producing secondary metabolites (Bhagyalakshmi et al., 2004; Shi and Kintzios, 2003; Allan et al., 2002; Murakami et al., 1998). In general, the metabolic patterns of hairy roots are found similar to that of mother plant roots. However, the profiles of secondary metabolites depend on the genetic make up of the hairy roots (Aoki et al., 1997). Hairy roots, in contrast to cell lines, were found genetically stable and could produce secondary metabolites concomitantly with the growth (Bourgaud et al., 1997). In different *Psoralea* species hairy root cultures were established and analysed for the production of flavonoids (Nguyen et al., 1992). Hairy root cultures of different *Psoralea* species produced more daidzein compared to callus cultures and whole plants (Bourgaud et al., 1999).

Amplified fragment length polymorphism (AFLP) markers are one of the recent innovations in genetic marker technologies. Using AFLP, DNA fingerprints can be generated without any prior sequence knowledge. Most AFLP fragments correspond to unique positions in the genome and as such could be used as landmarks in developing genetic and physical maps (Vos et al., 1995). Molecular markers were also used to establish phylogenetic relationships among species of *Dioscorea* (Asemota et al., 1996) and *Oryza* (Federici et al., 2001). Successful fingerprinting for malt in barley was done using AFLP markers (Faccioli et al., 1999).

Metabolomics is the large-scale phytochemical analysis or metabolic profiling of the plants for qualitative and quantitative measurement of metabolites present in a given sample. No single analytical method provides sufficient visualization of the metabolome; hence different techniques are needed for a comprehensive profiling (Hall et al., 2002). The selection of most suitable technique is generally a compromise between speed, selectivity and sensitivity (Sumner et al., 2003). Most of the methods used for the analysis of isoflavonoids were based on HPLC or capillary electrophoresis. Prasain et al. (2003) reported that HPLC coupled with UV detector failed to detect the unknown compounds in a crude extract, while mass spectrometry could be used to detect both known as well as unknown compounds. HPLC coupled with UV and MS was used to monitor changes in the profiles of isoflavonoids in *Lupinus albus* (Wang et al., 2002). HPLC methods with diode array or mass detection were used for profiling of flavonoids and their conjugates (Graham, 1991; Sumner et al., 1996); whereas, HPLC/MS and nuclear magnetic resonance (NMR) were used to identify cell culture metabolites in *Taxus* (Ketchum et al., 2003). Also, methods like ^{1}H NMR and multivariate analysis were used for metabolite profiling of *Strychnos* species (Frederich et al., 2004).

This study deals with the establishment of hairy root clones of *P. corylifolia* and their genomic and metabolomic profiling, using AFLP, GC/MS and HPLC/UV–MS analyses. An attempt has also been made to correlate the changes in the metabolite profiles with the alterations caused by Ri T-DNA in the genomes of hairy root clones. We report, herein, development of hairy root cultures and identification of an isoflavonoid, formononetin and its glycoside, for the first time, in *P. corylifolia*.

2. Results

2.1. Establishment and growth of hairy root clones

Hairy roots were observed after 12–15 days of infection by *Agrobacterium rhizogenes* from the cut ends of the hypocotyls. By contrast, control explants did not show any root formation even after four weeks. The hairy roots were allowed to grow to a length of about 3–4 cm, and then were cut from the explants and cultured on Murashige and Skoog's basal (MSB) medium supplemented with cefotaxime (250 mg/l). Each root originating from different pieces of the same hypocotyl of a seedling were selected and were labeled as individual clones. The two selected clones were morphologically similar in appearance, growth and lateral branching pattern. The hairy roots were thick-robust, creamish in colour and non-hairy with a large network of lateral branching (Fig. 1). Growth profiles of these roots

Fig. 1. Hairy root cultures of *Psoralea corylifolia* L. clone Ps I after four weeks of subculture grown in dark on MSB medium with 0.8% agar.

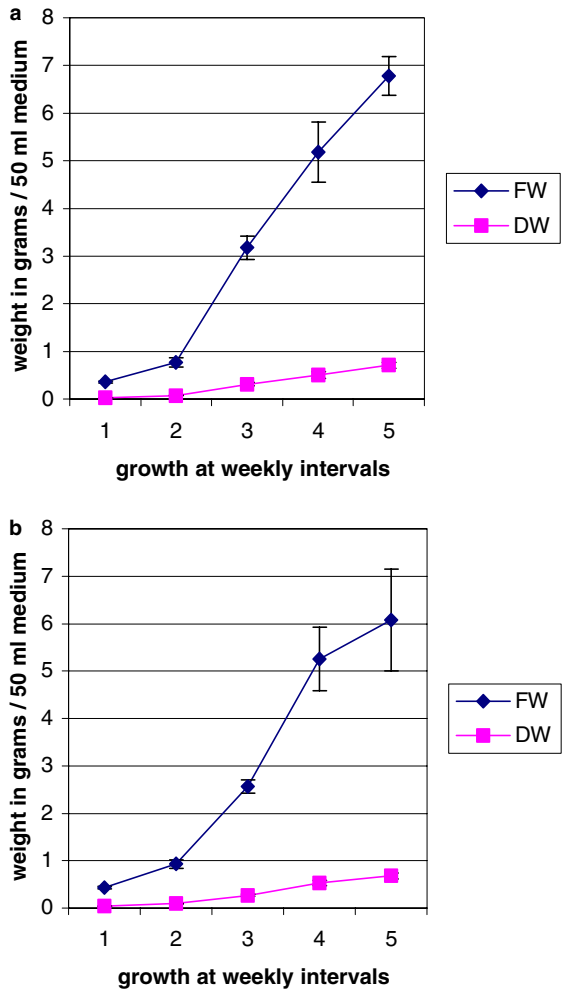

Fig. 2. Growth profiles of *Psoralea corylifolia* hairy root clones Ps I (a) and Ps II (b) for five weeks at weekly interval on MSB medium with 0.8% agar. FW, fresh weight; DW, dry weight; each point represents the mean of four replicates. Vertical bars represent standard error.

showed a typical sigmoid growth curve (Fig. 2(a) and (b)). The doubling-time of both the clones was found to be 5–7 days. As roots grew, they produced large number of laterals and secondary laterals all along the main root, and became indistinguishable.

2.2. AFLP analysis

AFLPs were carried out using five combinations of *Eco*RI and *Mse*I primers with three selective nucleotides. Each combination yielded a different pattern of amplified fragments (Fig. 3 and Table 1). AFLP analysis of the DNA from untransformed control, clones Ps I and Ps II using the primers E-AGG/M-CTC, gave a total of 28, 35 and 38 amplified fragments, respectively; out of these 17 amplified fragments were found to be common. In addition to these bands, clone Ps I exhibited three more common bands (737, 285 and 177 bp) and clone Ps II showed one common band (851 bp) with that of control. Both the clones also exhibited five plasmid-specific bands (715, 351, 279, 163 and 152 bp), which were absent in the control. Between the two clones, clone Ps I exhibited two more bands (159 and 130 bp) that were present in the plasmid. Clones Ps I and Ps II exhibited six common bands (534, 451, 217, 173, 171 and 168 bp) which were not observed in the control. Further, clones Ps I and Ps II also exhibited two (925 and 408 bp) and nine (1006, 984, 818, 767, 624, 422, 369, 319 and 240 bp) clone-specific bands, respectively; on the other hand, seven specific amplified bands (1016, 718, 636, 275, 183, 155 and 105 bp) were observed in the control.

The primers E-ACC/M-CTG exhibited a total of 13, 26 and 24 amplified fragments in untransformed control, clones Ps I and Ps II, respectively (Fig. 3 and Table 1). Eleven bands were found common in the control and the clones. In addition to these, clone Ps I showed one more common band (761 bp) with that of the control. However, both the clones exhibited three plasmid- specific bands (314, 247 and 147 bp) that were absent in the control. Besides these, Ps I and Ps II exhibited two (649 and 190 bp) and one (525 bp) additional bands which were present in the plasmid. The control displayed one specific band (300 bp) that was absent in both the clones. Whereas the clones exhibited five specific bands (723, 553, 235, 231 and 208 bp) which were absent in the control. In addition to these, clones Ps I (712, 606, 222 and 213 bp) and Ps II (728, 703, 564 and 202 bp) exhibited four clone-specific bands.

The primers E-AAC/M-CTC exhibited a total of 48, 47 and 54 amplified fragments in untransformed control, clones Ps I and Ps II, respectively; out of which 40 amplified bands were found common (Fig. 3 and Table 1). Additionally, clones Ps I and Ps II exhibited one (247 bp) and three (699, 461 and 278 bp) common bands with that of control. Both the clones showed two plasmid-specific bands (687 and 647 bp) that were absent in the control. Also, clones Ps I (185 bp) and Ps II (286 bp) exhibited one plasmid-specific band. The clones showed three common bands (444, 356 and 102 bp) which were absent in the control. In addition to these, clone Ps II exhibited five clone-specific (561, 549, 428, 383 and 258 bp) bands. Whereas, control displayed four specific bands (682, 588, 348 and 252 bp) that were absent in both the clones.

Analysis of untransformed control and clone Ps I with the primers E-ACC/M-CTA gave a total of 45 amplified fragments each, while clone Ps II showed 50 amplified fragments; out of these 36 amplified bands were found common (Fig. 3 and Table 1). Furthermore, clone Ps I exhibited one (802 bp) band and clone Ps II showed six (788, 647, 642, 337, 302 and 238 bp) bands that were similar to the control. Also, clones Ps I and Ps II showed five specific bands (670, 662, 482, 441

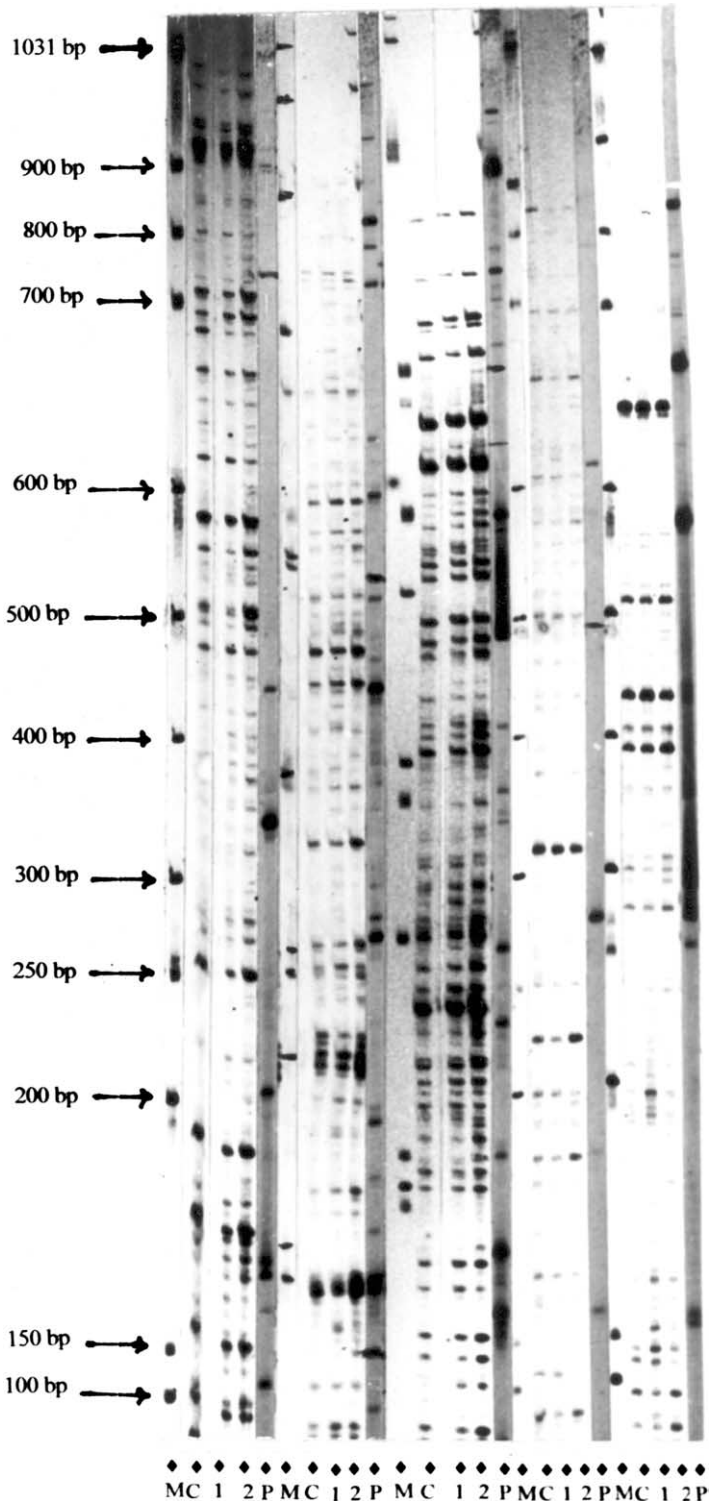

Fig. 3. AFLP profiles of *Psoralea corylifolia hairy root* clones Ps I , Ps II, untransformed control and the plasmid. 50 bp ladder was run along with the samples. In each combination lanes represent M – marker, C – control, 1 – clone Ps I, 2 – clone Ps II and P – plasmid. Combinations (from left to right): E-AGG/M-CTC; E-ACC/MCTG; E-AAC/M-CTC; E-ACC/M-CTA and E-ACG/M-CAA.

and 286 bp) which were absent in the control. Clone Ps II alone exhibited one (486 bp) plasmid-specific band. On the other hand, both Ps I and Ps II showed three (614, 377 and 178 bp) and two (594 and 169 bp) clone-specific bands, respectively, while control showed two (746 and 263 bp) specific bands.

The primers E-ACG/M-CAA exhibited a total of 34, 42 and 45 amplified fragments in untransformed control,

Table 1
AFLP profiles of hairy root clones Ps I, Ps II along with untransformed control and plasmid

E-AGG/M-CTC				E-ACC/M-CTG				E-AAC/M-CTC				E-ACC/M-CTA				E-ACG/M-CAA			
con	Ps I	Ps II	P	con	Ps I	Ps II	P	con	Ps I	Ps II	P	con	Ps I	Ps II	P	con	Ps I	Ps II	P
1016							781				778	977	977	977					1442
		1006					768	766	766	766	766	848	848	848		959	959	959	
		984		761	761			740	740	740		802	802			852	852	852	
944	944	944		734	734	734					733	788		788			839	839	
	925					728		721	721	721					752		833	833	
920	920	920			723	723					715	746				795	795	795	
851		851					718				711	738	738	738		698	698	698	
		818			712			706	706	706	706	695	695	695		693			
806	806	806				703		699		699		687	687	687				**687**	**687**
			781				662		**687**	**687**	**687**		670	670		682	682	682	
		767			**649**		**649**	682					662	662					673
737	737						628	662	662	662	662	657	657	657					657
			735		606				**647**	**647**	**647**	647		647					651
718				592	592	592					640	642		642		642	642	642	
	715	**715**	**715**			564					633		614					638	
			709		553	553		631	631	631	631	605	605	605		630	630	630	
694	694	694				**525**	**525**	612	612	612				594					629
684	684	684									609	582	582	582					625
663	663	663		516	516	516		600	600	600		571	571	571		622			
646	646	646		504	504	504		588				555	555	555				**603**	**603**
636							481	582	582	582		540	540	540		598	598	598	
		624		428	428	428				561					522				597
617	617	617					423	554	554	554		519	519	519		576	576	576	
580	580	580					411			549		501	501	501		561	561	561	
553	553	553					354				537			**486**	**486**	552	552	552	
540	540	540					327	534	534	534			482	482					537
	534	534			**314**	**314**	**314**	517	517	517		460	460	460		521	521	521	
508	508	508		300							485		441	441		509	509	509	
496	496	496					293	482	482	482		431	431	431					503
476	476	476					288				480	422	422	422					485
	451	451					282				474	399	399	399		484	484	484	
		422					275	469	469	469		389	389	389					478
	408						256	461		461			377			429	429	429	
388	388	388							444	444		371	371	371		405	405	405	
		369			**247**	**247**	**247**	435	435	435		353	353	353		388	388	388	
	351	**351**	**351**	240	240	240	240			428		337		337				373	
		319			235	235		421	421	421		331	331	331		357	357	357	
285	285				231	231		414	414	414		317	317	317					354
	279	**279**	**279**	226	226	226					410	302		302					345
			277		222			405	405	405		295	295	295			**335**	**335**	**335**
275				219	219	219		397	397	397			286	286		308	308	308	
255	255	255			213					383		280	280	280		300	300	300	
		240			208	208			356	356		263				292		292	
	217	217				202		348							255				286
			202		**190**		**190**				316	252	252	252		275	275	275	
191	191	191					187	298	298	298		238		238					263
183							174	288	288	288		225	225	225					259
177	177						163			**286**	**286**	200	200	200					255
	173	173					153	285	285	285		198	198	198					248
	171	171					151				281	194	194	194		234	234	234	
	168	168			**147**	**147**	**147**	278		278	278	186	186	186		214	214	214	
	163	**163**	**163**				143	275	275	275		181	181	181		211	211	211	
	159		**159**				140	267	267	267			178			208		208	
155				130	130	130		262	262	262		170	170	170		205	205		
	152	**152**	**152**				112			258				169			202		
	130		**130**	105	105	105		256	256	256		162	162	162		197	197	197	
105				100	100	100		252				158	158	158			194		
								250	250	250		155	155	155	155		192		
								247	247			115	115	115		191	191	191	
											246					179	179	179	
								242	242	242							174		

(*continued on next page*)

Table 1 (*continued*)

E-AGG/M-CTC				E-ACC/M-CTG				E-AAC/M-CTC				E-ACC/M-CTA				E-ACG/M-CAA			
con	Ps I	Ps II	P	con	Ps I	Ps II	P	con	Ps I	Ps II	P	con	Ps I	Ps II	P	con	Ps I	Ps II	P
								237	237	237								171	
								232	232	232								169	
								226	226	226							167	167	
											222						160	160	
								220	220	220							157	157	
								219	219	219							155	155	
								215	215	215							**151**	**151**	**151**
								213	213	213									150
								210	210	210						134	134	134	
								207	207	207						119	119	119	
								203	203	203									
									185		**185**								
								169	169	169									
								142	142	142									
											111								
									102	102									

The figures denote molecular sizes in base pairs. Each combination shows columns as: con – control; Ps 1 – clone Ps I; Ps II – clone Ps II and P – plasmid. Figures in bold denote matching bands of clones (either one or both) with that of the plasmid.

clones Ps I and Ps II, respectively (Fig. 3 and Table 1). Out of these, 29 bands were found common in control as well as the clones. In addition, clones Ps I and Ps II also showed one (205 bp) and two (292 and 208 bp) common bands with that of control. Both the clones exhibited two (335 and 151 bp) plasmid-specific bands that were absent in the control; while clone Ps II showed two additional (687 and 603 bp) plasmid-specific bands. Both the clones exhibited six common bands (839, 833, 167, 160, 157 and 155 bp) which were absent in the control. Control showed two specific bands (693 and 622 bp) which were not found in the clones. Furthermore, clones Ps I (202, 194, 192 and 174 bp) and Ps II (638, 373, 171 and 169 bp) exhibited four clone-specific bands.

2.3. Metabolite profiling by GC/MS

Solvent extracts of Ps I and Ps II along with untransformed control roots, leaves and seeds were subjected to qualitative analyses by GC/MS. The GC/MS total ion chromatograms of methanol extracts of Ps I, Ps II and untransformed controls are depicted in Fig. 4. The various compounds identified in hexane and methanol extracts of Ps I and Ps II and that of controls are presented in Table 2. A total of 28, 33 and 17 compounds were identified in the untransformed control roots, leaves and seeds, respectively; while clones Ps I and Ps II exhibited 25 compounds each. Out of a total of 27 hydrocarbons identified, three were found common in controls and hairy root clones. Eight hydrocarbons were specific to untransformed control roots, two were specific to control leaves and Ps I, while four were specific to Ps II. All the three fatty acids were observed in controls as well as in the clones. A furanocoumarin, psoralene, was present only in the controls while it was absent in the hairy root clones. An isoflavonoid, daidzein, was found in control roots as well as in hairy root clones, while it was absent in control leaves and seeds. An alkene, neophytadiene, was observed in control leaves and clone Ps I, and it was missing in control roots, seeds and clone Ps II.

2.4. Isoflavonoid identification

The methanol extracts of control and clones were analyzed using HPLC/UV–MS. The HPLC/UV profiles of control and clones are illustrated in Fig. 5, and the mass spectra (MS) are depicted in Fig. 6. In control, the MS data showed the presence of mainly two isoflavonoids and their glycosides, viz., daidzein (peak 4), its glycoside – daidzin (peak 1), genistein and its glycoside – genistin (peak 2). Further, the HPLC/UV analysis showed the presence of three minor unknown compounds with peaks at RT 6.63, 16.65 and 17.70 min (Fig. 5(a)). Whereas, clones Ps I and Ps II showed the presence of three isoflavonoids and their respective glycosides, viz., daidzein (peak 4), genistein (peak 7) , formononetin (peak 5), daidzin (peak 1), genistin (peak 2) and formononetin glycoside (peak 3) (Fig. 5(b) and (c)). Both the clones showed the presence of formononetin and its glycoside which were absent in the control. In Ps I, daidzein and daidzin together showed about 160% increase in the relative peak area (RPA), while 58% increase was observed in Ps II, when compared to the control (Figs. 7 and 8). Control showed a 2-fold increase in the production of daidzin than daidzein, whereas, Ps I and Ps II showed about 17- and 5-fold increases, respectively, in the production of daidzin compared to daidzein. Likewise, clone Ps I showed 78% increase in genistin production when compared to the control;

whereas, clone Ps II showed lesser genistin production than that of control. Two isoflavonoids, viz., formononetin and its glycoside were produced in both the hairy root clones which were absent in the control. Moreover, the clones Ps I and Ps II showed 9-fold and 3-fold increases, respectively, in the production of formononetin glycoside compared to aglycon formononetin (Fig. 8).

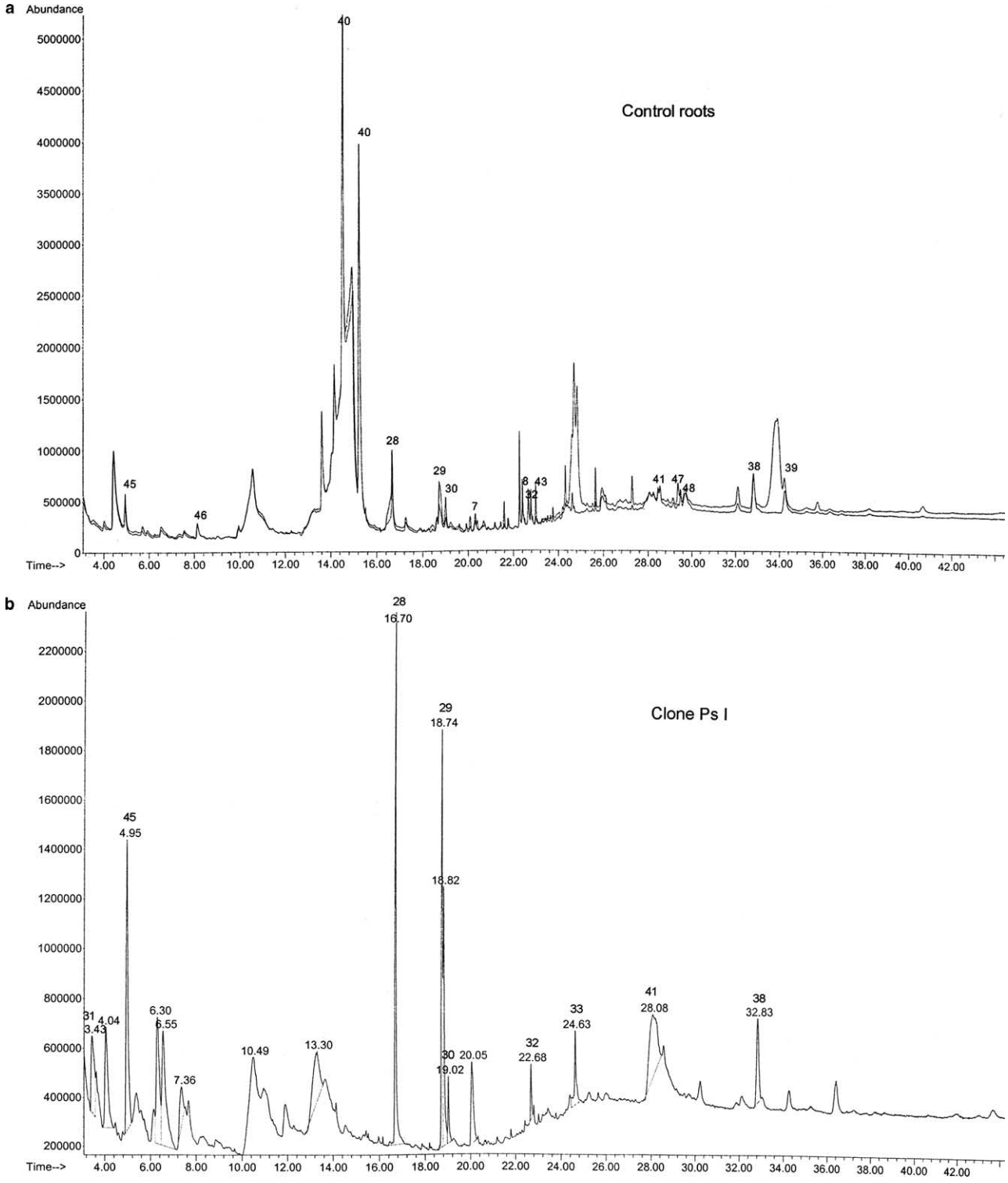

Fig. 4. GC chromatograms of methanol extracts of *Psoralea corylifolia* untransformed control roots (a), leaves (d) and seeds (e) along with transformed hairy root clones Ps I (b) and Ps II (c) after five weeks of culture. The peaks numbered are shown in Table 2 by an asterisk. The peaks are numbered as they appear in Table 2.

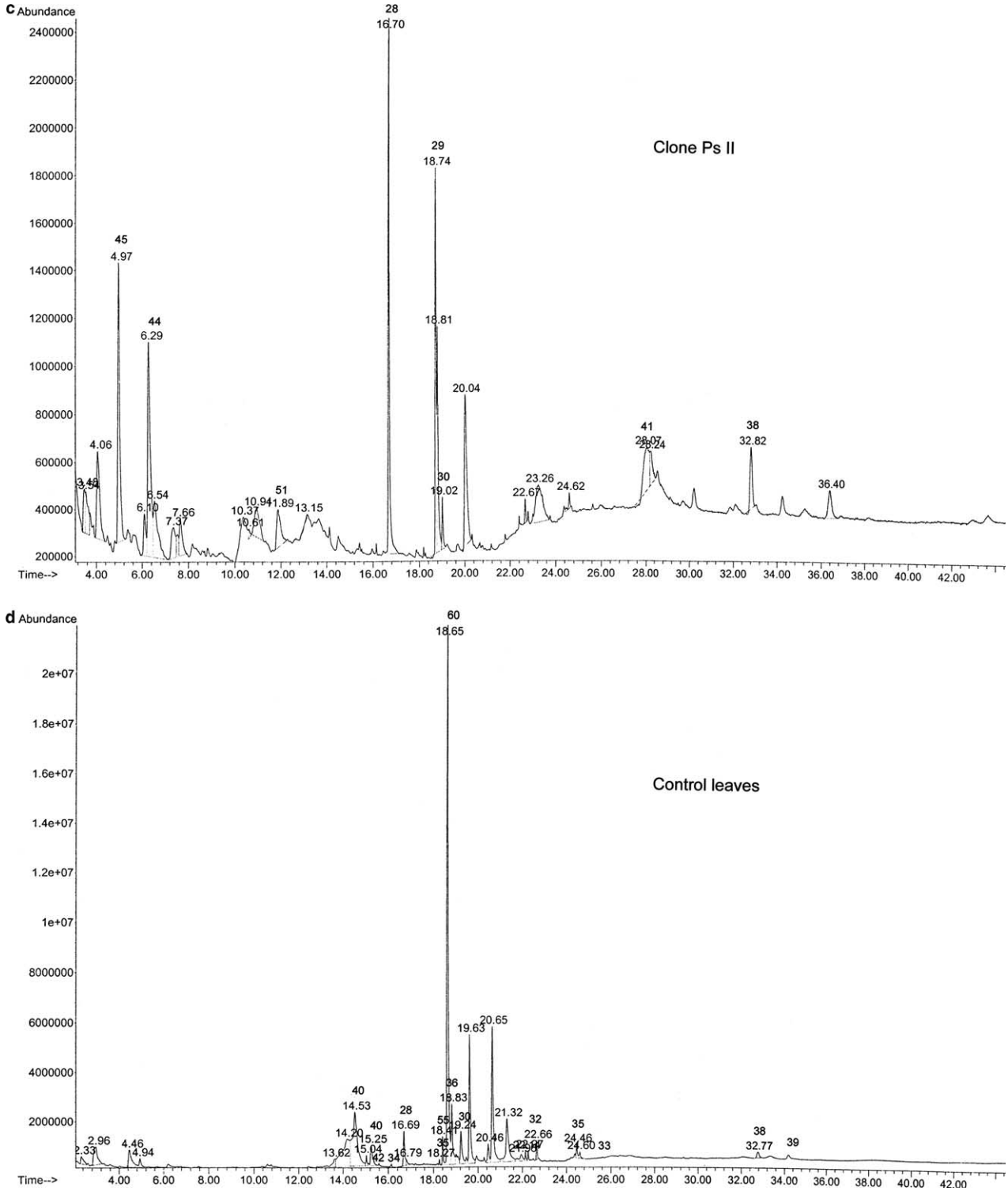

Fig. 4 (*continued*)

3. Discussion

Hairy roots induced by Ri T-DNA of *A. rhizogenes* are widely used for production of important pharmaceuticals from diverse medicinal plants (Babaoglu et al., 2004; Lodhi and Charlwood, 1996). In *P. corylifolia*, using hypocotyl explants, we have developed an efficient transformation protocol employing *A. rhizogenes* strain ATCC 15834. Among the different explants of carrot and *Nicotiana tabacum* tested for hairy root induction, only the calli of carrot tubers and stem segments of *N. tabacum* yielded hairy roots (Bercetche et al., 1987). In

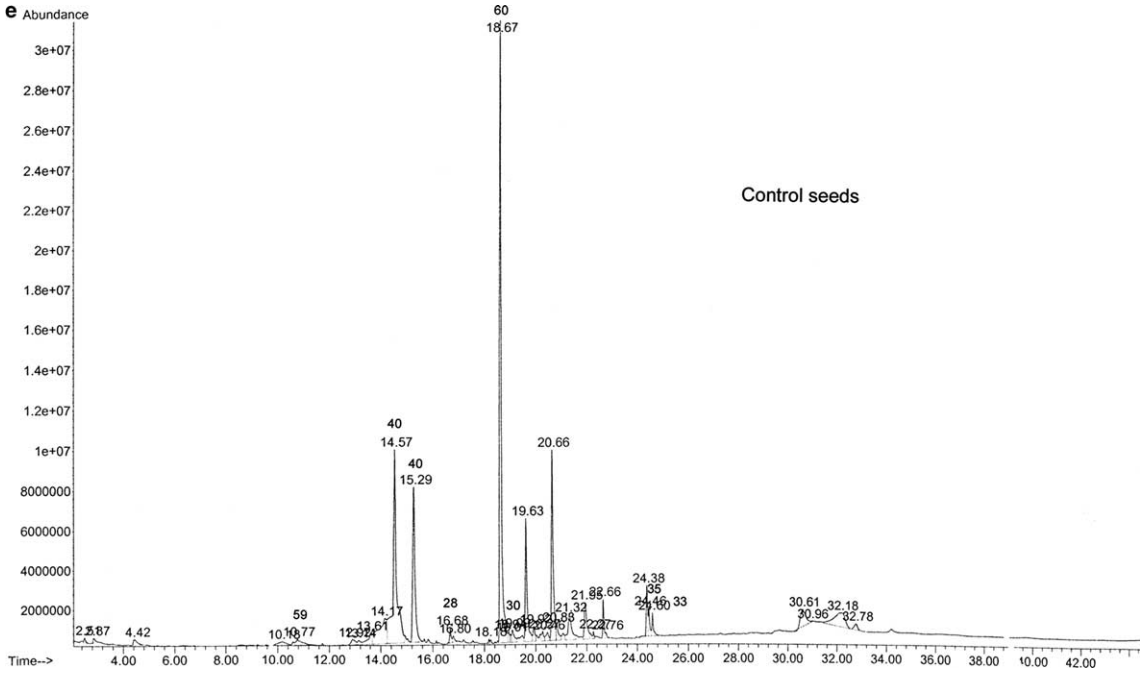

Fig. 4 (*continued*)

the present study, hypocotyl explants alone showed hairy root induction, while other seedling explants failed to produce any hairy roots, thus suggesting the importance of explant source in the induction of hairy root cultures. In *Peuraria phaseoloides* a similar explant specificity was observed in the induction of hairy root cultures (Shi and Kintzios, 2003). The profuse development of primary and secondary lateral roots along the main roots, observed herein, was distinctly different from the growth patterns observed in other *Psoralea* species which produced only a few secondary laterals (Bourgaud et al., 1999). In this investigation, the doubling-time of hairy roots was found to be 5–7 days and the hairy roots showed sigmoid growth pattern. The doubling-time of hairy roots observed was within the range of transformed roots obtained from several other plant species (Wilson et al., 1987). However, in other *Psoralea* species the doubling-time of hairy roots was observed to be less than two days (Nguyen et al., 1992). These differences may be attributed to the genotypic differences among different *Psoralea* species besides the type of plasmids harbored by the bacterial strains. In this investigation, no decline in growth of hairy roots was recorded even after five weeks of subculture. Whereas, the hairy root clones of *P. lachnostachys* showed deceleration of growth after 18 days of culture (Nguyen et al., 1992). An approximate 45–50 fold increase in fresh and dry weights of hairy roots was observed after five weeks of culture. These hairy roots were maintained for about 18 months without any callus formation or deceleration of growth. An overview of metabolite profiles, isoflavonoid analyses, AFLPs and growth profiles of hairy roots amply indicated the stability of the hairy root clones of *P. corylifolia*. These observations are comparable to hairy root clones obtained in several other plant species (Baiza et al., 1999).

A key feature of AFLP is its capacity for the simultaneous screening of different DNA regions distributed randomly throughout the genome. Further, AFLP markers are highly repeatable and are virtually free from artifacts (Mueller and Wolfenbarger, 1999). In five combinations of AFLP amplifications, a total of 168 bands were observed in the control, of which 140 and 145 bands were present in the clones Ps I and Ps II, respectively. This observation indicates that the major portion of the genomes of Ps I and Ps II remained unaltered. The absence of 28 bands in Ps I and 23 bands in Ps II (Fig. 3 and Table 1), as compared to control, is attributed to the integration of Ri T-DNA. Further, the 16 bands specific to control were missing in both the clones, suggesting that Ri T-DNA might have integrated in these regions and hence might be responsible for the absence of these bands. Twelve bands missing in Ps I and seven bands missing in Ps II, clearly reveal the clone independent integration of Ri T-DNA in the genomes of these two clones. Seventeen bands corresponding to Ri T-DNA were observed in clones Ps I and Ps II; out of these, twelve bands were found common to both the clones. Moreover, Ps I and Ps II each showed five different plasmid specific bands. These observations amply affirm that different regions of Ri T-DNA have stably integrated into the genomes of both the clones. Studies carried out in tobacco also showed variable number of Ri T-DNA integrations in different hairy

Table 2
GC/MS profiles of *Psoralea corylifolia* hairy root clones Ps I , Ps II and untransformed control roots, leaves and seeds

Sl No.*	Compound**	Control roots		Control leaves		Control seeds		Clone Ps I		Clone Ps II	
		H	M	H	M	H	M	H	M	H	M
	Hydrocarbons										
1	3-methyl hexane	+	–	–	–	–	–	–	–	–	–
2	Heptane	+	–	–	–	–	–	–	–	–	–
3	Pentadecane	+	–	–	–	–	–	–	–	–	–
4	Octadecane	+	–	–	–	–	–	–	–	–	–
5	Tetradecane	+	–	–	–	–	–	–	–	–	–
6	Eicosane	+	–	–	–	–	–	–	–	–	–
7*	5-Eicosene	–	+	–	–	–	–	–	–	–	–
8*	3-Eicosene	–	+	–	–	–	–	–	–	–	–
9	N-docosane	+	–	+	–	–	–	+	–	+	–
10	Pentacosane	+	–	+	–	+	–	+	–	+	–
11	Hexacosane	+	–	+	–	+	–	+	–	+	–
12	2,6,10,14,18,22-Tetracosahexane	+	–	+	–	–	–	+	–	+	–
13	Tetracosane	+	–	+	–	–	–	+	–	+	–
14	Nonacosane	+	–	+	–	+	–	+	–	+	–
15	Tricosane	+	–	+	–	–	–	+	–	–	–
16	Triacontane	–	–	+	–	–	–	+	–	+	–
17	Heptacosane	–	–	+	–	+	–	+	–	+	–
18	Octacosane	–	–	+	–	+	–	+	–	+	–
19	1-Nonadecene	–	–	–	–	–	–	+	–	–	–
20	Hentriacontane	–	–	–	–	–	–	+	–	–	–
21	Nonadecane	–	–	–	–	–	–	–	–	+	–
22	*n*-Heptadecylcyclohexane	–	–	–	–	–	–	–	–	+	–
23	Cyclohexane eicosyl-	–	–	–	–	–	–	–	–	+	–
24	Cyclopentane heneicosyl-	–	–	–	–	–	–	–	–	+	–
25	2-Pentadecanone, 6,10,14-trimet	–	–	+	–	–	–	–	–	–	–
26	Heptadecane	–	–	+	–	+	–	–	–	–	–
27	1-Eicosanol	–	–	+	–	–	–	–	–	–	–
	Fatty acids										
28*	Palmitic acid	+	+	+	+	–	+	+	+	+	+
29*	Linoleic acid	+	+	+	+	+	+	+	+	+	+
30*	Stearic acid	+	+	+	+	–	+	–	+	+	+
	Fatty acid esters										
31*	Monomethyl malonate	–	–	–	–	–	–	–	+	–	–
32*	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	–	+	–	+	–	–	+	+	–	–
33*	Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	–	–	+	+	–	+	+	+	+	–
34*	Hexadecanoic acid, methyl ester	–	–	+	+	–	–	–	–	–	–
35*	9,12,15-octadecatreinoic acid,...	–	–	+	+	–	+	–	–	–	–
36*	9,12,15-octadecatrienal	–	–	–	+	–	–	–	–	–	–
37	9-Octadecenoic acid (z)-, 2-hyd...	–	–	–	–	+	–	–	–	–	–
	Steroids										
38*	Stigmasta-5,22-dien-3-ol	–	+	+	+	+	–	+	+	+	+
39*	Stigmasta-5-en-3-ol	–	+	+	+	–	–	+	–	+	–
	Furnaocoumarin										
40*	Psoralene	+	+	+	+	+	+	–	–	–	–
	Isoflavonoids										
41*	Daidzein	–	+	–	–	–	–	–	+	–	+
	Alkene										
42*	Neophytadiene	–	–	+	+	–	–	+	–	–	–
	Aldehydes										
43*	14-Octadecenal	–	+	–	–	–	–	–	–	–	–
44*	2-Furancarboxaldehyde,5-(hydroxymethyl)	–	–	–	–	–	–	–	–	–	+

Table 2 (*continued*)

Sl No.*	Compound**	Control roots		Control leaves		Control seeds		Clone Ps I		Clone Ps II	
		H	M	H	M	H	M	H	M	H	M
	Phenolic and other compounds										
45*	2,3-Dihydro-3,5 dihydroxy-6-methyl-4H-pyran-4-one	−	+	−	−	−	−	−	+	−	+
46*	2,6-Dimethoxyphenol	−	+	−	−	−	−	−	−	−	−
47*	2,8-Diisopropyl-peri-xanthenoxanthene-4,10-quinone	−	+	−	−	−	−	−	−	−	−
48*	5β-Androstan-3α-diol-11,16-dione	−	+	−	−	−	−	−	−	−	−
49	2,4-Ditert butyl phenol	−	−	+	−	−	−	+	−	+	−
50	1,2-Benzenedicarboxylic acid	−	−	−	−	+	−	+	−	+	−
51*	4-Hydroxy-3-methoxybenzoicacid	−	−	−	−	−	−	−	−	−	+
52	2(4H)-Benzofuranone, 5,6,7,7a-t	−	−	+	−	−	−	−	−	−	−
53	Caryophyllene oxide	−	−	+	−	+	−	−	−	−	−
54	Myristic acid	−	−	+	−	−	−	−	−	−	−
55*	Phytol	−	−	+	+	−	−	−	−	−	−
56	6β-Hydroxy-1,10-dehydrofur	−	−	+	−	−	−	−	−	−	−
57	2-Monolinolenin	−	−	+	−	−	−	−	−	−	−
58	4H-pyran-4-one, 2,3-dihydro-3,5.	−	−	−	+	−	−	−	−	−	−
59*	Benzoic acid, 4-ethoxy-, ethyl...	−	−	−	−	−	+	−	−	−	−
60*	Bakuchiol	−	−	−	+	−	+	−	−	−	−

+, compound present; −, compound absent; H, hexane extract; M, methanol extract.

* Peaks numbered in Fig. 4.

** Compounds identified by library matching (>95%).

root clones (Tempe and Casse-Delbart, 1989). In each combination of selective nucleotides used for AFLP amplifications, one to seven bands of varied size, similar to that of plasmid profile, were observed in the hairy root clones (Fig. 3 and Table 1). These observations amply confirm the multiple integrations of Ri T-DNA into the genomes of hairy root clones. Furthermore, observation of several clone-specific bands in Ps I (13 bands) and Ps II (24 bands) validate the occurrence of independent transformation events in the clones. Petit et al. (1986) also showed multiple transformations and random integrations of Ri T-DNA in carrot and *Lotus corniculatus*. Recently, Batra et al. (2004) reported random and multiple integrations of Ri T-DNA regions into the genome of *Catharanthus roseus*.

Metabolome reflects more closely activities of the cells at the functional level. In the present investigation, the metabolite profiling of *P. corylifolia* hairy root clones was carried out to observe the various compounds formed in them and also to know the qualitative/quantitative differences between the clones in comparison to the control. GC/MS is the current popular method used for metabolite profiling of the plants. GC/MS analyses revealed the presence of a furanocoumarin, psoralene, and eight hydrocarbons in the untransformed control, which were absent in the clones. Total absence of psoralene in the hairy root clones is in agreement with the earlier report using different *Psoralea* species (Bourgaud et al., 1999). The presence of daidzein in the hairy root clones as well as in the untransformed control roots and its absence in the leaves and seeds, implicates that it is primarily synthesized in roots (Bourgaud et al., 1995). In this study, HPLC profiles displayed the presence of three isoflavonoids – daidzein, genistein, formononetin – and their respective glycosides. Failure to detect some of these compounds by GC/MS may be attributed to non-derivatisation of the compounds containing hydroxyl groups (Goodacre et al., 2004). Isoflavone glycosides were produced at increased levels than their respective aglycons in both the clones and untransformed control (Fig. 7). Isoflavones usually exist in conjugated forms like glucosides and malonyl glucosides, while free aglycons are rarely found in legumes. The enhanced levels of isoflavone glycosides produced in the hairy root clones are plausibly due to increased specific enzyme activities and/or increase in the precursor pool. The presence of higher levels of daidzein and daidzin, as compared to genistein and genistin, in the hairy root clones clearly suggest that these clones may be exploited for the production of daidzein and daidzin. Presently, isoflavones extracted mainly from soybean are being used in cancer therapy (Adlercreutz et al., 1991; Magee et al., 2004). However, soybean contains genistein as the most abundant isoflavone and daidzein is present in much lower quantities (Jung et al., 2003). Earlier studies indicated that daidzein could not induce any chromosomal damage in vitro even when present in high concentrations (Kulling et al., 2001); whereas genistein was found to induce genotoxic and mutagenic effects (Kulling et al., 2002). In view of this, the hairy roots of *P. corylifolia* might serve as a superior source of desirable isoflavones. The levels of isoflavones in naturally growing plants, in general, are quite variable and are affected very much by environmental conditions. This problem can be overcome by hairy root cultures grown under controlled conditions, as they are genetically and biochemically

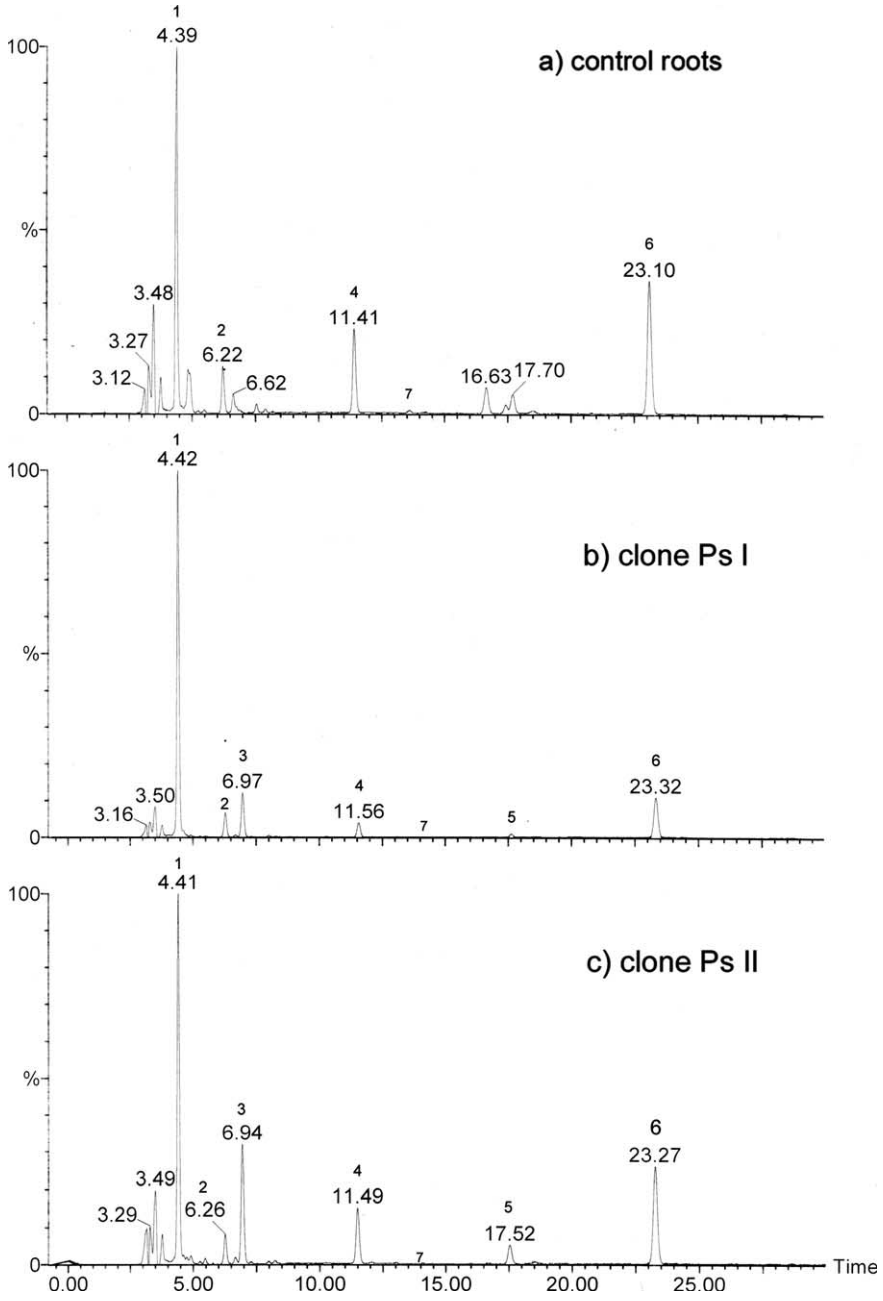

Fig. 5. HPLC chromatograms of *Psoralea corylifolia* control (a) and transformed hairy root clones Ps I (b) and Ps II (c) after five weeks of culture. The peaks numbered consecutively are: (1) daidzin, (2) genistin, (3) formononetin glycoside, (4) daidzein, (5) formononetin, (6) fluorescein (IS) and (7) genistein.

stable. Our results established that the total isoflavonoid levels are substantially higher in hairy root clones as compared to the control. The clone Ps I was found superior in the production of total isoflavonoids with 184% increase, while Ps II showed 94% increase when compared to the control (Fig. 7). Investigations in animal cells showed that isoflavonoid aglycons were readily metabolized into toxic compounds unlike glycosylated isoflavonoids (Schmitt et al., 2003). Hairy root clones produced higher levels of glycosylated isoflavonoids than their respective aglycons. Ps I showed a 17-fold increase in the production of isoflavonoid glycoside, daidzin, than its aglycon form, daidzein (Fig. 8). Daidzein is the major isoflavonoid found in *Psoralea* species and hairy root cultures of some *Psoralea* species produced upto 0.6% daidzein in the dry matter (Bourgaud et al., 1999). Whereas, our results revealed that, the clone Ps I produces >1.0 g/100 g dry weight (DW) of daidzin. The increased production of isoflavonoid glycoside, daidzin, is preferred over aglycon, daidzein, since daidzin reduces the risk of formation of toxic compounds by its further metabolism (Schmitt et al., 2003).

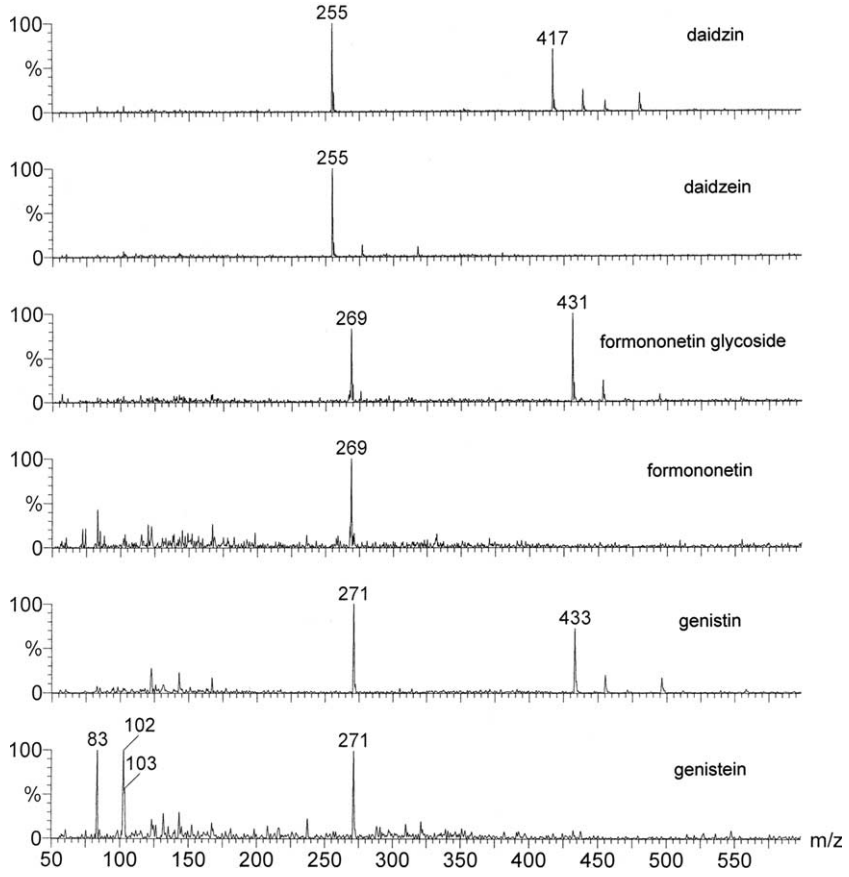

Fig. 6. Mass spectra of isoflavonoids identified in HPLC.

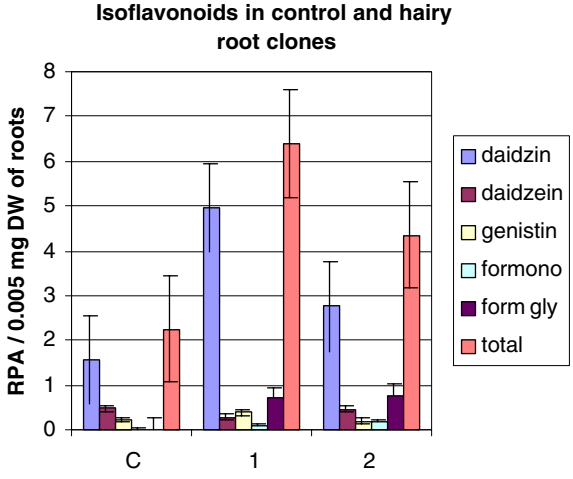

Fig. 7. Isoflavonoid production in control (C) and transformed hairy root clones Ps I (1) and Ps II (2) grown on MSB medium after five weeks of culture. The values are calculated in relation to the internal standard by the formula: relative peak area for sample a = peak area of the sample a/peak area of the IS, for each bar. Vertical bars represent standard error.

In this study, GC/MS revealed striking differences in the profiles of compounds formed in the hairy root clones Ps I and Ps II and the controls. Certain compounds present in the untransformed control were absent in the clones, while, compounds such as triacontane, heptacosane, octacosane, a fatty acid ester, neophytadiene and some phenol based compounds which were absent in control roots were present in the hairy root clones. Further, the clones showed distinct clone-specific compound profiles, proving that the clones are different in their secondary metabolism. The variation observed in the profiles may be attributed to differences in the Ri T-DNA copy number and position of integration in the genomes of hairy root clones. In *C. roseus*, independent hairy root clones accumulated variable amounts of ajmalicine and serpentine caused by random and multiple integrations of T-DNA; also, they reported that these differences in alkaloid accumulation might be due to differences in the integration sites of the T-DNA (Batra et al., 2004). Similar variations in the metabolite profiles of transformed clones were also reported in potato (Roessner et al., 2001) and tobacco (Choi et al., 2004).

In the transformed roots of *P. corylifolia* substantial alterations were observed in the secondary metabolite profiles with regard to the quantity and formation of new compounds. Both the hairy root clones showed the production of formononetin and its glycoside, which

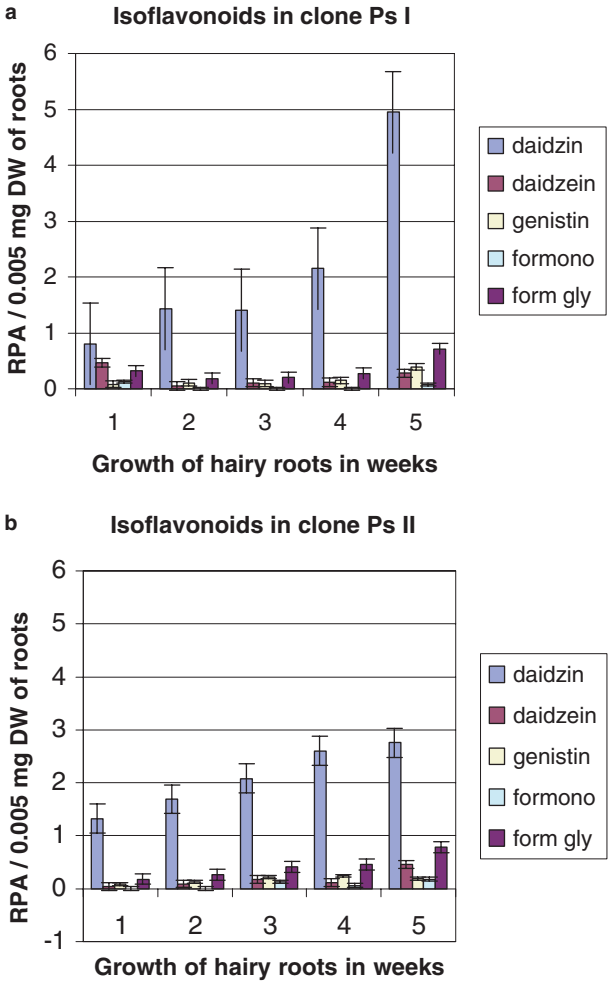

Fig. 8. Isoflavonoid production in hairy root clones of *Psoralea corylifolia* for five weeks at weekly intervals on MSB medium with 8 g/l agar. The values are calculated in relation to the internal standard by the formula: relative peak area for sample a = peak area of the sample a/peak area of the IS, for each bar. Vertical bars represent standard error.

were absent in the untransformed control. This may be attributed to the formation of isoflavone by dehydration of 2-hydroxy isoflavonone without methylation (Liu and Dixon, 2001) or the activation of isoflavone *O*-methyl transferase (IOMT) encoding genes. In plants, formononetin synthesis was attributed to the activity of enzyme IOMT (Liu and Dixon, 2001). The overall GC/MS profiles revealed that twelve compounds were specifically produced in the control roots. Absence of these compounds in hairy root clones may be attributed to the disruptions caused in the genomic DNA by Ri T-DNA integrations, as evidenced by the altered AFLP profiles. On the other hand, various clone-specific compounds, which were absent in the control roots, might have been synthesized by the expression of Ri T-DNA, resulting in altered metabolism. These observations further support the viewpoint that regulation of secondary metabolite pathways might be different in the transformed and normal roots (Batra et al., 2004).

In this study, an efficient protocol has been optimized for the production of hairy root cultures from the hypocotyl explants of *P. corylifolia* using *A. rhizogenes* strain ATCC 15834. AFLP analysis revealed diverse alterations in the genomes of hairy root clones, signifying random and clone-specific Ri T-DNA integrations. Clone-specific compound profiles, obtained through GC/MS and HPLC/UV–MS analyses, indicate that the clones are distinct and dissimilar in their secondary metabolite production. Further, HPLC/UV-MS analyses disclosed the production of two additional compounds, viz., formononetin and its glycoside, in the hairy root clones. Comparison between the clones revealed that Ps I is superior to Ps II in the production of total isoflavonoids.

4. Experimental

4.1. Plant material

Homozygous seeds of *P. corylifolia* were procured from Dr. Nutan Malpathak, Botany Department, Pune University, India. The material was sown in the nethouse and selfed seeds were collected. Progeny coming from single plant was employed in the present investigation. The seeds were treated with concentrated sulphuric acid for 20 min followed by thorough washing with running tap water for 2 h to remove the acid and the pericarp. Later, these seeds were washed with 3–4 drops of a detergent, labolene (Qualigens Fine Chemicals, Mumbai, India) and rinsed thoroughly with tap water followed by rinsing with distilled water. The washed seeds were surface sterilized with 0.1% mercuric chloride solution for 4 min followed by rinsing 4–5 times with sterile distilled water. The surface sterilized seeds were germinated in bottles on agar media and incubated in continuous light at 25 ± 2 °C and 80% relative humidity with an irradiance of 100 μmol m^{-2} s^{-2} provided by cool white fluorescent tubes. Epicotyl, hypocotyl and cotyledonary explants from one week old seedlings were used for *Agrobacterium* infections. Seedlings were grown on MS medium for five weeks and leaves and roots were collected from these seedlings and mature seeds collected from greenhouse grown plants were used for GC/MS analyses as untransformed controls. For HPLC/UV–MS, only untransformed roots along with hairy root clones were employed.

4.2. Transformation and establishment of hairy root clones

Hypocotyls and epicotyls were excised from the germinated seedlings and were cut into 3–4 pieces of the size of 7.0–8.0 mm each. Cotyledons were cut from

apical and distal ends. All these explant pieces were placed on Murashige and Skoog's basal medium (Murashige and Skoog, 1962) containing 30 g/l sucrose (MSB) solidified with 8 g/l agar along with 100 mM acetosyringone (Sigma Chemical Co, USA) and incubated for 2 days at 25 ± 2 °C in light for preculture.

A. rhizogenes strain ATCC 15834 (procured from Microbial Type Culture Collection, Chandigarh, India) was used for transformation studies. Bacterial cultures were raised by placing a loopful of bacteria in 20 ml liquid BYP medium (beef extract 1.0 g/l, yeast extract 2.0 g/l, peptone 5.0 g/l, sodium chloride 5.0 g/l) for 48 h. The bacterial cultures were grown on shaker at 29 °C and 225 rpm in dark. The bacterial culture showing absorbance of 1.0–1.2 at 600 nm was used for infection experiments. Bacterial culture was centrifuged at 8000 rpm for 6 min to pellet the bacteria. The pellet was resuspended in 1.0 ml of liquid MSB medium and used for infections. Ten microliters of bacterial suspension was placed on each precultured explant and cocultivated for 48 h. After 48 h, the explants were transferred on to MSB medium containing 250 mg/l cefotaxime (HiMedia Laboratories, Mumbai, India) and 250 mg/l carbenicillin (HiMedia Laboratories, Mumbai, India).

Hairy root clones were separated based on their origin and growth pattern. The hairy roots originating from different pieces of a single seedling hypocotyl were considered as separate clones. Two hairy root clones out of five from a single seedling explant were selected for this study. These clones were maintained on MSB medium containing cefotaxime (250 mg/l) for 2 months followed by atleast nine months on MSB medium without any antibiotics in dark with routine subculturing for every four weeks. The stabilized clones were employed for further analyses.

4.3. Growth analysis

Weighed inoculum (150 mg) of hairy roots was cultured on 50 ml MSB medium with 8 g/l agar for growth analysis. Growth pattern was analysed by recording fresh and dry weights of the hairy roots for five weeks at an interval of one week. Four replicates were used for fresh and dry weight analysis.

4.4. AFLP analysis

Genomic DNA was isolated using 200 mg of root tissue by the method of Zhang and Stewart (2000). Plasmid DNA was isolated from *A. rhizogenes* strain 15834 by standard methods (Sambrook and Russell, 2001). About 300 ng of DNA from each of the clones Ps I, Ps II, untransformed control and plasmid was used for AFLP reactions. AFLP was carried out mainly using the method of Vos et al. (1995) with some modifications. Amplification reactions were carried out using *Eco*RI/*Mse*I (E/M) as the enzyme combinations. Preamplification was carried out using *Eco*RI primers with an addition of a single base (A) and *Mse*I primers with addition of one base (C) at 3′ end. PCR conditions for preamplification were: 94 °C for 20 s, 56 °C for 30 s and 72 °C for 2 min (21 cycles). For selective amplification, 10 combinations of three nucleotides were used with *Eco*RI/*Mse*I primers. Out of these, only 5 combinations were presented in this paper. These are: E-AGG/M-CTC; E-ACC/M-CTG; E-AAC/M-CTC; E-ACC/M-CTA and E-ACG/M-CAA. Radioactive labeling was done using the ^{33}Pα dATP. The PCR conditions were: 94 °C for 20 s, followed by 30 s annealing step (annealing temperature in first cycle was 65 °C, subsequently it was reduced by 1 °C in each cycle for the next 10 cycles till it reached 56 °C), followed by extension step at 72 °C for 2 min. Once the annealing temperature reached 56 °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 sizes of the samples. The gel was then dried for 1 h at 80 °C and autoradiographed for 4 days on Kodak X-ray film at −70 °C. The fingerprinting pattern was analysed using the software SynGene Gene Tools, version 3.00.22 (SynGene Laboratories).

4.5. Metabolite profiling

Metabolite profiles were analysed by GC/MS method for hairy root clones (Ps I and Ps II) and also for untransformed roots, leaves and seeds as control. All the solvents used were of analytical grade. For extraction, hairy roots and control roots, leaves and seeds were dried at room temperature for 2–3 days, then kept at 50 °C for 2 h. The dried roots, leaves and seeds 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 Whatmann 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 Whatmann 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 5937 N mass selective detector (MSD).

GC conditions. Column DB-1701 (30 M, 0.32 mm ID and 0.25 μM film thickness), injection temperature 250 °C; interface temperature 280 °C; oven temperature was programmed from 40 °C, hold for 2 min, then increased by 4 °C/min till it reached 250 °C, and then hold for 10 min. Helium was used as a carrier gas at a flow rate of 1.2 ml/min. Injection was done in split mode (10:1) with volume of 1.0 μl.

MS conditions. Source temperature 230 °C, quadrupole temperature 150 °C, MS was scanned from *m/z* 40 to 800 at a scan rate of 1.2 scan/s. The experiment was repeated thrice with three replicates and false positives were avoided by considering the compounds which have >95% level of library match.

4.6. Isoflavone identification

Isoflavones were analysed from the dried hairy roots, collected after recording fresh weights for the growth profiles (at one week interval for five weeks), and untransformed control roots using HPLC/UV–MS. Extraction was done with 100 mg of root powder using 10 ml of methanol at room temperature for 2 days. The extract was filtered through Whatmann filter paper no. 1 and concentrated to 1.0 ml. All HPLC grade solvents were purchased from Merck (Mumbai, India). Deionized water was filtered through cellulose nitrate filter (Sartorius AG, Germany, 0.45 μm). The solvents were degassed before use. Fluorescein was used as an internal standard (IS). A stock solution of 100 ppm was prepared in 80% methanol. Five microliters of the extract was added with 495 μl of IS stock solution and 5 μl of this solution was injected into HPLC through autosampler. IS was added just before injection on HPLC to normalize the detector response with respect to concentration of IS for calculating the relative peak area (RPA) of interested compounds. The RPA is calculated in relation to the IS by the formula – relative peak area for sample X = peak area of sample X/peak area of IS.

4.6.1. HPLC–UV/MS analysis

Analyses were carried out using a Quattro LC triple quadrupole mass spectrometer (Micromass, Manchester, UK) coupled with HP 1100 series liquid chromatograph (Agilent, Palo Alto, USA). HPLC is equipped with a binary pump (model G1312A), auto liquid sampler (model G1313A) and a variable wavelength detector (model G1314A). All experiments were performed using Masslynx software (version 3.2). A Discovery® C18 reversed phase analytical column (25 cm × 4.6 mm, 5 μm particle size) was used. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The samples were eluted from HPLC column at a flow rate of 1.0 ml/min with a linear gradient. Initial conditions were maintained at 20% B and were increased to 40% B in 30 min. Over the next 5 min, it reached the initial conditions. Column was kept at ambient temperature. A Tee is connected after the column to split the flow such that 60% goes to UV and 40% to the MS. The wavelength of UV detection was kept at 254 nm. The UV detector data was used for quantification.

MS conditions. An electrospray ion source operating under positive ion mode was used for the analysis. The MS was scanned from *m/z* 40 to 800 at the rate of 2 scans/s. Capillary voltage was kept between 3.5 and 3.8 kV and the cone voltage was kept at 30 V. Nitrogen was used as the nebulizing and desolvation gas. The experiment was repeated three times with three replicates.

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