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ORIGINAL ARTICLE

Terpenoid and flavonoid spectrum of in vitro cultures of *Glycyrrhiza glabra* revealed high chemical heterogeneity: platform to understand biosynthesis

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Abstract Simultaneous qualitative and quantitative assessment of eight flavonoids and two terpenoids were performed in fourteen in vitro raised morphogenic cultures of *Glycyrrhiza glabra*. Our study revealed that the spectrum and production of ten compounds, under investigation, were higher in organized tissue than the undifferentiated mass, however, aerial portions of the in vitro raised plants (leaf and stem) were found to be devoid of therapeutically relevant triterpenoid, glycyrrhizin. A correlation was observed between cell maturation, morphological differentiation and glycyrrhizin accumulation. Mature stolons (4 months) were characterized by the maximum accumulation of glycyrrhizin (8.60 μg/mg) in in vitro plantlets. The cytotoxic effect of the extracts evaluated against a panel of human cancer cell lines (in vitro) indicated that the pancreatic cell line (MIA-PaCa-2) were sensitive to all the fourteen extracts investigated. To the best of our knowledge this is the first comprehensive report relating plant growth regulators to metabolite spectrum and cytotoxic assessment in in vitro raised *G. glabra* cultures. Overall, our findings demonstrated that the metabolite spectrum of in vitro raised morphogenetic lines, under different stages of maturation, might offer a platform to understand the regulatory aspects of the concerned metabolite pathway and their consequent role in differentiation.

Keywords In vitro · Morphogenic cultures · *Glycyrrhiza glabra* · Flavonoids · Terpenoids · Cytotoxicity · LC–MS · HR-MS

Introduction

Terpenoids and flavonoids are low-molecular-weight natural products ubiquitous in vascular plants encompassing a diverse range of structure-dependent biological functions. They have been accredited with a substantial role in the biochemistry, physiology and ecology of the plants. These secondary metabolites are acknowledged to provide a rich

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pool for exploring novel compounds that may be exploited commercially in various industries (Manach et al. 2013). Many of these compounds found in Licorice roots (*Glycyrrhiza glabra/Glycyrrhiza uralensis*) have received attention in the recent years owing to their multitude of biological activities (Asl and Hosseinzadeh 2008). The root extract has shown to possess anti-inflammatory, hepatoprotective, anti-ulcer, anti-allergy (Park et al. 2004), and antiviral activity against various DNA and RNA viruses (Fiore et al. 2008), including human immunodeficiency virus (Ito et al. 1988) and coronavirus (Cinatl et al. 2003). Recently focus on the plant has escalated due to a triterpenoid saponin, glycyrrhizin, exhibiting potent anticancer activity in human (Zhang et al. 2009) and mice (Khan et al. 2013).

Since almost all the licorice root used in mercantile, is collected from wild resources, therefore, its high demand and low supply has invited establishing alternative systems for production in renewable resources. However, selection of highly productive lines and optimization of chemical and physical culture milieu of the cells for copious production is a prerequisite for the commercial application of these systems. It has been demonstrated that the biosynthetic activity of cultured cells can be enhanced by regulating various factors like environment (Ayabe et al. 1990), selecting hyper–producing lines (Jiao et al. 2014; Mardamshin et al. 1994) precursor feeding (Yang et al. 2007a, 2010) and transformation method (Li et al. 2013). The expanding commercial importance of the plant based anti-cancer compound has kindled the researchers to look for their production in tissue culture system. Exploitation of the biosynthetic capabilities of different cell types under a defined set of parameters, would not only give higher yields of the desired compound but will also provide deeper insight into regulatory processes thereon. Various in vitro systems including callus, organ (Gupta et al. 2013), cell suspension (Yang et al. 2006, 2007b) root (Du et al. 2001) and shoot cultures (Kojoma et al. 2010), have been exploited and subjected to terpenoid and flavonoid investigations in the past. In-vitro culture based metabolite analyses have shown the presence of flavonoids, chalcones and isoflavonoids (Kobayashi et al. 1985). Flavonoids like formononetin, isoliquiritigenin, echinatin, glabridine (Man et al. 2012; Tamura and Oda 1996), liquiritigenin, p-hydroxy benzoic acid, 3′-hydroxy formononetin have been reported from the in vitro culture lines of *Glycyrrhiza* species. A recent study on adventitious roots of *G. uralensis* revealed production of flavonoids at par with the intact plant (Wang et al. 2013). Similar results on flavonoid hyper-producing cultures were reported by (Man et al. 2013) in suspended cells of *Glycyrrhiza*. However, reports on oleanane-type triterpenoids (glycyrrhizin and glycyrritinic acid) are few and recent (Gupta et al. 2013; Shirazi et al. 2012; Yang et al. 2006). On the contrary, lupane type triterpenoids (betulinic acid) and soya saponins, have been found in most of the earlier studies pertaining to tissue culture raised plants (Hayashi et al. 1988), exhibiting metabolite distribution pattern in various organs of the in vitro plant (Hayashi et al. 1988). Literature survey revealed most of the studies on terpenoids and flavonoids detection in in vitro plant cultures remain restricted to few compounds (Hayashi et al. 1988; Yang et al. 2007b), restraining a comprehensive picture of the metabolite composition. Until now, only a single report on the chemical analysis of six flavonoids and two terpenoids in suspension cells of *G. uralensis* has been accounted (Man et al. 2013). Here we are reporting the simultaneous assessment of ten compounds-eight flavonoids (butin, quercetin, isoliquiritigenin, formononetin, glabridin, 4′-*O*-methylglabridine, hispaglabridin A and hispaglabridin B) and two terpenoids (glycyrrhizin and 18α-glycyrritinic acid) in the licorice root and in vitro raised callus, organ culture and plantlets of *G. glabra*. Also, the in vitro cytotoxicity, against a panel of four human cancer cell lines, were evaluated for these samples. The present study broadens the concept to investigate the biosynthetic potential of in vitro raised *G. glabra* cultures.

Materials and methods

Plant material

Glycyrrhiza glabra rhizome, collected from selected clones of *G. glabra* obtained from Hissar, was cultivated in the experimental farm of IIIM Jammu in 2010 (32.73°N and 74.87°E). Plant raised under field conditions, following Good Agricultural Practices, was exploited as a source material. In the present study, three-year-old field grown plant was utilized for in vitro experiments. For chemical analysis, field grown intact plant was harvested and divided into aerial and underground parts and extracted for analyzing using LC–ESI–MS and HRMS techniques.

For in vitro studies, explants (leaf and nodal segment) from the same plant were employed. The explants were rinsed in detergent (0.5 % Tween 20) for 30 min and washed in running water for 4 h. After that, they were surface sterilized with sodium hypochlorite (3 %, v/v) for 2 min and dried on filter paper followed by thorough washing with autoclaved distilled water. Finally, the explants were cultured aseptically.

Initial culture establishment

For culture establishment, three basal media (Murashige and Skoog, White and Gamborg) with different PGR combinations were tried using leaf, stem and nodal segments as explants. The explants were dissected to the size of 4–5 mm each, and inoculated in their respective basal media (MS,

Gamborg and White). All the cultures were maintained at 25 ± 2 °C under continuous cool white fluorescent light (Phillips, India) having a light intensity of 40 μmol/m^2/s. Axenic root cultures and root based explants were incubated under dark conditions. The observations were taken after 2 weeks of inoculation. The cultures were sub-cultured after every 3 weeks. In each experiment, at least ten explants were used in triplicates in a Petri dish. The best response was observed in the nodal segments in MS medium. Subsequent culture development was done using nodal segment as explant.

Callus induction and plantlet regeneration

Nodal explant (~5 mm) was used for callus induction using MS medium supplemented with 3 % sucrose and 2, 4-dichloroacetic acid (1 M) and 6-benzylaminopurine (1 M). Shoot induction from the nodal explants was observed in ½ MS media having 2 % sucrose supplemented with BA (1 M) and NAA (0.4 M). Multiple shoots could be obtained after three sub-culturing in the same medium. Three months old shoots (in pairs) were transferred to rooting MS medium supplemented with 3 % sucrose and NAA (0.01 μM). Stolon regeneration from the root was performed according to Gupta et al. (2013). Growth index (fresh weight) was determined at the time of harvest of the callus, and chemical profiling of the callus was employed in 12-week cultures. For the chemical analysis of morphogenetic lines, 2 weeks old green stolons and 4 weeks old brown stolons were harvested, weighed and analyzed for the flavonoid and terpenoids. Twelve-week-old shoots and plantlet were harvested, divided into aerial and underground parts and analyzed for related flavonoids and terpenoids contents. The cyanocobalamine (1.5 ml/l) and calcium pantothenate (100 mg/l) were added to vitamins in R medium.

Organ culture

Roots from in vitro grown plantlets were subjected to axenic root culture establishment. Different PGR's with varying concentrations and combinations (NAA, IAA, and IBA) and vitamin composition (R7) in three basal media (MS, White's and Gamborg's) were tested for faster, healthier root initiation and development in *G. glabra*. After 2 weeks, the explants responded with different morphology and coloration. After two sub-culturing (6 weeks), three different morphologies could be observed (roots, root + callus, and callus). Best axenic root growth was seen in ½ MS medium supplemented with Indol-3-butyric acid (1 M). Roots, root with callus and callus were harvested and subjected to chemical analyses after 9 weeks.

Pharmacological activity

Cell culture

Human pancreatic cancer cell line MiaPaca-2, Human lung cancer line A549, Human breast cancer cell line Mcf-7 and human colon cancer cell line HCT-116 was purchased from Sigma-Aldrich, India (ECACC, type). Cells were grown in MEM medium containing 10 % FCS, 100 U/ml penicillin and 100 mg/ml of streptomycin medium, maintained in a CO2 incubator (Thermocon Electron Corporation, Houston, TX) at 370C with 95 % humidity and 5 % CO_2 gas environment. Cells treated with tested materials were dissolved in DMSO while the untreated control cultures received only the vehicle (DMSO < 0.2 %).

Cell proliferation assay

In-vitro cytotoxicity of the cancer cells were plated in 96-well plates at a density of 1.0 × 104 in 200 μl of medium per well as published in (Bhushan et al. 2006). Cultures were incubated with 100 μg/ml concentration of test material for 48 h. The medium was replaced with fresh medium containing 100 μg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 3 h. The supernatant was aspirated, and MTT-formazan crystals were dissolved in 200 μl DMSO and the OD of the resulting solution was measured at λ540 nm (reference wavelength, λ620 nm) on ELISA reader (Thermo Labs, USA). The percentage of cell viability and growth inhibition were calculated according to the following equation

$$\text{The \% of cell viability} = \text{Absorbance of treated cells} - \text{Absorbance of blank} \times 100$$

$$\text{Absorbance of control cells} - \text{Absorbance of blank}$$

$$\text{\% Growth inhibition} = 100 - \text{\% cell viability}$$

Since the assay was a quantitative colorimetric method for determination of cell survival and proliferation, the metabolic activity of viable cancer cells was assessed. Metabolically active cells reduced pale yellow tetrazolium salt (MTT) to dark blue DMSO soluble formazan crystals, which were quantified calorimetrically. The absorbance of the formazan directly correlates with the number of viable cells.

Phytochemical analysis

Chemicals and reagents: LCMS-grade solvents (Merck, Germany) and Milli-Q-plus filter systems (Millipore,

Bedford, MA, USA) were used for the study. Isolated, identified and authenticated investigated pure flavonoids(butin, quercetin, isoliquiritigenin, formononetin, glabridin, 4′-*O*-methylglabridine, hispaglabridin A, hispaglabridin B) and tri terpenoids(18-α glycyrrhetinic acid, glycyrrhizin) were analyzed after ascertaining the purity (≥98.70 %) by HPLC.

Stock solution of standard compounds

Stock solutions of the ten compounds under investigation namely, butin (0.6 mg/5 ml), buercetin (1.3 mg/5 ml),Glycyrrhizin (1.6 mg/5 ml), isoliquiritegenin (1.4 mg/5 ml), formononetin (1.2 mg/5 ml), glabridin (3.0 mg/5 ml), 4′-*O*-methylglabridine (1.7 mg/5 ml), 18-α glycyrrhetinic acid (1.5 mg/10 ml), hispaglabridin A (1.5 mg/5 ml) and hispaglabridin B (2.6 mg/5 ml) were prepared in volumetric flasks individually in mobile phase. Standard working solutions were then obtained by mixing and making appropriate dilutions of stock solutions using mobile phase (1:1 v/v). All the standard solutions were filtered through a 0.45-μm membrane filter (Millipore) and injected directly. The stock and working solution were stored at +4 °C.

Calibration curves

Before making calibration curve, one blank injection was run to check the noise level of the system. The calibration equation of butin, quercetin, glycyrrhizin, isoliquiritigenin, formononetin, glabridin, 4′-*O*-methylglabridine, 18-α glycyrrhetinic acid, hispaglabridin A and hispaglabridin B were obtained by plotting LC–MS peak area (y) versus the concentration (x, mg/ml) of calibrators as $y = 303.134x + 1638.738$ ($R^2 = 0.997$), $y = 398.445x + 1821.467$ ($R^2 = 0.997$), $y = 52.556x - 1318.368$ ($R^2 = 0.997$), $y = 770.804x + 43{,}571.728$ ($R^2 = 0.992$), $1818.008x + 136{,}132.115$ ($R^2 = 0.981$), $y = 779.232x + 57{,}387.530$ ($R^2 = 0.995$), $y = 1109.790x + 9935.280$ ($R^2 = 0.994$), $y = 1990.096x - 640.839$ ($R^2 = 0.996$), $y = 922.266x - 380.593$ ($R^2 = 0.997$) and $y = 668.044x + 7513.470$ ($R^2 = 0.987$), respectively. The equation showed very good linearity over the range.

Preparation of extract

Lyophilized samples of field grown intact plant and in vitro raised callus cultures, organ cultures and plantlets were extracted following protocol published in (Hayashi et al. 1988). For the simultaneous detection, identification and quantification of ten compounds under investigation LC–MS/MS analysis was performed on an Agilent 6410 LC/MS–MS (Agilent Technologies, USA) triple-stage quadrupole mass spectrometer equipped with the electrospray ionization (ESI) interface. Liquid chromatography was performed on an Agilent 1260 Infinity (Agilent, USA) quaternary pump equipped with an auto sampler, column heater, and online degasser. Analytical chromatographic separations of *G. glabra* extract was carried out on a chromolith performance RP-18e column (100 × 4.6 mm, Merck, Germany) protected by a chromolith guard column of the same company. The column temperature was maintained at 300 °C; flow rate was optimized to 0.4 mL/min and the sample injection volume was 10 μL. The solvent system optimized was a linear gradient of acetonitrile/water and formic acid. The mobile phase was programmed at acetonitrile 40 % for 4 min, 50 % for 6 min, B; 60 % for 15 min, 70 % for 5 min, 80 % for 5 min, and from 80 to 40 % in 15 min. ESI positive mode with single ion monitoring was chosen for the quantification of investigated compounds. Sample solutions of the extract of *G. glabra* were injected directly without pre-treatment under the optimum condition developed, for the simultaneous detection, identification and quantification of flavonoids and terpenoids from *G. glabra*.

Results and discussion

LC–ESI–MS analysis

Full-scan spectra obtained for the samples and pseudo-molecular ions were selected for the selected ion monitoring (SIM). Compound identities were confirmed by comparison of molecular ion (SIM) with that of standard and formula generated through High-Resolution Mass Spectroscopy (HRMS) analysis (supplementary Figure 1). Spectra generated for butin, quercetin, glycyrrhizin, isoliquiritigenin, formononetin, glabridine, 4′-*O*-methylglabridine, 18-α glycyrrhetinic acid, hispaglabridine A and hispaglabridine B, in positive ion detection, gave the protonated molecule [M + H] + 273.1, 303.2, 845 [M + Na] + , 257.1, 269.1, 325.1, 339.1, 471.3, 393.3 and 391.3, respectively. It can be seen from Fig. 1 that the retention time of ten (1–10) investigated compounds were 6.96, 8.00, 10.50, 12.02, 13.18, 24.81, 35.62, 37.02, 38.59 and 42.98 min., respectively. The procedure could be completed in a short analysis time (within 46 min), simultaneously separating all the ten investigated compounds. Further, it was also found that analysis of the protonated molecular ion [M + H]+ and [M + Na]+, of the compounds (1–10) yielded different retention time (6.96, 8.00, 10.50, 12.02, 13.18, 24.81, 35.62, 37.02, 38.59 and 42.98 min) indicating their presence. All the quantification were performed in triplicate (Fig. 1). The separated and identified ten peaks were subjected to HRMS analysis for confirmation.

In vitro chemical spectrum

The biosynthetic potential of plantlets and morphogenetically distinct culture lines were analyzed for the two

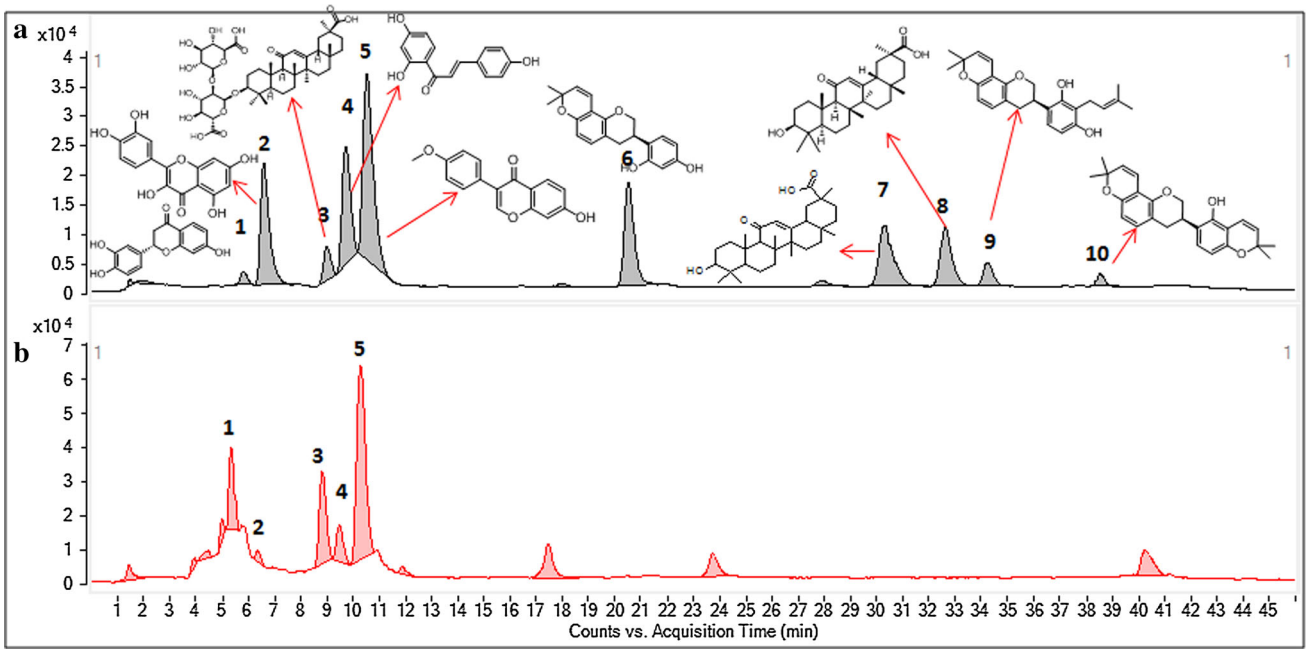

Fig. 1 LCMS profile of the ten compounds identified (*1*) Butin, (*2*) quercetin, (*3*) glycyrrhizin, (*4*) isoliquiritegenin, (*5*) formononetin, (*6*) glabridin, (*7*) 4′-*O*-methylglabridine, (*8*) 18-α glycyrrhetinic acid, (*9*) hispaglabridin A and (*10*) hispaglabridin B in (**a**) standard and (**b**) extract

triterpenoids and eight flavonoids in different stages of maturation and at various time intervals (Table 1) in the in vitro raised cultures of *G. glabra*. The metabolite spectrum of triterpenoids and flavonoids in the aerial and underground parts of plantlets were found to deviate significantly. The biologically active triterpenoid saponins (glycyrrhizin and 18-α glycyrrhetinic acid) were not detected in the aerial part of the 4 months old in vitro raised plantlet (TC1). However, underground rhizome showed presence of (8.60 µg/mg) glycyrrhizin. Among the organ culture analyzed, roots originating from in vitro plantlets, axenic root cultures (TR1 & TR2), yellow stolons (TC3), brown stolons (TC4) and brown root callus (TR2 & H7), produced glycyrrhizin under in vitro conditions. The glycyrrhizin content in these morphogenetic lines varied widely between 0.15 and 13.35 µg/mg. Analysis of the organ cultures revealed that the mature brown stolons (4 weeks) had a better capacity to form glycyrrhizin (1.9 µg/mg) than the yellow/green immature (2 weeks) stolons (0.19 µg/mg). Similarly, older roots showed more glycyrrhizin content (0.37 µg/mg) than the younger cultures (0.15 µg/mg). Further observations exhibited root cultures grown in solid media, in isolation, produced least glycyrrhizin. It seems certain amount of maturation along with differentiation is required for glycyrrhizin biosynthesis. Hayashi et al. (1988) had concluded similarly observing thickening of the roots and xylem tissues being tightly associated with glycyrrhizin accumulation (Hayashi et al. 1988). Literature has limited reports on glycyrrhizin production in cell cultures. Nevertheless, the results of the present study corroborated well with earlier studies on in vitro raised stolons of *G. glabra* (Gupta et al. 2013), *G. uralensis* (Kojoma et al. 2010), callus tissues (Wongwicha et al. 2008) and suspension cultures (Wang et al. 2013) in *Glycyrrhiza species*. The present study also detected glycyrrhitinic acid, aglycon of glycyrrhizin, in appreciable amount (4.6 µg/mg) in one of the axenic root culture (H2).

Among the eight flavonoids analyzed, four of them namely, hispaglabridine B (62.64 µg/mg), quercetin (1.42 µg/mg), butin (0.6 µg/mg) and formononetin (0.4 µg/mg) could be detected in aerial parts of the plant. The underground parts of the plantlet, however, had an appreciable amount of all the eight investigated flavonoids. The roots analyzed from mature plantlet (4 months old) had a moderate amount of all the investigated compounds, except quercetin and 18-α glycyrrhetinic acid which were not detected in the younger culture (2 months) also. The underground parts of the plantlet showed an appreciable amount of glabridine (18.6 µg/mg) and 4′-*O*-methylglabridine (16.5 µg/mg), other investigated flavonoids ranged between (0.3 µg/mg, quercetin to 6.8 µg/mg). Formononetin. Hispaglabridine B and glabridine showed interesting pattern of accumulation. Hispaglabridine B, which was present in the highest amount (62.6 µg/mg) in the aerial part, was appreciably lesser in quantity in the underground rhizome. While glabridine that was undetected in the aerial part was present in the maximum amount (18.6 µg/mg) in the underground rhizome of the

Table 1 Biochemical constituents and yield (average of three in ng/g) along with relative standard deviation (RSD %) of the ten compounds identified in the cultured tissues at different stages of morphogenetic differentiation in *Glycyrrhiza glabra*

Morphology	Gly. Acid RSD	18-α-Glycerrhitinic Acid RSD	Hispaglbridine A RSD	Hispaglbridine B RSD	Glabridin RSD	Formonomatin RSD	4-*O*-ME-Glabridine RSD	Butin RSD	Iso-liquiricitin RSD	Quercitin RSD
Ariel part of in vitro plantlet 4 months (TC1)	0.00	0.00	0.00	62.64	0.00	0.36	0.00	0.60	0.00	1.42
	0.00	0.0	0.0	0.31	0.0	0.34	0.0	0.09	0.0	0.26
In vitro plantlet root 4 months	8.60	0.00	4.58	4.58	18.65	6.81	16.49	2.30	2.19	0.28
	0.23	0.0	0.31	0.16	0.05	0.28	0.15	0.17	0.06	0.40
Green/yellow stolons 2 weeks (TC3)	0.19	0.00	0.00	0.06	0.83	2.88	0.57	0.30	2.28	0.0
	0.42	0.0	0.0	0.58	0.10	0.26	0.38	0.24	0.22	0.0
Brown stolons 4 wks (TC4)	1.90	0.00	0.34	1.20	6.61	10.34	3.72	0.40	1.22	0.0
	0.11	0.0	0.22	0.14	0.22	0.07	0.23	0.11	0.33	0.0
Root tissue 2 weeks (TR1)	0.15	0.00	0.00	0.40	1.53	6.01	0.84	0.50	3.66	0.00
	0.17	0.0	0.0	0.33	0.18	0.06	0.31	0.18	0.28	0.0
Root tissue 4 weeks (TR2)	0.37	0.00	0.00	0.38	3.87	3.03	1.36	0.41	2.27	0.00
	0.32	0.0	0.0	0.15	0.23	0.12	0.42	0.24	0.18	0.0
Area between stolon and root (TR6)	4.60	0.00	0.75	9.85	13.15	6.50	10.23	3.51	9.48	0.25
	0.22	0.0	0.16	0.09	0.11	0.20	0.07	0.37	0.17	0.23
Root + callus (R1)	16.50	0.00	35.58	0.00	0.00	1.45	0.00	1.26	0.00	0.00
	0.14	0.0	0.08	0.0	0.0	0.55	0.0	0.26	0.0	0.0
Root + callus (R6)	0.00	0.00	8.21	0.00	0.00	0.94	0.00	0.90	0.41	3.00
	0.0	0.0	0.21	0.0	0.0	0.31	0.0	0.11	0.09	0.43
Root + callus (R7)	0.00	0.00	3.96	0.00	0.0	0.00	0.00	0.33	0.00	0.27
	0.0	0.0	0.53	0.0	0.0	0.0	0.0	0.52	0.0	0.41
callus + root (H6)	0.00	0.00	14.80	0.00	0.00	2.25	0.00	0.71	0.00	2.01
	0.0	0.0	0.23	0.0	0.0	0.33	0.0	0.16	0.0	0.26
Callus + root (H7)	29.85	0.00	0.53	0.00	0.00	0.00	0.00	4.32	0.00	0.00
	0.11	0.0	0.22	0.0	0.0	0.0	0.0	0.29	0.0	0.0
Root (H2)	0.00	4.60	48.98	0.00	0.00	0.00	0.00	4.21	0.00	0.00
	0.0	0.32	0.42	0.0	0.0	0.0	0.0	0.38	0.0	0.0
Callus (F6)	13.35	0.00	26.01	0.00	0.00	2.76	0.00	0.31	0.60	0.71
	0.08	0.0	0.04	0.0	0.0	0.41	0.0	0.62	0.30	0.29

in vitro raised cultures under investigation. The stolon cultures and the axenic root tissues detected good amounts of formononetin (2.9–10.3 μg/mg) and isoliquiritigenin (1.2–3.6 μg/mg), and moderate amounts of other flavonoids, except quercetin, which was not detected in any of the stages under investigation. Contrary to observation in glycyrrhizin, no correlation could be deduced between flavonoid accumulation and maturation of the axenic root cultures. The part of tissue present between stem and root had a moderate amount of all the compounds except 18-α glycyrrhetinic acid. It showed maximum presence of glabridine (13.1 μg/mg) followed by 4′-*O*-methylglabridine (10.2 μg/mg) and isoliquiritigenin (9.5 μg/mg) and hispaglabridine B (9.8 μg/mg). Literature survey on flavonoid contents of in vitro raised plantlets/organ culture in *Glycyrrhiza species* gave scattered information. Most of the published studies have reported various flavonoids in cell suspension cultures of *Glycyrrhiza inflate* (Li et al. 2012), *Glycyrrhiza echinata*, *G. glabra* (Furuya et al. 1971, 1976) and *G. uralensis* (Wang et al. 2013). Many of the researchers are of the view that in vitro cultures of *Glycyrrhiza* has a better capability of production of flavonoids than the triterpenoids (Wang et al. 2013). However, reports about preferential accumulation and age-dependent synthesis of secondary metabolite are available in plants (Piatczak et al. 2015).

Effect of basal media and growth regulators on morphogenesis

Basal medium and plant growth regulators (PGRs) play a significant role in tissue morphogenesis, differentiation and secondary metabolite production (Raj et al. 2015). As most of the terpenoids and flavonoids were found in the licorice roots, the role of basal media composition and PGR effect on root tissue differentiation and secondary metabolite production was investigated in in vitro raised cultures of *G. glabra*. Five basal media namely, full strength Murashige and Skoog (FMS), half strength MS (HMS), R medium (MS with different vitamin composition), White's (W) and Gamborg's (B) media supplemented with seven (1–7) PGR combinations were used to assess the response of in vitro raised root explants. Simultaneous quantification of all the lines in various combinations demonstrated that irrespective of the basal medium, the root explants evoked three types of morphogenetic response, in 47 % of the cultures (16/35). Five out of sixteen cultures showed a very weak response, either due to the browning of the callus or very slow growth and hence were not studied further. Among the cultures responded, root + callus mixed morphology was observed in 8 cultures (50 %) while pure callus and pure root morphology were seen in 5 and 3 PGR combinations, respectively. Maximum response (5 out of 7 combinations) was observed in half strength MS medium (HMS), followed by R medium (4/7) and full MS and Gamborg's media (3/7). While White's medium responded only in one PGR combination (1/7), recorded after 2 weeks of culturing. A better response (4) was observed in MS medium when vitamin composition of full MS was changed to R vitamin combination. In the present study, the response of root explants in Gamborg's medium was noteworthy, as it responded in only 3 out of 7 combinations, and all the combinations produced only root type morphology, unlike the other basal media, where pure callus and root + callus morphology were dominant. In accordance with earlier observations of Gopitha et al. (2010), the present study also concludes that IBA augmented media is better in root induction than IAA. Browning was observed in FM6 & 7 cultures, Parsaeimehr and Mousavi (2009) also reported browning of callus when BAP and NAA growth hormones were used (Parsaeimehr and Mousavi 2009). Wongwicha et al. (2008) reported MS basal medium supplemented with 2, 4 D or NAA + kinetin growth regulators, to be a better medium for calli induction in *G. uralensis* (Wongwicha et al. 2008). The present study also observed better root multiplication in ½ MS medium supplemented with high concentrations of auxin (IAA/IBA) as was reported by Thengane et al. (1998). Findings of the study suggested growth hormone NAA in combination with IAA or IBA evoked callusing while NAA alone resulted in root initiation. Similar observations were recorded by (Elias et al. 2015) in shoot induction in *Echinocereus cinerascens* under in vitro conditions.

The biochemical spectrum of the root explants, grown in different cultures in various PGR combinations, varied widely (Table 1). Among the flavonoids, maximum flavonoids (5) were detected in root + callus cultures having the combination of NAA + BAP growth regulators (F6). As can be seen from the Table 1, butin (0.31–4.32 μg/mg) and hispaglabridine A (0.53–50.00 μg/mg) were found in all the cultures in varied quantities. Hispaglabridine B and glabridine were not detected in any of the cultures, while formononetin (0.94–2.76 μg/mg), isoliquiritigenin (0.41–0.60 μg/mg) and quercitin (0.27–3.00 μg/mg) were found in few cultures. 18-α glycyrritinic acid could not be detected in most of the samples except in axenic roots (H2) supplemented with ½MS media containing (1 mg/ml) IBA. The varied chemical spectrum is probably due to PGR effect as the root explants were of same age and origin and were grown under similar in vitro conditions. Recent works of on *Anacyclus pyrethrum* (Singh et al. 2015) and *Securinega suffruticose* (Raj et al. 2015) have demonstrated the effect of PGR combinations on plant differentiation and secondary metabolite accumulation.

The maximum number of compounds was reported from the mature stolons/roots of the plantlets (Table 1). All the investigated compounds, except the 18-α glycyrrhetinic acid, was present in the mature root of the in vitro culture,

and their quantity was found to increase with an increase in the maturity or age. Further, it was observed that hispaglabridine A was not found in 2 and 4-week old axenic root and stolon cultures, the compound could only be detected in the brown stolons and underground plant part (4 months), indicating a certain level of cellular organization or maturity of the tissue is a prerequisite for its biosynthesis. It was further noted that MS medium supplemented with auxins (NAA/IBA) had a positive effect on glycyrrhizin production. This is also supported by the observation that auxin predominately induced rooting, while its combination with kinetin or BAP resulted in callusing, in the present study. These findings are also in agreement with the culture medium composition recorded in earlier studies where glycyrrhizin was detected in *Glycyrrhiza species* (Gupta et al. 2013; Kojoma et al. 2010). Careful analysis of the quantification data (Table 1) showed mature stolon to be a good source of many flavonoids including hispaglabridine B, glabridine, 4′-*O*-methylglabridine, butin, and isoliquiritigenin, while aerial plant part was predominantly rich in hispaglabridine B (62.64 ng/mg) and quercitin (1.42 ng/mg). Secondary metabolites are synthesized in specific tissues at a certain developmental stage of the plant. Their production is significantly influenced by delicate physiological changes. With a better understanding of the cell culture behavior, their higher production through media modulation can be achieved.

Literature reports on flavonoid detection in *Glycyrrhiza species* are dispersed. Most of the reports refer to total flavonoid contents under in vitro conditions (Ayabe 1989; Jiao et al. 2014). However, few reports support formononetin production in suspension cultures of *G. glabra* (Arias-Castro et al. 1993), *G. echinata* (Ayabe et al. 1980) and *G. paddiflora* (Li et al. 2002) are available. Similarly, glabridine has been detected in the in vitro plantlet (Kinoshita et al. 1996) and suspension cells (Man et al. 2013; Tamura and Oda 1996).

Hispaglabridine A/B (Chin et al. 2007; Denisova et al. 2006) and Isoliquiritigenin, (Shirazi et al. 2012) were shown to be primarily distributed in the underground rhizomes of the naturally grown field plants. Isoliquiritigenin was similarly detected in the underground parts of the plantlets and organ cultures, in the present study. Its quantity, though, was found to be more in organized tissue than the undifferentiated mass. The isoliquiritigenin content varied between 1.22 µg/mg (mature stolon) to 9.48 µg/mg (the part between root and stolon), confirming the earlier report (Man et al. 2013) on isoliquiritigenin production in tissue culture raised plantlet of *G. glabra* (0.4–9.5 µg/mg). Our literature search revealed no reports on Hispaglabridine A/B, butin, a flavanone, and quercetin, flavonoid detection from the tissue cultures of *Glycyrrhiza species*. In the present study hispaglabridine A/B were found in most of the organ culture analyzed (Table 1) including underground plant parts and organ cultures. However, the compound was found to be absent in the aerial part and unorganized cell mass analyzed in the present study. The present LCMS-based analyses detected butin in all the in vitro raised samples analyzed, ranging between 0.31 µg/mg (F6) callus to 4.32 µg/mg (H7) in mixed morphology. The observation carries significance as flavonoids such as HispaglabridineA/B, glabridine and butin are being reported for the first time in *G. glabra* under tissue cultures conditions.

In vitro cytotoxic assay

Flavonoids and terpenoids have been known to possess anticancer properties (Fukai et al. 2000; Zhang et al. 2009). Preliminary in vitro cytotoxicity of all the tested compounds (1–10) was evaluated by performing a comprehensive screening at a single dose of 100 µg/ml, employing MTT assay, against various human cancer cell lines encompassing human breast cancer (MCF-7), pancreatic cancer cell lines (MIA PaCa-2), lung cancer cell line (A549) colon cancer cell line (HCT-116). The results are summarized in Table 2 and expressed as a percentage (%) of growth inhibition. The results demonstrated that extracts induced cell growth inhibition ranged between nil to 44 % in breast cancer, 43 % in lung cancer and 11 % in colon cancer cell lines. Pancreatic cancer cell lines showed inhibition ranging between 2–19 %. Out of the fourteen samples tested, pancreatic cancer cell lines responded to all the extracts examined, while the minimum response was observed in colon cancer cell lines (6). Field root, SPB shoot, and 11 PM extracts exhibited more than 40 % cell growth inhibitory effect in A549 and Mcf-7 cells. Root extract prepared from field grown plant was found to be the most potent amongst the series that exhibited around 44 % cell growth inhibition against breast cancer Mcf-7 cells (supplementary Figure 2). Further, extracts having a higher amount of glycyrrhizin notably showed a higher percentage of growth inhibition in breast cancer cell lines, while relatively more content of flavonoid displayed higher inhibitory activity in lung cancer cell lines in the present study.

The pattern and distribution of secondary metabolites in cell cultures are combinations of many interacting factors, including physiological status of culture, the origin of explants, age and degree of differentiation/developmental stage. Present results points towards a close association between cell differentiation and glycyrrhizin metabolism and also indicated the influence of the state of differentiation (under the influence of PGRs and basal media) on flavonoid spectrum. Metabolite spectrum of tissues at different stages of differentiation observed during the present study, offers a platform for understanding the inter-play between gene function and cellular regulatory processes involved in triterpenoid and flavonoid metabolism in *G. glabra*. It is a well-established fact that the functions of genes belonging to

Table 2 Screening of the in vitro cytotoxic activities against four human cancer cell lines of the in vitro raised morphogenetic lines of *Glycyrrhiza glabra*

Sample code	% Growth inhibition at concentration 100 μg/mL			
	MCF-7 (Breast cancer)	A549 (Lung cancer)	MIA-PaCa-2 (Pancreatic cancer)	HCT-116 (Colon cancer)
F6	15 ± 0.78	8 ± 0.57	10 ± 0.8	0
H2	19 ± 0.89	35 ± 2.3	15 ± 1.2	0
H6	8 ± 0.45	34 ± 2.7	15 ± 1.1	2 ± 0.01
H7	0	6 ± 0.45	2 ± 0.15	0
Field Leaf	7 ± 0.56	4 ± 0.31	5 ± 0.32	1 ± 0.08
R2	7 ± 0.6	2 ± 0.1	10 ± 0.84	2 ± 0.1
R6	0	0	10 ± 0.87	2 ± 0.15
Field stem	0	8 ± 0.58	11 ± 0.96	0
SPB shoot	0	41 ± 3.1	18 ± 1.1	0
11 PM	0	43 ± 2.9	19 ± 1.2	0
KG4	24 ± 1.2	23 ± 1.8	6 ± 0.45	11 ± 0.87
Field root	44 ± 3.1	13 ± 0.97	6 ± 0.42	0
SPB root	19 ± 0.9	1 ± 0.08	10 ± 0.78	2 ± 0.13
R1	13 ± 0.91	16 ± 0.59	5 ± 0.36	0

multi-gene families are widely divergent regarding substrate specificity and reaction sensitivity. In this context, medium modulation to regulate tissue differentiation could help in understanding the regulatory mechanisms involved in the production of the studied eight flavonoids and two terpenoids in the plant under investigation. Additionally, the study has further extended the biochemical spectrum of the in vitro raised morphogenetic lines, their metabolite flux and role in distribution pattern inside the tissue and cell differentiation.

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Author contribution SG, SK, PP& MM have executed the tissue culture experiments and chemical extraction. APG & MK performed the LCMS analysis; VA did HRMS analysis. Isolated, purified and authenticated chemical markers were provided by SJ. SB & did cytotoxicity work. RAV, AA &SG conceived and planned the experiments and wrote the MS. IIIM Publication No.: IIIM/1655/2014.

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