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Synthesis and Accumulation of Phytocompounds in Field-, Tissue-Culture Grown (Stress) Root Tissues and Simultaneous Defense Response Activity in *Glycyrrhiza glabra* L.

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Abstract: Harsh climates, i.e., drought, extreme temperatures, and toxic gases, pose issues to agriculture by altering plants' growth and yield. Biotechnology with biochemical defense approaches is beneficial for generating new plants/varieties with extra resilience to adverse conditions. In response to stress, cultures show an enriched level of secondary metabolite synthesis. Here, an efficient in vitro propagation method using axillary shoot proliferation, along with callus formation, was established in *Glycyrrhiza glabra* L. The phytochemical composition of in vitro and in vivo grown tissues was analyzed using a gas chromatography–mass spectrometry (GC–MS) technique, and the biochemical attributes were measured and compared in different investigated tissues. Callus formation from root explants was achieved with a frequency of 88.89% on MS medium containing 2.0 mg/L BAP and 0.5 mg/L 2,4-D. Axillary shoot proliferation was obtained from dormant buds when cultured onto MS supplemented with BAP alone, or in combination with, IAA. The maximum shoot proliferation (94.44%) was recorded on MS with 1.0 mg/L BAP with an average shoot length of 10.5 cm. The regenerated shoots were subcultured and transferred to the root induction medium, supplemented with various concentrations of IAA/IBA, wherein 2.0 mg/L IBA resulted in the best rooting frequency (88.89%). The GC–MS-based phytocompounds analysis of the methanolic extracts of root-derived callus and in vivo- and in vitro- grown root tissues was conducted. These samples revealed the presence of more than 35 therapeutically important bioactive compounds, such as methylglabridin, sitosterol, lupeol, squalene, stearic acid, linoleic acid, etc. The biochemical parameters, like total phenolic content, flavonoid content, DPPH scavenging activity, superoxide dismutase, and peroxidase activity were also measured. All the biochemical attributes were found to be higher in in vitro derived roots than the callus and in vivo grown root (donor) samples. These findings demonstrated that callus (root derived) and in vitro roots are a stable and potent source of multiple phytocompounds, encompassing medical significance with wide applications. This study may serve as an alternative opportunity in the sustained and continuous synthesis of important compounds without harming natural vegetation and normal environment.

Keywords: axillary shoot sprouting; antioxidant activity; biochemical attributes; defensive mechanism; GC-MS; harsh climatic conditions; metabolite profiling; secondary metabolism; resilient cultures

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1. Introduction

Glycyrrhiza glabra L. is widely known as licorice or mulethi, and is a popular member of the Fabaceae family. It is a perennial shrub, endemic to the Mediterranean region, but it has extended to parts of Central and Southwest Asia. The plant is now being introduced in Russia, the United Kingdom, the United States, Italy, France, Germany, Spain, China, and Northern India [1]. The plant is notable to the scientific community for its stoloniferous roots, which contain a wide array of phyto-compounds with diverse therapeutic applications [2]. The main bioactive compound present in the roots is glycyrrhizin (a triterpenoid saponin), which is a natural sweetener and flavoring agent. It has shown several biological activities, such as anti-inflammatory, anti-cancerous, anti-ulcer, anti-spasmodic, anti-allergic, and anti-viral properties [3]. It is also beneficial in the treatment of menstrual cramps, menopausal symptoms, fever, inflamed urinary and respiratory passages, hypoglycemia, and influenza [4].

G. glabra is propagated vegetatively using rhizomes, stolons, or other cuttings. The large-scale production of licorice is often impeded by incomplete seed sets, seed dormancy, low seed vigor, and unfavorable growing conditions [5]. Thus, micropropagation can be a good way to multiply high-quality plantlets under controlled and aseptic conditions [6]. In plant tissue culture, explants, culture mediums, culture conditions, and the application of plant growth regulators (PGRs) all affect the success of establishing an in vitro regeneration system of a plant species [6]. The tissue culture approach has various benefits over traditional methods, including rapid multiplication, germplasm preservation, the possibility of genetic modifications, and even yield improvement [7]. Furthermore, it may also ensure the uniform production of a higher yield of medically important phytocompounds [8].

In vitro conditions, such as the concentrations and combinations of PGRs, photoperiod, humidity, and gas exchange in vessels, and osmotic changes, are the major causes of stress inductions in cultures [9]. In response to these stresses, plant cells trigger a series of physiological reactions, including the increased production and accumulation of reactive oxygen species (ROS), phenolics, flavonoids, and other important phytocompounds [10]. These are detected by tracking various biochemical attributes and the phytochemical composition of cultured tissues. Phytochemical profiling by using the gas chromatography–mass spectrometric (GC–MS) approach has recently gained popularity in various research fields, such as biopharmaceutical and pharmacological sciences. This technique is suitable for the identification and quantification of compounds present in heterogenous mixtures of biological samples containing essential oils, fatty acids, alkaloids, terpenoids, saponins, etc. [11]. The GC–MS-based metabolite profiling has been carried out in various plant species, e.g., yellow elder (*Tecoma stans* L.) and Indian costus (*Saussurea costus*) [12,13]. In vitro regeneration studies have been attempted in *G. glabra* by using seeds, shoot tips, nodal, and leaf explants [14–16]. But, until now, no report has been available on the comparative metabolite profiling of in vivo- and in vitro-grown roots and root-derived callus in *G. glabra* by using the GC–MS method. Therefore, the purpose of this current study was to assess and compare the phytochemical profiles and biochemical variability of different cultured tissues, i.e., the callus- and in vivo- and in vitro-raised roots of *G. glabra*. The findings of the present investigation could help to provide a continual supply of therapeutically important bioactive compounds to the pharmaceutical sector by means of in vitro culture technology.

2. Material and Methods

2.1. Plant Material and Culture Establishment Conditions

Roots (1–2 cm) and nodal segments of *Glycyrrhiza glabra* L. were procured from a herbal garden in Jamia Hamdard, New Delhi, and were used as explants for in vitro experiments. Prior to culture establishment, the explants were subject to surface sterilization, following Sathish et al.'s [17] protocol, wherein the explants were initially treated with 30%

teepol solution for 8–10 min, followed by washing with running tap water, then surface sterilized with 70% ethanol and 0.1% mercuric chloride solution for 2–3 min, and finally rinsed with autoclaved double-distilled water to remove any particles of sterilizing agents. Later, the explants were aseptically placed on an MS medium [18], supplemented with varying concentrations and combinations of plant growth regulators (PGRs) (depending on the experiment as pictured below), along with 3% sucrose (w/v) and 0.8% agar (w/v). The cultures were grown at a temperature of 24 ± 2 °C under a light intensity of 60 $\mu molm^{-2}s^{-1}$ for 16 h light and 8 h dark photoperiods provided by white fluorescent tubes and with a relative humidity of 60%. The tissues were subcultured at every 3–4 week interval.

2.2. Callus Induction and Proliferation

Callus induction was initiated on disinfected root explants (1–2 cm in size) of *G. glabra* and was inoculated on the MS medium supplemented with different concentrations (0.5–2.0 mg/L) and combinations of auxins [2, 4-dichlorophenoxyacetic acid (2,4-D) and α-naphthalene acetic acid (NAA)] and cytokinin [6-benzylamino purine (BAP)]. In the control treatment, a PGR-free medium was used. Calli were subcultured regularly on the medium with the same PGR every 4 weeks. In each experiment, six explants were used (one explant/culture tube), and every experiment was repeated thrice. After 4 weeks of inoculation, callus induction frequency (%) and callus fresh biomass (in gm) were recorded.

$$\text{Callus induction frequency (\%)} = \frac{\text{Number of explants showing callusing}}{\text{Total number of explants inoculated}} \times 100$$

2.3. Axillary Shoot Sprouting and Growth

Surface sterilized nodal segments were cultured onto a shooting medium containing different concentrations of BAP (0.5–4.0 mg/L) alone, or in combination with, indole-3-acetic acid (IAA) at a concentration range of 0.5–1.0 mg/L. The in vitro shoots were then subcultured on the same medium for shoot proliferation every 4 weeks. Each experiment contained six explants, and each of the experiments were repeated three times. The shoot sprouting percentage (%) and the length of the shoots were measured (cm) after 4 weeks of culturing.

2.4. Root of Axillary Shoots

In vitro axillary shoots (4-week-old) were then excised and transferred to both solid and liquid rooting media containing indole acetic acid (IAA) or indole-3-butyric acid (IBA) with a concentration range of 1.0–3.0 mg/L. In each experiment, six explants were used, and every experimental set was repeated thrice. After 4 weeks of culture, the root induction rate (%) and the number of roots per shoot were observed. The healthy in vitro grown roots were then washed with sterile double-distilled water to remove the adhering agar and were subjected to further biochemical and metabolite profiling analyses.

2.5. Preparation of Extracts

The root derived callus and roots of *G. glabra* grown in vivo and in vitro were collected and shade dried at room temperature for 3 days. About 1.0 g (dry weight) of each shade dried sample was ground into a fine powder using a mortar and pestle and was then individually extracted with a methanol solvent (10 mL) in a rotary shaker for 48 h. After that, the methanolic extracts were filtered using Whatman No. 1 filter paper. Finally, the filtered samples were centrifuged at 10,000 rpm for 5 min and the collected supernatant was kept at a temperature of 4 °C until further use.

2.6. GC–MS Analyses

The GC–MS analysis of methanolic extracts of callus and roots was performed using the equipment GC-MS-QP-2010 (Shimadzu, Tokyo, Japan), following program specifications: helium gas, as a carrier gas, was kept at a constant flow of 1.21 mL/min; the injection temperature of 260 °C; the initial oven temperature was 100 °C with a holding time of 3 min, which was gradually increased to 300 °C for 17 min. The column used in the separation of compounds by GC–MS was the Rxi-5Sil MS GC Capillary Column, 30 m, 0.25 mm ID, 0.25 µm df. The ion source and interface temperatures were operated at 220 °C and 270 °C, respectively, with a solvent cut time of 2.5 min and the GC–MS running time for all samples was 35 min. The bioactive compounds present in each sample were identified by using the mass spectral database of NIST (National Institute of Standards and Technology) library as well as comparing the retention indices, peak area, and peak area % with already identified phytocompounds using GCMS solution software (Version 4.45 SP 1).

2.7. Biochemical Analyses

2.7.1. Estimation of Total Phenolic Content

The total phenolic content (TPC) of the extracts was determined using the Folin–Ciocalteu method [19]. About 0.5 mL of the extract was mixed well with 2.5 mL of 10% (*v*/*v*) Folin–Ciocalteu (FC) reagent (Sigma-Aldrich, New York, NY, USA). After the mixture was incubated for 5 min at room temperature, 2.0 mL of 7% sodium carbonate solution was added and subsequently incubated at room temperature for 90 min. Later, absorbance was measured using a UV-Vis spectrophotometer (Biolinkk, BL-295, Delhi, India) at 765 nm against the blank with no extract. The measurement was carried out in triplicates and the total phenolic content was determined by utilizing the calibration curve equation of the standard gallic acid solution. The outcome data were presented as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW).

2.7.2. Estimation of Total Flavonoid Content (TFC)

The total flavonoid content (TFC) was assayed according to the method described by Aryal et al. [20]. Firstly, 0.2 mL of 10% aluminum chloride solution and 0.2 mL of 1 M potassium acetate solution were mixed with 1.0 mL of extract solutions. The final reaction volume was made up to 5.0 mL by adding 3.6 mL distilled water with an incubation period of 30 min at room temperature. The solution was thoroughly mixed, followed by a measurement of their respective absorbance at 415 nm against a blank. The measurement was carried out in triplicates. A standard graph of quercetin of different concentrations was plotted against their respective absorbance. The samples' TFC were expressed as milligrams of quercetin equivalents per gm of dry weight (mg QE/g DW).

2.7.3. Determination of Free Radical Scavenging Activity by DPPH Assay

Free radical scavenging activity (FRSA) of extract samples of *G. glabra* was measured using a stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) by using Baliyan et al.'s [19] method. An amount of 0.1 mL of extract solutions were briefly added into each test tube containing 3.0 mL of DPPH (0.024% *w*/*v*) and 0.1 mL of methanol mixed with 3.0 mL of DPPH was used as a standard. Afterwards, the samples were kept in total darkness for a 90 min incubation period at room temperature. Finally, the absorbance was recorded at 517 nm. The anti-oxidant potential of each sample was checked using the following formula [10]:

$$\text{Scavenging activity \%} = (A_C - A_S/A_C) \times 100$$

where A_C = absorbance of control and A_S = absorbance of sample.

2.7.4. Determination of Peroxidase (POD; EC: 1.11.1.7) Activity

The sample preparation and POD assay were conducted following Haida et al.'s [21] protocol, with slight modifications. Around 1.0 g of fresh samples (callus, in vivo, and in vitro root tissues) were homogenized with 10 mL of 0.1 M extracting phosphate buffer (pH = 6.0). Acquired extracts of samples were then filtered and centrifuged at 12,000 rpm for 30 min. The supernatant was collected and heated at 65 °C for 1 min and stored until further use. For the peroxidase assay, the reaction mixture was composed of 1.0 mL of 10 mM potassium-phosphate buffer (pH = 7.0), 0.5 mL of 1% guaiacol solution, 0.5 mL hydrogen peroxide solution (0.4%), and 0.5 mL of enzyme extract, along with 2.5 mL of distilled water. The control group was prepared with all the reagents, excluding the enzyme extract. Afterwards, the increase in absorbance was checked at 470 nm within a 30 min period, indicating the formation of tetraguaiacol. The enzymatic activity was calculated by the following formula [10]:

$$A = ELC$$

where, A = absorbance, E = extinction coefficient (6.39 $mM^{-1}cm^{-1}$), L = path length (1.0 cm) and C = enzyme concentration (mM/g FW), and FW = fresh weight of samples.

2.7.5. Determination of Superoxide Dismutase (SOD; EC: 1.15.1.1) Activity

The enzyme extract preparation and SOD assay were carried out according to the protocol proposed by Mujib et al. [22]. Firstly, the enzyme extract was prepared by homogenization of 1.0 g of fresh tissue samples (callus, in vivo and in vitro root tissues) in 10 mL of 0.5 M of sodium phosphate buffer (pH 7.3) which contained 3.0 mM ethylenediaminetetraacetic acid (EDTA), 1.0% (*w*/*v*) polyvinylpyrollidone (PVP), and 1.0% (*v*/*v*) Triton X-100. Later, the homogenate was filtered and centrifuged at 11,800 *g* for 15 min at 4 °C, and finally, the supernatant was collected. For the SOD assay, the final reaction mixture of 3.0 mL contained 50 mM potassium phosphate buffer (pH 7.8), 45 µM methionine, 1.0 M Na_2CO_3, 2.25 mM Nitro blue tetrazolium (NBT) solution, 3.0 mM EDTA, 10 µM riboflavin, 10 µL of enzyme extract, and distilled water. A control group was taken without any enzyme extract. The mixture was then incubated at 25 °C for 10 min in the presence of 15 W fluorescent lamps. After 10 min of incubation, the absorbance of each sample was recorded at 560 nm using a spectrophotometer. One unit of SOD activity is defined as the amount of enzyme utilized causing 50% inhibition of NBT reduction (units (U)/mg FW).

2.8. Statistical Analysis

All the in vitro culture experiments were performed in a completely randomized design (CRD) in triplicates with six explants per treatment and the biochemical analyses were also replicated thrice. Each data set is represented as mean ± standard error. To statistically analyze the data, one-way analysis of variance (ANOVA) was employed using SPSS software (Ver. 25). The significant differences among the means of samples were determined using Duncan's multiple range test (DMRT) at $p < 0.05$ level [23]. GC–MS chromatograms were prepared by GCMS solution software (Shimadzu Corp., Columbia, MD, USA) and biochemical graphs were generated by Microsoft Office Excel ver16.0 (Microsoft Corp., Redmond, WA, USA).

3. Results

3.1. Callus Induction and Proliferation

The roots of *G. glabra* were used as explants for inducing callus. Within 4 weeks of culturing , white friable callus started to emerge from the surface of explants (Figure 1A). Control treatment (without PGR) did not induce any callus; whereas callus induction was noticed in all MS media containing PGRs. The BAP and 2,4-D combinations were proved

to be more efficient in inducing callus (61.11–88.89%) compared to BAP and NAA treatments showing the callus induction range from 11.11–44.44% (Table 1). The highest callus induction frequency (88.89%) was achieved on the MS medium with 2.0 mg/L of BAP + 0.5 mg/L of 2,4-D and the highest fresh biomass (4.4 g/explant) was obtained on the same medium (Figure 1B,C). On the contrary, the lowest callusing rate (11.11%) and fresh biomass (0.8 g/explant) were observed on MS containing 1.0 mg/L BAP and 2.0 mg/L NAA.

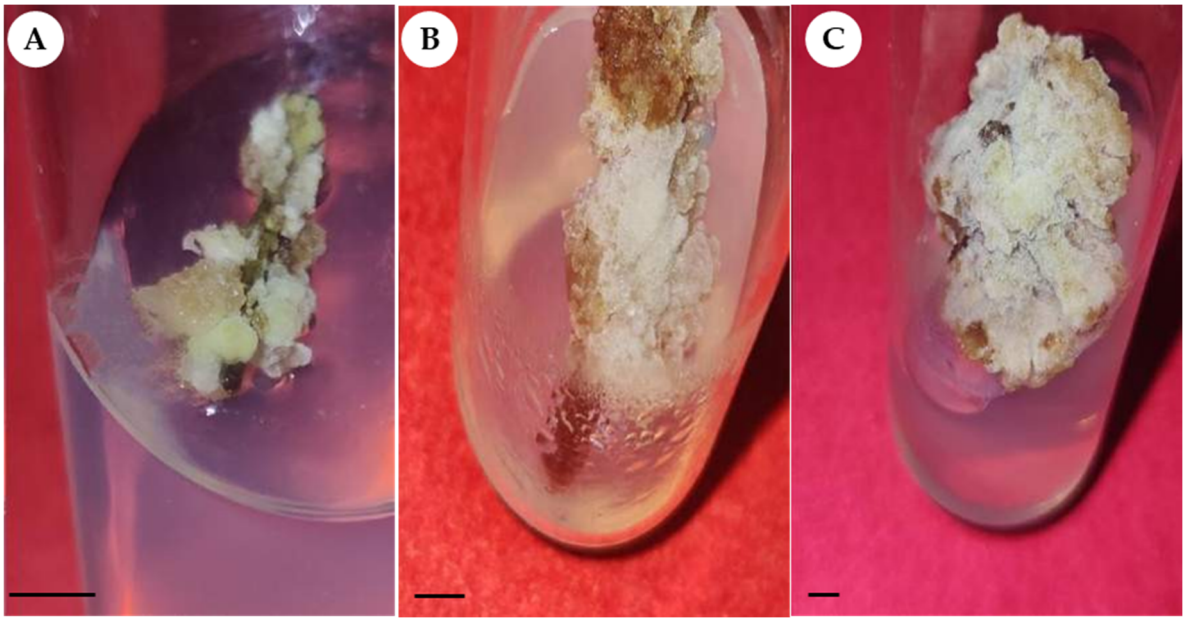

Figure 1. Callus induction and proliferation on the root explant of *G. glabra* on MS medium supplemented with 2.0 mg/L BAP and 0.5 mg/L 2,4-D. Callus initiation after 4 weeks of inoculation (Bar = 0.5 cm) (**A**). Callus proliferation after regular subculture (Bars (**B**) = 1.0 cm, (**C**) = 1.5 cm).

Table 1. Effect of combinations of cytokinin (BAP) and auxins (2,4-D/NAA) on callus induction frequency and growth of callus using root explants of *G. glabra* L. after 4 weeks of inoculation.

PGRs	Concentration (mg/L)	Callusing Frequency (%)	Fresh Biomass (g)
Control	0	0 ^{e}	0 ^{d}
BAP + 2,4-D	1.0 + 0.5	72.21 ± 5.56 ^{ab}	3.9 ± 0.6 ^{ab}
	1.0 + 2.0	61.11 ± 5.55 ^{bc}	3.6 ± 0.4 ^{ab}
	2.0 + 0.5	88.89 ± 11.11 ^{a}	4.4 ± 0.7 ^{a}
BAP + NAA	1.0 + 0.5	27.77 ± 5.56 ^{de}	2.1 ± 0.1 ^{c}
	1.0 + 2.0	11.11 ± 5.55 ^{e}	0.8 ± 0.1 ^{d}
	2.0 + 1.0	44.44 ± 5.56 ^{cd}	2.9 ± 0.2 ^{bc}

Values represent mean ± S.E. of six replicates per treatment in three repeated experiments. Mean values with different letters within each column are significant at $p \leq 0.05$ level as per DMRT.

3.2. Axillary Shoot Sprouting and Growth

Nodal explants with dormant buds were placed on MS containing either BAP alone, or in combination with IAA. After 6–7 days of culturing, the buds showed the signs of swelling (Figure 2A) and later, the axillary shoots emerged (Figure 2B,C). The highest axillary shoot sprouting frequency (94.44%) was observed in 1.0 mg/L BAP treatment, and this frequency declined significantly when the BAP level was increased to 2.0 mg/L in media (Table 2). The BAP and IAA combinations had a moderate stimulating effect on axillary shoot sprout and growth. The longest axillary shoots (10.5 and 8.7 cm) developed on the MS medium were supplemented with 1.0 mg/L and 0.5 mg/L of BAP, respectively. The axillary shoots were later excised and transferred to the BAP-containing medium for further proliferation (Figure 2D).

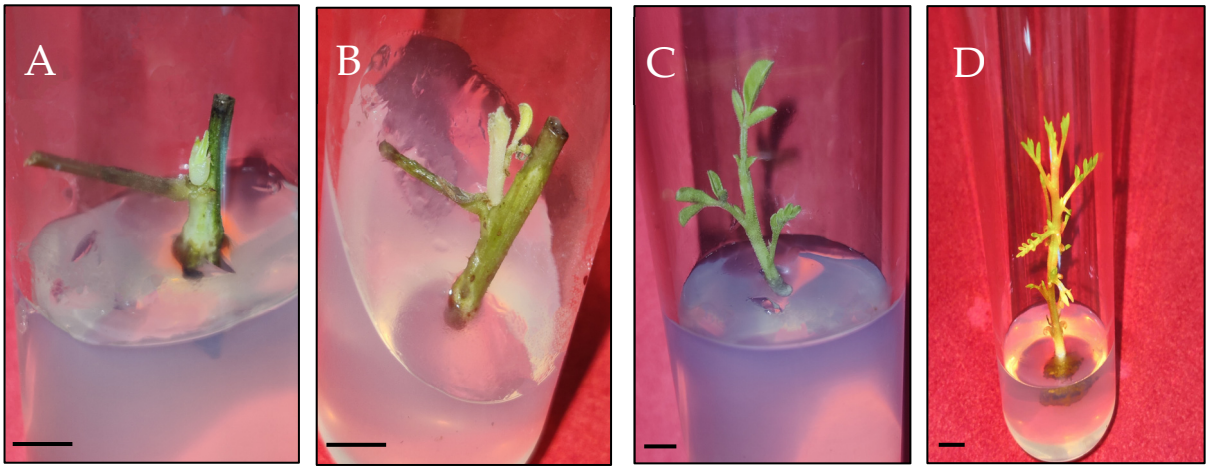

Figure 2. In vitro axillary shoot sprouting in *G. glabra*.: Swelling of the axillary bud after 7 days of inoculation (Bar = 0.5 cm) (**A**). Axillary bud sprouting on 1.0 mg/L BAP fortified MS medium (Bar = 0.5 cm) (**B**). Elongation of in vitro sprouted axillary shoot on medium with 1.0 mg/L BAP (Bars (**C**) = 1.0 cm, (**D**) = 2.0 cm) (**C**,**D**).

Table 2. Effect of BAP alone, or in combination with, IAA on the axillary shoot sprouting from axillary buds of *G. glabra* L. after 4 weeks of inoculation.

PGRs	Concentration (mg/L)	Shooting Frequency (%)	Shoot Length (cm)	Mean Shoot Number
Control	0	0 ^{e}	0 ^{f}	0 ^{d}
BAP	0.5	77.77 ± 5.56 ^{ab}	8.7 ± 0.4 ^{b}	3.33 ± 0.67 ^{ab}
	1.0	94.44 ± 5.56 ^{a}	10.5 ± 0.3 ^{a}	4.67 ± 0.88 ^{a}
	2.0	38.88 ± 14.70 ^{cd}	4.4 ± 0.6 ^{d}	2.67 ± 0.88 ^{bc}
	4.0	22.21 ± 5.55 ^{de}	2.1 ± 0.2 ^{e}	1.33 ± 0.33 ^{cd}
BAP + IAA	1.0 + 0.25	55.55 ± 11.11 ^{bcd}	6.2 ± 0.4 ^{c}	3.33 ± 0.33 ^{ab}
	1.0 + 0.50	61.12 ± 14.69 ^{abc}	6.8 ± 0.8 ^{c}	3.33 ± 0.33 ^{ab}

Values represent mean ± S.E. of six replicates per treatment in three repeated experiments. Mean values with different superscripts within a column indicate significant difference between them at $p \leq 0.05$ level as per DMRT.

3.3. Rooting of Axillary Shoots

The healthy in vitro axillary shoots were transferred to the MS medium, supplemented with IAA or IBA (1.0–3.0 mg/L) for rooting. Successful root induction was observed within 3–4 weeks of culturing (Figure 3); 2.0 mg/L of IBA proved to be the best in inducing roots which showed 88.89% rooting frequency and average 7.33 root numbers/shoot, followed by 1.0 mg/L of IBA that had 6.67 mean root numbers/shoot with 72.22% rooting frequency (Table 3). On the other hand, with increasing IAA concentrations, there was a significant reduction in rooting and, upon reaching up to 3.0 mg/L IAA, no root development was observed in the axillary shoots.

Figure 3. In vitro root induction of the axillary shoots in *G. glabra* on liquid root induction medium (3.0 mg/L IBA) (Bar = 2.0 cm).

Table 3. Effect of auxins (IBA/IAA) on in vitro root induction of the axillary shoots of *G. glabra* L. after 4 weeks of inoculation.

PGRs	Concentration (mg/L)	Rooting Frequency (%)	Mean Root Numbers/Shoot
Control	0	0 ^{c}	0 ^{d}
IBA	1.0	72.22 ± 14.70 ^{ab}	6.67 ± 1.20 ^{ab}
	2.0	88.89 ± 11.11 ^{a}	7.33 ± 1.33 ^{a}
	3.0	61.12 ± 14.70 ^{ab}	4.67 ± 0.88 ^{abc}
IAA	1.0	49.99 ± 9.62 ^{b}	3.67 ± 0.33 ^{bc}
	2.0	38.89 ± 11.11 ^{b}	2.33 ± 0.67 ^{c}
	3.0	0 ^{c}	0 ^{d}

Values represent mean ± S.E. of six replicates per treatment in three repeated experiments. Mean values with different superscripts within a column indicate significant mean difference with one another at $p \leq 0.05$ level as per DMRT.

3.4. GC–MS Analysis

In this current study, metabolite profiling of methanolic extracts of in vivo and in vitro grown roots, as well as root derived callus, was performed using the GC–MS approach, and their respective chromatograms are presented in Figure 4. A comparative chromatographic examination of in vivo and in vitro derived roots revealed the presence of 38 and 36 phytocompounds, respectively (Table 4). The heatmap cluster analysis shown in Figure 5 is based on phytocompounds peak area % measured by GC–MS, and it was found that mome inositol, beta-monoolein, methyl ester of oleic acid, guanosine, dipalimitin, glycidyl oleate, methyl ester of palmitic acid and stearic acid are some of the majorly occurring metabolites in both samples. In total, 24 bioactive compounds have been found to be present in in vivo and in vitro grown samples at varied levels, which include guanosine, glycidyl palmitate, glycidyl oleate, dipalmitin, gamma-sitosterol, stigmasta-3,5-diene, methyl esters of linoleic acid, oleic acid, stearic acid, alpha-spinosterol acetate, etc.

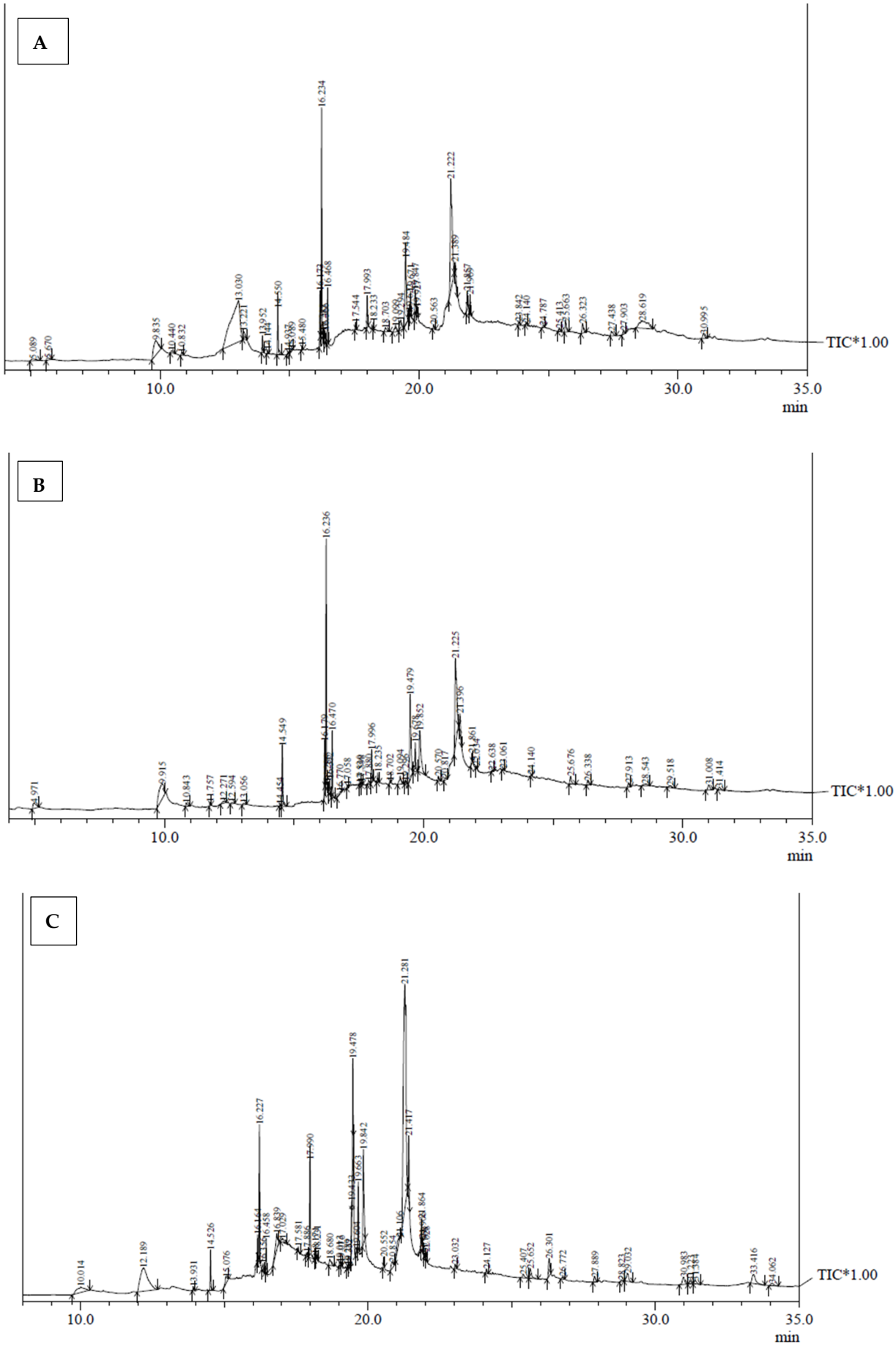
A
16.234
21.222
13.030
14.550
17.993
19.484
21.389
TIC*1.00
10.0
20.0
30.0
35.0
min
B
16.236
21.225
19.479
14.549
16.129
16.470
21.396
TIC*1.00
10.0
20.0
30.0
35.0
min
C
21.281
19.478
16.227
17.990
19.842
21.417
14.526
12.189
TIC*1.00
10.0
20.0
30.0
35.0
min

Figure 4. GC–MS chromatograms of methanolic extracts of in vivo grown root tissue (**A**), in vitro derived root tissue (**B**), and root derived callus of *G. glabra* (**C**).

Table 4. GC–MS analysis of phytocompounds present in the methanolic root extracts of *G. glabra* plants grown in vivo and in vitro.

S.No.	Name of the Compound	Retention Time (min)		Peak Area %		Molecular Formula	Molecular Weight
		In Vivo Root	In Vitro Root	In Vivo Root	In Vitro Root		
1	Pyranone	5.089	4.971	0.68	1.89	$C_6H_8O_4$	144
2	2-piperidinemethanol	5.670	-	0.47	-	$C_6H_{13}NO$	115.00
3	Guanosine	9.835	9.915	4.80	8.59	$C_{10}H_{13}N_5O_5$	283
4	Xanthosine	10.440	-	0.37	-	$C_{10}H_{12}N_4O_6$	284
5	1-Propylpentyl butyrate	10.832	-	0.28	-	$C_{12}H_{24}O_2$	200
6	Hexadecanoic acid	-	10.843	-	0.71	$C_{16}H_{32}O_2$	256
7	cis-Sesquisabinene hydrate	-	11.757	-	0.28	$C_{15}H_{26}O$	222
8	4-Methylmannitol	-	12.271	-	0.55	$C_7H_{16}O_6$	196
9	Mome inositol	13.03	12.594	27.79	0.27	$C_7H_{14}O_6$	194
10	Ethyl 3-(4-fluorophenyl)-3-oxopropanoate	-	13.056	-	0.35	$C_{11}H_{11}FO_3$	210
11	4-octanol	13.221	-	1.12	-	$C_8H_{18}O$	130
12	1-pentadecanol	14.144	-	0.2	-	$C_{15}H_{32}O$	228
13	Butylated Hydroxytoluene	-	14.454	-	0.21	$C_{15}H_{24}O$	220
14	Palmitic acid, methyl ester	14.55	14.549	3.03	5.09	$C_{17}H_{34}O_2$	270
15	Isopropyl palmitate	15.48	-	0.15	-	$C_{19}H_{38}O_2$	298
16	Linoleic acid, methyl ester	16.173	16.179	1.92	3.19	$C_{19}H_{34}O_2$	294
17	Oleic acid, methyl ester	16.234	16.236	10.01	16.26	$C_{19}H_{36}O_2$	296
18	Methyl elaidate	16.285	16.286	0.12	0.17	$C_{19}H_{36}O_2$	296
19	Linolenic acid, methyl ester	16.366	16.372	0.72	1.21	$C_{19}H_{32}O_2$	292
20	Stearic acid, methyl ester	16.468	16.47	2.46	4.48	$C_{19}H_{38}O_2$	298
21	Glycol myristate	-	17.53	-	0.15	$C_{16}H_{32}O_3$	272
22	Tributyl acetylicitrate	17.544	-	0.3	-	$C_{20}H_{34}O_8$	402
23	2-Monopalmitin	-	17.598	-	0.16	$C_{19}H_{38}O_4$	330
24	Glycidyl palmitate	17.993	17.996	1.65	2.72	$C_{19}H_{36}O_3$	312
25	Eicosanoic acid, methyl ester	18.233	18.235	0.25	0.69	$C_{21}H_{42}O_2$	326
23	9-octadecenamide	18.703	18.702	0.59	0.21	$C_{18}H_{35}NO$	281
27	Oleoyl chloride	19.099	19.094	0.89	1.58	$C_{18}H_{33}ClO$	300
28	alpha-Monostearin	-	19.296	-	0.35	$C_{21}H_{42}O_4$	358
29	Glycidyl oleate	19.484	19.479	7.51	11.64	$C_{21}H_{38}O_3$	338
30	cis-8,11,14-Eicosatrienoic Acid	19.622	-	0.26	-	$C_{20}H_{34}O_2$	306
31	Glycidyl stearate	19.671	19.678	1.14	3.01	$C_{21}H_{40}O_3$	340
32	Dipalmitin	19.847	19.852	1.69	7.92	$C_{35}H_{68}O_5$	568
33	Pentadecyl hexanoate	20.563	20.57	0.29	0.51	$C_{21}H_{42}O_2$	326
34	.beta.-Monoolein	21.222	21.225	15.13	16.53	$C_{21}H_{40}O_4$	356
35	Methyl 12-oxo-9-dodecenoate	21.389	21.396	1.48	2.21	$C_{13}H_{22}O_3$	226
36	Oleic acid, 3-hydroxypropyl ester	21.857	21.861	1.02	0.89	$C_{21}H_{40}O_3$	340
37	Squalene	21.969	-	0.76	-	$C_{30}H_{50}$	410
38	Isopropyl linoleate	-	22.638	-	0.13	$C_{21}H_{38}O_2$	322
39	4′-O-Methylglabridin	23.842	-	0.32	-	$C_{21}H_{22}O_4$	338
40	Stigmasta-3,5-diene	24.14	24.14	0.25	0.11	$C_{29}H_{48}$	396
41	Heneicosanoic acid, 3-ethyl-3-methyl-, methyl ester	24.787	-	0.18	-	$C_{25}H_{50}O_2$	382
42	24-Epicampesterol	25.413	-	0.30	-	$C_{28}H_{48}O$	400
43	(22E)-Stigmasta-4,22-dien-3-ol	25.663	25.676	1.20	1.35	$C_{29}H_{48}O$	412
44	gamma.-Sitosterol	26.323	26.338	1.04	0.74	$C_{29}H_{50}O$	414
45	Lupeol	27.438	-	0.70	-	$C_{30}H_{50}O$	426
46	Ethylcyclodocosane	-	27.913	-	0.65	$C_{24}H_{48}$	336
47	alpha-Spinosterol acetate	28.619	28.543	4.65	0.79	$C_{31}H_{50}O_2$	454
48	N,N-Dimethylcholestan-6-amine	30.995	-	0.75	-	$C_{29}H_{53}N$	415
49	N-[2-(tetradecyloxy)phenyl]acetamide	-	31.008	-	1.2	$C_{22}H_{37}NO_2$	347
50	cis-15-Tetracosenoic acid, propyl ester	-	31.414	-	0.68	$C_{27}H_{52}O_2$	408

The content of guanosine was found to be almost doubled (8.59%) in in vitro grown roots compared to in vivo grown roots (4.80%). Similarly, the methyl ester content of palmitic acid was found to be higher in the in vitro root (16.26%) sample than in the in vivo

grown root sample (10.01%). A similar difference was noted for glycidyl oleate (7.51% in field grown roots and 11.64% in in vitro derived root tissue) as well. On the other hand, some of the metabolites were observed to be lower in in vitro root tissue than the in vivo derived root sample; examples of this are 9-octadecenamide, stigmasta-3,5-diene, gamma-sitosterol and alpha-spinosterol acetate. Certain bioactive compounds are exclusively present in each sample of in vivo grown roots, such as xanthosine (0.37%), squalene (0.76%), 4′-O-methylglabridin (0.32%), 24-epicampesterol (0.30%) and lupeol (0.70%), whereas, in vitro grown roots contained cis-sesquisabinene hydrate (0.28%), 4-methylmannitol (0.55%), 2-monopalmitin (0.16%), alpha-monostearin (0.35%), isopropyl linoleate (0.13%) exclusively.

Figure 5. Heat-map cluster analysis displaying the relative abundance of phytocompounds identified and quantified by GC–MS technique in methanolic root extracts of *G. glabra* plants grown in vivo and in vitro.

The phytochemical profile of root derived callus of *G. glabra* was similarly determined and analyzed. The obtained result of methanolic callus extract depicted a total of 44 phytocompounds; many of which are detected in trace quantities (Table 5). Oleic anhydride (35.65%), methyl galactoside (10.73%), 2,3-dihydroxypropyl laurate (5.96%), Glycidyl palmitate (5.19%), methyl ester of oleic acid (5.18%) were observed as the versatile phytocompounds present in major proportions. Some of the important phytochemicals like palmidrol, 1-monostearin, beta-stigmasterol, 24-epibrassicasterol, squalene, beta-saccharostenone were quantified in minute amounts.

Table 5. Phytocompounds identified in the methanolic extract of root derived calli of *G. glabra* by GC-MS analysis.

S.No.	RT (min)	Peak Area %	Name of the Compound	Molecular Formula	Molecular Weight
1	10.014	2.44	Guanosine	$C_{10}H_{13}N_5O_5$	283
2	12.189	10.73	Methyl galactoside	$C_7H_{14}O_6$	194
3	14.526	1.59	Palmitic acid, methyl ester	$C_{17}H_{34}O_2$	270
4	15.076	0.88	Palmitinic acid	$C_{16}H_{32}O_2$	256
5	16.164	0.90	Linoleic acid, methyl ester	$C_{19}H_{34}O_2$	294
6	16.227	5.18	Oleic acid, methyl ester	$C_{19}H_{36}O_2$	296
7	16.356	0.45	Linolenic acid, methyl ester	$C_{19}H_{32}O_2$	292
8	16.458	1.26	Stearic acid, methyl ester	$C_{19}H_{38}O_2$	298
9	16.839	2.26	cis-9,cis-12-Octadecadienoic acid	$C_{18}H_{32}O_2$	280
10	17.581	0.15	2-Monopalmitin	$C_{19}H_{38}O_4$	330
11	17.990	5.19	Glycidyl palmitate	$C_{19}H_{36}O_3$	312
12	18.171	0.19	Palmidrol	$C_{18}H_{37}NO_2$	299
13	18.231	0.31	Eicosanoic acid, methyl ester	$C_{21}H_{42}O_2$	326
14	18.680	0.68	9-octadecenamide	$C_{18}H_{35}NO$	281
15	19.076	0.14	Oleoyl chloride	$C_{18}H_{33}ClO$	300
16	19.289	0.09	2-Formylhexadecane	$C_{17}H_{34}O$	254
17	19.433	1.05	1,3,14,16-Nonadecatetraene	$C_{19}H_{32}$	260
18	19.478	3.88	Glycidyl oleate	$C_{21}H_{38}O_3$	338
19	19.604	0.18	Ethyl. alpha.-linolenate	$C_{20}H_{34}O_2$	306
20	19.663	2.75	1-Monostearin	$C_{21}H_{42}O_4$	358
21	19.842	5.96	2,3-dihydroxypropyl laurate	$C_{15}H_{30}O_4$	274
22	20.552	0.48	Pentadecyl hexanoate	$C_{21}H_{42}O_2$	326
23	20.854	0.84	Linalool oxide, trimethylsilyl ether	$C_{13}H_{26}O_2Si$	242
24	21.106	1.20	.beta.-Stigmasterol	$C_{29}H_{48}O$	412
25	21.281	35.65	Oleic anhydride	$C_{36}H_{66}O_3$	546
26	21.417	2.43	2-Stearoylglycerol	$C_{21}H_{42}O_4$	358
27	21.864	1.27	Oleic acid, 2-hydroxyethyl ester	$C_{20}H_{38}O_3$	326
28	21.906	0.25	Methyl 2-hydroxy-octadeca-9,12,15-trienoate	$C_{19}H_{32}O_3$	308
29	21.968	0.15	Squalene	$C_{30}H_{50}$	410
30	22.028	0.35	Octadecyl hexanoate	$C_{24}H_{48}O_2$	368
31	23.032	0.19	Dodecyl 3-(trifluoromethyl)benzoate	$C_{20}H_{29}F_3O_2$	358
32	24.127	0.26	Stigmasta-3,5-diene	$C_{29}H_{48}$	396
33	25.407	0.43	24-Epicampesterol	$C_{28}H_{48}O$	400
34	25.652	1.11	(22E)-Stigmasta-4,22-dien-3-ol	$C_{29}H_{48}O$	412
35	26.301	1.51	gamma.-Sitosterol	$C_{29}H_{50}O$	414
36	26.772	0.19	beta.-Saccharostenone	$C_{29}H_{46}O$	410
37	27.889	0.51	Ethylcyclodocosane	$C_{24}H_{48}$	336
38	28.823	0.19	Dihydroagarofuran	$C_{15}H_{26}O$	222
39	29.032	1.18	3-Oxo-9.beta.-lanosta-7,22,24-trien-26,23-olide	$C_{30}H_{42}O_3$	450
40	30.983	1.02	N-[2-(tetradecyloxy)phenyl]acetamide	$C_{22}H_{37}NO_2$	347
41	31.223	0.30	Methyl hexadecatrienoate	$C_{17}H_{28}O_2$	264
42	31.384	0.47	cis-15-Tetracosenoic acid, propyl ester	$C_{27}H_{52}O_2$	408
43	33.416	1.95	2-Methylpregn-4-ene-3,20-dione	$C_{22}H_{32}O_2$	328
44	34.062	0.45	24-Epibrassicasterol	$C_{28}H_{46}O$	398

3.5. Total Phenolic Content (TPC), Total Flavonoid Content (TFC) and DPPH Scavenging Activity

The total phenolic content of callus and root extract was estimated by the Folin–Ciocalteu method, using gallic acid as standard. The highest phenolic content was noted in the in vitro grown root, followed by the in vivo grown roots and the least was recorded in the callus extract (Table 6). The TPC value of the in vitro derived root was 9.76 ± 0.21 mg GAE/g DW and the in-field grown root extract was 7.49 ± 0.54 mg GAE/g DW, whereas the lowest TPC was found in the callus extract (3.64 ± 0.45 mg GAE/g DW). The total flavonoid content of different tested samples was determined by the aluminium chloride method using quercetin as the standard and the results were expressed as quercetin equivalent (QE) per gram of dry weight (Table 6). The flavonoid content of extracts ranged from 0.72 to 1.58 mg QE/g DW, showing for about two-fold variation. The flavonoid content of in vivo and in vitro grown root extracts are nearly equal (1.44 and 1.58 mg QE/g DW, respectively), and the lowest TFC was noted to be 0.72 mg QE/g DW in root derived calli. The antioxidant activity of each extract was determined using a DPPH free radical scavenging assay and the activity is presented in Table 6. The antioxidant potential of tested tissues showed a similar trend with TPC and TFC. The level of scavenging activity of the in vitro raised root extract was found to be higher (40.63%) as compared to the in vivo root (31.34%). The lowest scavenging activity percentage (22.90) was observed in callus extract.

Table 6. Total phenolic content, total flavonoid content, and DPPH scavenging activity of callus and root tissues of *G. glabra*.

Sample Type	TPC (mg GAE/g DW)	TFC (mg QE/g DW)	DPPH Scavenging Activity (%)
Root derived callus	3.64 ± 0.45 ^{c}	0.72 ± 0.36 ^{b}	22.9 ± 0.31 ^{c}
In vivo root	7.49 ± 0.54 ^{b}	1.44 ± 0.15 ^{a}	31.34 ± 1.35 ^{b}
In vitro root	9.76 ± 0.21 ^{a}	1.58 ± 0.67 ^{a}	40.63 ± 2.11 ^{a}

Note: TPC: total phenolic content, TFC: total flavonoid content, GAE: gallic acid equivalent, QE: quercetin equivalent, DW: dry weight. Values represent mean ± S.E. of three repeated experiments. Mean values with different superscripts within a column indicate significant mean difference with one another at $p \leq 0.05$ level as per DMRT.

3.6. Antioxidant Enzyme Activities

The peroxidase (POD) activity was assessed in callus, in vivo, and in vitro grown roots of *G. glabra*. Peroxidase activity was found to be significantly higher in root tissue when compared to the root derived callus (Figure 6A). The callus showed the lowest POD activity (0.28 mM/g FW), followed by the in vivo grown root (0.51 mM/g FW). The highest POD activity was displayed by in vitro roots (0.60 mM/g FW), with a two-fold increment in activity as compared to the root derived calli. The superoxide dismutase (SOD) activity was measured in different tissue samples to check the antioxidant potential (Figure 6B). Like POD, a similar trend in SOD activity was detected in different tissues. Considering SOD, callus showed the lowest activity, i.e., 1.11 U/mg FW, followed by the field grown root sample (1.33 U/mg FW). The highest SOD activity was found in roots of tissue cultured shoots (1.47 U/mg FW).

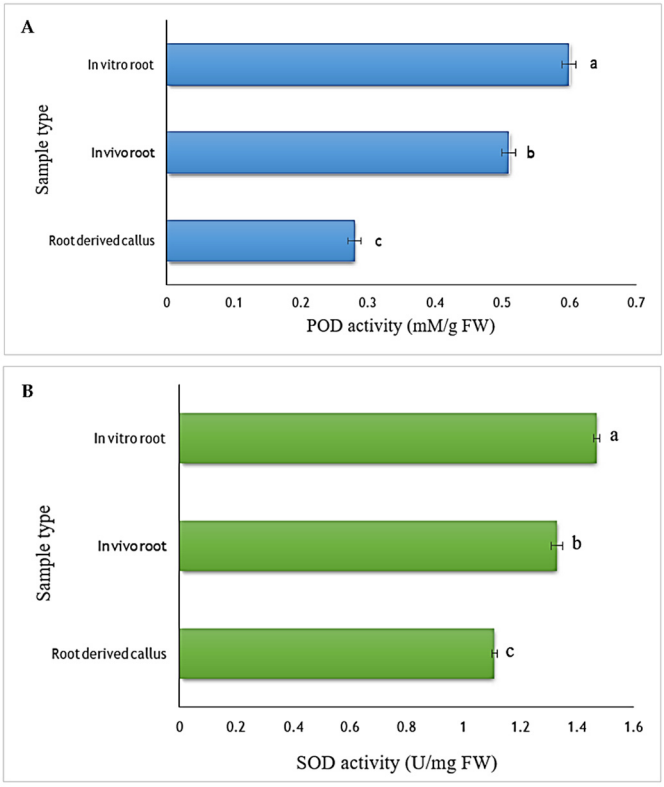

Figure 6. Activities of peroxidase (POD) (**A**) and superoxide dismutase (SOD) (**B**) in callus, and in vivo or in vitro root tissues of *G. glabra* L. Values represent mean ± S.E. of three repeated experiments. Mean values with different letters within the horizontal bar indicate significant mean difference with one another at $p \leq 0.05$ level as per DMRT.

4. Discussion

This study was conducted to study the effects of PGRs, their concentrations, and combinations on the success of some in vitro propagation steps such as callus induction, axillary shoot sprouting, and rooting of in vitro shoots of *Glycyrrhiza glabra* L. The phytochemical and biochemical profiles of in vitro regenerated tissues were also evaluated. Firstly, the root explants were cultured on MS with varying concentrations of PGRs for callus induction, and the results indicated that BAP, along with 2,4-D, showed good callus growth with the highest fresh biomass as compared to the BAP and NAA combinations. A previous study of callus induction from leaf and stem explants of *G. glabra* also reported similar observations [5]. The positive effect of 2,4-D with BAP was previously observed in several other plant species, like *Digitalis ferruginea* [24] and *Dioscorea nipponica* Makino [25]. Axillary buds are normally dormant under wild conditions, However, shoot formation and proliferation can be induced with the help of cytokinins under in vitro conditions [26]. Here, we demonstrated that the BAP alone, or in combination with IAA, showed better efficiency in breaking axillary bud dormancy leading to high shoot formation. Consistent with our observations, similar effects of BAP on shoot induction from dormant buds were noticed in several plants [27,28]. BAP, along with IAA, also showed moderate effects here, in inducing shoots from dormant axillary buds. In addition to cytokinin, auxins are often useful in inducing shoots as these signaling elements are known to overcome the excessive effects of cytokinins [29]. The promotive effect of auxin with cytokinin in shoot formation has also been documented in previous reports of *Glycyrrhiza glabra* L. [1] as well as in different plant species such as *Curcuma zedoaria* [30], *Basella rubra* L. [31] and *Santalum album* L. [32]. The axillary shoots were later transferred to a rooting medium containing different concentrations of IAA and IBA. Shoots cultured on MS supplemented with IBA showed a higher rate of rooting than those of shoots treated with IAA. Ayangla et al. [33]

reported superior effects of IBA over other auxin treatments in inducing roots in organogenic derived shoots of *G. glabra* L. IBA showed similar effects on rooting under in vitro conditions in other plants species such as *Dracaena sanderiana* [34] and *Vaccinium corymbosum* [35]. The high root induction rate induced by IBA is attributed to its stability and easy translocation to different tissues [36].

GC–MS, a popular analytical technique, is widely utilized in the identification of several phytocompounds, such as volatile compounds, long chain hydrocarbons, sugar alcohols, esters, alkaloids, flavonoids, saponins, etc. [12]. Moreover, this technique also provides impactful insights into the role of various in vitro factors on growth and development by detecting changes in peak area % in metabolite profiles of regenerants and their wild relatives [37]. In this investigation, the obtained chromatographic results showed the presence of more than 35 important bioactive compounds in each tested samples. The comparative metabolite profiling of in vivo and in vitro grown root tissues displayed variation in several detected phytocompounds level. The increased production of phytocompounds in the in vitro derived root (compared to intact in vivo plant tissues) may be attributed to various factors such as media composition, hormonal concentration, genotype, photoperiod, temperature, etc., [13,38] and thus, could be a more potent and reliable source of phytocompounds for pharmacological uses. Previously, Vijayalakshmi and Shourie [39] reported the presence of variety of phytochemicals in leaf, stem, and leaf derived callus in *G. glabra*. The identification and quantification of bioactive compounds in various plant parts (root, leaf, stem, anther, callus) by GC–MS have recently been described in different plant varieties like *Tanacetum sinaicum* [40], *Catharanthus roseus* L.,[41] and *Amomum nilgiricums* [42]. A wide number of therapeutically active phytoconstituents have been detected in *G. glabra* samples. Squalene, a terpenoid, possesses various biological activities like anti-oxidant, anti-cancerous, detoxifying and moisturizing agents [43]. Glabridin belongs to flavonoid, showing a diverse range of medicinal potentials, such as protection against cardiovascular diseases, diabetes, bacterial infections and possess estrogenic, anti-inflammatory, anti-cancerous effects [44]. Lupeol is a pentacyclic triterpenoid, known to have anti-inflammatory, anti-microbial, anti-angiogenic, anti-diabetic and anti-protozoal agents [45]. Stigmasterol, found in callus samples, was reported to be associated with anti-tumor, anti-osteoarthritis, antibacterial, anti-oxidant, immunomodulatory, anti-parasitic, anti-fungal and neuroprotective properties [46]. Similarly, n-hexadecanoic acid, which was exclusively found in the in vitro derived root tissue, is known to exhibit anti-inflammatory, anti-bacterial and anti-oxidant properties [47,48]. Consistent with our findings, Khan et al. [3] reported the presence of important flavonoids and triterpenoids in the in vitro cultures of *G. glabra.*

In vitro culture conditions often induce stress in cell lines/regenerated tissues and thereby reduce their rate of survival [9]. Therefore, it is crucial to assess the cellular physiology by monitoring biochemical attributes of tissues at regular interval. The biochemical and antioxidant attributes of in vitro derived tissues were investigated and compared with donor (mother) plant in *G. glabra*. Various factors like different PGRs used in culturing are involved in up- and down-regulation of phenolics and flavonoid biosynthesis [49]. In our study, the phenolic, as well as flavonoid, synthesis was found to be higher in in vitro root tissues as compared to mother plants. This is in accordance with earlier reported biochemical studies conducted in different plant species [50,51]. The antioxidant activities of in vitro raised tissues were tested using three antioxidant, i.e., DPPH, SOD and POD assays, and the data obtained by these studies revealed that the in vitro derived root tissue showed higher anti-oxidant activities than the field grown root and callus samples. Under stressful conditions, the reactive oxygen species (ROS) production in plant tissues exceeds the normal level, which may be reduced by a number of anti-oxidant enzymes like catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), glutathione reductase (GR), etc. [52]. DPPH is a dark colored, stabilized, organic free radical, which transformed into a light yellow color whenever it accepts an electron or a free radical, indicating the scavenging activity [53]. SOD is a scavenger of superoxide radical and helps to manage H_2O_2 levels

[54], whereas POD oxidizes co-substrates, and promotes decomposition of H_2O_2 [55]. In this study, the higher degree of antioxidant potential of laboratory grown root tissue is due to the positive correlation of phenolics and flavonoids with antioxidant activity as these molecules donate hydrogen atoms to free radicals, leading to their deactivation [20]. Similar results of antioxidant potential were observed in different plant varieties like *Salvia hispanica* [56], *Zingiber officinale* [57], *Tylophora indica* [58], *Thalictrum foliolosum* [59], etc. The above evidences elucidates that in vitro derived tissues, such as callus and root, possess immense pharmacological values in terms of phytoconstituents and identifying such compounds could pave the way for novel drug discovery in shorter period of time with minimal cost. Further studies, like molecular docking and bio-prospecting, could be carried out in the future to understand the ligand-protein interaction and biological activities of these significant bioactive compounds.

5. Conclusions

This work focused on the comparative metabolite profiling of in vivo and in vitro derived root tissues of *G. glabra* for the first time using the GC–MS technique. The analyses displayed a wide variety of phytocompounds ranging from alkaloids, flavonoids, phenolics, terpenoids, sugars, sterols, etc. The root derived callus showed the presence of 44 phytochemicals, which all have diverse therapeutic importance. The biochemical and antioxidant assessment of callus, in vivo, and in vitro grown root tissues were compared, wherein in vitro roots showed a higher presence of phenolics and flavonoids, as well as more antioxidant potential than the other tested tissues. This study reports the presence of various medically beneficial bioactive compounds from in vitro derived plant tissues, which can be utilized by the pharmaceutical industry for their large-scale production.

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