

Establishment of *Gypsophila paniculata* root culture for biomass, saponin, and flavonoid production

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Abstract

Baby's breath (*Gypsophila paniculata* L.) roots are valuable as pharmaceuticals due to the content of triterpenoid saponins and other bioactive phytochemicals. However, the long root harvest period and fluctuation of these constituent's content are among the constraints to traditional agricultural production. The present study shows an efficient and reliable liquid root culture of *G. paniculata* cv. 'Perfecta' established in a shake flask system using different auxins, media, and sugars. Hairy root (HR) induction in *G. paniculata* through *Rhizobium rhizogenes*-mediated transformation was also investigated. α -Naphthaleneacetic acid (NAA) was optimized for adventitious roots (AR) biomass, saponin, and flavonoid production at 1 or 2 mg/L compared to IBA. Full strength Gamborg's medium (B5) recorded higher saponin content; however, the highest yield of total saponin and total flavonoids was achieved by full strength Murashige and Skoog's (MS) medium. Sucrose was more essential for root growth and accumulated total saponins and flavonoids rather than fructose and glucose. Polymerase chain reaction (PCR) analysis showed that *G. paniculata* HR carried *rolC* gene of *R. rhizogenes* A4 strain in its genome but not *virD2* gene. Compared to non-transformed root, saponin content of leaf and stem-derived HR was 2.7 and 2.3-fold, while total flavonoid content was 2.1 and 2.0-fold, respectively. The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was also higher in HR extracts than in non-transgenic roots. This study established an efficient protocol for *G. paniculata* root cultures for sustainable production of important natural saponins and flavonoids.

Keywords: adventitious roots; baby's breath; DPPH; flavonoid; hairy root; saponin

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Introduction

Gypsophila paniculata L. (baby's breath) is a perennial plant that is well known for its decorative, medicinal, and industrial applications. The genus *Gypsophila* (Caryophyllaceae) contains 150 species native to the temperate regions of Eurasia (Li *et al.*, 2022). *G. paniculata* is globally grown and represents a significant cut flower plant in commercial floriculture production and floral arrangements with fresh or dried flowers (Li *et al.*, 2020). It ranks as one of the 10 best-selling cut flower species globally (Vettori *et al.*, 2015). *G. paniculata* is an herb that can reach 1-1.5 m in height with erected shoots and greyish green leaves (5-8 cm long). The elongate bipartite cymes are very dense inflorescences, with small round flowers. The roots, which are the most important part both medically and industrially, are branched into thick and long branches (Vettori *et al.*, 2015).

Phytochemical examination of *G. paniculata* proved the existence of different groups of biologically active secondary metabolites, e.g., saponins, flavonoids, and phenolic compounds (Zdraveva *et al.*, 2015; Kołodziej *et al.*, 2019). The flavonoid saponarin was reportedly detected in *G. paniculata*, *G. elegans*, *G. altissima*, *G. trichotoma*, and *G. glomerata*. The reports have shown that saponarin possesses antioxidant, hypoglycemic, antimicrobial, and hepatoprotective activities (Zdraveva *et al.*, 2015; Popova *et al.*, 2022). The triterpenoid saponins are the main phytoconstituents in *G. paniculata* roots (Henry *et al.*, 1991). The roots of *G. paniculata*, *G. pacifica*, and *G. scorzonrifolia* exhibited high antifungal and antioxidant activity (Kołodziej *et al.*, 2019). Moreover, a saponin from *Gypsophila* species is used in food and chemical industries and as shampoo and soap materials (Kołodziej *et al.*, 2019). Gevrenova *et al.* (2014) showed that gypsogenin 3-*O*-glucuronide derivatives, a saponin found in the root extract of *G. paniculata*, was responsible for the cytotoxicity in macrophage cells. Furthermore, natural saponins have become a potential drug delivery system carrier (Liao *et al.*, 2021). In this context, triterpenoid saponins of *G. paniculata* and *G. arrostii* improved the selective cytotoxic effect of targeted toxins (Korchowiec *et al.*, 2022). These considerations led to the demand of increase saponin content from *G. paniculata* roots.

During the vegetative cycle of *G. paniculata* plant, the biosynthesis and accumulation of saponin only occur in the root, reaching 4% of dry weight (DW), as gypsogenin 3-*O*-glucuronide, in the fourth vegetative cycle (Henry *et al.*, 1991). Although this saponin is accumulated in a considerable amount in *G. paniculata* roots, 4% of DW, the long period of harvest - after 3-4 years (Kołodziej *et al.* 2019) and the fluctuation of saponin content due to seasonal variations and plant development stages may hinder the production of these essential bioactive compounds (Henry *et al.*, 1991). Moreover, Saponinum album, a commercial crude mixture (Merck) of triterpenoid saponin of *G. paniculata* and *G. arrostii*, cannot be used for further biological studies because Merck discontinued its production in the 1990s (Korchowiec *et al.*, 2022). Therefore, alternative options should be considered to solve such a problem. Hence, the production, extraction, and application of *G. paniculata* active ingredients can be facilitated using *in vitro* culture techniques (Gevrenova *et al.*, 2010).

Compared with conventional cultivation, plant cell, tissue, and organ cultures offer alternative systems to produce various bioactive metabolites (Elateeq *et al.*, 2020; Elshahawy *et al.*, 2022). Among biotechnological approaches, *in vitro* root culture is an alternative technique and is considered a complementary method to entire plant cultivation. This technique supplies plant roots in large quantities without being restricted to a specific season, under constant conditions, in limited time, and a small space, as well as without being influenced by environmental stress factors (Gabr *et al.*, 2019; Murthy *et al.*, 2021). Moreover, root culture shows high stability, high proliferation with fast growth, and a copious content of bioactive metabolites (Matvieieva *et al.*, 2022). In recent reports, *in vitro* root cultures produced greater amounts of phytoconstituents than donor plant, as well as compounds not located in the intact plants (Gehlot *et al.*, 2022). Root cultures can be divided into two groups –non-transformed and transformed or transgenic root (hairy

root) cultures which are transformed by *Rhizobium rhizogenes* bacteria (formerly known as *Agrobacterium rhizogenes*) (Li *et al.*, 2016; Gabr *et al.*, 2019). Successful attempts to produce phytochemical compounds on a large scale have been developed in industrial bioreactors for hairy root (HR) and adventitious root (AR) cultures (Jiang *et al.*, 2015; Wu *et al.*, 2018; Pandey *et al.*, 2022a), which will allow industrial production of natural bioactive compounds from plant roots. Studies show that root growth and their capability for the biosynthesis of various biologically active compounds are affected by types and levels of carbohydrate source, plant growth regulators (PGRs), culture media, elicitation as well as culture condition (Jiao *et al.*, 2014; Wu *et al.*, 2018; Cui *et al.*, 2020; Jiao *et al.*, 2020).

To our knowledge, few reports on *in vitro* production of saponin from *G. paniculata* are available in multiple shoots, callus cultures (Pauthe-Dayde *et al.*, 1990; Henry, 1993; Hanafy and Abou-Setta, 2007), and excised root cultures (Henry *et al.*, 1991; Fulcheri *et al.*, 1998; Herold and Henry, 2001; Gevrenova *et al.*, 2010) but not in AR and HR cultures. In these previous studies, various treatments such as different explants and culture media, subculture number, and growth regulators have been tested. As roots of *G. paniculata* are considered the primary organ for saponin production, the present study was carried out to establish the root suspension culture of *G. paniculata* under the influence of auxins, culture media, and sugar levels for the accumulation of root biomass, saponin, and flavonoid. HR induction in *G. paniculata* through *R. rhizogenes*-mediated transformation was also studied.

Materials and Methods

Plant material and explant preparation

Shoots (10 cm) of *G. paniculata* cv. 'Perfecta' (white flower), three months old, were kindly gifted from Floramix Egypt Company, Giza, Egypt. Shoot tips (1 cm) were washed with running water, then immersed for 1 min in 70% ethanol followed by 20 min in 1% sodium hypochlorite solution containing two drops of Tween 20. Further, the sterile explants were washed with sterile distilled water and cultured in jars containing Murashige and Skoog's (1962) (MS) basal medium and 30 g/L sucrose, solidified with 8 g/L agar and supplemented with 6-benzyl adenine (BA) (Sigma-Aldrich Co. Ltd., Dorset, UK) and NAA (Sigma-Aldrich Co. Ltd., Dorset, UK) each at 0.5 mg/L for shoot production. For *in vitro* rooting, healthy shoots were excised after four weeks of incubation and transferred to half strength MS medium ($\frac{1}{2}$ MS) supplemented with 1 mg/L NAA + 3 mg/L indole-3-butyric acid (IBA) (Sigma-Aldrich Co. Ltd., St. Louis, MO, USA) according to our previous experiments (Toaima *et al.*, 2016). The *in vitro* rooted shoots provided the roots required to establish AR cultures. Roots were excised after four weeks of culture.

Establishment of adventitious root cultures

Effect of auxin

The excised roots (1-2 cm long) were transferred into flasks (Erlenmeyer; 250 mL) containing 50 mL of MS liquid medium with 30 g/L sucrose and amended with NAA or IBA at 0.25, 0.5, 1, and 2 mg/L. MS-free auxin was used as a control treatment. After four weeks of culture, the obtained AR was used in the further experiments of culture media and sugar type.

Effect of culture media and sugar

Roots [about 0.7 g fresh weight (FW)], established on MS with 2 mg/L NAA, were inoculated in a flask containing 50 mL of the examined liquid medium and 2 mg/L NAA (one explant was cultured per flask). The tested media were MS [full strength (1MS), half strength ($\frac{1}{2}$ MS), and quarter strength ($\frac{1}{4}$ MS)] and Gamborg *et al.* (1968) B5 [full strength (1B5), half strength ($\frac{1}{2}$ B5), and quarter strength ($\frac{1}{4}$ B5)].

For the effect of sugar, the roots were cultured on IMS supplemented with 2 mg/L NAA. Sucrose, fructose, and glucose were tested at 15, 30, and 45 g/L. For control culture, MS medium devoid of any sugar was used.

Flasks were placed on a shaker at 110 rpm. Cultures were incubated in a growth chamber under 25±2 °C and completely dark.

R. rhizogenes-mediated transformation of *G. paniculata*

Preparation of *R. rhizogenes*

R. rhizogenes A4 strain was initiated from glycerol stock and maintained on a YEB-solid medium (1 g/L yeast extract, 5 g/L beef extract, 5 g/L sucrose, 5 g/L peptone, 300 mg/L MgSO₄·7H₂O, and 18 g/L agar) for 48 h at 28 °C in the dark. A single clone was re-growth for 24 h in 20 mL YEB-liquid medium at 28 °C on a rotary shaker at 100 rpm in the dark.

Establishment of hairy root culture

Leaf and stem explants excised from healthy shoot cultures were used to establish HR cultures. The explants were immersed in bacterial suspension (OD_{600 nm} = 1.0) for 5 min. Subsequently, the infected explants were dried on sterile filter paper to remove excess bacteria and co-cultivated on solid or liquid hormone-free MS medium with 30 g/L sucrose. Liquid cultures were kept on a rotary shaker at 100 rpm under dark conditions at 25±2 °C and a solid medium without shakings for 2 days. After that, the infected explants were transferred to the same fresh medium with 500 mg/L cefotaxime to eliminate bacteria and incubated under the same conditions for 14 days. Next, the explants, including HR were subcultured into a hormone-free MS solid medium with 300 mg/L cefotaxime and incubated under the same conditions. Roots from uninfected stems were used as control (non-transformed culture).

HR from stem and leaf explants and non-transformed roots were separated and transferred to jars containing 40 mL of hormone-free MS medium and 30 g/L sucrose, solidified with 7 g/L agar, and subcultured three times (four weeks intervals). After the third subculture, roots were collected, and the FW, DW, saponin, and flavonoid content were determined.

The pH value of all tested media was fitted to 5.8 with NaOH or HCl (1N) and autoclaved at 121 °C and 1.2 kg.F./cm² for 20 min.

Measurements and determinations

PCR detection of transgenic hairy root culture

Transformation of HR cultures was confirmed using polymerase chain reaction (PCR). Genomic DNA of control stem root and HR cultures was extracted using the CTAB (Cetyltrimethyl Ammonium Bromide) method (Doyle and Doyle, 1987). The sequences of the primers used to amplify a fragment of the *rolC* gene were as follows: *rolC* forward, 5'- TGTGACAAGCAGCGATGAGC3', reverse, 5'- GATTGCAAACCTTGCACTCGC-3', product size 490 bp. In addition, the *virD2* gene was detected using 5'-ATGCCCGATCGAGCTCAAGT-3' and 5'-CCTGACCCAAACATCTCGGCT-3' forward and reverse primers, respectively, amplifying a fragment of 338 bp (Medina-Bolivar *et al.*, 2007). A PCR (BIO-RAD C1000 Touch™ Thermal Cycler, USA) protocol was performed for *rolC* gene under the following condition (Gabr *et al.*, 2018): initial denaturation at 95 °C for 4 min, followed by 30 cycles of amplification (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min) and 5 min at 72 °C. For the *virD2* gene, the program was: initial denaturation at 95 °C for 3 min, followed by 30 cycles of amplification (95 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s) and 10 min at 72 °C. Amplified products were analysed on 1.5% agarose gel.

Determination of root biomass

Roots were collected from liquid media after the incubation period (4 weeks), and the FW was recorded after rinsing with distilled water and blotting dry with tissue paper. DW of roots was determined after drying at 45 °C in the oven for 24 h. Dry matter percentage, growth index, and relative growth rate were determined as follows according to Ho *et al.* (2018):

$$\text{Dry matter (\%)} = (\text{final DW} / \text{final FW}) \times 100$$

$$\text{Relative growth rate (RGR)} = [(\ln \text{ final DW}) - (\ln \text{ initial DW})] / \text{incubation period,}$$

where ln: natural log, and the incubation period is 4 weeks.

$$\text{Growth index} = (\text{final DW} - \text{initial DW}) / \text{initial DW}$$

Determination of total saponin and total flavonoid

Total saponin content (TSC) was determined by following the published procedure of Zhang and Wang (2009) with minor modifications. Roots (250 mg DW) were extracted with 80% methanol (10 mL) and incubated at 25 °C for 12 h, then filtered and evaporated to dryness (not higher than 40 °C). The dried residue was re-extracted with water-saturated n-BuOH (5 mL). After evaporating the n-BuOH phase to dryness (not exceeding 45 °C), methanol (10 mL) was added to dissolve the residue. Vanillin solution (0.5 mL; 8%, dissolved with 99.9% ethanol) was mixed with 0.25 mL of methanol extraction solution and 5 mL H₂SO₄ (72%, on the inner side wall of the tube) and left for 3 min after mixing. The tubes were warmed to 60 °C in a water bath for 10 min. After rapid cooling in iced water, the absorbance of the reaction mixture was measured at 544 nm against blank with a JENWAY 6800 UV/Vis. spectrophotometer (Bibby Scientific Ltd., UK) after the solution reached room temperature. Standard solutions of saponin (BDH Chemicals Ltd., Poole, England) were used to determine the calibration curve. The TSC was expressed as mg/g DW.

Total flavonoid content (TFC) was determined using the colorimetric method of aluminum chloride, according to Chang *et al.* (2002) and Madaan *et al.* (2011). Roots (100 mg DW) were extracted with 80% methanol (5 mL), incubated at 25 °C for 24 h, and then filtered. 1.5 mL of methanol (95%) was mixed with 0.5 mL of methanol extraction solutions, 0.1 mL of potassium acetate (1M), 0.1 mL of aluminum chloride (10%) and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm against blank with a JENWAY 6800 UV/Vis. spectrophotometer (Bibby Scientific Ltd., UK). Standard solutions of quercetin (Sigma-Aldrich Co. Ltd., Germany) were used to determine the calibration curve. The TFC was expressed as mg quercetin/g DW.

$$\text{Saponin and flavonoid yield (mg/L)} = \text{TSC or TFC (mg/g DW)} \times \text{root biomass yield (DW g /L)}$$

Determination of free radical scavenging activity

Dried roots (100 mg) were extracted with 5 ml ethanol (95%) for 24 h at 25±2 °C. After filtration, the antioxidant activity of root extracts was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) test according to Elateeq *et al.* (2021). Three ml of DPPH ethanol solution (200 µM) (Phygene Biotechnology Co. Ltd., China) was mixed with ethanol extract (0.7 ml). The mixture was shaken and incubated for 30 min at 25±2 °C in the dark. The absorbance was detected at 517 nm using JENWAY 6800 UV/Vis. spectrophotometer (Bibby Scientific Ltd., UK). The percentage of DPPH radical scavenging activity was calculated using the following formula:

$$\text{DPPH radical scavenging activity (\%)} = [(A \text{ control} - A \text{ sample}) / A \text{ control}] \times 100$$

where, A control is the absorbance of the DPPH solution with 0.7 ml ethanol (95%) free sample, while A sample is the absorbance of the root extract mixed with DPPH solution.

The statistical analysis

All experiments were conducted in a complete randomized design (CRD). Each treatment comprised 5 flasks (for AR) or 5 jars (for HR) and was repeated twice. The statistical analysis of data was subjected to Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) (Duncan, 1995) at

$P < 0.05$ for the significant differences between treatments using COSTAT package ver. 6.4 (CoHort Software Monterey, Pacific Grove, CA, USA), and the results are represented by means with a Standard Error (\pm SE).

Results and Discussion

Establishment of adventitious root culture

Effect of auxins on root biomass, saponin and flavonoid production

Root biomass production

The establishment of *G. paniculata* root cultures affected by auxins application in the mode of liquid culture was studied. After the incubation period, AR proliferated and turned into tangled and round shapes. Roots have grown significantly in the surrounding outer areas with yellowish-white color and brownish-yellow color in the central interior area (Figure 1), consistent with previous observations of Zhang *et al.* (2013) on *Psammosilene tunicoides* (Family Caryophyllaceae). The changes in root colour could be due to the sufficient supply of oxygen and nutrients to surrounding tissues, but on the contrary, they are not adequately provided for the interior roots (Zhang *et al.*, 2013).

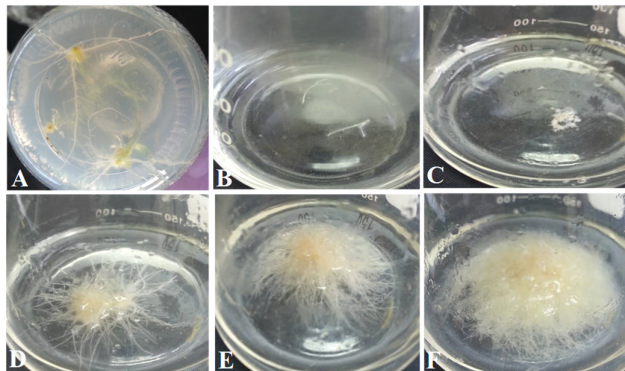


Figure 1. Development of *G. paniculata* root cultures, (A) *in vitro* rooting, (B) initial root culture, (C) 1 week, (D) 2 weeks, (E) 3 weeks and (F) 4 weeks of cultivation

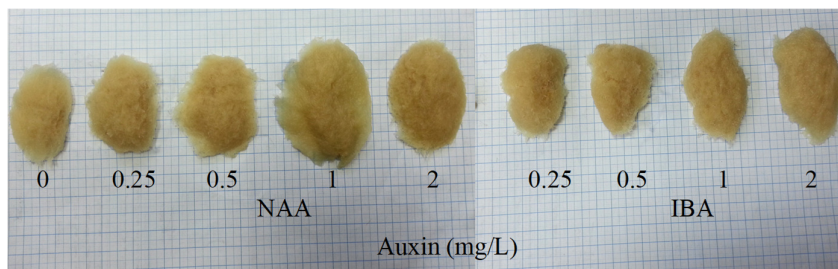
Root biomass accumulation was of highly significant value enhanced by auxin supplementation (Table 1 and Figure 2). In total, NAA improved the root biomass FW and DW compared to IBA. Moreover, the % dry matter was higher under most NAA levels than IBA, with the highest percentage (5.84%) at 2 mg/L NAA. The FW and DW were increased by increasing the concentration of both auxins (except for FW at higher level of NAA; 2 mg/L). The highest significant FW (12.11 g/flask, equivalent to 242.27 g/L medium) was achieved with 1 mg/L NAA. However, the highest DW (0.59 g/flask, equivalent to 11.80 g/L medium) was harvested from the medium enriched with a higher level of NAA (2 mg/L), followed by 1 mg/L NAA compared to auxin free medium (control).

Table 1. Effect of NAA and IBA levels on biomass production of *G. paniculata* root cultures after 4 weeks of incubation period

Growth regulators	Levels (mg/L)	Root biomass FW		Root biomass DW		Dry matter (%)
		(g/flask)	Yield (g/L)	(g/flask)	Yield (g/L)	
Control	0	6.11±0.35 ^f	122.13±6.93 ^f	0.32±0.01 ^c	6.40±0.23 ^c	5.25±0.11 ^{bc}
NAA	0.25	8.49±0.23 ^c	169.87±4.62 ^c	0.48±0.02 ^b	9.60±0.35 ^b	5.67±0.36 ^{ab}
	0.5	9.40±0.23 ^{cd}	187.93±4.62 ^{cd}	0.50±0.01 ^b	10.00±0.23 ^b	5.32±0.01 ^{bc}
	1	12.11±0.17 ^a	242.27±3.47 ^a	0.57±0.01 ^a	11.40±0.23 ^a	4.71±0.03 ^d
	2	10.11±0.23 ^{bc}	202.20±4.62 ^{bc}	0.59±0.01 ^a	11.80±0.12 ^a	5.84±0.08 ^a
IBA	0.25	8.90±0.06 ^{de}	178.00±1.15 ^{de}	0.40±0.02 ^d	8.00±0.46 ^d	4.49±0.23 ^d
	0.5	9.00±0.29 ^{de}	180.00±5.77 ^{de}	0.43±0.02 ^{cd}	8.60±0.35 ^{cd}	4.78±0.04 ^d
	1	10.02±0.15 ^{bc}	200.33±2.96 ^{bc}	0.46±0.01 ^{bc}	9.20±0.23 ^{bc}	4.59±0.05 ^d
	2	10.17±0.15 ^b	203.46±2.92 ^b	0.50±0.01 ^b	10.00±0.23 ^b	4.91±0.04 ^{cd}

Data are presented as mean±SE, n=3. Different characters in the same column indicate significant differences and the same characters indicate insignificance according to DMRT at $P<0.05$

Similarly, Reis *et al.* (2011) reported that AR of *Stevia rebaudiana* was established by transferring root explants to MS media with 2 mg/L NAA. NAA has also been superior to IBA for AR culture in *Cleome rosea* (Cordeiro *et al.*, 2015) and *Valeriana jatamansi* (Pandey *et al.*, 2022b). Under *in vitro* system, NAA is rapidly absorbed and utilized more by the plant cell than IBA and indole-3-acetic acid (IAA) resulting in greater cell and tissue biomass (Pandey *et al.*, 2022b).

**Figure 2.** Effect of NAA and IBA levels on root production of *G. paniculata* after 4 weeks incubation period

Total saponin production

AR harvested from media augmented with NAA accumulated total saponin higher than the hormone-free medium (Figure 3A). Unlike NAA, only 0.5 and 1 mg/L of IBA enhanced saponin accumulation. The highest TSC (7.22 mg/g DW) was recorded for the higher supplementation of NAA (2 mg/L) as against the control roots (5.11 mg/g DW).

Total saponin productivity was calculated by considering the TSC (mg/g DW) and the dry root yield (g/L medium). Although exogenous IBA only enhanced the TSC at 0.5 and 1 mg/L than control, total saponin yield was higher than control at all tested concentrations of IBA (Figure 3A), because of the dry matter at all IBA concentrations was higher than that recorded for control (Table 1). The highest saponin yield (85.2 mg/L medium) was calculated for the roots grown in a 2 mg/L NAA-containing medium, while hormone-free cultures produced the lowest yield of saponin (32.7 mg/L). Our findings are in harmony with that of Kumar *et al.* (2015) on *Hibiscus cannabinus*, who reported that efficient root biomass and ascorbic acid production were recorded for roots cultivated on MS with 2 mg/L NAA.

Total flavonoid production

The data illustrated in Figure 3B show that IBA was more suitable than NAA for flavonoid production. The lower rate of IBA (0.25 mg/L) optimized the TFC in root cultures which recorded the most significant

response (5.77 mg/g DW). However, this response was markedly suppressed when the medium was amended with >0.25 mg/L IBA. Although NAA cannot enhance the TFC in *G. paniculata* root cultures, the productivity of total flavonoid reached the highest yield (52.8 mg/L) with 1 mg/L NAA versus hormone-free culture (29.0 mg/L) – Figure 3B.

It shows that the accumulation of AR biomass and bioactive secondary metabolites (saponin and flavonoid) in *G. paniculata* is auxins dependent. The process of PGRs decarboxylation results in the accumulation of free radicals and reactive oxygen species (ROS) in the plant tissue, which may explain the increase of secondary metabolites synthesis under auxin addition (Folkes and Wardman, 2001). It was proved that auxins supplementation to plant tissue culture medium enhances the synthesis of *de novo* proteins (Teale *et al.*, 2006), which may help stimulate the biosynthesis of various metabolites.

DPPH radical scavenging activity

Root extracts of *G. paniculata* exhibited 68.36 – 82.02% scavenging activity of DPPH compared to the positive control (ascorbic acid), which showed 97.82% (Figure 4A). All tested levels of NAA positively enhanced the antioxidant activity compared to control (75.29%) and IBA treatments. This may be related to the pronounced effect of NAA on saponin accumulation, which enhances the antioxidant capacity. NAA at 0.25 mg/L recorded the highest value of antioxidant activity (82.02%), followed by other levels of NAA. Our findings confirmed the results of Ataslar *et al.* (2019) and Kołodziej *et al.* (2019) that gypsophila saponins can act as natural antioxidants to protect cells from oxidative damage.

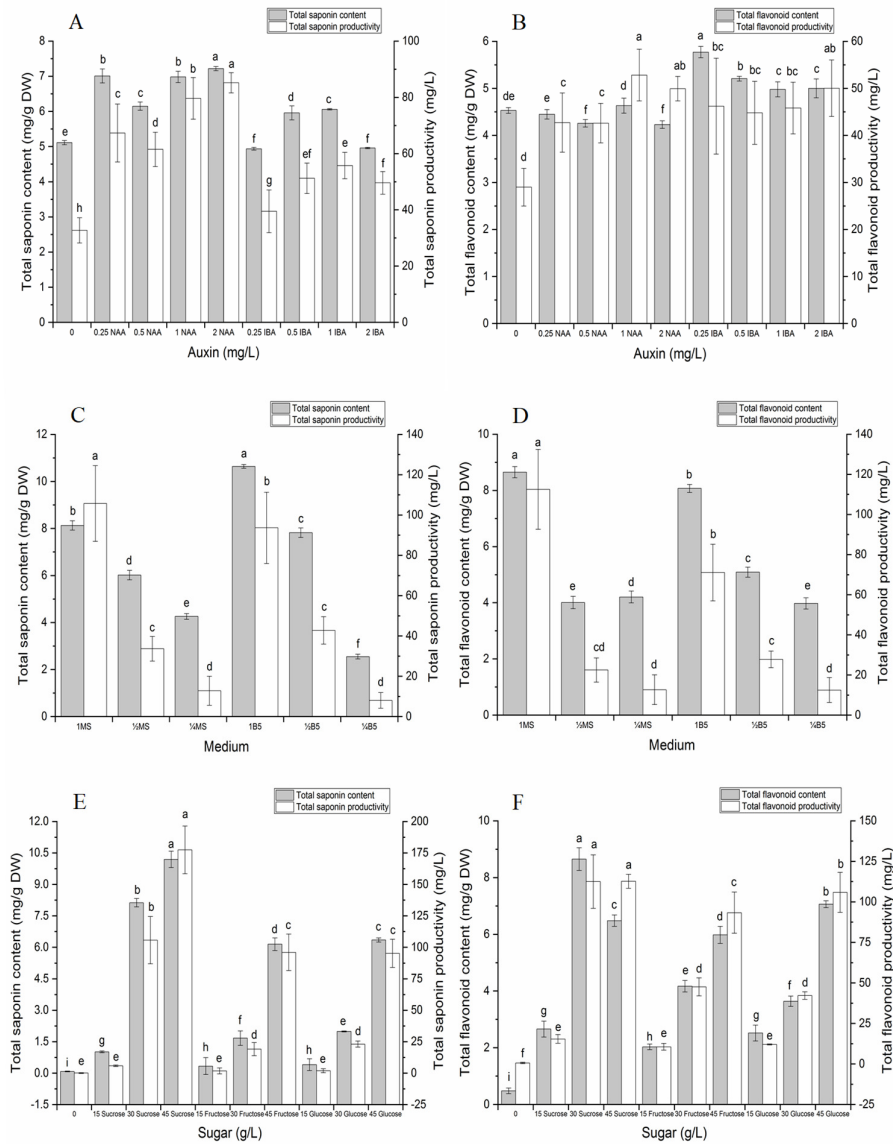


Figure 3. Effect of auxin, culture media, and sugar on total saponin production (A, C, and E, respectively) and total flavonoid production (B, D, and F, respectively) of *G. paniculata* root cultures after 4 weeks incubation period. The bars represent SE, n=3. Columns annotated with different characters are significantly different according to DMRT at $P < 0.05$

Effect of culture media on root biomass, saponin and flavonoid production

Root biomass production

It is crucial to find the optimum type and concentration of the nutrient medium for AR cultures which is considered a critical factor for root growth and phytochemicals accumulation (Cui *et al.*, 2020). The data of root growth parameters (FW, DW, % dry matter, RGR, and growth index) are compiled in Table 2. It could be observed that root biomass production is significantly influenced by both type and strength of culture media (Table 2 and Figure 5). In this concern, MS media was superior to B5 for biomass FW. This may be owing to a higher level of inorganic nutrients in the MS medium than in B5 one (Pakdin Parizi *et al.*, 2015). The favourable media strength for root biomass accumulation was 1MS which exhibited the maximum FW (343.73

g/L medium), DW (13.00 g/L medium), RGR (0.69), and growth index (14.85). Correspondingly, Yin *et al.* (2013b) on *Pseudostellaria heterophylla* (Caryophyllaceae) found that 1MS recorded the highest growth rate, FW, and DW of AR compared to other levels.

A pronounced decline in root fresh and dry masses was observed when the strength level of each media was decreased to half ($\frac{1}{2}$) or to quarter ($\frac{1}{4}$) levels, which is attributed to a deficiency in essential components needed for cellular anabolism. However, the highest percentages of root dry matter (5.46 and 4.57%) were achieved with $\frac{1}{2}$ B5 and $\frac{1}{4}$ B5, respectively. In general, the initial inoculum was approximately 0.82 g DW/L medium (0.041 g DW/flask). Here, the biomass production of root DW was increased to 3.00-13.00 g/L, equivalent to 3.66 - 15.85 times higher than the initial inoculum DW.

Total saponin production

In contrast to root biomass production, TSC was significantly enhanced in *G. paniculata* roots grown on a B5 medium (Figure 3C). The TSC was optimized and reached the maximum significant value (10.64 mg/g DW) with 1B5 compared to 1MS (8.13 mg/g DW). B5 medium contains higher levels of vitamins (nicotinic acid, thiamine HCl, and pyridoxine HCl) than MS, which are essential for *in vitro* growth and active metabolites biosynthesis. In conformity with these results, Lee *et al.* (2011) reported that MS was the best medium for root growth while aloe-emodin production is better using B5 in AR cultures of *Aloe vera*.

Even though root cultures grown on 1B5 accumulated the highest TSC, roots cultivated in 1MS media yielded the highest total saponin compared to 1B5 (105.8 mg/L medium versus 93.6 mg/L medium) (Figure 3C), as the DW of roots harvested from 1MS was higher than that from 1B5 (Table 2).

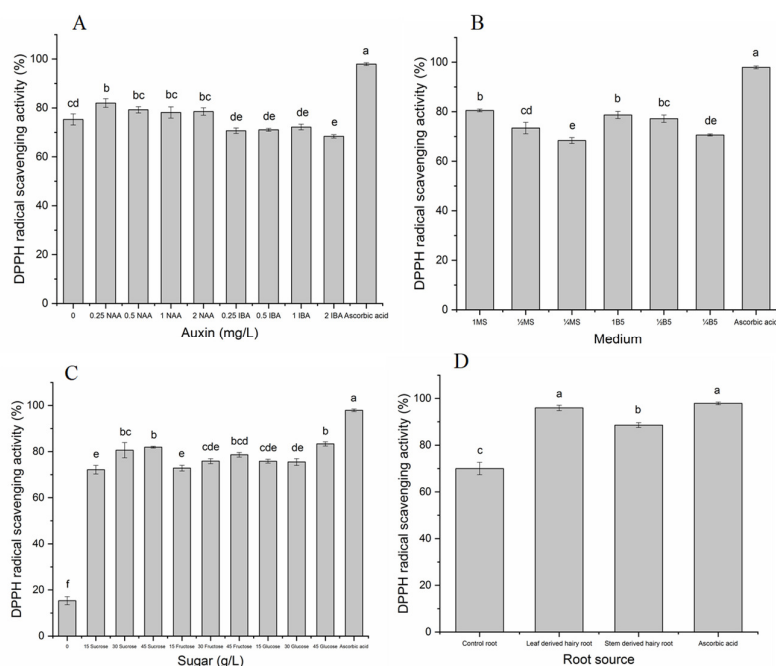


Figure 4. Effect of auxin, culture media, sugar, and root source on DPPH radical scavenging activity (A, B, C, and D, respectively) of *G. paniculata* root cultures after 4 weeks incubation period. The bars represent SE, n=3. Columns annotated with different characters are significantly different according to DMRT at $P < 0.05$

Table 2. Effect of MS and B5 media strength levels on biomass production of *G. paniculata* root cultures after 4 weeks of incubation period

Media type	Strength level	Root biomass FW Yield (g/L)	Root biomass DW Yield (g/L)	Dry matter (%)	Relative growth rate	Growth index
MS	1	343.73±8.09 ^a	13.00±0.58 ^a	3.78±0.08 ^{bc}	0.69±0.01 ^a	14.85±0.70 ^a
	½	191.93±11.55 ^c	5.60±0.35 ^c	2.92±0.01 ^c	0.48±0.02 ^c	5.83±0.42 ^c
	¼	103.73±8.09 ^d	3.00±0.44 ^d	2.86±0.23 ^c	0.32±0.04 ^d	2.66±0.54 ^d
B5	1	223.67±6.94 ^b	8.80±0.46 ^b	3.93±0.09 ^{bc}	0.59±0.01 ^b	9.73±0.56 ^b
	½	103.00±9.81 ^d	5.47±0.29 ^c	5.46±0.82 ^a	0.47±0.01 ^c	5.67±0.35 ^c
	¼	68.00±4.62 ^c	3.13±0.41 ^d	4.57±0.30 ^{ab}	0.33±0.03 ^d	2.82±0.49 ^d

Data are presented as mean±SE, n=3. Different characters in the same column indicate significant differences and the same characters indicate insignificance according to DMRT at $P<0.05$

Total flavonoid production

Figure 3D show that full concentrations of both media (1MS and 1B5) were favourable for flavonoid production. The highest significant TFC and flavonoid yield were recorded for roots grown on 1MS (8.65 mg/g DW and 112.5 mg/L medium, respectively) followed by 1B5 (8.07 mg/g DW and 71.1 mg/L medium, respectively). More recently, the TFC in *G. glomerata* shoot and callus cultures grown on MS medium were 0.36 and 2.00 mg/g DW, respectively (Popova *et al.*, 2022). Our results correspond to the results of Jiao *et al.* (2014) on *Astragalus membranaceus* HR. They concluded that 1MS recorded the highest biomass DW and total isoflavonoids production as against other media and various levels of MS. Also, in AR of *Glycyrrhiza uralensis*, 1MS exhibited higher yields of root DW, glycyrrhizic acid, total flavonoid, and polysaccharide (Li *et al.*, 2016). On the other hand, saponin and flavonoid production were significantly dropped when the roots were grown on media reduced to half or quarter strengths. These lower levels of culture media seem unsuitable for root biomass production and bioactive compound accumulation because the essential nutrient components are insufficient to support root growth and metabolism.

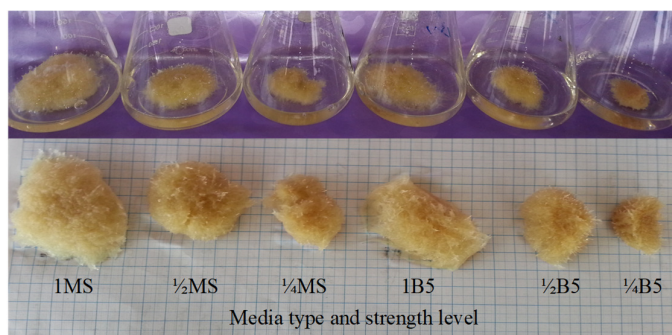


Figure 5. Effect of MS and B5 media strength on root production of *G. paniculata* root cultures after 4 weeks incubation period

DPPH radical scavenging activity

As shown in Figure 4B, the highest significant antiradical activity was reported for roots cultivated on 1MS, 1B5, and ½B5 media (80.51, 78.72, and 77.19%, respectively). Accordingly, these levels of culture media produced copious amounts of saponin and flavonoid higher than roots grown on low-strength media, which enhanced their antioxidant capacity. The antiradical activity of an extract is mainly due to the ability of the hydroxyl groups in the extract compounds to donate hydrogen. The presence of *ortho*-dihydroxy groups in the B-ring of the flavonoid structure is the most critical determinant for antioxidant activity (Krstić-Milošević *et*

al., 2013). Moreover, likely, the differences in the levels of antioxidant capacity of the roots are related to their different levels of saponins and flavonoids.

Effect of sugar types and levels on root biomass, saponin, and flavonoid production

Root biomass production

Carbohydrates are molecules that can act as a source of carbon and energy. Additionally, it can alter the expression of the gene and the activity of some enzymes involved in cell metabolism, development, and growth (Hossain *et al.*, 2017). The results suggest that carbon sources (sucrose, fructose, and glucose) and their levels (15, 30, and 45 g/L) are critical determinants for AR growth of *G. paniculata* (Table 3). The root growth was almost stopped in the sugar-free culture (control). Root biomass production was increased gradually by increasing the sugar level in the culture medium. In particular, the highest root FW (470.00 g/L medium), DW (17.41 g/L medium), RGR (0.76), and growth index (20.22) were obtained from cultures fed with 45 g/L sucrose. Undoubtedly, sucrose is considered one of the essential carbon sources for the plant; easily catabolized by plant tissues into glucose and fructose, provides energy rapidly, and participates in cell growth and development (Gehlot *et al.*, 2022). The study of Sivanesan and Jeong (2009) also reported a decline in root growth of *Plumbago zeylanica* when sucrose was replaced with fructose or glucose in the root culture medium.

Comparatively, less root biomass accumulation was observed at lower sugar (15 g/L), which may be related to the earlier sugar depletion by roots. According to the data in Table 3, from 4.80-17.41 g of roots, DW/L were harvested after 4 weeks of incubation, representing 5.85-21.23-fold higher than the initial inoculum (0.82 g DW/L medium).

Table 3. Effect of sucrose, fructose, and glucose levels on biomass production of *G. paniculata* root cultures after 4 weeks of incubation period

Sugar type	Level (g/L)	Root biomass FW yield (g/L)	Root biomass DW yield (g/L)	Dry matter (%)	Relative growth rate	Growth index
Control	0	40.00±2.31 ^g	1.20±0.12 ^g	2.99±0.12 ^{bc}	0.09±0.02 ^f	0.46±0.14 ^f
Sucrose	15	210.00±11.55 ^c	5.81±0.12 ^c	2.77±0.10 ^{bc}	0.49±0.01 ^d	6.07±0.15 ^c
	30	343.73±8.09 ^d	13.00±0.46 ^c	3.78±0.08 ^a	0.69±0.01 ^{bc}	14.85±0.70 ^c
	45	470.00±11.54 ^a	17.41±0.35 ^a	3.71±0.16 ^a	0.76±0.00 ^a	20.22±0.42 ^a
Fructose	15	213.33±12.13 ^c	5.20±0.23 ^{cf}	2.47±0.25 ^c	0.46±0.01 ^{dc}	5.34±0.28 ^c
	30	385.87±3.47 ^c	11.41±0.22 ^d	2.95±0.03 ^{bc}	0.66±0.01 ^c	12.90±0.28 ^d
	45	417.80±6.93 ^b	15.60±0.46 ^b	3.74±0.17 ^a	0.74±0.01 ^a	18.02±0.56 ^b
Glucose	15	170.00±11.55 ^f	4.80±0.12 ^f	2.86±0.26 ^{bc}	0.44±0.01 ^c	4.85±0.13 ^c
	30	381.33±6.36 ^c	11.61±0.35 ^d	3.04±0.04 ^b	0.66±0.01 ^c	13.15±0.42 ^d
	45	376.13±10.39 ^c	15.00±0.58 ^b	4.00±0.27 ^a	0.73±0.01 ^{ab}	17.29±0.70 ^b

Data are presented as mean±SE, n=3. Different characters in the same column indicate significant differences and the same characters indicate insignificance according to DMRT at $P<0.05$

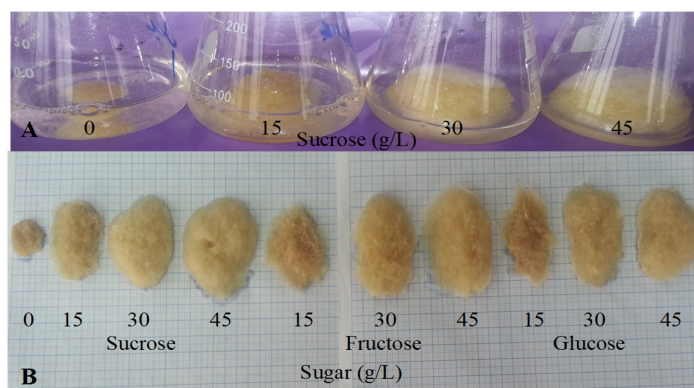


Figure 6. Effect of (A) sucrose and (B) different sugar (sucrose, fructose, and glucose) levels on root production of *G. paniculata* root cultures after 4 weeks incubation period

Total saponin production

Saponin biosynthesis in *G. paniculata* root culture was significantly enhanced by sugar feeding, and sucrose represented the most favourable sugar. TSC was increased by increasing sugar levels in the culture media regardless of sugar types (Figure 3E).

Total saponin yield took the same pattern as TSC because the yield of biomass DW was also generally taking the same pattern as TSC (Table 3 and Figure 3E). Of the various tested levels of sucrose, 45 g/L sucrose was the best concentration, where total saponin reached the highest significant content and yield (10.19 mg/g DW and 177.4 mg/L medium, respectively), followed by 30 g/L sucrose (8.13 mg/g DW and 105.8 mg/L medium, respectively). Lower levels of sucrose, fructose, and glucose (15 g/L) negatively contributed to saponin biosynthesis (1.02, 0.34 and 0.41 mg/g DW, respectively) compared to control (0.08 mg/g DW). Sucrose contains a large carbon skeleton. Therefore, cell metabolic activities may be enhanced upon increasing sucrose feeding and, subsequently, the biosynthesis of saponin and other secondary metabolites may also be improved (Krstić-Milošević *et al.*, 2013). Our results are close to the findings of Yin *et al.* (2013a) and Li *et al.* (2016) on *P. heterophylla* and *G. uralensis*, respectively, where MS medium fortified with 40 g/L sucrose maximized root growth and saponin production in AR cultures.

Total flavonoid production

In contrast to TSC, the TFC was increased by raising the sucrose level to 30 g/L and then declined at a higher consumption level (45 g/L). This reduction in TFC may be due to high media osmosis with a high sucrose concentration, causing cell dehydration and the diffusion of flavonoids from roots into the liquid media (Jiao *et al.*, 2014). On the contrary, a gradual increase in TFC was recorded by increasing the fructose and glucose from 0 to 45 g/L (Figure 3F), indicating that the impact of different sugar types and levels on the production rate of secondary metabolites depends on the type of metabolite. The highest TFC (8.65 mg/g DW) was recorded for 30 g/L sucrose, followed by 45 g/L glucose (7.06 mg/g DW). In accordance, Nagella and Murthy (2010) on *Withania somnifera* reported the superiority of sucrose to glucose, fructose, and maltose for biomass production, and the highest accumulation of withanolide-A was recorded for 30 g/L sucrose enriched medium. Gai *et al.* (2015) also found that sucrose was superior to fructose, glucose, and maltose for HR growth and flavonoid accumulation in *Isatis tinctoria*. Furthermore, sucrose regulates flavonoid and polyphenol biosynthesis by altering the expression of pathway genes and transcription factors genes (Qian *et al.*, 2018).

The highest flavonoid productivity (112.7 mg/L medium) was achieved with 45 g/L sucrose, closely followed by 30 g/L sucrose (112.5 mg/L medium) without a significant difference between them (Figure 3F). Corroborating to our results, Jiang *et al.* (2015) noticed that the production of root biomass, polysaccharides,

phenolic, and flavonoids of *Oplopanax elatus* were stimulated by increasing sucrose level to 50 g/L. The experimental data presented here also proved the importance of initial sucrose concentration added, which affects various parameters for *in vitro* cultures, e.g., root growth and the biosynthesis of important bioactive secondary metabolites from *G. paniculata* plant. Furthermore, a reverse relation of the liquid media volume to root FW was noticed, which was linked to water and nutrients from culture media (Figure 6A).

DPPH radical scavenging activity

Indeed, roots cannot grow without a source of carbohydrates in the cultural media. Likewise, the levels of secondary metabolic products, including saponins and flavonoids, decrease; thus, the antioxidant efficacy decreases with the absence or low sugar concentration in the growth medium. In general, the antioxidant activity of gypsophila root extract increased with an increase in the sugar level in the culture media, most likely due to the increased accumulation of saponins and flavonoids (Figure 4C). Hence, the topmost values of radical scavenging activity were found for roots that received 45 g/L of sucrose, fructose, or glucose and 30 g/L of sucrose (81.92, 78.62, 83.36, and 80.61%, respectively).

R. rhizogenes-mediated transformation of *G. paniculata*

Growth kinetics of transformed root cultures

Leaf and stem explants from *in vitro* stock culture of *G. paniculata* (Figure 7A) were successfully transformed with *R. rhizogenes* strain A4 resulting in the formation of HR (Figure 7B, C, D, and E). HR was coming into view on stem and leaf surfaces after 8-10 days from the co-cultivation periods - 2 days (Figure 7B), while non-transformed roots (adventitious roots) appeared on stem explants after six weeks. Leaf explants in the control culture did not form any roots. Hyperhydricity was observed when the infected explants were co-cultivated on a liquid medium; therefore, no root formation was detected. Stem explants were found to be superior to leaf in term of root formation percentage (60.3%) and HR number (4.0 roots/explant), while root length was higher for leaf explants (3.0 cm) (data not shown).

PCR detection of *R. rhizogenes* rolC gene

It is well known that each root loci (*rol*) gene (*rolA*, *rolB* and *rolC*) of the Ri-plasmid in *R. rhizogenes* is responsible for the induction of HR during the plant-*R. rhizogenes* interaction (Gabr *et al.*, 2016; 2018; Sarkar *et al.*, 2018), but this needs to be confirmed at the molecular level. To determine the insertion of *rol* genes, PCR-based analysis of *rolC* gene was conducted to assess the genetic transformation of HR transformed lines. Additionally, the *virD2* gene was used to confirm the complete absence of *R. rhizogenes* in HR transformed lines. Figure 8 shows PCR analysis of the reference gene. *G. paniculata* HR carried *rolC* gene in its genome but not the *virD2* gene, which confirmed the genetic transformation in *G. paniculata* HR explants (leaf and stem) and the complete elimination of bacteria.

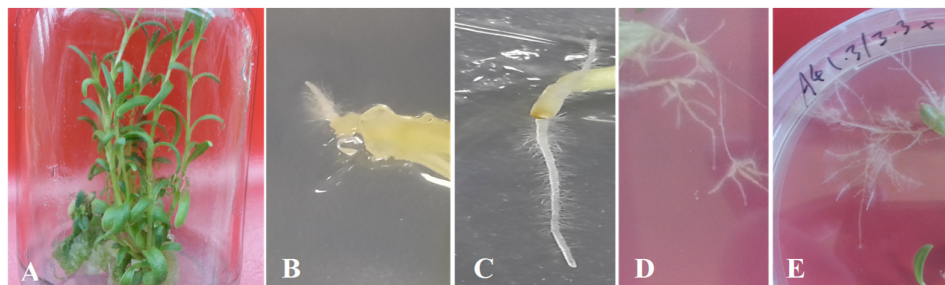


Figure 7. *G. paniculata* hairy root (HR); (A) *in vitro* stock cultures, (B) HR induction 8-10 days after co-cultivation, (C and D) stem-derived HR, (E) leaf-derived HR

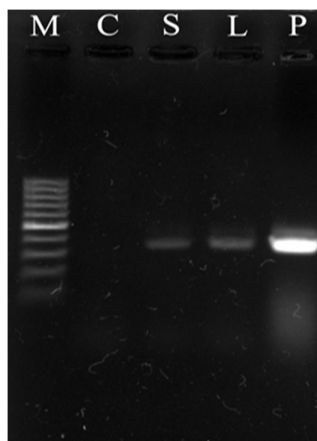


Figure 8. PCR amplification of the *rolC* gene from genomic DNA isolated from different hairy root cultures of *G. paniculata*, stem (S), leaf (L), non-transformed roots (C), and (P) plasmid DNA from *R. rhizogenes* (positive control); *rolC* (490 bp)

Accumulation of biomass, saponin and flavonoid in hairy root cultures

Biomass accumulation of hairy root cultures

Genetic changes caused by *R. rhizogenes* conditioned a higher root biomass formation (Table 4). Thence, biomass FW and DW of the different HR lines (leaf and stem) were significantly higher than that of non-transformed roots. The highest significant FW (195.00 g/L medium) was recorded for stem-derived HR. In this concern, the FW of stem and leaf HR lines were found to be 2.7 and 1.8-fold higher than non-transformed roots. Moreover, stem-derived HR also recorded the highest DW value (10.75 g/L) and was closely followed by leaf HR (10.00 g/L) without significant differences between them, which represented, respectively, 2.5 and 2.4-fold higher than non-transformed root cultures (4.17 g/L). Also, Tusevski *et al.* (2019) reported higher root elongation, lateral branching, and biomass production of *Hypericum perforatum* HR in comparison to non-transgenic roots.

Increasing root biomass production of *G. paniculata* HR cultures over non-transformed roots may be related to the presence of *rolC* of *R. rhizogenes* in the plant genome. In this context, Sarkar *et al.* (2018) reported that *rol* genes are the most exciting genes for biotechnological studies because it can stimulate both the growth of transformed cells and the biosynthesis of secondary metabolites. Usually, non-transformed root cultures need an exogenous PGRs supply, mainly auxins, to grow well. Therefore, some species exhibit slow growth in their root cultures, resulting in the poor or negligible synthesis of active ingredients. In contrast, the HR system is characterized by rapid growth and extensive branching under PGR-free medium conditions (Pandey *et al.*, 2022a).

Total saponin accumulation of hairy root cultures

The analysis of variance for the data displayed in Table 4 proved that TSC in the transformed HR of *G. paniculata* was significantly enhanced when compared with non-transformed root. Moreover, a significant difference between HR lines (leaf and stem) in TSC was noticed. Leaf-derived HR accumulated the highest significant TSC (9.73 mg/g DW). TSC in leaf and stem HR represented 2.7 and 2.3-fold higher than non-transformed root, respectively. In conformity with these results, Nasiri *et al.* (2022) found that the diosgenin content in the transgenic roots of *Trigonella foenum-graceum* was three times higher than that of the non-transgenic roots.

No significant difference was recorded between leaf-derived HR and stem-derived HR in terms of total saponin yield (97.23 and 89.96 mg/L medium, respectively). The productivity of total saponin from leaf and

stem HR cultures was 6.5 and 6.0-fold higher than non-transformed roots (15.02 mg/L medium). Zheleznichenko *et al.* (2018) recorded that the biosynthetic rate of saponins, flavonoids, hydroxycinnamic acids, pectins, and protopectins in HR cultures of *Nitraria schoberi* was higher than that of the intact plant roots.

Table 4. Biomass, saponin, and flavonoid production in *G. paniculata* hairy root (HR) cultures

Root source	Root biomass FW yield (g/L)	Root biomass DW yield (g/L)	TSC (mg/g DW)	Total saponin yield (mg/L)	TFC (mg/g DW)	Total flavonoid yield (mg/L)
Control root	72.50±5.77 ^c	4.17±0.22 ^b	3.58±0.23 ^c	15.02±1.73 ^b	3.64±0.052 ^c	15.19±1.01 ^b
Leaf-derived HR	127.50±7.22 ^b	10.00±0.29 ^a	9.73±0.12 ^a	97.23±1.65 ^a	7.49±0.064 ^a	74.94±2.80 ^a
Stem-derived HR	195.00±11.55 ^a	10.75±0.58 ^a	8.38±0.12 ^b	89.96±3.60 ^a	7.29±0.052 ^b	78.42±4.77 ^a

Data are presented as mean±SE, n=3. Different characters in the same column indicate significant differences and the same characters indicate insignificance according to DMRT at $P<0.05$

Total flavonoid accumulation of hairy root cultures

The flavonoid production in transformed HR cultures was significantly higher than in non-transformed roots (Table 4). About 2.1 and 2.0-fold increase in the TFC was observed in leaf and stem HR lines (7.49 and 7.29 mg/g DW, respectively) compared to control roots (3.64 mg/g DW). Increasing flavonoid accumulation in *G. paniculata* HR may be correlated to the role of *rolC* of *R. rhizogenes* on the induction of enzymes involved in the phenylpropanoid pathway leading to flavonoid biosynthesis as hypothesized by Gabr *et al.* (2016). Sahayarayan *et al.* (2020) also recorded an enhancement in the production of total phenolics and total flavonoids in *Cucumis anguria* HR compared to non-transformed roots.

Non-statistical differences were detected between both HR lines concerning the flavonoid yield (74.94 and 78.42 mg/L medium for leaf and stem HR cultures, respectively). Overall, the total flavonoid yield of stem and leaf HR was 5.2 and 4.9-fold higher than the non-transformed root one (15.19 mg/L medium).

The enhanced accumulation of saponin and flavonoid in the transformed HR compared with non-transformed roots might be due to the alteration of hormonal metabolism and transport, which generally happens during transformation with *rol* genes (Sarkar *et al.*, 2018). However, evidence indicated that the *rolC* gene mediated uncommon signal transduction pathways. It acts on the production of secondary metabolites independently of plant defense hormones and the calcium-dependent NADPH oxidase pathway (Bulgakov *et al.*, 2013). In transformed plants and plant cell cultures, *rolC* gene induced the production of copious contents of secondary metabolites such as flavonoids (Gai *et al.*, 2015), steroidal saponin (Zolfaghari *et al.*, 2020), and rosmarinic acid (Dowom *et al.*, 2022).

Radical scavenging activity of hairy root cultures

HR extracts of *G. paniculata* showed higher antioxidant potential than non-transformed control roots (Figure 4D). The superiority of HR over non-transformed roots in the bower of the antioxidant activity has been proven in previous studies (Jiao *et al.*, 2014; Sahayarayan *et al.*, 2020). Tusevski *et al.* (2019) found that the antioxidant ability and phenolic content of *H. perforatum* HR were significantly higher than non-transgenic root. Leaf-derived HR exhibited the highest scavenging activity that follows that of ascorbic acid (95.97 and 97.91%, respectively). In addition, the genetic changes caused by *R. rhizogenes* that occurred in the HR led to a higher accumulation of saponins and flavonoids, which caused an increase in antioxidant activity compared to non-transgenic roots (70.01%). A positive correlation of phenolic compounds with antioxidant properties was also observed in HR cultures of *H. perforatum* (Tusevski *et al.*, 2019).

Conclusions

Root suspension culturing is a successful method to produce valuable phytoconstituents. In the current investigation of a shake flask technique, the results confirm that the composition of root culture media has efficient effects on root growth and the augmentation production of bioactive ingredients from *G. paniculata* plant. NAA at 1 or 2 mg/L, MS medium at full strength, and 45 g/L of sucrose were the most suitable treatments to optimize the root culture for biomass, saponin, and flavonoid production. A successful transformation protocol by *R. rhizogenes* A4 strain using stem and leaf explants was established for hairy root transformation of *G. paniculata* plant. Production of biomass, saponin, and flavonoid was found to be high in hairy root lines obtained compared to non-transformed roots, showing that the genetic changes caused by the *R. rhizogenes* transformation conditioned a higher biomass and active ingredients production. The presented experiments can be helpful in the commercial production of valuable saponins and flavonoids from *G. paniculata* roots on a large scale using the bioreactor technique. It is noted that the emerging private companies used bioreactor technology to produce some bioactive compounds commercially. Moreover, due to the fast growth, and biochemical and genetic stability of the adventitious root and hairy root cultures, this system can be effectively used in the future for studying the metabolic pathways of triterpenoid saponins and flavonoids.

Authors' Contributions

Conceptualization: NMT, HAB, and YF; Data curation: AMA, MMZ, HP, and SU; Formal analysis: AAE, SU; Investigation: AMMG, NMT, and HAB; Methodology: AAE, AMMG, AMA, MMZ, JJ, and HP; Resources: AMMG, HSE, JJ, and SU; Software: AAE, HSE, and HP; Supervision: NMT, HAB, and YF; Writing - original draft: AAE, AMMG, AMA, SU, and HP; Writing - review and editing: AAE, HSE, JJ, and YF. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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