Establishment of hairy root cultures by *Agrobacterium rhizogenes* mediated transformation of *Gardenia jasminoides* Variegata for enhancing of phenolic and antioxidant accumulation capacity

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Background
The gardenia is an aromatic medicinal plant belonging to the coffee family (Rubiaceae). Because of its high concentration of phenolic compounds, including caffeic acid, ferulic acid, and chlorogenic acid, it can be used to treat inflammatory illnesses and relieve pain.
Objective
It is the first study on *Gardenia jasminoides* Variegata hairy root cultures to increase the levels of Phenolic, flavonoid compounds, and accumulation of antioxidants.
Patients and methods
Gardenia jasminoides Variegata shoots were infected with the *Agrobacterium rhizogenes* A4 strain. Total phenolics and flavonoid content has been estimated. The content of phenolic and flavonoid compounds in the cultures was quantified using high-performance liquid chromatography. To emphasize and clarify the results the high-performance thin-layer chromatography were used. Antioxidant activity were demonstrated by 2,2′- azino-bis (3-ethylbenzothiazoline-6-sulfonate) radical cation and 2,2-Diphenyl-1-picrylhydrazyl.
Results and conclusion
The transgenic culture was confirmed through PCR using *rol* genes primers. Total phenolics content has been reported to be higher in hairy roots than in control samples recorded (1.674 as versus 1.073 mg/g DW, respectively). Additionally, the total flavonoid content in the transgenic stem cultures showed higher levels than the nontransgenics (2.824 versus 1.553 mg/g dry weight, respectively). Hairy root cultures recorded more antioxidant activity than the nontransgenics. Chlorogenic acid, ferulic acid, and caffeic acid were accumulated in high values in transgenic cultures (3248.4, 2948.2, and 452.2 μg/g DW, respectively). So, transgenic culture can accumulate as more as about 100 folds the nontransgenic ones for the chlorogenic acid and ferulic acid.

Keywords:
Gardenia jasminoides Variegata, *Agrobacterium rhizogenes* mediated-transformation, Secondary metabolites, HPLC, HPTLC

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Introduction

Hairy roots are considered an interesting system for producing important secondary metabolites because they typically exhibit rapid growth, possess genetic stability, and often, but not always, mimic the biochemical profiles of plant roots. Because of their stability in terms of both genetics and biosynthesis, it can enhance the activities of growth regulators [1,2] and play a crucial role in plant hormonal growth development. The T-DNA is inserted into and inherited within the plant's genomic DNA, is a portion of the Ri (root inducing) plasmid that determines the *Agrobacterium rhizogenes* capability to induce hairy roots. Auxin biosynthesis genes and genes necessary for the synthesis of non-protein amino acids those called opines are encoded by the T-DNA [3,4]. A large variety of compounds can be developed, maintained, and produced by plant roots, and transformed root cultures can undergo a variety of biosynthetic processes [5]. It has been shown that hairy root cultures of several plants can elevate the production of a large number of metabolites [6–10].

Since roughly 25% of medications are thought to be derived from medicinal plants, plants are seen as a major source of bioactive chemicals that are beneficial in drug manufacture or employing plants directly as herbal medicine [11,12]. More than 350 000 species were evaluated in recent years for the production of

bioactive substances, which plants produce in the form of flavors, food additives, or biochemicals with medicinal uses [13,14].

Tropical, subtropical, and temperate regions all cultivate gardenia as an ornamental and medicinal plant. Gardenia is utilized both as a cut flower and a garden shrub for borders, screens, and hedges. The genus *Gardenia* contains more than a hundred different species, including the species *jasminoides*, which has two subspecies (*Ellis* and *Variegata*) [15]. It is essential in traditional Chinese medicine [16]. Gardenia has shown that it can be utilized to treat inflammatory diseases and reduce pain. Because it is rich in beneficial phenolic compounds, such as Ferulic acid and chlorogenic acid, also a large number of anti-inflammatory flavonoids, such as rutin and Apigenin [17,18]. According to [19] chlorogenic acid (CGA) is considered as a phenolic compound. It is recognized as an ester derived from both caffeic acid and (–)-quinic acid as a derivative of cinnamic acid with biological benefits mainly related to its anti-inflammatory effects (Fig. 1). Risks related to type 2 diabetes, Alzheimer's disease, and also to cardiovascular diseases is reduced by chlorogenic acid in recent years [20–22]. It has also been shown to have anti-inflammatory and antibacterial properties [23,24].

Patients and methods

Plant material

The plant material source of *Gardenia jasminoides* Variegata was the in vitro-grown plantlets. Three subcultures of these plantlets were performed on MS media. [25]. The culture required a supplement of 2 mg/l BA and 0.5 mg/l NAA for shoot multiplication.

Agrobacterium rhizogenes Preparation

The glycerol stock served as the source of the *A. rhizogenes* A4 strain. It was grown in YEBS liquid culture, which contained 0.5 g/l magnesium sulfate, 1.0 g/l yeast extract, 5.0 g/l sucrose, 5.0 g/l bacto-peptone, and 5.0 g/l beef extract and pH 7.0. This liquid culture was incubated at 28°C, and it was shaken at 150 rpm during that time. The suitable antibiotic (Rifampicin) was added to the cultures.

Agrobacterium rhizogenes mediated-transformation

Using the protocol outlined by [10,26], 2 cm-long stem explants with three nodes and petiole-long leaves were inoculated with the *A. rhizogenes* A4 strain (OD_{600} nm = 1.0). It treated each explant independently. After being infected for five minutes in a bacterial suspension, the explants were placed on sterile filter paper to get rid of any remaining bacteria. Following sterilization, the sample was incubated in a basal MS liquid medium with 30 g of sucrose per liter for two days. The mixture was shaken at 100 rpm while being kept completely dark. After co-cultivation, the infected explants were moved to new basal MS liquid medium containing 30 g of sucrose per liter and 250 mg/l of cefotaxime (cefotaxime sodium salt, Sigma-Aldrich). To remove *A. rhizogenes*, these explants were then cultured under the same conditions for 14 days. The transformed hairy root cultures were collected for biochemical and PCR analysis after three weeks of sub-culturing. As controls, uninfected stem explants (nontransformed cultures) were employed.

PCR detection

The Plant DNA Preparation Kit (Jena Bioscience), a solution-based kit, was used to extract genomic DNA from the cultures that were the subject of the investigation. The *A. rhizogenes*; *rolA*, *rolB*, and *rolC*

Figure 1

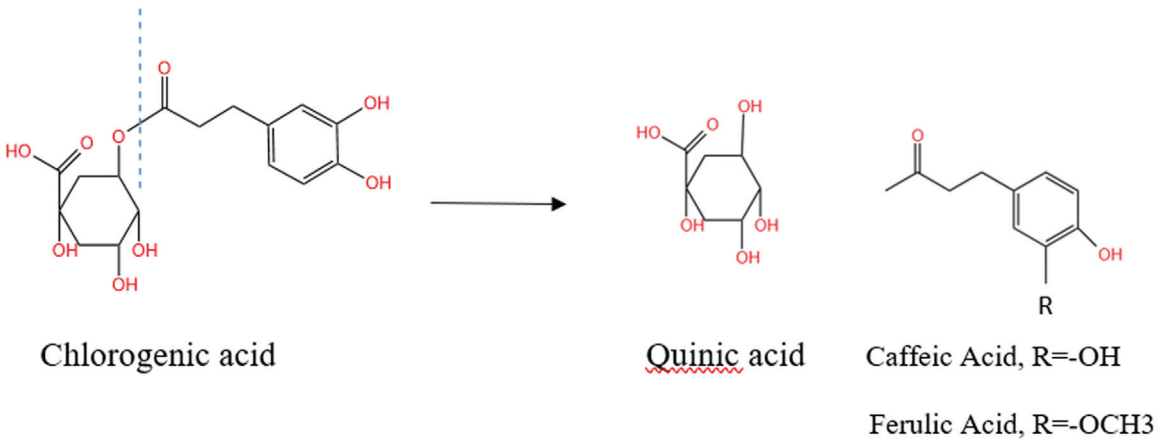


Diagram shows Chlorogenic acids (CGAs); an ester between trans-cinnamic acid (such as caffeic, ferulic, and quinic acid).

genes were used as targets in a PCR analysis of the transformed hairy root cultures to verify the transformation activity. The *virD2* gene was utilized to confirm that the tissue was free of Agrobacteria infection. The T100 thermal cycler (Bio-Rad, USA) facilitated the polymerase chain reaction amplification. Primers for the *rolA* gene, 5′-GTTGTCGGAAT GGCCCAGAC-3′ and 5′-CGTAGGTCTGAATA TTCCGGTC-3′, amplified a 245 bp fragment. For the *rolB* gene, the primers 5′-ATGCGCTTTCGC GAAATCCAA-3′ and 5′-TTCAGGTTTACTGC AGCAGGC-3′ produced a 564 bp fragment and for the *rolC* gene, 5′-TGTGACAAGCAGCGATG AGC-3′ and 5′-GATTGCAAACTTGCACTC GC-3′ primers yielded a 490 bp fragment [26,27]. Additionally, the *virD2* gene was detected using 5′-CCTGACCCAAACATCTCGGCT-3′ and 5′-AT GCCCGATCGAGCTCAAGT-3′ primers, which amplified a 338 bp fragment [26]. The *aux1* gene was amplified using the primers 5′-CCAAGC TTGTCAGAAAACTTCAGGG-3′ and 5′-CCG GATCCAATACCCAGCGCTTT-3′, resulting in a 1000 bp fragment [26]. The PCR was run under the following conditions: 4 min of initial denaturation at 95°C, 30 cycles of amplification (1 min at 94°C, 1 min at 55°C, and 1 min at 72°C), and a final 5 min of extension at 72°C. The *virD2* gene underwent three minutes of initial denaturation at 95°C, thirty cycles of amplification (30 s at 95°C, 30 s at 56°C, and 45 s at 72°C), and a final extension lasting 10 min at 72°C. A 1.5% agarose gel was used to evaluate the amplified products.

Sample extraction

According to Gabr *et al.* [28] over the course of 24 h, 1.5 ml of 80% methanol was used to extract 100 mg of ground, dried resources. The extracts were then sonicated in an ultrasonic water bath for 20 min (Grant, United Kingdom). Next, the samples underwent a 5 min centrifugation at 6000 rpm (Sigma, 2–16 PK, Germany). Following centrifugation, the pellets were extracted twice more using 500 µl of the solvent, and the supernatants were gathered. The extracts were then kept until they were needed again at −20°C.

Biochemical analysis

Total phenol content

Total phenols were quantified using the Folin–Ciocalteu micro-method as described by [29,30]. The method used was adding 300 µl of a 200 g/l Na2CO3 solution after 20 µl of the extract solution, 1.16 ml of distilled water, and 100 µl of Folin-Ciocalteu reagent had been combined. Following a 30 minute incubation period at 40 °C in a water bath, the mixture's absorbance at 760 nm was determined. An extract-free control sample was also investigated. The calibration curve's standard was gallic acid. The following formula was used to determine the total phenolic content, expressed as gallic acid equivalent: A= 0.98 C+9.0925×10–3 (R2= 0.9996), where A stands for gallic acid absorption and C for its concentration.

Total flavonoid content

The approach by Ordon *et al.* 2006 [31] was used to calculate the total flavonoid content. An $AlCl_3$ methanolic solution (20 g/l) was added in 0.5 ml volumetric measure to 0.5 ml extract solution. The absorbance at 420 nm was determined following an hour of incubation at room temperature. Flavonoids can be identified by the appearance of a yellow coloration. Also, the sample was examined as a control that did not include the extract. Y= 0.0255 X (R2= 0.9812), where X is the absorbance and Y is the concentration (mg QE g^{-1} DW), was used to compute the total flavonoid content, expressed as quercetin equivalent (QE). This equation was generated using the calibration curve.

The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{\bullet+}$) Radical – scavenging activity

The Re *et al.* 1999 [32] method was used to perform the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) assay. Potassium persulfate (2.4 mmol/l) and ABTS (7 mmol/L) were the stock solutions. Equal parts of the two stock solutions were combined to create the working solution, which was then left to react for 12 to 16 h at room temperature in the dark. The absorbance at 734 nm was then measured with a spectrophotometer after 1 ml of the ABTS solution was diluted with 60 ml of methanol. For every test, a fresh ABTS solution was made. The absorbance at 734 nm was measured after varying the quantities of extract and synthetic antioxidants (t-butyl-hydroxyquinone (TBHQ), butyl-hydroxyanisol (BHA), and butyl-hydroxytoluene (BHT) in ethanol) solutions (1 ml each) were combined with 1 ml of ABTS solution and allowed to react for 7 min. An extract-free control group was also subjected to analysis. Following is the calculation of the scavenging activity: [(Abs control – Abs sample)/ Abs control] x 100 is the ABTS radical-scavenging activity (%).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

According to Gabr *et al.* 2017 [28] the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay was used, albeit with

some adjustments. After combining 0.1 ml of methanolic extracts from transgenic and non-transgenic explants with 1.9 ml of DPPH solution, the mixture was vortexed for 30 s. Following a half-hour reaction period, the liquid was tested for absorbance at 515 nm. An extract-free control group was also examined. This is how the scavenging activity was determined: (A control – A sample / A control) x 100 is the DPPH radical-scavenging activity (%). where A is the 515 nm absorbance.

Determination of phenolics and flavonoids content by High Performance Liquid Chromatography (HPLC)

The phenol and flavonoid contents were measured using the Agilent 1100 series High-Performance Liquid Chromatography (HPLC) system, manufactured by Agilent Technologies, located in Palo Alto, California, was utilized for the HPLC determination. It was equipped with a UV detector, a quaternary pump G1311A, a degasser G1322A, and Agilent ChemStations Rev. B. 04.03. The separation was performed using a LiChrospher RP-18 HPLC column (250 mm 4.6 mm, 5 m; Merck, Germany). Methanol and water were combined and acidified with 0.3% orthophosphoric acid w/v to create the mobile phase. At 1.4 ml/min, the flow rate was adjusted. Absorption at λ=288 nm was used for detection, and substances such as ferulic acid, vanillic acid, p-coumaric acid, syringic acid, caffeic acid, p-hydroxybenzoic acid, and chlorogenic acid were identified by comparing their absorption spectra and retention times with a standard phenol complex. The criteria for flavonoids are dihydrokaempferol, catechin, and quercetin. The manufacturers of the standards used were Sigma Aldrich. The regions of the sample peaks and a known concentration of the standard were used to determine the sample content, which was represented in micrograms per gram of material.

Determination of phenolics and flavonoids content by High-Performance Thin-Layer Chromatography (HPTLC)

Reagents, solvents, and standards

Ultra-pure water (Millipore), HPLC-grade methanol, ethyl acetate, acetic acid, formic acid, toluene, and analytical-grade standards, chlorogenic acid and ferulic acid (Sigma), were among the solvents, reagents, and standards used. These were employed in the creation of calibration standards curves in methanol at 100 μg/ml^{-1}, which were then contrasted with extracts derived from micro-propagated plants.

Sample application on High-Performance Thin-Layer Chromatography (HPTLC) plates

Using Camag equipment located in Muttenz, Switzerland, High-Performance Thin-Layer Chromatography (HPTLC) analysis was carried out. TLC silica gel 60 F_{254} glass plates (10×20 cm) from Merck, Germany, were used for the TLC procedure. Eight-millimeter bands holding standards and samples were applied, spaced eight millimeters from the plate's bottom border. A 50 μg ml^{-1} ferulic acid solution was spotted in quantities of 2 and 6 μl next to 2 and 6 μl of the sample's reference extract. The mixture of toluene, ethyl acetate, and formic acid in a ratio of 5: 5 : 0.2 v: v: v made up the 10 ml mobile phase for ferulic acid. In an automated development chamber ADC2, the plates were produced at 22.9°C and 44.5% humidity. During 12.5 min, the mobile phase traveled 70 mm. Chromatograms were seen and recorded using Visualizer 2 at wavelengths of 254 and 331 nm after development. The dry weight of sample (mg g^{-1}) was used to quantify the amounts of ferulic acid. Samples of chlorogenic acid were placed on 20×10 cm HPTLC glass Silica Gel 60 F_{254} Plates from Merck in Darmstadt, Germany. Standard solutions of 50 μg ml^{-1} chlorogenic acid were spotted against 2 and 6 μl of the extracts from the samples. Ethyl acetate, acetic acid, formic acid, and water were combined in the following ratios to create a 10 ml mobile phase for chlorogenic acid: 24.1 : 2.651 : 2.651 : 5.543 (v: v: v: v). In an ADC2 chamber, plates were grown at 19.6°C and 52.7% humidity. In 25 min, the mobile phase traveled 70.8 mm. After that, chromatograms at 254 and 366 nm were seen and captured on camera using Visualizer 2. Chlorogenic acid concentration in the samples was given as mg g^{-1} DW.

Statistical analysis

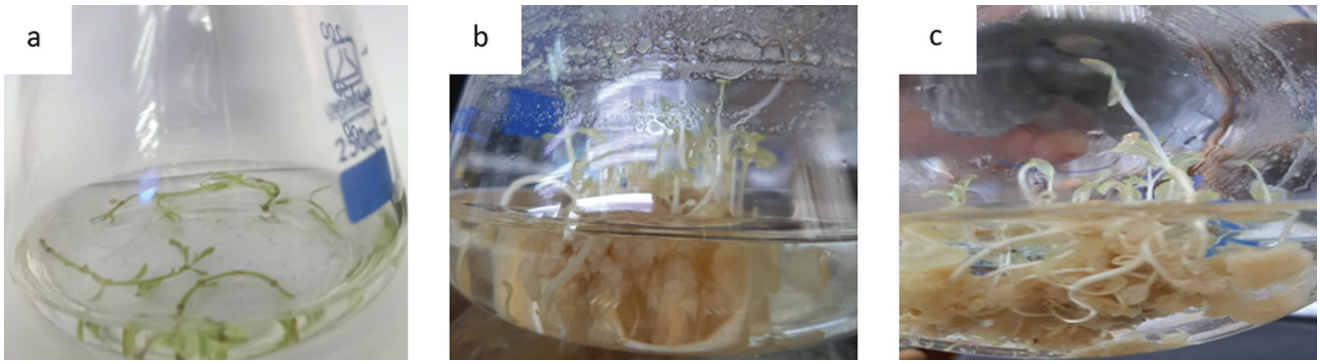
All analyses were conducted in five replicates. The means±standard deviations of the data are displayed. A *t*-test was performed using GraphPad Prism version 5.01 to determine the *P* value and significance. We use KingDraw program to draw chemical structures.

Results and discussion

Hairy root transformation

In a trial to increase the phenolic compounds content, transformed hairy root cultures from stem cultures were established of *Gardenia jasminoides* Variegata. These cultures were infected with *A. rhizogenes* A4 strain and subjected as plant material. Explants were transferred to free plant growth regulators liquid (MS-medium) with 250 mg/l cefotaxime after two days of co-cultivation to eliminate *A. rhizogenes*. Visible roots started to grow after 5 to 7 days. Ten to 14 days later, the roots started to grow more quickly. The thick hairy roots were visible 2–3 weeks after inoculation with appearance of calli in the base of the infected transgenic stems. In the control shoots

Figure 2



Stem cultures of *Gardenia jasminoides* Variegataafter four weeks of infection with *Agrobacterium rhizogenes*: a. Control stem segments without infection. b. and c. Transgenic stem cultures showing thick roots with calli in the base.

there were no any roots even there was no any difference observed (Fig. 2). The calli observed on the base of the infected stems may be a result for the presence of auxin biosynthesis genes (*aux1*).

PCR analyses to *rolA*, *rolB*, *rolC*, and *aux1* genes

It is widely recognized that the *rol* genes of the Ri-plasmid in *A. rhizogenes* are responsible for inducing hairy root formation, yet this assertion requires molecular level confirmation. DNA was isolated from transformed stem cultures. In PCR analysis specific primers for the *rolA*, *rolB*, *rolC*, and *aux1* genes (sequences were detailed in material and methods). Furthermore, the *virD2* gene served as a confirmation of the total absence of *A. rhizogenes* in the transformed cultures. DNA fragments of the expected size were amplified from the total DNA of the transgenic culture. However, these fragments were not detected in the DNA of nontransformed plants. Figure 3 shows PCR analysis of the reference genes. The PCR analysis clearly demonstrated the insertion of TL-DNA, evidenced by the presence of *rolB* and *rolC* gene fragments, and TR-DNA, indicated by the *aux1* gene fragment. The *rolB* gene (652 bp), *rolC* gene (500 bp), and *aux1* gene (1000 bp) which were inserted into hairy roots from the Ri-plasmid in *A. rhizogenes* according to molecular analysis performed by PCR amplification. There were found to be responsible for the induction of hairy roots in plant species.

PCR was conducted to verify the presence of *rolB* and *rolC* genes in the transformed hairy root cells, which are crucial for inducing the hairy root phenotype. Although extensive research has been carried out, the biochemical and molecular functions of these genes in altering plant development are still not well understood.

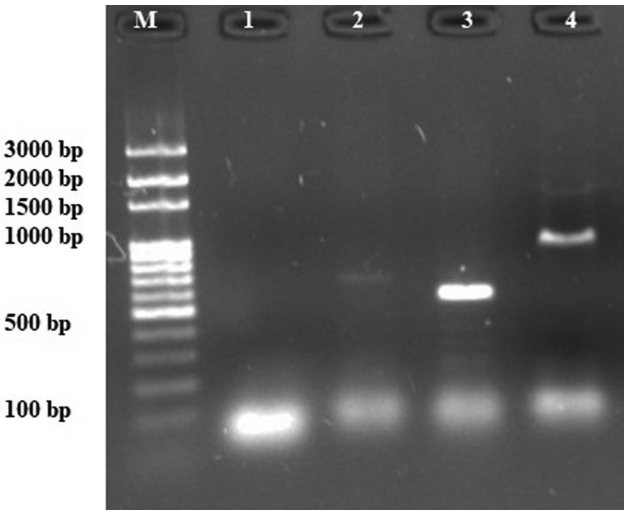
In this study, we have successfully established an appropriate and efficient protocol for the hairy root transformation of *Gardenia jasminoides* Variegata using stem segments to accumulate secondary metabolites in plants. Our protocol could be beneficial for future studies on the biosynthesis of phenolic compounds in transgenic cells of *Gardenia jasminoides* Variegata for the aim of elevating useful secondary metabolites, such as pharmaceuticals and food additives. Hairy root cultures of numerous plant species were thoroughly investigated [26,33–37].

Biochemical analysis

Total phenolic content

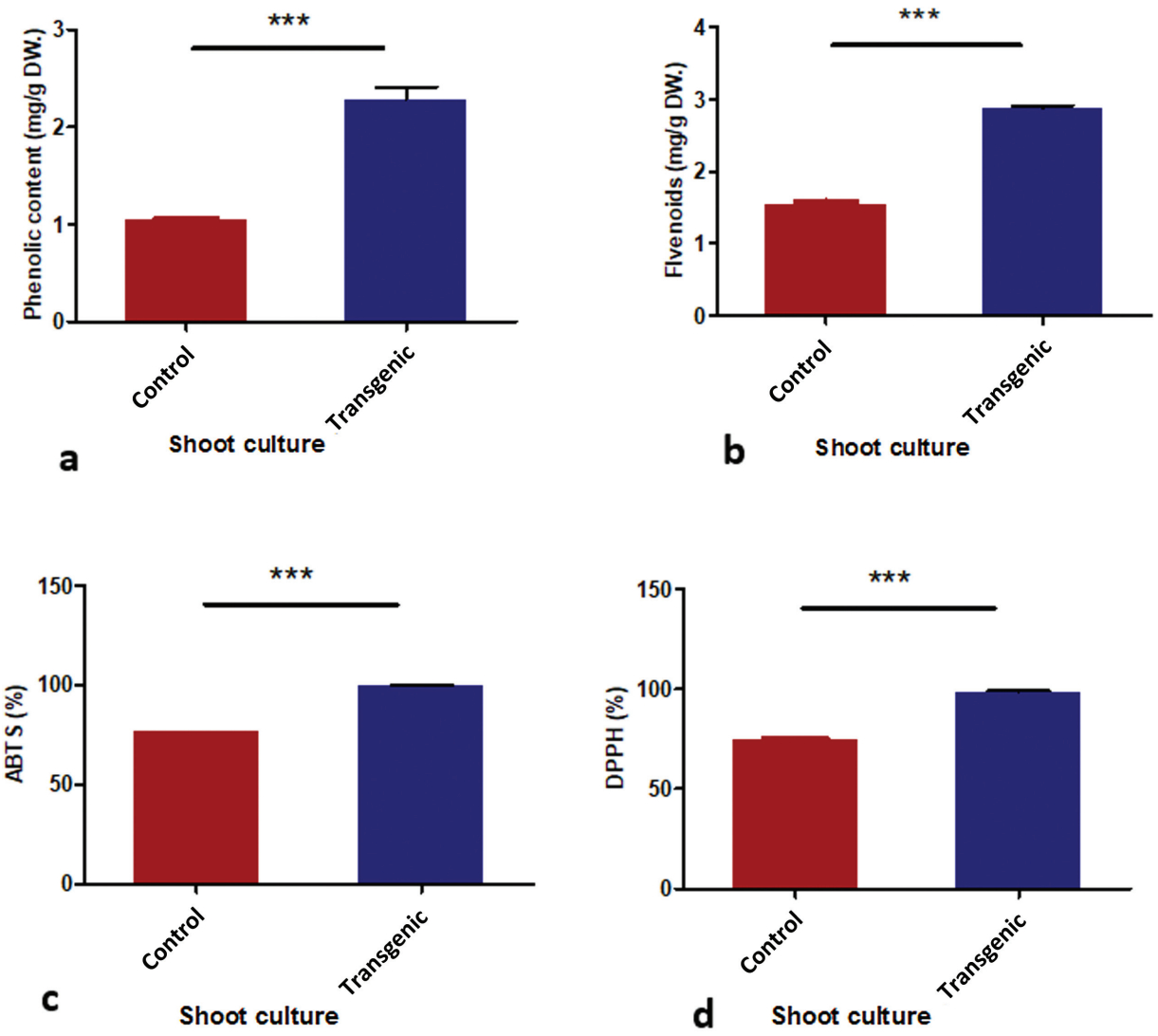
Phenolic acids accumulation was determined in hairy roots and control samples after 4 weeks (Fig. 4a). Hairy roots noticeably increased the total phenolic acid

Figure 3



Confirmation PCR of *rol* genes Lane1: *rolA*, Lane 2: *rolB*, Lane 3: *rolC* Lane, 4: *aux1*. M: 100 bp DNA ladder. *rolB:* appeared faintly approximately at 625 bp, *rolC:* approximately 500 bp, and *aux1:* approximately 1000 bp.

Figure 4



Effect of hairy root transformed stem cultures of *Gardenia jasminoides* Variegata on: a; the total phenolic content (mg/g Dry weight), b; the total flavonoid content (mg/g Dry weight), c; the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonate) radical scavenging activity (%) and d; the 2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity (%).

content than the control samples (1.0462±0.013 vs. 2.284±0.128 mg/gr dry weight, respectively).

It has been well reported that *A. rhizogenes* different strains have different capabilities to stimulate various secondary metabolites in hairy root cultures [38–40]. The primary phenolics, chlorogenic acid, and hyperoxide, exhibit significant antioxidant activity [41]. Our findings are consistent with those reported by [42], who stated that methanolic extracts of *Cucumis anguria* L. hairy roots contained more phenolic compounds than the nontransformed root (124.46 ±6.13 mg GA/g), and with that reported with Min Chung *et al.*, [43], who reported that hairy roots of turnip (*Brassica rapa* ssp. rapa) had higher amounts of total phenol and flavonoid contents than non-transformed roots, according to colorimetric measurements.

Total flavonoid content

Flavonoid accumulation was determined in hairy roots and control samples after 4 weeks. The transgenic stem cultures recorded higher total flavonoid content than the control ones (1.539±0.042 vs. 2.876±0.035 mg/g dry weight, respectively). Our findings are consistent with the findings of Min Chung *et al.* [43], who found that colorimetric measurements revealed that hairy roots of turnips (*Brassica rapa* ssp. rapa) have larger quantities of total phenol and flavonoid contents than non-transformed roots. Additionally, Sawy *et al.*, [44] observed that the transgenic hairy roots of *Lactuca seriola* L. showed an approximately 76.2% increase in

Table 1 Effect of hairy root transformation on the phenolic compounds' accumulation (μg /g DW.) in Gardenia *jasmonide* variegate stem cultures

	Phenolic compounds (μg/g DW)							
Treatments	Chloro-genic acid	Ferulic acid	Caffeic acid	*P* hydroxy-benzoic acid	Syringic acid	Vanillic acid	*p*-coumaric acid	Sinapic acid
Control	39.61±0.02	26.84±0.05	N.D.	N.D.	N.D.	N.D.	41.64±0.05	32.76±0.05
Transgenic	3248.4±0.55	2948.2±0.45	452.2±0.45	738.52±0.47	153.76±0.05	78.28±0.41	N.D.	N.D.

N.D, not detected.

total flavonoid content. Furthermore, Matvieieva *et al.* [45] reported that the flavonoid content of *Artemisia vulgaris* hairy roots may be increased by the transformation mediated by *A. rhizogenes*.

The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical cation (ABTS$^{\bullet+}$) Radical – scavenging activity

As stated by Dorman *et al.*, 2003 [46], there is no one method that is suitable for assessing the antioxidant capacity because many approaches can produce wildly inconsistent results. It is necessary to employ multiple strategies based on various mechanisms. The ABTS and DPPH radical-scavenging activity tests were utilized in this instance. By taking a look on (Fig. 4c) it was found that the transgenic stem cultures exhibited much more antioxidant activity (99.824%) compared with the nontransgenic stem cultures (76.658%).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Free radicals involved in lipid peroxidation are believed to play a significant role in various chronic diseases, including cancer and cardiovascular disorders [46]. DPPH is regarded as a stable radical capable of accepting a hydrogen radical or an electron to become a stable molecule. which make it easily used for the determination of the antioxidant activities of different compounds. This method is considered as a rapid method. Because of that the DPPH has been used widely for the detection of different antioxidants [47,48]. The scavenging activity of hairy roots cultures against the nontransgenic culture is represented in (Fig. 4d). It was observed that the hairy root cultures exhibited more antioxidant activity compared with the nontransgenic culture (98.618 and 74.988%). Our findings are consistent with those reported by Gabr *et al.* [49], who found that the highest scavenging capacity was achieved with extracts from hairy root cultures of buckwheat stem, showing a 78.3% inhibition of DPPH, surpassing its control. In our study, a correlation between ABTS and DPPH was observed, with the scavenging capacity of the ABTS radical by the hairy root cultures being higher than that of the DPPH radical. This aligns with the findings of Gabr *et al.* [49] and Awika *et al.* [50], who noted a significant correlation between ABTS and DPPH and the antioxidant activity of sorghum.

Determination of phenolics and flavonoids by high-performance liquid chromatography (HPLC)

By taking into consideration data presented in Table 1. It could be concluded that the transgenic stem cultures accumulate more chlorogenic acid than nontransgenic ones it can accumulate as more as 80-fold the nontransgenic ones since they accumulate (3248.4 ±0.548, 39.616±0.024 μg/g dry weight, respectively). It also accumulates about 100-fold the control cultures from ferulic acid (2948.2±0.444, 26.84±0.055 μg/g dry weight, respectively). It accumulates different phenolic compounds which were not detected in the nontransgenic cultures like *p* –hydroxybenzoic acid, caffeic acid, syringic acid, and vanillic acid (738.52 ±0.466, 452.2±0.447, 153.76±0.055, and 78.28 ±0.409 μg/g dry weight, respectively). While the non-transgenic cultures accumulated some phenolic compounds, which were not detected in the transgenic cultures in small amounts like sinapic acid and *p* – coumaric acid (32.76±0.055 and 41.64 ±0.055 μg/g dry weight, respectively).

Data presented in Table 2, it could be reported that the transgenic stem cultures accumulate dihydrokaempferol since it recorded 1898.2 ±0.447 μg/g dry weight while it is not detected in the nontransgenic cultures.

Our results are in line with those found by Tuan *et al.* [51] who reported that *A. rhizogenes*-mediated introduction of AtPAP1 raised the mRNA levels of all investigated CGA biosynthesis genes and caused a 900% up-regulation of CGA accumulation in hairy roots in comparison to controls in *P. grandiflorum* hairy roots. Also, it is in the same line with what was reported by Xiao *et al.*, 2015 [52] who reported that the total content of these three compounds (3-caffeoylquinic acid, 3-CQA), 3,5-dicaffeoylquinic acid (3,5-CQA), and 4,5-dicaffeoylquinic acid (4,5-CQA) were the major chlorogenic acid derivative compounds detected in the hairy root cultures of *Stevia rebaudiana*. Under optimal culture conditions, the

Table 2 Effect of hairy root transformation on the flavonoid compounds' accumulation (µg/g DW) in *Gardenia jasmonide* variegate stem cultures

	Flavonoids compounds (µg / g DW.)		
Treatments	Dihydrokaempferol	Catechin	Quercetin
Control	N.D.	294.4±0.55	30.64±0.05
Transgenic	1898.2±0.45	N.D.	N.D.

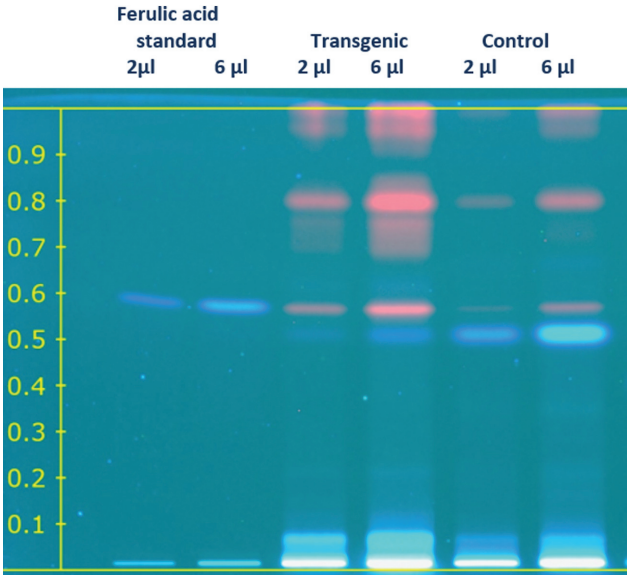
N.D, not detected.

total content of these three compounds reached 105.58 mg/g. Our results are also on line with those reported by Gabr *et al.*, 2012,50, who reported the elevation of chlorogenic acid content from 0.035 to 0.103 mg/g in the transgenic stem culture of buckwheat compared with the control and the elevation of p-hydroxybenzoic acid from 0.398 to 0.438 mg/g in the transgenic stem culture of buckwheat compared with the control. These results can be explained aschlorogenic acid is a highly prevalent metabolite found in plants; also, it seems to offer defense against some types of stress [53]. Chlorogenic acid was found in high concentration (0.93 mg/g DW) in methanolic extracts from the hairy root culture of Echinacea purpurea, according to HPLC tests [54]. Data reported in the literature shows that p-hydroxybenzoic acid enhances the cell wall's impermeability, enhancing resistance to pathogen infection [55]. After 7 days of incubation, a noticeable amount of soluble p-hydroxybenzoic acid accumulation (390 µg/g DW) was seen in the hairy root culture of *D. carota* [56].

Determination of phenols and flavonoids content by High Performance Thin-Layer Chromatography (HPTLC)

The retardation factor of the 50 µg ml^{-1} standard during ferulic acid measurement was 0.586 (Fig. 5). A linear calibration curve was created using the standard's two reference volumes (2 and 6 µl). The linear equation curve was $Y=2.984\times10^{-7}$ X $+4.15\times10^{-4}$ with R=1.00000 and CV = 0.00%. According to the final results, the transgenic shoot extracts displayed a ferulic acid content of 13.72 µg/ml. Ferulic acid content could be separated and determined using the sensitive, repeatable, and straightforward HPTLC method. As for using the HPTLC for estimating the chlorogenic acid, it was found that, the retardation factor of the 50 µg ml^{-1} standard during chlorogenic acid measurement was 0.605 (Fig. 6). A linear calibration curve was created using the standard's two reference volumes (2 and 6 µl). The linear equation curve was Y =5.053×10-7 X–5.279×10^{-3}with R=1.00000 and CV = 0.00%. According to the final results, the transgenic shoot extracts displayed 94.65 µg/ml. Ferulic acid and

Figure 5



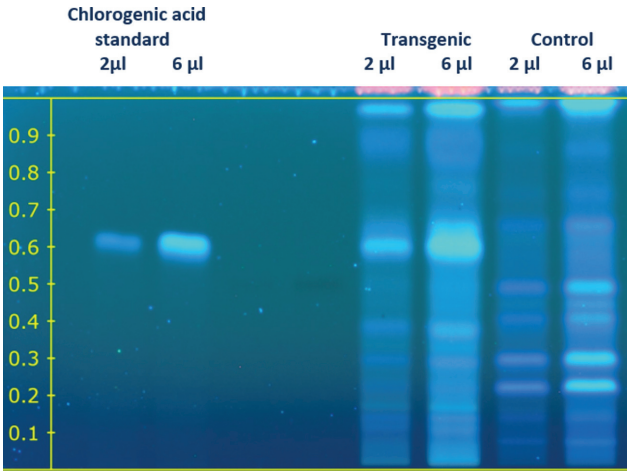
High-Performance Thin-Layer chromatogram of *Gardenia jasminoides* Variegata, variegate transgenic and non-transgenic stem extracts against standard of ferulic acid captured at 366 nm. Tracks 1,2 ferulic acid 50 µg ml^{-1} standard volumes 2, 6 µl. Tracks 3,4 extracts of transgenic shoots volumes 2, 6 µl. Track 5,6 extracts of nontransgenic shoots volumes 2, 6 µl.

Chlorogenic acid concentrations in *Lycium schweinfurthii* [57] and *Setaria italica* [58] were estimated using it.

Conclusion

Our research is the first study on *Gardenia jasminoides* Variegata hairy root cultures to increase the levels of

Figure 6



High-performance thin-layer chromatogram of *Gardenia jasminoides* Variegata transgenic and non-transgenic stem extracts against standard of chlorogenic acid captured at 366 nm. Tracks 1,2 chlorogenic acid 50 µg ml^{-1} standard volumes 2, 6 µl. Tracks 5,6 Sample extracts of transgenic stem volumes 2, 6 µl. Tracks 7,8 sample extracts of nontransgenic shoots volumes 2, 6 µl.

phenolic and flavenoid compounds. Their stem cells were infected with the *A. rhizogenes* A4 strain, resulting in transformed hairy root cultures. It can accumulate as more as about 100 folds the non-transgenic ones for the chlorogenic acid and ferulic acid. These Phyto-active compounds have positive health effects on humans and recommended the use of standardized extracts in pharmaceutical products. While the total concentration of polyphenols, phenolic acids, and flavonoids is positively correlated with antioxidant activity, certain actions, such anti-inflammatory actions, are likely the result of specific compound(s) to which extracts should be standardized. Thin layer chromatography offers a straightforward and accessible method for achieving such goals.

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Conflicts of interest
There are no conflicts of interest.

References

1 Giri A, Narasu ML. Transgenic hairy roots: recent trends andapplications. Biotechnol Adv 2000; 18:1–22.

2 Arafa NM, Gabr AMM, Ibrahim MM, Shevchenko Y, Smetanska I. Study the effect of hairy root transformation on rapid growth (growth morphology) of Nepeta cataria *in vitro* cultures. J Innov Pharm Biol Sci 2015; 2:439–450.

3 Petit A, David C, Dahl GA, Ellis JG, Guyon P, Casse-Delbart F, Tempe J. Further extension of the opine concept: Plasmids in *Agrobacterium rhizogenes* cooperate for opine degradation. Mol Gen Genet 1983; 190: 204–214.

4 Jung G, Tepfer D. Use of genetic transformation by the Ri T-DNA of *Agrobacterium rhizogenes* to stimulate biomass and tropane alkaloid production in Atropa belladonna and Calystegia septum roots grown in vitro. Plant Sci 1987; 50:145–151.

5 Flores HE, Curtis WR. Approaches to understanding and manipulating the biosynthetic potential of plant roots. Ann N Y Acad Sci 1992; 665:188–209.

6 Goel MK, Mehrotra S, Kukreja AK. Elicitor-induced cellular and molecular events are responsible for productivity enhancement in hairy root cultures: an insight study. Appl Biochem Biotechnol 2011; 165:1342–1355.

7 Mehrotra S, Goel MK, Rahman LU, Kukreja A. Molecular andchemical characterization of plants regenerated from Ri-mediated hairy root cultures of Rauwolfia serpentina. Plant Cell, Tissue Organ Cult 2013; 114:31–38.

8 Gabr AMM, Ghareeb H, El Shabrawi HM, Smetanska I, Bekheet SA. Enhancement of silymarin and phenolic compound accumulation in tissue culture of Milk thistle using elicitor feeding and hairy root cultures. J Genet Eng Biotechnol 2016; 14:327–333.

9 Alok A, Shukla V, Pala Z, Kumar J, Kudale S, Desai N. In vitro regeneration and optimization of factors affecting Agrobacterium mediated transformation in Artemisia Pallens, an important medicinal plant. Physiol Mol Biol Plants 2016; 22:261.

10 Gabr AMM, Sytar O, Ghareeb H, Brestic M. Accumulation of amino acids and flavonoids in hairy root cultures of common buckwheat (*Fagopyrum esculentum*). Physiol Mol Biol Plants 2019; 25:787–797.

11 De Luca V, Salim V, Atsumi SM, Yu F. Mining the biodiversity of plants: a revolution in the making. Science. 2012; 336:1658–61.

12 Wurtzel ET, Kutchan TM. Plant metabolism, the diverse chemistry set of the future. Science. 2016; 353:1232–6.

13 Raskin I, Ribnicky DM, Komarnytsky S. Plants and human health inthe twenty-first century. Trends Biotechnol 2002; 20:522–31.

14 Hall RD, Brouwer ID, Fitzgerald MA. Plant metabolomics and its potential application for human nutrition. Physiol Plant 2008; 132:162–175.

15 Mizukam H. XII Gardenia jasminoides Ellis: In Vitro Propagation and the formation of iridoid glucosides. In: Biotechnology in Agriculture and Forestry, Vol. 7 Medicinal and Aromatic Plants II (ed. by Y. P. s. Bajaj). XX: Springer-Verlag Berlin Heidelberg 1989.

16 Dharmananda S. Gardenia: Key herb for dispelling dampness and heat via the triple burner, Institute for Traditional Medicine, Portland, Oregon 2003. (http://www.itmonline.org/arts/gardenia.htm).

17 Uddin R, Saha MR, Subhan N, Hossain H, Jahan IA, Akter R, Alam A. Advanced Pharmaceutical Bulletin 2014; 4:273–281.

18 El-Ashry AAL, Gabr AMM, Arafa NM. Rutin accumulationin gardenia calli cultures as a response to phenyl alanine and salicylicacid. Bull Natl Res Cent 2019; 43:141.

19 Boerjan W, Ralph J, Baucher M. 'Lignin biosynthesis'. Annual Review of Plant Biology 2003; 54:519–546.

20 Ranheim T, Halvorsen B. Coffee consumption and human health: beneficial or detrimental Mechanisms for effects of coffee consumption on different risk factors for cardiovascular disease and type 2 diabetes mellitus. Mol Nutr Food Res 2005; 49:274–84.

21 Salazar-Martinez E, Willett WC, Ascherio A, Manson JE, Leitzmann MF, Stampfer MJ, Hu FB. Coffee consumption and risk for type 2 diabetes mellitus. Ann Intern Med 2004; 140:1–8.

22 Lindsay J, Laurin D, Verreault R, Hebert R, Helliwell B, Hill GB, McDowell I. Risk factors for Alzheimer's disease: a prospective analysis from the Canadian Study of Health and Aging. Am J Epidemiol 2002; 156:445–53.

23 Almeida AA, Farah A, Silva DAM, Nunam EA. Gloì ria MBA. Antibacterial activity of coffee extracts and selected coffee chemical compounds against enterobacteria. J Agric Food Chem 2006; 54:8738–43.

24 Santos MD, Almeida MC, Lopes NP, Souza GEP. Evaluation of the antiinflamatory, analgesic and antypiretic activity of the natural polyphenol chlorogenic acid. Biol Pharm Bull 2006; 29:2236–40.

25 Murashigue T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol., Planta. 1962; 15:473–497.

26 Gabr AMM, Mabrok HB, Ghanem KZ, Blaut M, Smetanska I. Lignan accumulation in callus and Agrobacterium rhizogenes-mediated hairy root cultures of flax (Linum usitatissimum). Plant Cell, Tissue Organ Cult 2016; 126:255–267.

27 Bonhomme V, Laurain-Mattar D, Fliniaux MA. Effects of the rolC gene on hairy root: induction development and tropane alkaloid production by Atropa belladonna. J Nat Prod 2000; 63:1249–1252.

28 Gabr AM, Arafa NM, El-Ashry AA, El-Bahr MK. Impact of zeatin and thidiazuron on phenols and flavonoids accumulation in callus cultures of gardenia (Gardenia jasminoides). Pak J Biol Sci 2017; 20:328–335.

29 Slinkard K, Singleton T. Total Phenolic Analyses: Automation and Comparison with Manual Method. Am J Enol Viticul 1997; 28:49–55.

30 Saeedeh A-D., Asna U. Antioxidant properties of various solvent extracts of mulberry (*Morus indica* L.) leaves. Food Chem 2007; 102:4.

31 Ordon Ez AAL, Gomez JD, Vattuone MA, Isla MI. Antioxidant activities of Sechium edule (Jacq.) Swart extracts. Food Chem 2006; 97:452–458.

32 Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Evans CR. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biol Med 1999; 26:1231–1237.

33 Christey MC, Braun RH. Production of hairy root cultures and transgenic plants by Agrobacterium rhizogenes-mediated transformation. Methods Mol Biol 2005; 286:4–60.

34 Georgiev MI, Pavlov AI, Bley T. Hairy root type plant in vitro systems as sources of bioactive substances. Appl Microbiol Biotechnol 2007; 74: 1175–1185.

35 Srivastava S, Srivastava AK. Hairy root culture for mass production of high-value secondary metabolites. Crit Rev Biotechnol 2007; 27:29–43.

36 Chandra S, Chandra R. Engineering secondary metabolite production in hairy roots. Phytochem Rev 2011; 10:371–395.

37 Syklowska-Baranek K, Pietrosiuk A, Gawron A, Kawiak A, Lojkowska E, Jeziorek M, Chinou I. Enhanced production of antitumour naphthoquinones in transgenic hairy roots lines of Lithospermum canescens. Plant Cell Tissue Organ Cult 2012; 108:213–219.

38 Thwe A, Valan Arasu M, Li X, Park CH, Kim SJ, Al-Dhabi NA, Park SU. Effect of different *Agrobacteriumrhizogenes* strains on hairy root induction and phenylpropanoid biosynthesis in Tartary Buckwheat (Fagopyrum tataricum Gaertn). Front Microbiol 2016; 7:318.

39 Park WT, Baskar TB, Yeo SK, Il Park N, Park JS, Park S. Response of different *Agrobacterium Rhizogenes* strains for *in vitro* hairy root induction and accumulation of rosmarinic acid production in agastache rugosa. Online J Biol Sci 2017; 17:136–142.

40 Tavassoli P, Safipour, Afshar A. Influence of different *Agrobacterium rhizogenes* strains on hairy root induction and analysis of phenolic and flavonoid compounds in marshmallow (*Althaea officinalis* L). Biotech 2018; 8:351.

41 Azuma K, Nakayama M, Koshioka M, Ippoushi K, Yamaguchi Y, Kohata K, *et al.* Phenolic antioxidants from the leaves of Corchorus olitorius L. J Agric Food Chem 1999; 47:3963–3966.

42 Sahayarayan JJ, Udayakumar R, Arun M, Ganapathi A, Alwahibi MS, Aldosari NS, Abubaker MA. Effect of different Agrobacterium rhizogenes strains for in-vitro hairy root induction, total phenolic, flavonoids contents, antibacterial and antioxidant activity of (*Cucumis anguria* L.). Saudi. J Biol Sci 2020; 27:2972–2979.

43 Min Chung M, Rekha1 K, Rajakumar G, Thiruvengadam M. Production of glucosinolates, phenolic compounds and associated gene expression profiles of hairy root cultures in turnip (Brassica rapa ssp. rapa). Biotech 2016; 6:175.

44 El Sawy MA, El Shiekh A, El Ansary HO, Ali H, El Shiekh M, Witczak MJ, Ahmed M. Genetic transformation and hairy root induction enhance the antioxidant potential of *Lactuca serriola* L. Oxidative Medicine and Cellular Longevity 2017; 8:5604746.

45 Matvieieva N, Drobot K, Duplij V, Ratushniak Y, Shakhovsky A, Kyrpa-Nesmiian T, Mickevičius S, Brindza J. Flavonoid content and antioxidant activity of Artemisia vulgaris L. hairy roots. Prep Biochem Biotechnol 2019; 49:82–87.

46 Dorman HJD, Koşar M, Kahlos K, Holm Y, Hiltunen R. Antioxidant properties and composition of aqueous extracts from mentha species, hybrids, varieties, and cultivars. J Agric Food Chem 2003; 51:4563–4569.

47 Grayer RJ, Eckert MR, Veitch NC, Kite GC, Marin PD, Kokubun T, Simmonds MS, Paton AJ. The chemotaxonomic significance of two bioactive caffeic acid esters, nepetoidins A and B, in the Lamiaceae. Phytochem 2003; 64:519–528.

48 Georgiev M, Heinrich M, Kerns G, Pavlov A, Bley T. Production of Iridoids and Phenolics by Transformed Harpagophytum procumbens root cultures. Eng Life Sci 2006; 6:593–596.

49 Gabr AMM, Sytar O, Ahmed AR, Smetanska I. Production of phenolic acid and antioxidant activity in transformed hairy root cultures of common buckwheat (Fagopyrum Esculentum M). Aust J Basic Appl Sci 2012; 6: 577–586.

50 Awika JM, Rooney LW, Wu X, Prior RL, Cisneros-Zevallos L. Screening methods to measureantioxidant activity of sorghum (sorghum bicolor) and sorghum products. J Agric Food Chem 2003; 51:6657–6662.

51 Tuan PA, Kown DY, Lee S, Arasu MV, Al-Dhabi NA, Park N, Park SU. Enhancement of chlorogenic acid production in hairy roots of *Platycodon grandiflorum* by over-expression of an *Arabidopsis thaliana* transcription factor AtP AP1. Int J Mol Sci 2014; 15:14743–14752.

52 Fu X, Yin ZP, Chen JG, Shangguan XC, Wang X, Zhang QF, Peng DY. Production of Chlorogenic Acid and Its Derivatives in Hairy Root Cultures of *Stevia rebaudiana*. J Agric Food Chem 2015; 63:262–268.

53 Grace SC, Logan BA. Energy dissipation and radical scavenging by the plant phenylpronanoid pathway. PhilTrans R Soc Lond B 2000; 355: 1499–1510.

54 Liu C-Z., Abbasi BH, Gao M, Murch SJ, Saxena PK. Caffeic Acid Derivatives Production by hairy root cultures of *Echinacea purpurea*. J Agric Food Chem 2006; 54:8456–8460.

55 Horváth E, Pál M, Szalai G, Páldi E, Janda T. Exogenous 4-hydroxybenzoic acid and salicylicacid modulate the effect of short-term drought and freezing stress on wheat plants. Biologia Plantarum 2007; 51:480–487.

56 Sircar D, Roychowdhury A, Mitra A. Accumulation of p-hydroxybenzoic acid in hairy roots of Daucus carota. J Plant Physiol 2007; 164:1358–66.

57 Mamdouh D, Mahgoub HAM, Gabr AMM, Ewais EA, Smetanska I. Genetic Stability, Phenolic, Flavonoid, Ferulic Acid Contents, and Antioxidant Activity of Micropropagated *Lycium schweinfurthii*. Plants Plants 2021; 10:2089.

58 Goudar G, Sathisha GJ. Effect of Processing on Ferulic Acid Content in Foxtail Millet (*Setaria italica*) Grain Cultivars Evaluated by HPTLC. Orient J Chem 2016; 32:2251–2258.