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FATMA PEHLİVAN KARAKAŞ

GÜNCE CİNGÖZ

ARZU TÜRKER

TÜBİTAK

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Enhancement of direct shoot regeneration and determination of bioactive secondary metabolites in leaves of *Galega officinalis* L.

Fatma PEHLİVAN KARAKAŞ[1,2,*], Günce ŞAHİN[2], Arzu TÜRKER[2]

[1]Department of Field Crops, Faculty of Agriculture and Natural Sciences, Abant İzzet Baysal University, Bolu, Turkey
[2]Department of Biology, Faculty of Science and Arts, Abant İzzet Baysal University, Bolu, Turkey

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Abstract: *Galega officinalis* L. is a medicinal plant being used as a galactagogue and an antidiabetic in folk medicine. A new effective micropropagation protocol was established from leaf, stem, node, and root explants of *G. officinalis* for in vitro plant regeneration and callus induction systems. Murashige and Skoog (MS) medium supplemented with 55 various concentrations and 4 different combinations [benzyl adenine (BA)/naphthalene acetic acid (NAA), BA/indole-3-acetic acid (IAA), thidiazuron (TDZ)/indole-3-butyric acid (IBA), and TDZ/IAA] of plant growth regulators was used for the regeneration systems. The regenerated shoots were observed on only nodal explants through direct organogenesis at the 35th day. The highest shoot number (7.13 ± 1.12 shoots per explants) was obtained from nodal explants on MS medium supplemented with a 1.0/0.25 mg L^{-1} BA/NAA combination. The largest (2 cm in diameter) green and compact callus formations were induced using root explants on MS medium supplemented with 2.0/2.0 mg L^{-1} BA/NAA. A total of 16 and 11 phenolic compounds from wild-grown and in vitro-grown leaves was detected by using liquid chromatography-electrospray ionization-multistage/mass spectrometry (LC-ESI-MS/MS) analysis, respectively. Levels of apigenin, luteolin, and chlorogenic acid in in vitro-grown leaves were higher than those in wild-grown leaves. These findings showed that micropropagation of *G. officinalis* can be potentially used as a new protocol for the production of beneficial secondary metabolites in pharmaceutical and supplemental food industries.

Key words: Micropropagation, plant growth regulators, callus, phenolic compounds, LC-ESI-MS/MS

1. Introduction

Galega officinalis L. (goat's rue) is a perennial forage plant in the family Leguminosae that grows wild in damp and low-lying regions. It is a native plant for southeastern Europe (Pundarikakshudu et al., 2001). Goat's rue has been used as a remedy against diabetes, malignant fever, and parasitic infections and was commonly cultivated as cattle feed in ancient times (Grieve, 1982; Chevallier, 1996). It has been generally used to increase milk output of goats and most other mammals in folk medicine (Castroviejo, 1999; Leporatti and Ivancheva, 2003; Rasekh et al., 2007). A highly lactogenic effect of *G. officinalis* forage was demonstrated with experimental studies in sheep (Gonzalez et al., 2004) and cows (Bikbulatov et al., 1997). This plant was especially described as an antidiabetic herb, having the ability to reduce blood sugar levels (Chevallier, 1996; Oubre et al., 1997). Galegine is a guanidine alkaloid and a major compound isolated from *G. officinalis* to reduce the blood glucose levels (Oubre et al., 1997; Palit et al., 1999; Vuksan and Sievenpiper, 2005). Metformin, a synthetic form of galegine, is an antihyperglycemic agent used in the treatment of noninsulin-dependent type 2 diabetes mellitus and causes significant effects in reducing the risk of diabetes mellitus development (Yeh et al., 2003). Atanasov and Spasov (2000) showed that the crude aqueous extract of *G. officinalis* has an inhibitory activity on platelet aggregation. Some different extracts of aerial parts of *G. officinalis* exhibited a broad spectrum of antibacterial effects against both gram-positive (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and gram-negative (*Serratia marcescens*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Enterobacter cloacae*, and *Escherichia coli*) bacteria (Pehlivan Karakaş et al., 2012). *G. officinalis* contains some important secondary metabolites such as alkaloids (e.g., galegine), saponins, flavonoids (e.g., sativan and medicarpin) (Le Bail et al., 2000), phytoestrogens (e.g., flavonol triglycosides, kaempferol, and quercetin) (Champavier et al., 1999; Peiretti and Gai, 2006), tannins (Chevallier, 1996), and fatty acids (e.g., α-linolenic acid, palmitic acid, and linoleic acid) (Peiretti and Gai, 2006).

* Correspondence: fatmapehlivankarakas@gmail.com

Increasing use of forage legumes ensures possibilities to drop the costs of milk manufacturing, adorn the environment, and expand the effective field use (Pahlow et al., 2000; Peiretti and Gai, 2006). Forages found in the family Leguminosae have significant potential in efficient sustainable agriculture areas owing to their role in nitrogen (N) fixation and their nutritional rate (Halling et al., 2000). *Galega* species has been used as forage herbs like fresh green feed and winter forage for some animals (Adamovich, 2000; Peiretti and Gai, 2006) because of their high organic nutrient content. Moreover, they have an effect in the decrease of plant diseases, pests, and some weeds (Varis, 1986; Gonzalez et al., 2004).

Notwithstanding the fact that *G. officinalis* is a beneficial medicinal plant and useful forage, there are no comprehensive reports on a tissue or callus culture protocol for this important antidiabetic plant species. The present study provides, for the first time, efficient in vitro regeneration and callus formation methods for *G. officinalis*. At the same time this protocol could be used for *G. officinalis* micropropagation in order to conserve and provide commercial natural antidiabetic drug production. The high frequency of regeneration and callus formation showed the potential of *G. officinalis* for secondary metabolite production in the pharmaceutical industry. The present study aimed to screen the phytochemical compositions of 20 various phenolic standards in the leaves of *G. officinalis* obtained from wild-grown and in vitro-grown plants by liquid chromatography-electrospray ionization-multistage/mass spectrometry (LC-ESI-MS/MS) analysis.

2. Materials and methods

2.1. Plant material, seed sterilization, and germination

Seeds of *G. officinalis* were collected from the campus of Abant İzzet Baysal University, Bolu, Turkey, in September 2015 (Figure 1a). Identification of the species was done using *Flora of Turkey and the East Aegean Islands* (Davis, 1970) and voucher specimens (AUT-1912) were deposited at the herbarium of Abant İzzet Baysal University.

The seed surface sterilization procedure was performed as described by Egamberdieva et al. (2013) with some modifications. The seeds were dipped in distilled water for 24 h after they were placed in a refrigerator at 4 °C for 7 days. Subsequently, the seeds were washed with an antibacterial soap (Protex®), rinsed with distilled water and surface-sterilized by shaking in concentrated sulfuric acid for 5 min, and rinsed twice with sterilized distilled water. Sterilization was accomplished with 70% ethanol (EtOH) for 3 min. Finally, the seeds were washed with sterile distilled water 4 or 5 times (Figure 1b). Furthermore, the seeds were germinated in sterile, disposable petri dishes containing 1% water agar in the dark at 28 °C for 1 week (Figure 1c). After 7 days of incubation on this medium, seedlings (Figure 1d) were transferred to glass jars containing MS medium (4.43 g/L MS, Sigma Chemical Co., St. Louis, MO, USA; Murashige and Skoog, 1962) with 30 g/L sucrose and 8.0 g/L Bacto-agar (Difco®) for an additional 3 weeks (Figure 1e). The pH of the culture medium was adjusted to 5.8. Sterilization of the culture medium was performed in an autoclave at 121 °C for 20 min. All prepared culture vessels were maintained at 22 ± 2 °C with a long day photoperiod (16/8 light/dark regime) under cool white light at 22–28 μmol m^{-2} s^{-1} and 55%–60% relative humidity.

2.2. Explants, plant growth regulators (PGRs), and culture conditions

Leaf lamina (5 × 5 mm pieces), stem (5–6 mm segments), node (6–8 mm segments), and root (8–10 mm segments) explants (Figure 1f) were excised from 4-week-old sterile seedlings and placed into sterile disposable petri dishes (90 × 15 mm) containing 20 mL of MS medium supplemented with various combinations and concentrations of PGRs: BA (0.25, 0.5, 1.0, 2.0, 3.0, or 5.0 mg L^{-1}) and NAA (0.1, 0.25, 0.5, 1.0, 2.0, or 3.0 mg L^{-1}); BA (0.1, 0.5, 1.0, 3.0, or 5.0 mg L^{-1}) and IAA (0, 0.1, 0.25, or 0.5 mg L^{-1}); TDZ (0.5 or 1.0 mg L^{-1}) and IBA (0, 0.5, or 1.0 mg L^{-1}); and TDZ (0.25, 0.5, or 1.0 mg L^{-1}) and IAA (0, 0.5, or 1.0 mg L^{-1}). After 35 days, the callus formation diameters (-: no callus formation, +: callus formation only on edges of explant, ++: callus formation is 0.5–1.0 cm in diameter, +++: callus formation is 1.0–1.5 cm in diameter, ++++: callus formation is 1.5–2.0 cm in diameter), the numbers of shoot formations, and the percentage (%) of explants forming shoots were recorded.

2.3. Rooting process of the sterile regenerated shoots

Well-developed shoots (≥3 cm in length) from nodal explants were separated and transferred to MS medium supplemented with 0.5, 1.0, and 3.0 mg L^{-1} IAA, IBA, and NAA or 0.05, 0.5, and 1.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). After 30 days, effective root formation was observed and the numbers of roots per shoot and % of shoots producing roots were recorded.

2.4. Acclimatization

Rooted plantlets, namely regenerants, were washed with sterile distilled water under sterile conditions to clean the rooting medium from roots. The rooted plantlets were planted in transparent Magenta containers including sterile vermiculite (Agrekal®) and water. Seven days later, surviving plantlets were transferred to plastic pots including sterile potting soil and vermiculite (4:1) and maintained under high humidity (55%–60% relative humidity) for 15 days. Following this process, all pots were

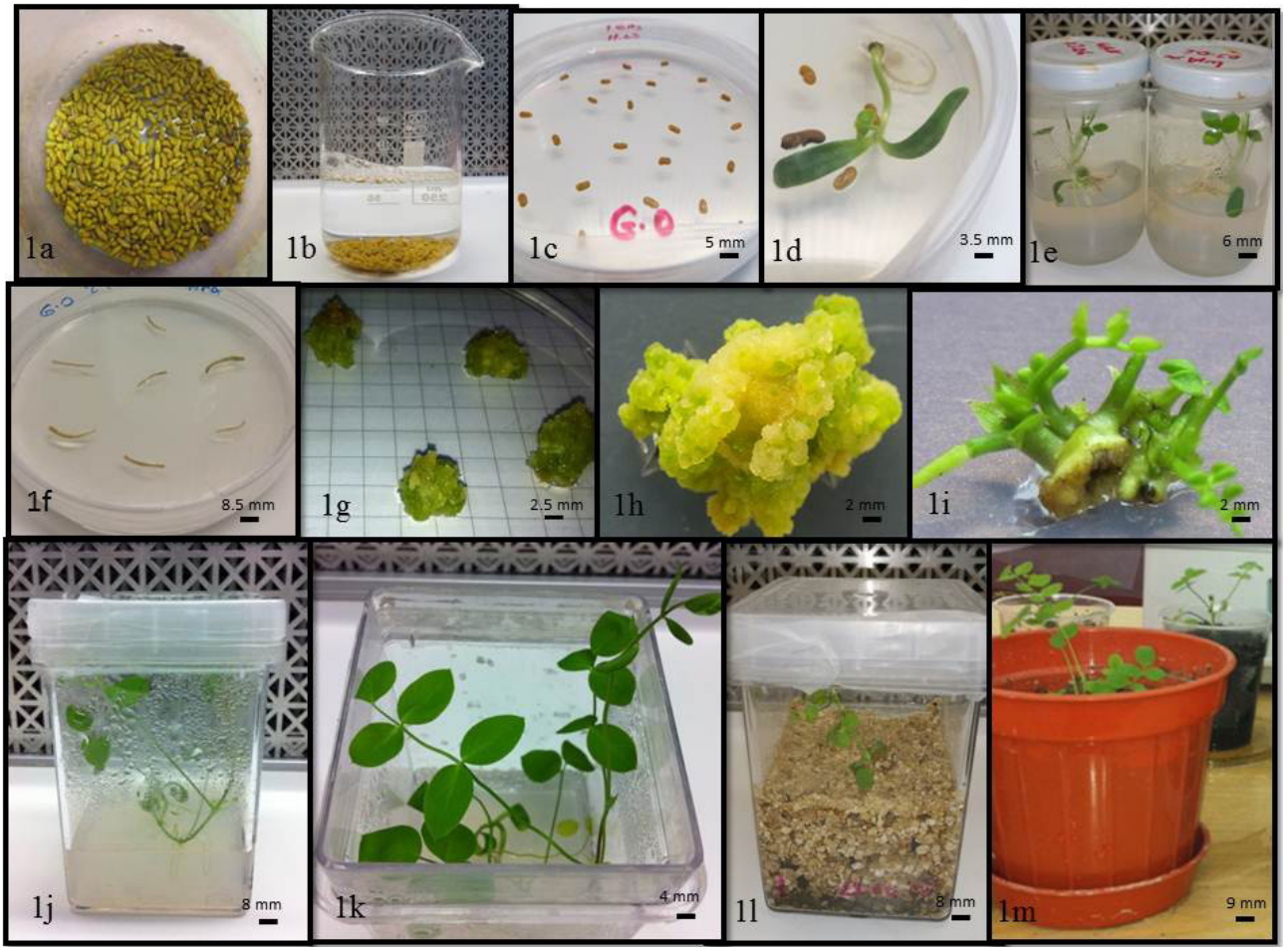

Figure 1. In vitro plant regeneration process of *G. officinalis*: a) Seeds of *G. officinalis*. b) The surface sterilization of seeds. c) Seeds on the germination medium. d) Germinated seed. e) Shoot elongation in MS medium. f) Root explants. g) Callus formations. h) The largest callus formation developed from root explant on MS medium containing 2.0/2.0 mg L^{-1} BA/NAA after 4 weeks in culture conditions. i) Adventitious shoots formed from nodal explants on MS medium containing 1.0/0.25 mg L^{-1} BA/NAA after 4 weeks. j) Rooting of the regenerated shoots on MS medium containing 0.5 mg L^{-1} IAA after 4 weeks in culture condition. k) Before regenerated plantlets were transferred for acclimatization. l) Acclimatization. m) Surviving plantlets placed into pots with sterile soil and vermiculite (4:1) under plant growth room conditions.

maintained in plant growth room conditions at 20–22 °C with low humidity (25%–35%).

2.5. Plant material and extract preparation for analysis of phenolic compounds

Wild-grown leaves of *G. officinalis* were obtained from Abant, Bolu, Turkey, in May 2015. In vitro-grown leaves of *G. officinalis* were collected from in vitro-cultured plants that were previously propagated in vitro in laboratory conditions. Freeze-dried powdered plant parts (10 g) of *G. officinalis* were extracted with 100 mL of methanol (MeOH) using a water bath (at 35 °C) for 16 h. After the extraction process, residues were filtrated with Whatman filter paper (Grade 1) and extraction solvents were evaporated at 38 °C using a rotary evaporator.

2.6. Phenolic compound determination by LC-MS/MS analysis

The quantification of the 20 chosen phenolic compounds (apigenin, caffeic acid, *p*-coumaric acid, gallic acid, genistein, kaempferol, luteolin, myricetin, procyanidin-*C1*, quercetin, rutin hydrate, vanillic acid, ferulic acid, salicylic acid, sinapic acid, chlorogenic acid, hesperidin, naringenin, rosmarinic acid, and isorhamnetin) in MeOH extracts of wild-grown and in vitro-grown leaves was measured using the LC-ESI-MS/MS method. Analysis was performed by the METU Central Laboratory, Molecular Biology-Biotechnology Research and Development Center, Mass Spectroscopy Laboratory, Ankara, Turkey, with an Agilent 6460 Triple Quadrupole System (ESI + Agilent Jet Stream) coupled with Agilent 1200 Series HPLC. The stock

standards were prepared with methanol (MS grade) and dilutions were done with 50% methanol. A mobile phase consisting of a mixture of 0.05% formic acid + 5 mM ammonium formate (solvent A) and methanol (MS grade, Merck) (solvent B) was delivered at a flow rate of 0.3 mL/min for 13 min (run time). All analysis conditions were established according to Karakas and Turker (2013).

2.7. Statistical analysis

Each experiment was repeated in triplicate and 15 explants were evaluated for each replicate. The significance of differences among means was performed using Duncan's multiple range tests at $P < 0.05$ (ANOVA). The results were calculated as mean ± SD (standard deviation). Small letters (i.e. a, b, c, d, e) were used to show statistical differences between means. All results were statistically analyzed using SPSS 22.0 (IBM Corp., Armonk, NY, USA).

3. Results and discussion

3.1. In vitro plant regeneration of *G. officinalis*

Considering the importance of *G. officinalis* as feed for some animals with high nutritional value, and as a natural antidiabetic plant for humans, its in vitro tissue culture protocol has not been previously reported in the literature. In the present study, multiple shoot formation was achieved from node explants of *G. officinalis*.

After surface sterilization of the seeds, they were germinated on 1% water agar for 6–7 days. High seed viability and high germination rate (92%) were observed for obtaining sterile seedlings. The sterile seedlings were transferred to MS medium for an additional 3 weeks. Explants (leaf, petiole, stem, node, and root) were excised from sterile seedlings (4 weeks old) and cultured on MS medium including different levels of BA/NAA or BA/IAA and TDZ/IBA or TDZ/IAA (Table 1). Shoot regeneration was observed only with nodal explants. Shoot propagation was not observed with leaf, stem, and root explants with the tested PGR combinations (data not shown). The most efficient treatment was the combination of 1.0/0.25 mg L^{-1} BA/NAA with an average of 7.13 ± 1.12 shoots per tested explant and 100% shoot production frequency (Table 1). When node explants were cultured on media containing 1.0 mg L^{-1} BA plus NAA, the shoot production and frequency were at nearly the same levels as those seen with NAA. While increasing the NAA concentration from 0.25 mg L^{-1} to 2.0 mg L^{-1}, shoot number decreased from 7.13 to 3.0 shoots. Likewise, shoot formation frequency decreased from 100% to 80. On the other hand, increasing the NAA concentration from 2.0 mg L^{-1} to 3.0 mg L^{-1}, shoot formation was not observed (Table 1). Different levels of kinetin (KIN) plus NAA combinations were also tested for shoot production for all explant types excised from sterile seedlings. This combination did not prove efficient for shoot propagation (data not shown). The TDZ with IBA or IAA combination was examined for shoot generation for all types of explants. Shoot propagation was only ensured from nodal explants, with 100% shoot formation frequency on medium supplemented with both 0.5 and 1.0 mg L^{-1} TDZ in combination with 0.5 mg L^{-1} IAA (Table 1). Control treatments without PGRs, as well as treatments with BA (0.25, 0.5, 1.0, 2.0, or 3.0 mg L^{-1}) and TDZ (0.25, 0.5, or 1.0 mg L^{-1}) combined with various concentrations of auxins (NAA and IAA), produced no shoots at all (Table 1). Callus formation was observed for stem, root, petiole, and leaf explants (Figure 1g). The callus formation results are shown in Table 1. Optimum callus formation was obtained from root explants with 100% callus formation frequency on the medium supplemented with 2.0 mg L^{-1} BA plus 2.0 mg L^{-1} NAA (Table 1; Figure 1h).

It is well known that both endogenous contents of the hormones and exogenous PGRs affect direct and indirect shoot regeneration processes in plants (Trigiano and Gray, 2000; Karakas and Turker, 2013). Many studies showed that various plant tissue samples (i.e. leaf, root, stem, node, petiole, and flower) may have various amounts of endogenous plant hormones. Accordingly, explant origin has an important effect on successful shoot propagation. The source and the part of the plant material have a critical role in the performance of micropropagation processes (Karakas and Turker, 2013). In the present study, when node, stem, root, petiole, and leaf explants were used, it was evident that only node explants of *G. officinalis* were fruitful for shoot production (Table 1; Figure 1i). Likewise, the nodal explants of some plant species were noticed as being the most effective explants on axillary shoot multiplication with *Gleditsia triacanthos* L. (Basbaa et al., 1995), *Bauhinia variegata* L. (Papafotiou et al., 2010), *Desmodium gangeticum* L. (Preeti et al., 2013) and *Ajuga multiflora* (Sivanesan and Park, 2015) in the family Leguminosae. However, plant hormones and PGRs provided in the medium affected the findings acquired with in vitro plant tissue cultures (Pintos et al., 2002). In our research, when exogenous auxin or cytokinin was not added to the MS culture medium, shoot multiplication was not observed in any tested explant types. It is clear that the existence of endogenous and exogenous auxins is mandatory for shoot multiplication induction, since they can arrange biosynthesis of cytokinin and decrease cytokinin concentrations in plant tissues (Nordstrom et al., 2004; Karakas and Turker, 2013). It is well known that cytokinins regulate many cellular processes, but the control of cell division is central to the growth and development of many plants. Therefore, an equilibrium between endogenous plant hormone concentrations and PGR treatment in the culture medium is needed (Mallon et al., 2011). The helping action of BA on in vitro growth has

Table 1. Shoot regeneration from nodal explants and callus formation from stem, root, petiole, and leaf explants cultured on MS medium containing different combinations and concentrations of BA/NAA, BA/IAA, TDZ/IBA, and TDZ/IAA. Means in a column with different letters (superscripts) are significantly different according to Duncan's multiple range test ($P < 0.05$). "Control" means that no PGRs were supplemented to the MS medium.

PGRs (mg L^{-1})		Nodal explant		Callus formation			
		Mean number of shoots/per explant (±SD)	Shoot induction rate (%)	Stem explant	Root explant	Petiole explant	Leaf explant
Control		-	-	-	-	-	-
BA	NAA						
0.25	0.1	2.06 ± 0.59 ^{h}	93.34	++	+++	++	+
0.25	0.25	1.86 ± 0.35 ^{hijk}	100	-	-	-	-
0.25	0.5	-	-	-	-	-	-
0.25	1.0	-	-	-	-	-	-
0.25	2.0	-	-	-	-	-	-
0.25	3.0	-	-	-	-	-	-
0.5	0.1	2.33 ± 0.48 ^{fgh}	86.67	++	++	++	-
0.5	0.25	0.93 ± 0.25 ^{m}	93.34	+	+	+	-
0.5	0.5	2.8 ± 0.77 ^{defg}	100	+	+	+	-
0.5	1.0	1.13 ± 0.35 ^{klm}	100	+	-	-	-
0.5	2.0	1.0 ± 0.0 ^{lm}	100	++	++	+	-
0.5	3.0	-	-	-	-	-	-
1.0	0.1	2.46 ± 0.63 ^{efgh}	100	++	++	++	+
1.0	0.25	7.13 ± 1.12 ^{a}	100	-	+	-	-
1.0	0.5	5.6 ± 1.05 ^{b}	100	-	+	-	-
1.0	1.0	4.13 ± 1.35 ^{c}	86.67	++	+	++	-
1.0	2.0	3.0 ± 0.51 ^{def}	80	++	++	+	-
1.0	3.0	-	-	-	-	-	-
2.0	0.1	1.13 ± 0.35 ^{klm}	100	+	++	++	-
2.0	0.25	1.33 ± 0.46 ^{ijklm}	100	+	++	+	-
2.0	0.5	2.06 ± 0.59 ^{hijkl}	100	++	+	+	-
2.0	1.0	1.0 ± 0.53 ^{lm}	86.67	++	++	+	-
2.0	2.0	0.86 ± 0.63 ^{m}	80	+	++++	+	+
2.0	3.0	-	-	-	-	-	-
3.0	0.1	1.26 ± 0.59 ^{jklm}	100	++	+	+	-
3.0	0.25	4.2 ± 0.56 ^{c}	100	-	+	-	-
3.0	0.5	4.4 ± 1.2 ^{c}	100	+++	++	++	+
3.0	1.0	1.93 ± 0.70 ^{hij}	100	-	-	-	-
3.0	2.0	1.2 ± 0.70 ^{klm}	93.34	+	-	+	-
3.0	3.0	-	-	-	-	-	-
BA	IAA						
0.1	0.1	-	-	-	-	-	-
0.1	0.25	-	-	-	+	-	-
0.1	0.5	-	-	+	++	-	-
0.5	0.1	-	-	-	-	-	-
0.5	0.25	1.0 ± 0.36 ^{lm}	93.34	+	+++	-	-
0.5	0.5	2.06 ± 0.59 ^{h}	100	+	++	-	-
1.0	0.1	3.13 ± 0.51 ^{de}	100	+	+	-	-
1.0	0.25	4.2 ± 1.37 ^{c}	93.34	+	+	-	-
1.0	0.5	-	-	-	-	-	-
3.0	0.1	2.0 ± 0.92 ^{hi}	86.67	-	-	-	-
3.0	0.25	2.93 ± 0.67 ^{def}	100	-	-	-	-
3.0	0.5	3.26 ± 0.70 ^{d}	100	-	-	-	-
5.0	0.1	-	-	+	+	-	-
5.0	0.25	0.86 ± 0.51 ^{m}	80	-	-	-	-
5.0	0.5	1.06 ± 0.45 ^{lm}	93.34	-	-	-	-
TDZ	IBA						
0.5	0.5	-	-	-	-	-	-
0.5	1.0	1.06 ± 0.59 ^{lm}	86.67	-	+	-	-
1.0	0.5	0.73 ± 0.45 ^{m}	73.34	-	++	-	-
1.0	1.0	1.13 ± 0.63 ^{klm}	86.67	-	+++	-	-
TDZ	IAA						
0.25	0.5	-	-	-	-	-	-
0.25	1.0	-	-	-	-	-	-
0.5	0.5	3.2 ± 0.41 ^{d}	100	++	+++	+	+
0.5	1.0	-	-	+	-	-	-
1.0	0.5	1.26 ± 0.59 ^{jklm}	100	-	-	-	-
1.0	1.0	-	-	-	-	-	-

been recently noticed for some plant species (Sivanesan and Park, 2015). A combination of BA with NAA was more effective than the combinations of TDZ/IAA or TDZ/IBA for shoot formation in this study (Table 1). This result corroborated the findings of Sivanesan and Park (2015), who reported that the highest number of shoots was obtained with a combination of BA (2.0 mg L^{-1}) and NAA (0.5 mg L^{-1}) from nodal explants of *Ajuga multiflora*. Similarly, the shoot induction effect of BA plus NAA has been shown in different medicinal plant species such as *Mucuna pruriens* (Fasial et al., 2006), *Vitex negundo* (Ahmad et al., 2013), and *Swertia corymbosa* (Mahendran and Narmatha Bai, 2014) in previous studies.

Multiplied shoots were separated singularly and maintained on MS medium including various types and concentrations of auxins (IAA, IBA, 2.4-D, or NAA) for the rooting process of *G. officinalis*. Root formation was observed within 4 weeks. IAA was the best rooting PGR among the tested auxins. The highest number of roots per shoot (3.6 ± 0.57 roots generated for each regenerated shoot with a 100% frequency of root formation production) was obtained on MS medium supplemented with 0.5 mg L^{-1} IAA (Table 2; Figures 1j and 1k). Similarly, IAA was the most efficient auxin for root formation with *Lathyrus sativus* L. belonging to Leguminosae (Barpete et al., 2014). However, increasing the IAA concentration from 0.5 to 1.0 mg L^{-1}, less root formation was observed. In addition, enhancing IAA concentration from 1.0 to 3.0 mg L^{-1}, frequency of root formation was decreased from 100% to 0% (Table 2). Auxins are commonly known for obtaining meristematic ability of susceptible cells. However, when this capability has been accomplished, higher auxin concentrations generally show inhibitory effects for adventitious root formation (Gurel and Wren, 1995; Karakas and Turker, 2013). Our results showed that increasing levels of IBA did not modify the rate of root formation. On the other hand, increasing IBA concentration from 0.5 to 3 mg L^{-1}, rooting frequency was decreased from 86.66% to 0%. NAA, 2.4-D (data not shown), and the auxin-free MS control group were not effective for root formation (Table 2).

After 4 weeks, the rooted seedlings were placed in plastic pots with commercial soil under high humidity condition for 2 weeks (Figure 1l). Acclimatized plants (80%) were placed into bigger pots and held in conditions of low humidity (20%–25%) (Figure 1m).

An in vitro multiplication protocol for *G. officinalis* was obtained in this study for the first time. This protocol would give considerable assistance for the production of large amounts of certain secondary metabolites such as galegine, apigenin, luteolin, chlorogenic acid, medicarpin, and sativan from *G. officinalis* and can supply natural plant products for future food industry research and medicinal drug studies.

3.2. Phenolic composition of *G. officinalis*

Twenty phenolic compounds of MeOH extracts of wild-grown and in vitro-grown leaves obtained from *G. officinalis* were analyzed using LC-ESI-MS/MS analysis. Our findings showed that total phenolic content in the methanol extract of wild-grown plant leaves [36.6859 µg/g of dry extract (de)] was fourfold higher than that of in vitro-grown plant leaves (9.3166 µg/g of de) (Table 3). Considerable amounts of *trans*-ferulic acid (6.8120 µg/g of de), *p*-coumaric acid (6.8120 µg/g of de), rutin hydrate (5.5526 µg/g of de), isorhamnetin (4.0190 µg/g of de),

Table 2. Effects of different auxins on root induction from regenerated shoots. Different superscripts in the same column indicate significant differences within treatments according to Duncan's multiple range test ($P < 0.05$).

PGRs (mg L^{-1})	Mean root numbers/shoot (±SD)	Root induction rate (%)
Control	-	-
IAA		
0.5	3.6 ± 0.57 [a]	100
1	1.1 ± 0.31 [b]	80
3	-	-
IBA		
0.5	0.93 ± 0.35 [c]	86.66
1	-	-
3	-	-

Table 3. The amount of the tested phenolic compounds examined in tissue culture-grown and wild-grown leaves from *G. officinalis* using LC-ESI-MS/MS analysis. Values are means ± SD of three measurements. Nd: not detected.

Phenolic compounds		Retention time (min)	Amount of phenolics (µg/g of dry extract)	
			In vitro-grown leaves	Wild-grown leaves
1	Apigenin	6.998	0.3177 ± 0.0017	0.0374 ± 0.0006
2	Caffeic acid	3.150	Nd	2.0488 ± 0.0061
3	*p*-Coumaric acid	3.959	0.6275 ± 0.0028	6.8120 ± 0.0149
4	Gallic acid	0.943	Nd	0.1379 ± 0.0044
5	Genistein	6.493	0.3116 ± 0.0074	0.3076 ± 0.0033
6	Kaempferol	6.864	0.0287 ± 0.0017	1.2319 ± 0.0142
7	Luteolin	6.489	1.7884 ± 0.0100	0.0117 ± 0.0002
8	Myricetin	5.347	Nd	Nd
9	Procyanidin-C1	3.181	Nd	Nd
10	Quercetin	6.135	Nd	2.0664 ± 0.0558
11	Rutin hydrate	4.985	1.0067 ± 0.0065	5.5526 ± 0.0587
12	Vanillic acid	3.120	Nd	0.2938 ± 0.0030
13	Ferulic acid	4.271	0.6275 ± 0.0028	6.8120 ± 0.0149
14	Salicylic acid	3.933	0.2899 ± 0.0080	3.3763 ± 0.0328
15	Sinapic acid	4.377	Nd	0.1475 ± 0.0127
16	Chlorogenic acid	2.807	4.2206 ± 0.0075	2.1316 ± 0.1168
17	Hesperidin	5.169	Nd	Nd
18	Naringenin	6.235	0.3599 ± 0.0002	1.7368 ± 0.0373
19	Rosmarinic acid	4.949	Nd	Nd
20	Isorhamnetin	7.003	0.0558 ± 0.0022	4.0190 ± 0.0471
Total phenolics			9.3166	36.6859

salicylic acid (3.3763 µg/g of de), chlorogenic acid (2.1316 µg/g of de), quercetin (2.064 µg/g of de), caffeic acid (2.0488 µg/g of de), naringenin (1.7368 µg/g of de), kaempferol (1.2319 µg/g of de), genistein (0.3076 µg/g of de), vanillic acid (0.2938 µg/g of de), sinapic acid (0.1475 µg/g of de), gallic acid (0.1379 µg/g of de), apigenin (0.0374 µg/g of de), and luteolin (0.0117 µg/g of de) were detected in MeOH extract of wild-grown leaves (Table 3). The large amount of phenolic compound ingredients of wild-grown leaves of *G. officinalis* might be based on the changes occurring in biotic and abiotic natural environment conditions. The biosynthesis of some phenolic molecules, accompanied by enhanced activities of phenylalanine ammonia-lyase (PAL), is stimulated when plants are injured or infected by some microorganisms (Takahama and Oniki, 2000), or under abiotic stress conditions such as low nutrient supply, heavy metals, low or high temperatures, and UV-radiation (Ruiz et al., 2003). The most dominant phenolic compounds were chlorogenic acid (4.2206 µg/g of de), rutin hydrate (1.7884 µg/g of de), *trans*-ferulic acid (0.6275 µg/g of de), *p*-coumaric acid (0.6275 µg/g of de), naringenin (0.3599 µg/g of de), apigenin (0.3177 µg/g of de), genistein (0.3116 µg/g of de), salicylic acid (0.2899 µg/g of de), isorhamnetin (0.0558 µg/g of de), and luteolin (0.0117 µg/g of de) in in vitro-grown leaves (Table 3). Sixteen of the 20 phenolic compounds were defined within the limit of detection (LOD). The myricetin, procyanidin C1, hesperidin, and rosmarinic acid levels were lower than the LOD in both tested extracts. Furthermore, caffeic acid, gallic acid, quercetin, vanillic acid, and sinapic acid were lower than the LOD in MeOH extract of in vitro-grown leaves (data not shown). The methanol extract of in vitro-

grown leaves had tenfold higher apigenin, hundredfold higher luteolin, and twofold higher chlorogenic acid than wild-grown leaves (Table 3). Some secondary metabolites' production or biosynthesis may need specific PGRs or non-stressed conditions.

Recent studies showed that only two isoflavones (sativan and medicarpin) were purified and identified from aerial parts of *G. officinalis* (Le Bail et al., 2000) until now. The *trans*-ferulic acid, *p*-coumaric acid, rutin hydrate, isorhamnetin, salicylic acid, chlorogenic acid, quercetin, caffeic acid, naringenin, kaempferol, genistein, vanillic acid, sinapic acid, gallic acid, apigenin, and luteolin were determined in the MeOH extract of wild-grown leaves of *G. officinalis* for the first time in our present study (Table 3).

The existence of some important phenolic molecules in wild-grown and in vitro-grown leaves of *G. officinalis* can be beneficial as a basis for future studies of *G. officinalis*. These findings could probably be useful for commercial secondary metabolite production from natural plant materials.

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