Original Paper

In vitro proliferation and triterpenoid characteristics of licorice (*Glycyrrhiza uralensis* Fischer, Leguminosae) stolons

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Abstract Dried roots and stolons of licorice plants (*Glycyrrhiza uralensis*) are among the most important drugs (*Glycyrrhizae radix*) in traditional oriental medicine; they are also important commercial products used worldwide in sweetening and flavoring. Here, we describe the establishment of an *in vitro* stolon culture system for *G. uralensis*. Stolon formation was induced in single node stems (with axillary buds) grown in Murashige and Skoog (MS) liquid culture medium, supplemented with 0.01 μM α-naphthaleneacetic acid (NAA). Stolons were cultured at 26°C in the dark on a rotary shaker, gyrating at 100 rpm. The same NAA concentration produced the highest rates of stolon proliferation (6.58-fold in 4 weeks). A 6% sucrose concentration also enhanced stolon proliferation (6.34-fold in 4 weeks). GC/MS analysis confirmed the accumulation of small amounts of glycyrrhizin (14 μg g^{-1} dry weight) in cultured stolons. Interestingly, betulinic acid and oleanoic acid production *in vitro* were higher than in field-grown stolons. Adventitious root and shoot regeneration from cultured stolons were readily achieved under illuminated conditions in MS medium containing 0.01 μM of NAA and 0.2% gerlite. Regenerated plants produced glycyrrhizin (7,600 μg g^{-1} dry weight) in their roots. Our *in vitro* stolon culture system is suitable for studying glycyrrhizin biosynthesis and for rapid propagation of elite licorice clones.

Key words: *Glycyrrhizae radix, Glycyrrhiza uralensis*, glycyrrhizin, licorice, stolon, tissue culture.

Dried roots and subterranean stems (stolons) of the *Glycyrrhiza* species *Glycyrrhza uralensis* Fisch. and *G. glabra* L. (licorice plants, Leguminosae) comprise the crude drug *Glycyrrhizae radix* (Japanese Pharmacopoeia, 2006). *G. inflata* and other *Glycyrrhiza* species are also used as natural medicines. *Glycyrrhizae radix* has important uses in traditional oriental medicine because of its anti-inflammatory (Matsui et al. 2004), immunomodulatory (Takahara et al. 1994), anti-ulcer (He et al. 2001) and anti-allergenic properties (Park et al. 2004). It also has antiviral activity against HIV (Ito et al. 1988, Hirabayashi et al. 1991) and acute respiratory syndrome (SARS)-associated *Coronavirus* (Cinatl et al. 2003). Roots and stolons of licorice plants are also an important commercial product, used as a sweetener and flavoring in the many food, tobacco, and confectionary products. Roots and stolons contain a number of interesting compounds, the most important of which is glycyrrhizin (glycyrrhizic acid; Figure 1), a triterpenoid saponin. Licorice plants grow in only a few regions of the world, primarily semi-arid zones of southern Europe and Asia. In recent decades, wild-grown resources in these regions have been exhausted, and they are undergoing desertification.

Most licorice roots and stolons are obtained from the harvest of wild plants or extensive farming. The quantity and the quality of the products are not sufficiently stable for use in drugs. Because the sexual fertility of licorice plants is low, stolon segments are usually used for vegetative propagation. However, even if an elite clone were to be isolated, obtaining sufficient quantities of stolon segments for propagation would be difficult, as stolon growth is very slow. Plant tissue culture is an alternative route for *in vitro* vegetative propagation.

There are several reports of *in vitro* licorice plant micro-propagation. Single shoots of *G. glabra* have been

Abbreviations: BAP, 6-benzylaminopurine; GA_3, gibberellic acid; NAA, α-naphthaleneacetic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; MS, Murashige and Skoog; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; GC/MS, gas chromatograph mass spectrometer; LC/MS, liquid chromatograph mass spectrometer; TMS, trimethylsilyl; TOF/MS, time of flight mass spectrometer; MS/MS, tandem mass spectrometer.

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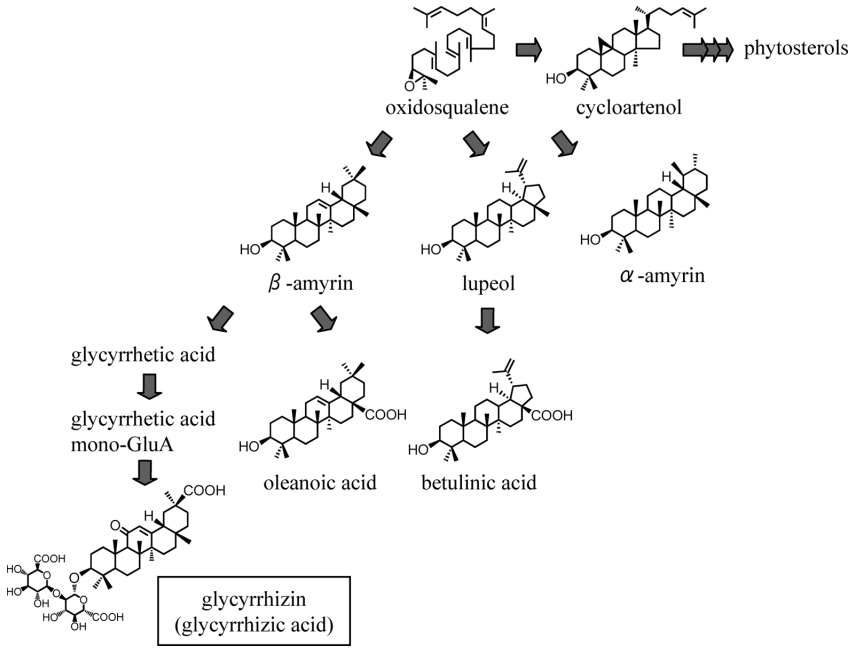

Figure 1. Chemical structures and the deduced biosynthetic pathway for glycyrrhizin (glycyrrhizic acid) and related triterpenoids in licorice plants.

produced from nodal stem segments with axillary buds (Shah and Dalal 1980). Henry et al. (1991) produced single shoots and obtained plantlet regeneration in *G. uralensis*, *G. glabra* var. *typica,* and *G. glabra* var. *glandulifera*. Multiple shoot formation from an axillary bud and plantlet regeneration have been achieved in *G. glabra* (Kohjyouma et al. 1995). A systematic single shoot clonal propagation of *G. glabra* has also been reported (Mousa et al. 2006). Somatic embryogenesis has been reported for *G. echinata* and *G. squamulosa* (which produce no glycyrrhizin; Kakutani et al. 1999). Mousa et al. (2007) reported plantlet regeneration of the embryogenic callus from leaf segments of selected clones of *G. glabra*. Shoot regeneration from callus culture has also been reported in *G. uralensis*, *G. glabra* and *G. inflata* (Wongwicha et al. 2008). *G. inflata* had the highest rate of shoot formation.

There have been many attempts to obtain glycyrrhizin from *in vitro* tissue cultures. Mousa et al. (2007) detected a low level of glycyrrhizin using TLC-tentative analysis of cell suspension cultures of selected *G. glabra* clones. Wongwicha et al. (2008) reported glycyrrhizin production in callus cultures of *G. uralensis* (36.52 μg g^{-1}), *G. glabra* (14.39 μg g^{-1}), and *G. inflata* (16.14 μg g^{-1}). Wongwicha et al. detected glycyrrhizin using a competitive ELISA system (Shan et al. 2001). However, many attempts to produce glycyrrhizin from cultured tissues have been unsuccessful (Wu et al. 1974; Hayashi et al. 1988; Saito et al. 1990; Arias-Castro et al. 1993; Toivonen and Rosenqvist 1995).

In the intact plant, glycyrrhizin is localized primarily in the roots and stolons, but it also occurs in the leaf, stem, and seeds (Hayashi et al. 1993). We selected stolons for the propagation of *G. uralensis* and study of glycyrrhizin and other secondary metabolite production. To our knowledge, this is the first report of the culture of *G. uralensis* stolons in an *in vitro* system. Xu et al. (1998) produced potato stolons from the *in vitro* culture of single-node cuttings.

Here, we report the development of a stolon culture system for *G. uralensis*, from which we obtained whole plant regeneration. Additionally, we tested for glycyrrhizin and other related compounds (triterpenoids and sterols) in the cultured stolons.

Materials and methods

Plant materials

Seeds of *G. uralensis* (Accession No. 13905) were obtained from the agricultural research field of the Division of Hokkaido Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation (NIBIO), Japan. Seeds were washed with tap water for 3 h and surface-sterilized in 70% (v/v) EtOH for 1 min and then in a 2% (v/v) sodium hypochlorite solution (10% commercial bleach), containing 0.02% (v/v) Tween 20 for 15 min, followed by three rinses in sterile water. Seeds were placed on Murashige and Skoog (MS) medium (Murashige and Skoog 1962), containing 3% (w/v) sucrose and solidified with 0.2% (w/v) gelrite (San-Ei Gen F.F.I., Inc. Osaka, Japan) in plastic tubes (ϕ 30×115 mm) containing 10 ml of medium (Figure 2A). The medium was adjusted to pH 5.8 and autoclaved at 121°C under a pressure of 1.2 kg cm^{-2} for 15 min. The cultures were incubated at 23°C with 40 μmol photons m^{-2} s^{-1} of fluorescent light (16 h d^{-1}). After one month of seed culture, one germinated plantlet was selected for the propagation of clonal explants for all subsequent experiments, to exclude genetic differences among the seeds. Single-node cuttings from stem segments with

axillary buds were cultured, essentially as described by Kohjyouma et al. (1995). The medium consisted of MS containing 3% sucrose and 0.2% gelrite, supplemented with 0.1 μM α-naphthaleneacetic acid (NAA). Culture conditions were as above.

Effects of GA_3 and NAA on stolon formation

We examined the effects of the phytohormones gibbelleric acid (GA_3) and NAA on *in vitro* stolon induction. Procedures for producing stolons followed those of Xu et al. (1998) for potato. Stem segments (1 cm) with axillary buds were cultured in 100 ml MS liquid medium containing 3% sucrose (15 segments per polycarbonate culture bottle, ϕ 80×102 mm; Figure 2B). The medium was supplemented with GA_3 (0, 0.01, 0.1, 1, and 10 μM) or NAA (0, 0.01, 0.1, 1, and 10 μM; Table 1). Segments were cultured at 26°C in the dark on a rotary shaker gyrating at 100 rpm. Each treatment was applied in duplicate. Samples were transferred to fresh medium (same composition) after two weeks in culture. After two further weeks (total four weeks) in culture, analyses were conducted.

Effects of NAA on stolon proliferation

We examined effects of NAA on the proliferation of cultured stolons. We used stolon segments produced in MS liquid medium, containing 3% sucrose and 0.01 μM NAA. The stolons (2–3 cm long) were inoculated (as before) into MS liquid medium containing 3% sucrose in polycarbonate culture bottles. The medium was supplemented with NAA (0, 0.01, 0.1, 1, and 10 μM; Table 2). Ten segments (total 2 g fresh weight) were cultured in each bottle. Cultures were maintained at 26°C in the dark on a rotary shaker gyrating at 100 rpm. Each treatment was replicated four times. The samples were transferred to fresh medium (same composition) after two weeks in culture. After two further weeks (total four weeks) in culture, analyses were conducted.

Effects of sucrose on stolon proliferation

We examined effects of sucrose on the proliferation of cultured stolons. We used stolon segments produced in MS liquid medium containing 3% sucrose and 0.01 μM NAA. The stolons (2–3 cm long) were inoculated (as before) into MS liquid medium (without phytohormones in this case) in polycarbonate culture bottles. The medium was supplemented with sucrose (1, 3, 6, and 9%; Table 3). Ten segments (total 2 g fresh weight) were cultured in each bottle. The cultures were maintained at 26°C in the dark on a rotary shaker gyrating at 100 rpm. Each treatment was replicated four times. The samples were transferred to fresh medium (same composition) after two weeks in culture. After two further weeks (total four weeks) in culture, analyses were conducted.

Effects of auxin on rooting and shoot regeneration from stolons

We examined the effects of auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and NAA on adventitious root induction and plant regeneration from cultured stolons. Stolon segments (about 5 cm long), each with one bud primordium, were transferred to MS medium with 3% sucrose and 0.2% gelrite in a polycarbonate culture vessel (80.5×80.5×100 mm, Technopot® Sumitomo Bakelite, Co. Ltd. Tokyo, Japan) containing 80 ml medium (Figure 3A). The medium was supplemented with 0.01 or 0.1 μM of 2,4-D, IAA, IBA, or NAA (Table 4). Five tissue segments were examined for each treatment. Each treatment was replicated three times. Cultures were incubated at 23°C under 40 μmol photons $m^{-2}\,s^{-1}$ fluorescent lights (16 h d^{-1}).

Analysis of glycyrrhizin in regenerated plants

After acclimation, regenerated plants were cultivated at 23°C under 40 μmol photons $m^{-2}\,s^{-1}$ fluorescent lights (16 h d^{-1}; Figure 3C). Roots that formed on six-month-old regenerated plants (Figure 3D) were used for HPLC quantitative analysis of glycyrrhizin (according to procedures of The Japanese Pharmacopoeia, 2006). Roots were dried (50°C, 12 h) and ground to a fine powder. The dried root samples (*ca.* 200 mg) were extracted with 50% EtOH (5 ml) for 30 min at room temperature. These were then treated with ultrasonication for 10 min at room temperature. This operation was repeated twice, and the extracts were made up to a final volume of 10 ml. The extract (20 μl) was subjected to HPLC analysis. The HPLC system used consisted of an LC-2000 Plus system (JASCO, Tokyo, Japan) and a TSKgel ODS-80T_S QA column (150×4.6 mm, TOSOH). The column temperature was maintained at 30°C. A solvent system consisting of 2.1% (v/v) acetic acid-CH_3CN (3 : 2) was used at a flow rate of 0.6 ml min^{-1}. The eluent was monitored by absorption at 254 nm. A glycyrrhizin standard was purchased from Tokyo Chemical Industry (Tokyo, Japan). Three regenerated plants were analyzed.

Triterpenoid and sterol analysis of stolons

We quantitatively analyzed glycyrrhizin and other triterpenoids (oleanolic acid and betulinic acid), triterpenols (β-amyrin, α-amyrin, and lupeol), and sterols. Freeze-dried plant materials were extracted twice with $CHCl_3$-MeOH (1 : 1) and twice with MeOH-H_2O (1 : 1) at 80°C. Internal standards [25,26,26,26,27,27,27-$^2H_7$]cholesterol (98% D, Cambridge Isotope Laboratories, Inc., Andover, MA, USA), [3,28,28,28-$^2H_4$] β-amyrin, [28,28,28-$^2H_3$]α-amyrin, and [28,28,28-$^2H_3$] lupeol (Ohyama et al. 2007) were added to the extracts. The extract was injected into a polyamide cartridge column (Discavery® DPA-6S, 500 mg, SUPELCO) and eluted successively with MeOH, 50% MeOH, H_2O, and 1% NH_3. The MeOH eluent was dried *in vacuo*, and the residue was separated on silica gel preparative TLC plates and developed twice using hexane–ethyl acetate (3 : 1), to yield triterpenol, sterol, oleanolic acid, and betulinic acid fractions. Each fraction was trimethylsilylated with *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide at 80°C for 30 min and analyzed using GC/MS. Sterol and triterpenol quantifications were carried out using previously described methods (Ohyama et al. 2007). Oleanolic acid and betulinic acid were quantified using standard calibration curves with coefficients of determination, r^2>0.999. The curves were constructed using the peak area ratio (*m*/*z* 482) of TMS derivatives.

After drying, water and 1% NH_3 eluents were analyzed using LC/MS. We used a mass spectrometer (QSTAR Pulsar, API) connected to an HPLC (1100 series, Agilent Technologies) for identifications. For glycyrrhizin identification, LC/MS analyses were performed with two solvent systems and with both negative and positive ion scans. For HPLC, we used a

Table 1. Effects of GA_3 and NAA on stolon formation of *Glycyrrhiza uralensis*

Concentration of growth regulator (μM)		Frequency of induction (%)[a]	Length of stolon (cm)[a]	Morphological characteristics
GA_3	NAA			
0	—	26.7	6.73 ± 7.34[b]	normal stolon
0.01	—	16.7	2.60 ± 1.48	enlarged bud
0.1	—	36.7	3.57 ± 3.90	normal stolon
1	—	23.3	4.10 ± 3.45	thin stolon
10	—	10.0	3.33 ± 1.92	thin stolon
—	0	26.7	6.73 ± 7.34	normal stolon
—	0.01	40.0	10.31 ± 4.07	normal stolon
—	0.1	36.7	6.85 ± 4.82	normal stolon
—	1	13.3	1.93 ± 0.87	normal stolon
—	10	0*	—	callus

[a] Stolons were counted over 10 mm in length.
[b] Each value represents mean±standard deviation. (n=30)
* Data differ significantly ($P<0.05$) compared with the "0" NAA or "0" GA_3-treated respectively.

SenshuPak ODS-II 25 cm×0.2 mm column with a flow rate of 0.2 ml min^{-1}. System 1: Eluent A was 0.1% (w/v) acetic acid in acetonitrile; eluent B was 0.1% (w/v) acetic acid in water. After an 80% B hold for 2 min, the gradient was linear from 80% to 5% B in 10 min; this was followed by a 5% B hold for a further 8 min and a re-equilibration period of 5 min. The column temperature was 35°C. System 2 operated under the same conditions as System 1, but with Eluent A being 0.1% (w/v) acetic acid in MeOH. Mass spectrometer settings were optimized using authentic glycyrrhizin in TOF/MS scan mode, with electrospray ionization; the resulting ion, $[M-H]^-$ ($[M+H]^+$), was readily detected. Thus, product ion scan (MS/MS) analyses were performed by scanning at *m/z* 821.3 (823.3). Glycyrrhizin quantifications were calculated from the ratio of the peak area at *m/z* 821.3 from product ion scans using a calibration curve of authentic compound (with coefficient of determination: $r^2>0.999$).

Data were analyzed statistically using Student's *t*-test or a test comparing two proportions (proliferation rates). Values with an asterisk differ significantly ($P<0.05$ or 0.01).

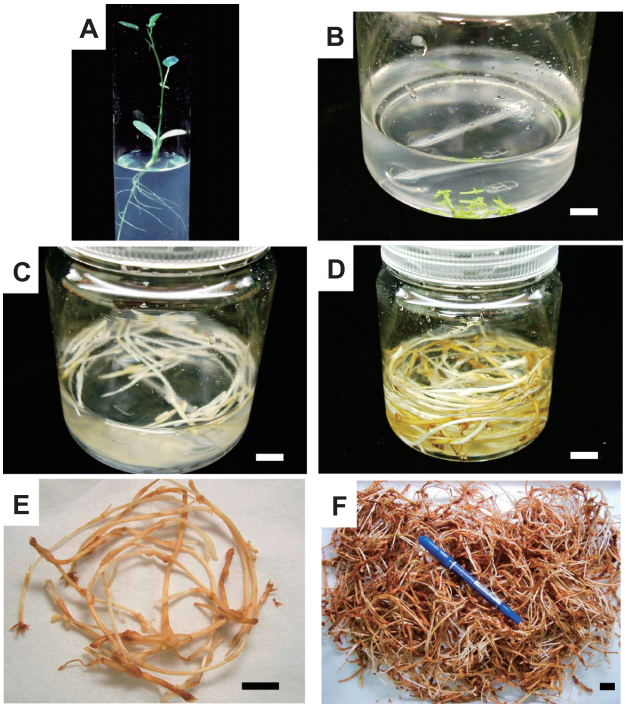

Figure 2. *In vitro* stolon organogenesis in *Glycyrrhiza uralensis*. (A) Aseptically germinated plantlet. (B) Node segments in MS liquid medium. (C, D, E, F) *In vitro* cultured stolon formed in MS liquid medium with 0.01 μM NAA and 3% sucrose (F; 0.01 μM NAA, 6% sucrose). Bars: 1 cm

Results and discussion

Effects of GA_3 and NAA on stolon formation

Results are summarized in Table 1. The highest frequency of stolon induction occurred using 0.01 μM NAA (40.0%; Figure 2C), which also produced the longest stolon lengths after four weeks in culture (10.31±4.07 cm). Higher NAA concentrations tended to decrease the frequency of stolon induction and elongation. No stolons were induced using 10 μM NAA, although callus formed. We observed few cases of induction or elongation of stolons using GA_3 treatments, but did observe abnormal stolon morphologies and enlarged stolon buds (0.01 μM GA_3), as well as thin stolons (1 and 10 μM).

Effect of NAA on stolon proliferation

Results are summarized in Table 2. The largest effect

Table 2. Effects of NAA on stolon proliferation of *Glycyrrhiza uralensis*

Concentration of NAA (μM)	Fresh weight of stolon/bottle (g)	Proliferation rate (fold)[b]
0	10.83 ± 0.30[a]	5.45
0.01	12.94 ± 0.40**	6.58**
0.1	10.74 ± 0.82	5.39
1	8.48 ± 0.41**	4.29**
10	8.30 ± 0.03**	4.15**

[a] Each value represents mean±standard deviation. (n=4)
[b] Fresh weight of stolon after 4 weeks in culture/Fresh weight of stolon segments at the start.
** Data differ significantly ($P<0.01$) compared with the "0" NAA-treated.

response was obtained in medium supplemented with 0.01 μM NAA (12.94±0.40 g fresh weight/bottle). There was a 6.58-fold increase in proliferation per bottle after four weeks in culture. Under these conditions, stolons grew vigorously (Figure 2D, E). However, higher concentrations of NAA reduced growth rates. The mean fresh weights of stolons per bottle treated with 1 μM and 10 μM NAA were significantly lower ($P<0.01$) than that of stolons without NAA treatment. Stolon growth at 10 μM NAA equaled that at 1 μM NAA, but at 10 μM NAA some short adventitious roots (2–5 mm) formed.

Effects of sucrose on stolon proliferation

Results are summarized in Table 3. The largest effect was obtained in medium supplemented with 6% sucrose (12.82±0.17 g fresh weight/bottle). Stolons proliferated 6.34-fold in four weeks of culture. Higher concentrations of sucrose reduced stolon growth: the mean fresh weight of stolons per bottle at 9% sucrose was significantly ($P<0.01$) lower than that of stolonns treated with 3% sucrose. Lower concentrations of sucrose also reduced stolon growth: the mean of fresh weight of stolon per bottle in 1% sucrosewas significantly ($P<0.01$) lower than that of stolons treated with 3% sucrose. The stimulatory effect of sucrose on stolons was unaffected by the presence of 0.01 μM NAA (data not shown). Stolons cultured in 6% sucrose were slightly thickened in comparison with those grown in 3% sucrose.

Table 3. Effects of sucrose on stolon proliferation of *Glycyrrhiza uralensis*

Concentration of Sucrose (%)	Fresh weight of stolon/bottle (g)	Proliferation rate (fold)[b]
3	10.36 ± 0.43[a]	5.09
1	6.34 ± 0.23**	3.11**
6	12.82 ± 0.17**	6.34**
9	6.09 ± 0.15**	2.98**

[a] Each value represents mean±standard deviation. (n=4)
[b] Fresh weight of stolon after 4 weeks in culture/Fresh weight of stolon segments at the start.
** Data differ significantly ($P<0.01$) compared with the "3%" sucrose-treated.

Effects of auxin on stolon rooting and shoot regeneration

Results are summarized in Table 4. After four weeks of culture, there was a high frequency of rooting in medium with NAA (0.01 and 0.1 μM), IBA (0.01 and 0.1 μM), and without phytohormones (>87%). Elongation of roots and shoots was also vigorous under these conditions. Generally shoot and root development was most vigorous in 0.01 μM NAA. However, adventitious roots that formed in 0.1 μM NAA and 0.1 μM IBA developed a slightly abnormal thickened shape. Callus induction was abundant in medium supplemented with 2,4-D (0.01 and 0.1 μM). Adventitious roots and shoots were not observed at 0.1 μM 2,4-D. The continuing culture of plantlets in phytohormone-free medium resulted in a gradual loss of vigor in growth and shoot development (data not shown). Hence, medium supplemented with 0.01 μM NAA was the most suitable for the adventitious root formation and shoot development required to achieve whole plant regeneration (Figure 3B). Cultured stolons from which whole plants regenerated on solid medium retained a pale whitish or light-brown color similar to that of intact field-grown stolons. Regenerated plants were easily acclimated to pot cultivation after *in vitro* culture (Figure 3C). The regenerated plants grew vigorously and formed thickened roots similar to those of field plants (Figure 3D). Regenerated plants contained glycyrrhizin in the roots, (7,600 $\mu g\,g^{-1}$ dry weight), despite the short cultivation period of 6 months.

Mousa et al. (2006) propagated clonal plants using *in vitro* shoot culture of *G. glabra*. Shoot elongation, initiated in the dark, increased the number of explants (micronodes). Clonal propagation from stolon culture has been reported in fern (Padhya et al. 1982) and potato (Dragicevic et al. 2008). The present study is the first report of clonal propagation of licorice plants (*G. uralensis*) using stolon culture, and our stolon culture

Table 4. Effects of auxin on rooting and shoot regeneration from cultured stolons of *Glycyrrhiza uralensis*

Concentration of auxins (μM)		Frequency of rooting (%)[a]	Number of root[a]	Length of root (cm)[a]	Frequency of shoot regeneration (%)[a]	Length of shoot (cm)[a]
phytohormone free		87	2.3 ± 1.5[b]	7.7 ± 6.0[b]	87	4.6±1.5[b]
NAA	0.01	93	1.8 ± 1.1	8.8 ± 5.4	93	6.3 ± 2.1*
	0.1	87	1.9 ± 1.3	4.6 ± 5.1	87	4.2 ± 1.6
IAA	0.01	60	1.0 ± 1.1*	5.3 ± 7.0	60	3.5 ± 2.4
	0.1	67	1.2 ± 1.6	7.4 ± 6.4	67	3.7 ± 2.3
IBA	0.01	87	2.1 ± 1.6	7.3 ± 4.9	87	5.2 ± 1.7
	0.1	93	1.7 ± 1.0	8.6 ± 4.5	93	4.8 ± 1.6
2,4-D	0.01	60	0.9 ± 0.9**	7.4 ± 7.4	60	3.1 ± 2.1
	0.1	0	—	—	0	—

[a] Adventitious roots and shoots were counted over 5 mm in length.
[b] Each value represents mean±standard deviation. (n=15)
* Data differ significantly ($P<0.05$) compared with the "phytohormone free"-treated.
** Data differ significantly ($P<0.01$) compared with the "phytohormone free"-treated.

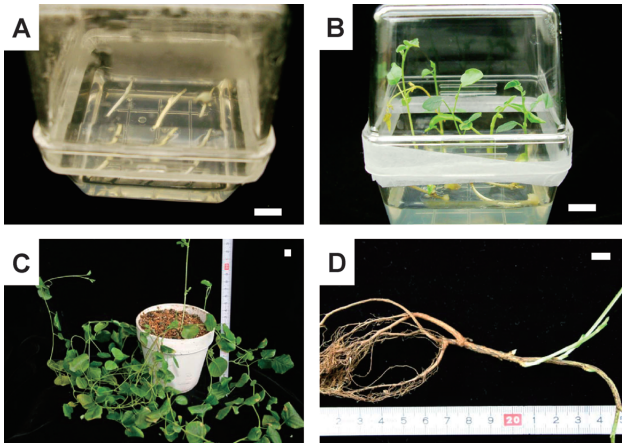

Figure 3. Plant regeneration from *in vitro* stolons of *Glycyrrhiza uralensis*. (A) Segments of cultured stolons on the regeneration medium. (B) Shoot and adventitious root formation on MS medium with 0.01 μM NAA. (C, D) Regenerated plant pot-cultivated for six months after acclimatization (C; above ground, D; below ground). Bars: 1 cm

Table 5. Contents of glycyrrhizin and related triterpenoids and sterols in cultured stolons of *Glycyrrhiza uralensis*

	Contents (μg g^{-1} dry weight)	
	Cultured stolon[a]	Intact stolon[b]
glycyrrhizin	14.00[c]	36500.00
glycyrrhetic acid mono-GluA	n.d.	
glycyrrhetic acid	n.d.	
β-amyrin	12.51	15.00
α-amyrin	0.18	n.d.
lupeol	13.60	27.40
oleanolic acid	23.80	1.30
betulinic acid	160.90	46.70
cycloartenol	n.d.	2.50
cholesterol	12.30	3.10
24-methylene cycloartanol	4.00	1.60
campesterol	75.60	107.60
brassicasterol	0.70	n.d.
sitosterol	249.90	742.20
stigmasterol	521.10	397.80
campestanol	4.60	4.60
sitostanol	16.40	26.30

[a] The cultured stolons were used in MS medium with 6% sucrose and 0.01 μM NAA.
[b] Intact field-grown stolon of the same strain of *G. uralensis* for culterd stolon was used.
[c] The data of glycyrrhizin content of culterd stolon was the mean of three analysis (1.40±0.05).

system proved to be suitable for the micro-propagation of *G. uralensis*.

Triterpenoid and sterol analysis of stolons

Results are shown in Table 5. HPLC charts of authentic glycyrrhizin and the extract from cultured stolons using Systems 1 and 2 (see Materials and Methods, *Triterpenoid and sterol analysis of stolons*) are shown in Figure 4. The product ion spectra of authentic glycyrrhizin and the extract from cultured stolons at two

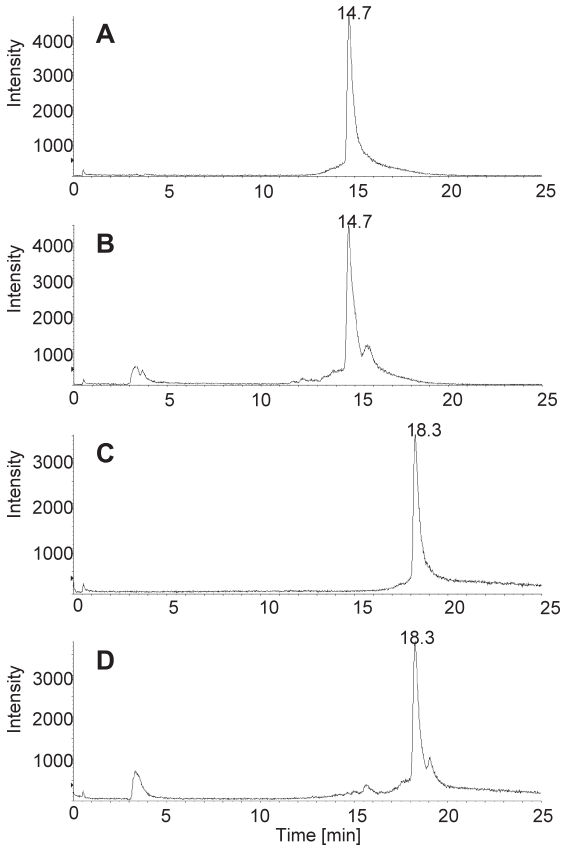

Figure 4. LC/TOF-MS/MS sum ion chromatograms of authentic glycyrrhizin and the extract of cultured stolons as the precursor ion of the parent ion in negative ion scan mode. HPLC chart of (A) authentic glycyrrhizin in System 1 HPLC condition, (B) the extract of the cultured stolon in System 1, (C) authentic glycyrrhizin in System 2, and (D) cultured stolon in System 2. Conditions of Systems 1 and 2 are described in Materials and Methods under "Triterpenoid and sterol analysis of stolons".

different ion scan modes (negative and positive) are shown in Figure 5.

We confirmed glycyrrhizin production in cultured stolons ($14.0 \pm 0.50\,\mu g\,g^{-1}$ dry weight) by GC/MS, although many researchers have reported a lack of glycyrrhizin production in cultured *Glycyrrhiza* cells (Wu et al. 1974; Hayashi et al. 1988; Saito et al. 1990; Ayabe et al. 1990; Arias-Castro et al. 1993; Toivonen and Rosenqvist 1995). Compared with the glycyrrhizin content of intact field-grown stolons ($36{,}500\,\mu g\,g^{-1}$), the concentration in cultured stolons was very low. Glycyrrhetic acid and glycyrrhetic acid monoglucronide were not detected in cultured stolons, although small amounts were detected in intact field-grown stolons (data not shown). Interestingly, the content of betulinic acid (biosynthesized from lupeol, Figure 1) in cultured stolons ($160.9\,\mu g\,g^{-1}$) was 3.4-fold higher than that of intact field-grown stolons. Furthermore, the content of oleanolic acid (biosynthesized from β-amyrin, as is glycyrrhizin) in cultured stolons ($23.8\,\mu g\,g^{-1}$) was 18-fold higher than in intact stolons ($1.3\,\mu g\,g^{-1}$).

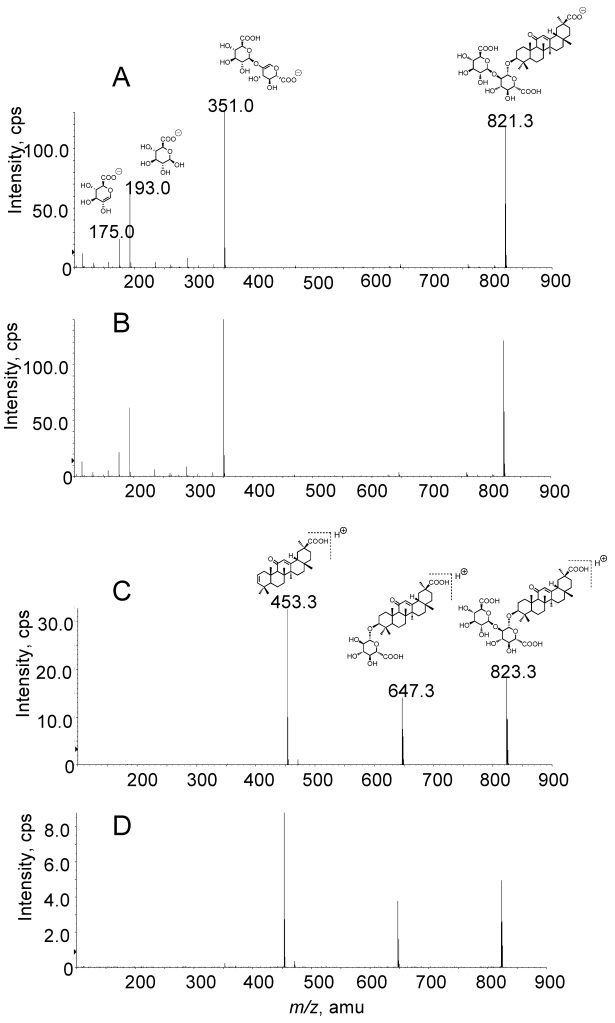

Figure 5. Product ion spectra of precursor ions *m/z* 821.3, $[M-H]^-$ and 823.3, $[M+H]^+$. (A) authentic glycyrrhizin in negative ion scan mode, (B) main peak of the extract of the cultured stolon in negative ion scan mode, (C) authentic glycyrrhizin in positive ion scan mode, (D) main peak of the extract from cultured stolons in positive ion scan mode.

Despite previous reports of a lack of glycyrrhizin in tissue cultures of licorice plants (Wu et al. 1974; Hayashi et al. 1988; Saito et al. 1990; Ayabe et al. 1990; Arias-Castro et al. 1993; Toivonen and Rosenqvist 1995), we confirmed glycyrrhizin production in cultured stolons by using highly sensitive analyses. The chemical composition and content of cultured and field-grown stolons differed, as reported above. We also analyzed cultured stolons from only one culture condition. We expected the chemical composition and content of stolons to change when cultured under different conditions. Some compounds that are precursors of triterpenoids probably transfer from above-ground parts, such as leaves, in field-grown plants. However, this organization is lacking in cultured stolons. Thus, we suspect that this is the cause of the difference between field-grown and cultured stolons in chemical composition and content, as some influences appear necessary to the generation of secondary metabolites such as glycyrrhizin. A convincing explanation for the chemical differences must await future biosynthetic studies. Our results indicate that cultured stolons have the gene set for glycyrrhizin biosynthesis, although the regulation of gene expression appears to differ from that of intact field-grown stolons. Recently, Sudo et al. (2009) analyzed the expressed sequence tag (EST) of *G. uralensis* stolons (the first critical gene for glycyrrhizin biosynthesis, β-amyrin 11-oxidase, was identified by Seki et al. 2008). Evidence that cultured stolons have the ability to produce glycyrrhizin led us to study glycyrrhizin biosynthesis in our culture system.

In conclusion, we developed an *in vitro* stolon culture system for *Glycyrrhiza uralensis*. MS liquid medium supplemented with 0.01 μM of NAA was most effective at inducing stolons from a stem node. Under these conditions, stolons proliferated extensively; proliferation also occurred in a 6% sucrose medium. Adventitious root and shoot regeneration from the stolons was achieved easily in illuminated cultures on MS medium supplemented with 0.01 μM NAA and 0.2% gerlite. Regenerated plants produced glycyrrhizin (7,600 $\mu g\, g^{-1}$ dry weight). The cultured stolons contained glycyrrhizin, but at very low concentrations. Glycyrrhetic acid and glycyrrhetic acid monoglucronide were not detected in cultured stolons. We believe our stolon culture system may be useful for the micro-propagation of *G. uralensis*. The system is also likely suitable for studying metabolism of glycyrrhizin or other secondary metabolites.

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