Production of Glycyrrhizin in Callus Cultures of Licorice

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Licorice plants, *Glycyrrhiza glabra, G. uralensis*, and *G. inflata*, were investigated for callus induction using Murashige and Skoog (MS) medium combined with auxins and cytokinins. After 4 weeks of culture, 33–100% of leaf or stem explants formed calli. Maximum of shoot induction from callus cultures was achieved by *G. inflata* stem explants cultured on MS medium supplemented with 1 mg/l α-naphthaleneacetic acid (NAA) and 0.5 mg/l 6-benzyladenine (BA) (67%) which also gave maximum shoot formation per explant (two shoots per explant). These results indicated that all three *Glycyrrhiza* species regenerated shoots from callus cultures on MS medium combined with NAA and BA or only thidiazuron (TDZ; 0.1 and 0.5 mg/l). Glycyrrhizin contents of *G. uralensis* calli induced using MS medium in combination with NAA and BA [(27.60 ± 8.47) μg/g DW] or TDZ alone [(36.52 ± 2.45) μg/g DW] were higher than those found in other combinations.

Key words: Glycyrrhizin, Licorice, Callus Cultures

Introduction

The ordinary botanical sources of licorice are *Glycyrrhiza glabra* L., which is geographically distributed from Southern Europe to Western China, and *G. uralensis* Fisch, found from Central Asia to Eastern China (Rauchensteiner *et al*., 2005). The use level of licorice is high due to many pharmacological properties, whereas licorice sources are limited regions in the world. In many studies *in vitro* culturing of these plants was attempted including callus cultures, suspension cultures and hairy root cultures (Arias-Castro *et al.*, 1993a; Ayabe *et al.*, 1990; Toivonen and Rosenqvist, 1995; Henry and Marty, 1984; Hayashi *et al.*, 1988; Li *et al.,* 2000; Yoo and Kim, 1976). There is only one report of glycyrrhizin detection in the literature (Yoo and Kim, 1976). In the present study, we investigated the efficiency of callus induction and developed a regeneration system of three licorice plants, *G. glabra, G. uralensis* and *G. inflata*. We have successfully detected glycyrrhizin in callus cultures by the competitive enzyme-linked immunosorbent assay (ELISA) using anti-glycyrrhizin monoclonal antibody (MAb), a highly specific and rapid method. We also established a protocol to multiply these plants by shoot regeneration from callus cultures.

Materials and Methods

Chemicals

6-Benzyladenine (BA), α-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxy acetic acid (2,4-D) were purchased from Fluka Chemical (Buchs, Switzerland). Kinetin (Kin) and *N*-phenyl-*N*′-1,2,3-thidiazol-5′-yl urea (thidiazuron, TDZ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glycyrrhizin was purchased from Wako Pure Chemical (Osaka, Japan). Glycyrrhizin-HSA and anti-glycyrrhizin MAb were obtained from the Department of Medicinal Plant Breeding, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan. All other chemicals were standard commercial products of analytical grade.

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Plant materials

G. glabra, G. uralensis and *G. inflata* seeds were obtained from the Institute of Botany, Mongolian Academy of Sciences, Ulaanbaatar, Mongolia. The seeds were washed with sterile distilled water and were surface-sterilized in 10% sodium hypochlorite for 15–20 min. After being washed three times with sterilized water, the seeds were immersed in 70% ethanol for 1 min and then germinated on hormone-free Murashige and Skoog (MS) medium containing 3% sucrose (w/v), pH 5.5. Germination started within 5 d and was carried out at (25 ± 1) °C under 16 h light/day. Plantlets were subcultured on the same medium every 4 weeks.

Callus induction and maintenances of callus cultures

Leaf and stem segments (0.5 cm) from 2 weeks fully grown *in vitro* plantlets were cultured on MS medium with growth regulators, *i. e.* combinations of NAA (0.5–1 mg/l), 2,4-D (0.5–1 mg/l), BA (0.5–1 mg/l) and Kin (0.5–1mg/l), and TDZ alone (0.1–1 mg/l). After 4 weeks, the initial calli were subcultured on the same medium. The regenerated shoots were subcultured on MS medium without hormones for elongation and rooting. Shoot regeneration was observed for rooting at the end of the sixth week. After the fourth subculture, calli were maintained on MS medium containing the same combination of hormones for glycyrrhizin analysis. The regenerated shoots were maintained on MS medium without hormones at (25 ± 1) °C under 16 h light/day and sulcultured every 8 weeks.

Extraction of callus samples and glycyrrhizin analysis

Dried samples (50 mg) of 4-week-old calli from *G. glabra, G. uralensis,* and *G. inflata* were powdered and extracted five times with 0.5 ml methanol with sonication. The extracts were combined, evaporated and then redissolved in 1 ml methanol. The extracted solutions were diluted with 20% methanol and glycyrrhizin analysis was performed by competitive ELISA using anti-glycyrrhizin MAb as described previously (Shan *et al.,* 2001).

Result and Discussion

Callus induction was evaluated on MS medium at a light intensity of 70 W/m^2, 16 h/day, with 3% sucrose, 0.9% agar, combinations of auxins (NAA and 2,4-D) and cytokinins (BA, Kin and TDZ) like NAA and BA (NB series), 2,4-D and BA (DB series), 2,4-D and Kin (DK series) and TDZ alone (TDZ series). After 4 weeks of culture, we succeeded to induce callus formation as shown in Fig. 1 (A–D). We observed the contact point between the pieces of explants and the medium and found that the dark substances appeared and diffused into the medium from that point especially in the TDZ series. The initial calli were subcultured for further studies after a 4-week induction. The calli from the NB series grew very well and easy due to its loosed texture. On the other hand,

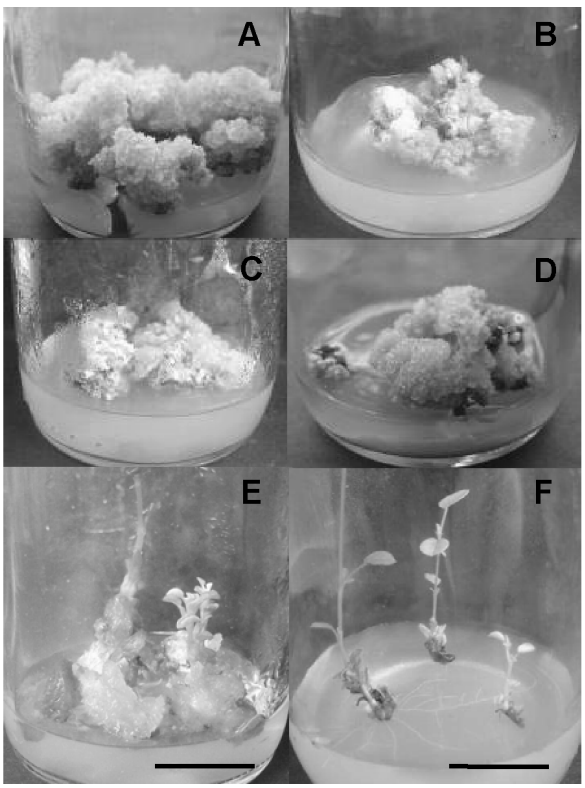

Fig. 1. Callus from *G. glabra* cultured on MS medium with combinations of hormones (A–D) and regenerated shoots of *G. inflata* (E, F); (A) combination with NAA and BA; (B) combination with 2,4-D and BA; (C) combination with 2,4-D and kinetin; (D) TDZ alone; (E) shoot organogenesis on MS medium with 0.1 mg/l TDZ; (F) shoot growth after 6 weeks of subculture on MS medium without hormones. Bar: 2.5 cm.

Table I. Percentage of callus and shoot induction, number of shoots per explant and glycyrrhizin (GC) content in calli from *Glycyrrhiza* species.

Hormone[a]	Stem[b]/leaf[b]	*G. uralensis*				*G. glabra*				*G. inflata*			
		Callus induction (%)	GC content [μg/g DW]	Shoot induction (%)	Number of shoots/ explant	Callus induction (%)	GC content [μg/g DW]	Shoot induction (%)	Number of shoots/ explant	Callus induction (%)	GC content [μg/g DW]	Shoot induction (%)	Number of shoots/ explant
$N_1B_{0.5}$	Stem	76.92	21.68 ± 0.48	15.38	1.5	100.00	8.56 ± 0.76	30.77	1.25	100.00	8.77 ± 1.02	66.67	2
	Leaf	91.67	ND[c]	–	–	100.00	8.65 ± 0.79	–	–	92.86	ND	–	–
N_1B_1	Stem	92.31	14.92 ± 1.21	50.00	1.17	100.00	4.46 ± 1.09	8.33	1	92.86	4.38 ± 0.54	50.00	1.14
	Leaf	76.92	12.41 ± 0.34	–	–	100.00	9.43 ± 0.82	–	–	100.00	6.40 ± 0.80	–	–
$N_{0.5}B_1$	Stem	100.00	27.60 ± 8.47	41.67	1	83.33	6.30 ± 1.22	50.00	1	76.92	4.86 ± 0.47	46.15	1.5
	Leaf	91.67	7.87 ± 1.96	–	–	92.31	4.28 ± 0.63	–	–	85.71	8.37 ± 1.30	–	–
$D_1B_{0.5}$	Stem	91.67	9.30 ± 2.32	–	–	83.33	ND	–	–	100.00	3.45 ± 1.17	25.00	1.5
	Leaf	92.86	11.24 ± 1.15	–	–	83.33	ND	–	–	90.91	4.99 ± 1.02	–	–
D_1B_1	Stem	83.33	6.50 ± 1.05	–	–	66.67	ND	–	–	100.00	ND	20.00	1
	Leaf	100.00	6.32 ± 0.25	–	–	75.00	ND	–	–	100.00	ND	–	–
$D_{0.5}B_1$	Stem	100.00	3.56 ± 0.10	15.38	1	75.00	ND	–	–	91.67	6.70 ± 0.59	33.33	1
	Leaf	100.00	ND	–	–	33.33	ND	–	–	100.00	ND	–	–
$D_1K_{0.5}$	Stem	100.00	3.21 ± 0.92	–	–	100.00	ND	–	–	100.00	ND	–	–
	Leaf	46.15	ND	–	–	71.43	ND	–	–	100.00	ND	–	–
D_1K_1	Stem	100.00	ND	–	–	100.00	ND	–	–	100.00	ND	–	–
	Leaf	90.00	ND	–	–	58.33	ND	–	–	100.00	ND	–	–
$D_{0.5}K_1$	Stem	100.00	3.20 ± 0.79	–	–	78.57	ND	–	–	85.71	2.68 ± 0.74	–	–
	Leaf	75.00	4.18 ± 0.83	–	–	100.00	ND	–	–	91.67	ND	–	–
$TDZ_{0.1}$	Stem	83.33	36.52 ± 2.45	58.33	1	50.00	14.39 ± 2.00	50.00	1.33	76.92	16.14 ± 1.48	46.15	1
	Leaf	66.67	ND	–	–	0.00	ND	–	–	50.00	ND	–	–
$TDZ_{0.5}$	Stem	100.00	ND	8.33	1	92.31	10.04 ± 0.93	30.77	1	84.62	12.27 ± 0.47	61.54	1.25
	Leaf	100.00	29.97 ± 0.40	–	–	0.00	ND	–	–	62.50	6.25 ± 0.85	–	–
TDZ_1	Stem	66.67	27.01 ± 7.24	–	–	69.23	7.15 ± 0.91	30.77	1	85.71	10.19 ± 1.06	28.57	1
	Leaf	50.00	13.17 ± 4.24	–	–	0.00	ND	–	–	77.77	8.29 ± 0.90	–	–

Values represent the mean ± S.D. (n = 3).
[a] N, NAA; B, BA; D, 2,4-D; K, Kin.
[b] n = 12.
[c] ND, not detectable.

the calli culture in the DB, DK and TDZ series were dwarfed, showing symptoms of severe necrosis and poor growth.

The percentages of callus induction and shoot induction are shown in Table I indicating that 33–100% of leaf or stem explants had formed calli. In the case of the induction in the NB series, the percentage of callus induction was ranging from 77–100% while in the DB and DK series, it was 33–100%, and in the TDZ series, the percentage of induction was ranging from 0–100%.

Fig. 1 (E–F) shows the shoot regeneration from callus cultures of *G. inflata*. This is the first report regarding the shoot formation from a callus culture of licorice plants. The maximum of shoot induction was achieved from the stem explant of *G. inflata* cultured on medium supplement with 1 mg/l NAA and 0.5 mg/l BA ($N_1B_{0.5}$, 67%) which also gave maximum shoots per explant (2 shoots per explant) (Table I). All three *Glycyrrhiza* species could produce shoot regeneration from calli cultured on medium with a combination of NAA and BA or only TDZ (0.1 and 0.5 mg/l). The initial regenerated shoots were subcultured after 4 weeks of callus induction on MS medium without hormones. After being subcultured for 4 weeks, the regenerated shoots were rooted. Efficiencies of callus and shoot induction from each combination of hormones were not similar. The different results were due to the fact that auxins and cytokinins have their major effects on callus induction and regeneration; varying of hormone concentration in the medium causes differences in amount, rate and growth pattern of the explants (Ekiz and Konzak, 1997).

The 4-week-old calli were harvested for determination of glycyrrhizin contents by competitive ELISA using anti-glycyrrhizin MAb. Glycyrrhizin level of *G. uralensis* calli cultured in the NB and TDZ series was high [(27.60 ± 8.47) and (36.52 ± 2.45) μg/g DW, respectively] while in another series, it was lower (Table I). Similar to previous reports, the result also showed that in many of the calli in the DK series no glycyrrhizin could be detected (Arias-Castro *et al*., 1993a, b; Ayabe *et al.,* 1990).

There have been a few reports on callus and suspension cultures of *G. glabra* (Hayashi *et al.*, 1988; Henry and Marty, 1984; Yoo and Kim, 1976). Only in one study the presence of glycyrrhizin in callus cultures could be detected by TLC analysis (Yoo and Kim, 1976), while others failed to detect glycyrrhizin in callus and suspension cultures. The reason why no glycyrrhizin could be determined in the past may be due to the sensitivity of analytical methods or the differentiation of cell lines.

As mentioned above, callus induction in TDZ series was ranging from 0–100%. Calli were dwarfed, poor in growth and slowly changing to brown colour due to callus necrosis, showing dark substances which diffused into the medium from the contact point between explants and medium. These substances might be due to the production of an enormous quantity of phenol-like substances that could also inhibit callus growth. Licorice cells produce flavonoids and polyphenols (Kovalenko and Kurchii, 1998). Continuous exposure of explants to high concentration of TDZ was reported to inhibit cell growth (Huetterman and Preece, 1993; Hutchinson *et al.*, 1996). Therefore, calli were dwarfed, growed poorly and slowly changed to brown colour. However, calli from the TDZ series contained a high level of glycyrrhizin compared with the DB and DK series, which may be due to stress conditions caused by the phenol-like substances.

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