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CONSTITUENTS OF REGENERATED SHOOT AND CULTURED ROOT TISSUE OF *REHMANNIA GLUTINOSA*

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Key Word Index—*Rehmannia glutinosa*; Scrophulariaceae; callus culture; regeneration; root culture; iridoid glycoside; caffeoyl glycosides.

Abstract—Callus formation from leaf segments of *Rehmannia glutinosa* var. *purpurea* was obtained after the addition of 1 mg/l 2,4-D. When the calli were subcultured embryogenesis occurred. Mature somatic embryos developed normal shoots when transferred to 1/2 MS medium containing 1 mg/l GA and BAP. Root differentiation were cultured in a MS liquid medium supplemented with 1 mg/l IBA. Root tissues produced two iridoid glycosides, melittoside and rehmannioside D. A caffeoyl glycoside, acetoside, and ethyl-β-D-glucose were detected in the regenerated shoots.

INTRODUCTION

The root of *Rehmannia glutinosa* Libosch. var. *purpurea* Makino is an important Chinese drug which is prescribed together with other herbal drugs for anaemia, haemoptysis and gynaecological diseases. The dried roots of this plant contain sugars [1], amino acids [2], iridoid glycosides [3–6], cerebroside [4], acteoside [4], ionone glycosides [6] and polysaccharides [7]. However, the relationships between these constituents and the pharmacological activities of the drug are still obscure, except in the case of the, iridoid glycosides [6] and polysaccharides [7].

In a continuing study of tissue culture of *R. glutinosa* var. *purpurea*, four caffeoyl glycosides and ethyl-β-D-glucose have been isolated from callus cultures induced from leaf segments. None of the iridoid glycoside, however, has been found in callus tissue [8]. Furthermore, we have already reported on the clonal micropropagation of virus-free plants by shoot tip tissue culture [9, 10]. This paper describes the regeneration of this plant from callus via embryogenesis and axillary bud formation and a comparison of the constituents found in cultured roots and regenerated shoots.

RESULT AND DISCUSSION

Callus induction from leaf segments was investigated by methods similar to those described in a previous paper [8]. When a leaflet was incubated with 2,4-D (0.25, 0.5 or 1 mg/l) and kinetin (0, 0.5, 1 and 2 mg/l) callus formation occurred on the edge of leaf and then extended to the whole leaf over a period of three weeks. After six weeks, the callus had grown into a friable pale yellowish mass of cells. The frequency of callus formation was perfect with all combinations of 2,4-D and kinetin and 1 mg/l 2,4-D only. It fell to 90% when supplemented only with 2,4-D at 0.25–0.5 mg/l. Somatic embryogenesis of a globular type occurred on the surface of the callus over a 6-week period. In order to increase the number of embryos and later stages, such as heart-shaped or cotyledonary forms, further subculture was carried out on the media described above. Regeneration from somatic embryos was investigated next. The addition of GA and BAP (each 1 mg/l) favoured shoot regeneration from matured embryos. In this investigation, the effect of callus-inducing condition for regeneration was observed. The callus induced by the supplement of 1 mg/l 2,4-D gave the best regeneration ratio (80%) (Table 1). When leaf segments were inoculated with a medium supplemented with 1 mg/l NAA and 2.5 mg/l BAP and subcultured, axillary bud formation with a regeneration ratio of 10%. Although the regeneration of *R. glutinosa* var. *hueichingensis* from callus was previously reported [11, 12], this is the first example of the formation of regenerated shoots from callus culture via both embryogenesis and axillary bud formation by hormone control.

Table 1. Effect of callus-inducing conditions on regeneration from somatic embryos of *R. glutinosa* var. *purpurea*

Callus inducing hormone (mg/l)		Regeneration ratio (%)	No. of shoots per culture
2,4-D	Kinetin		
	0	10	20
0.25	0.5	20	12.5
	1	70	7.9
0.5	0	25	3.8
	0.5	30	3.0
	0	80	20.9
1	0.5	67	25.5
	1	0	0

Culture conditions: 1/2 MS medium containing 1 mg/l GA and BAP, continuous light, 25±1°, 5 weeks.

The shoots regenerated were transferred to hormone free media resulting in root formation as previously described [9]. The roots were transferred to a liquid Murashige and Skoog (MS; 13) medium supplemented with 1 mg/l IBA and subcultured every four weeks.

The roots of *R. glutinosa* contain iridoid glycosides [3–6]. However, the calli produce only caffeoyl glycosides and ethyl-β-D-glucose [8]. As it is known that the production of secondary metabolite can be enhanced by the induction of organogenesis [14], the shoots regenerated from embryos and the root tissue cultures were analysed.

The methanol extract of the root tissues were partitioned with organic solvent and then repeatedly chromatographed on Sephadex LH-20 and MCI GEL CHP-20P to give compounds **1**–**3**. These were identified as melittoside (**1**), rehmannioside D (**2**) [3, 15] and acteoside (**3**) [16] by ^{1}H NMR, ^{13}C NMR and FABMS (see Experimental). The methanol extract of regenerated shoots was analysed by HPLC and TLC to detect acteoside and ethyl-β-D-glucose. cf, callus tissue [8]. This is the first example of the isolation of iridoid glycosides from root tissue culture of *R. glutinosa* var. *purpurea*.

EXPERIMENTAL

Mps: uncorr. ^{1}H NMR: 270 and 400 MHz, TMS as int. standard; ^{13}C NMR: TMS as int. standard. HPLC: a column (4 mm × 250 mm) packed with either Nucleosil 5C-18 (Nagel) (for phenolic glycoside) or inatosil ODS (Gasukuro Kogyo) (for iridoid glycoside), column temp: room temp; solvent: 35% MeCN (for phenol glycoside) or 20% MeOH (for iridoid glycoside); flow rate: 0.5 ml/min; detector: 325 nm (for phenol glycoside) or 212 nm (for iridoid glycoside) and photodiodoaray using Spectrophotometric Detector SPD-M6A (Shimadzu). CC was carried out with Sephadex LH-20 and MCI GEL CHP-20P using a mixture of MeOH and H_2O. TLC: solvent 1, *n*-BuOH–HOAc–H_2O (4:1:5); solvent 2, $CHCl_3$–MeOH–H_2O (6:4:1).

Tissue culture. A leaf of *R. glutinosa* var. *purpurea* obtained by shoot tip tissue culture as previously described [8] was aseptically cut into square pieces (5 × 5 mm). Ten segments were cultured individually for 3 months. The basal medium and culture condition were the same as those described in the previous paper [8] except for the culture period. In order to induce shoots, an embryo cluster was transferred to 1/2 MS medium supplemented with 1 mg/l GA and 1 mg/l BAP and cultured for 5 weeks. The callus segment induced from a medium inoculated with NAA and kinetin was subcultured in the same medium every 4 weeks. Regenerated shoots were cut, transferred to hormone-free medium to give plantlets [8]. Roots were transferred to liquid MS medium supplemented with 1 mg/l IBA and subcultured every 4 weeks at 25 ± 1° under 16 hr light.

Isolation and identification of components in callus and differentiated organs. Accumulated fresh root tissue (236 g) was extracted with MeOH to give an extract (9.9 g) which was partitioned with Et_2O, EtOAc and *n*-BuOH, successively. The *n*-BuOH-soluble fraction (1.9 g) was subjected to Sephadex LH-20 CC using MeOH and H_2O as solvent to give **1** (11.6 mg). The residual aq layer (5.1 g) was purified by Sephadex LH-20 and MCI GEL CHP-20P CC and prep. HPLC to give **2** (14.7 mg) and **3** (13.5 mg).

Compound **1.** Amorphous substance; $[\alpha]_D^{20}$ −6.5° (MeOH; *c* 1.0); FD-MS *m/z*: 647, 625; it was directly identified with authentic acteoside by comparison of the ^{1}H NMR and ^{13}C NMR spectra.

Compound **2.** White powder; mp 176–177°; $[\alpha]_D^{22}$ −28.9° (H_2O; *c* 1.1); FABMS *m/z*: 547 $[M+Na]^+$; ^{1}H NMR (D_2O): 4.23 (2H, *m*, H-10), 4.57 (1H, *br s*, H-6), 5.15 (1H, *d*, *J* = 6.3 Hz, H-4), 5.40 (1H, *d*, *J* = 5.3 Hz, H-1), 5.83 (1H, *br s*, H-7), 6.49 (1H, *d*, *J* = 6.3 Hz, H-3); ^{13}C NMR (D_2O): 53.3 (C-9), 62.3 (C-10), 63.2 (glc-6′, 6″), 72.0 (glc-4″), 72.1 (glc-4′), 75.5 (glc-2′), 75.8 (glc-2″), 78.1 (glc-5″), 78.4 (glc-3″, 5′), 78.9 (glc-3′), 82.5 (C-6), 83.4 (C-5), 97.8 (C-1), 100.5 (glc-1″), 100.7 (glc-1′), 107.4 (C-4), 130.3 (C-7), 146.1 (C-8), 147.3 (C-3). It was identified with authentic melittoside [3].

Compound **3**: White powder; $[\alpha]_D^{22}$ −26.7° (H_2O; *c* 1.0); negative FABMS *m/z*: 685 $[M-H]^-$; ^{1}H NMR (D_2O): 3.77 (2H, *m*, H-10), 4.22 (1H, *br s*, H-6), 5.21 (1H, *d*, *J* = 6 Hz, H-4), 5.36 (1H, *d*, *J* = 5 Hz, H-1), 5.81 (1H, *br s*, H-7), 6.48 (1H, *d*, *J* = 6 Hz, H-3); ^{13}C NMR (D_2O): 54.0 (C-9), 62.4 (C-10), 63.2 (glc-6′), 63.3 (glc-6‴), 63.7 (glc-6″), 72.1 (glc-4‴), 72.2 (glc-4′), 72.4 (glc-4″), 75.6 (glc-2′), 76.7 (glc-2‴), 78.3 (glc-5″, 5‴), 78.4 (glc-5′), 78.8 (glc-3″), 79.0 (glc-3′), 79.1 (glc-3‴), 82.7 (glc-2″), 83.0 (C-6), 83.9 (C-5), 98.5 (C-1), 99.1 (glc-1‴), 100.9 (glc-1′), 105.7 (glc-1″), 106.9 (C-4), 130.5 (C-7), 146.5 (C-8), 147.3 (C-3). It was directly identified with authentic rehmannioside D [3].

Accumulated fresh shoots (74 g) were extracted with MeOH to give an extract (2.4 g) which was partitioned with Et_2O, EtOAc and *n*-BuOH, successively. The *n*-BuOH-soluble fraction and the residual aq. layer were analysed by HPLC and TLC, respectively. R_t (min): rehmannioside D (12.8), melittoside (7.6), aucubin (6.6), acteoside (9.0). Acteoside was detected in the *n*-BuOH-soluble fraction and ethyl-β-D-glucose was detected in the residual aq. layer by TLC.

REFERENCES

1. Tomoda, M., Kato, S. and Onuma, M. (1971) *Chem. Pharm. Bull.* **19**, 1455.
2. Tomoda, M., Tanaka, M. and Kondo, N. (1971) *Chem. Pharm. Bull.* **19**, 2411.
3. Oshio, H. and Inouye, H. (1982) *Phytochemistry* **21**, 133.
4. Kitagawa, I., Fukuda, Y., Taniyama, M. and Yoshikawa, M. (1986) *Chem. Pharma. Bull.* **34**, 1399.
5. Yoshikawa, M., Fukuda, Y., Taniyama, T., Cha, B. C., Kitagawa, I., Nakase, K., Kimura, I. and Kimura, M. (1986) *The 6th Symposium on the Development and Application of Naturally Occurring Drug Materials Abstract*, p. 31.
6. Yoshikawa, M., Fukuda, Y., Taniyama, T., Cha, B. C. and Kitagawa, I. (1986) *Chem. Pharm. Bull.* **34**, 2294.
7. Endo, K., Matsuura, A., Kanazawa, K., Onosato, A., Konno, C. and Hikino, H. (1984) *Abstract Paper of the 104th Annual Meeting of the Pharmaceutical Society of Japan*, p. 165.
8. Shoyama, Y., Matsumoto, M. and Nishioka, I. (1986) *Phytochemistry* **25**, 1633.
9. Shoyama, Y., Nagano, M. and Nishioka, I. (1983) *Planta Med.* **48**, 124.
10. Matsumoto, M., Shoyama, Y., Nishioka, I., Iwai, H. and Wakimoto, S. (1989) *Plant Cell Rep.* **7**, 636.
11. Mao, W. Y., Li, Z. G. and Zhu, B. M. (1983) *Chin. Bull. Bot.* **1**, 44.
12. Xu, Z. H. and Davey, M. R. (1983) *Plant Cell Rep.* **2**, 55.
13. Murashige, T. and Skoog, T. (1962) *Physiol. Plant.* **15**, 473.
14. Hagimori, M., Matsumoto, T. and Obi, Y. (1982) *Plant Cell Physiol.* **23**, 1205.
15. Matsumoto, M., Shoyama, Y., Nishioka, I. and Irino, N. (1987) *Abstract Paper of the 107th Annual Meeting of the Pharmaceutical Society of Japan*, p. 360.
16. Nonaka, G. and Nishioka, I. (1977) *Phytochemistry* **16**, 1265.