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Shoot Regeneration from Mesophyll Protoplasts and Leaf Explants of *Rehmannia glutinosa*

X.-H. Xu[1] and M. R. Davey

Plant Genetic Manipulation Group, Department of Botany, University of Nottingham, University Park, Nottingham NG7 2RD, UK

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ABSTRACT

Mesophyll protoplasts obtained from leaves of shoot cultures of *Rehmannia glutinosa* were cultured in Murashige and Skoog (1962) liquid or liquid-over-agar medium containing 2.0 mg L^{-1} naphthaleneacetic acid and 0.5 mg L^{-1} benzylamino purine. An amino acid mixture of glutamine, arginine, glycine, and aspartic acid promoted sustained protoplast division, with an average plating efficiency of 27%. Protoplast-derived colonies formed callus which readily regenerated shoots on transfer to Murashige and Skoog based agar medium with 2.0 mg L^{-1} indoleacetic acid and 1.0 mg L^{-1} benzylamino purine. Leaf explants also showed a marked capacity for shoot regeneration in culture.

Abbreviations: BAP, 6-benzylamino purine
NAA, α-naphthaleneacetic acid
IAA, 3-indoleacetic acid
MS, KM, UM, CPW, see Methods

INTRODUCTION

Rehmannia glutinosa (Scrophulariaceae) is a useful Chinese medicinal herb, the main functions of which include enrichment of the blood, stimulation of the heart, and a diuretic effect. In China, the herb has suffered serious deterioration, mainly as a result of virus infection. There are difficulties associated with the routine breeding of this plant, such as incompatibility during inbreeding. Some attempts have been made to apply tissue culture methods to this herb, including *in vitro* fertilization, apical meristem and anther culture (W-Y. Mao, personal communication), together with plant regeneration from callus tissues induced from seedling root, stem, and leaf explants (Jiang and Mao 1979).

To date, protoplasts have been isolated and cultured from only two members of the Scrophulariaceae, namely *Antirrhinum majus* (Poirier-Hamon et al. 1974) and *Digitalis purpurea* (Dieffrich and Luckner 1980; Dieffrich et al. 1980), with somatic embryoids being reported in *Antirrhinum*. This paper extends the studies in the Scrophulariaceae. It summarises conditions for the isolation and culture of *Rehmannia* mesophyll protoplasts, together with plant regeneration from tissues of protoplast and leaf explant origin.

MATERIALS AND METHODS

Plant material - Seeds of *Rehmannia glutinosa* Libosch. f. *hueichingensis* (Chao et Schih) Hsiao were surface sterilised with 0.1% w/v mercuric chloride in 0.1% w/v sodium lauryl sulphate (10 m), washed with sterile water (3 changes), and germinated on agar-solidified (0.6% w/v; Sigma) hormone-free Murashige and Skoog (1962) medium (MSO). After 4 weeks, the shoots were excised and transferred to MS agar medium with 1.0 mg L^{-1} zeatin (MSZ) to stimulate multiple shoot production. Sterile shoots were maintained by sub-culture every 4 weeks on MSO agar medium.

Mesophyll protoplast isolation and culture - Expanding leaves were used from 3 to 4 weeks old cultured shoots. The lower epidermis was removed, and the leaves plasmolysed (1 h) in CPW salts solution (Frearson et al. 1973) containing 13% w/v mannitol (CPW13M), followed by incubation (28°C, dark, 16 h) in an enzyme solution of 2.5% w/v Meicelase (Meiji Seika Kaisha Ltd, Tokyo, Japan) and 0.25% w/v Macerozyme (Yakult Biochemicals Co. Ltd, Nishinomiya, Japan) in CPW13M, pH 5.8. Protoplasts were processed using a routine procedure employing floatation on CPW solution containing 21% w/v sucrose (Power and Davey, 1980).

Protoplasts were cultured at 5 X 10^4 ml^{-1} at 28°C in the dark for 2 to 3 weeks, and subsequently at 23°C in diffuse light (700 lux). 2.0 ml of liquid or 1.0 ml of liquid over 1.0 ml agar solidifed medium were used in 56 X 14 mm Nunclon plastic dishes. The media employed were :

(1) MSP19M, based on the MS formulation with 2.0 mg L^{-1} NAA, 0.5 mg L^{-1} BAP, and 9% w/v mannitol.
(2) MSP19M-AA with NH_4NO_3 and KNO_3 of the MS formulation replaced with 2940 mg L^{-1} KCl, and supplemented with an amino-acid mixture of L-glutamine, L-arginine, L-glycine and L-aspartic acid at 877, 288, 75, and 266 mg L^{-1} respectively. In some cases, m-inositol (1000 mg L^{-1}) was added to both MSP19M and MSP19M-AA (designated MSP19M-I and MSP19M-AAI). Both the amino-acid mixture and m-inositol were added to the medium following filter sterilisation.
(3) KM8P/KM8 (2:1, v:v) (Kao and Michayluk 1975).

Cultures were diluted at 7 to 10 days intervals by addition of 0.5 ml aliquots of either MSP1 lacking mannitol or KM8 to the dishes.

[1] *Permanent address:* Institute of Plant Physiology, Academia Sinica, Shanghai, China

Callus culture and plant regeneration - Protoplast-derived tissues were transferred when 1.0 mm in size to MSD3 agar medium containing 2.0 mg L^{-1} IAA and 1.0 mg L^{-1} BAP for shoot induction. Regenerated shoots formed roots when transferred to hormone-free MS agar medium or to MSP2 medium with 0.2 mg L^{-1} NAA. Rooted shoots survived transfer to compost. Undifferentiated protoplast-derived tissues were maintained on MSP1 agar medium.

Leaf explant cultures - Leaf explants, similar to those used as a source of protoplasts, were cultured on the following agar soldified media:

(1) MSP1 and MSP1-AA.
(2) UM (Uchimiya and Murashige 1974) with 2.0 mg L^{-1} 2,4-D, 0.25 mg L^{-1} kinetin and 2000 mg L^{-1} casein hydrolysate.
(3) MSD3 with 2.0 mg L^{-1} IAA and 1.0 mg L^{-1} BAP.

RESULTS

Protoplast culture - Protoplast yields ranged from 1.5 to 3.0 X 10^6 per g fresh weight of leaf tissue. Isolated *Rehmannia* mesophyll protoplasts (Fig 1) entered division within 2 days of culture in the amino-acid supplemented medium (MSP19M-AA) (Fig 2), with those in unsupplemented MSP19M taking longer to divide (4 to 5 days). The amino-acid mixture promoted sustained protoplast division and colony formation. However, m-inositol had no apparent advantageous effect on protoplast division (Table 1). Plating efficiencies estimated after 7 days of culture were higher in liquid media compared to liquid-over-agar, reaching 33% in the amino-acid supplemented medium (MSP19M-AA). However, liquid-over-agar was the best culture system for supporting longer term growth of protoplast-derived colonies. Regular dilution ensured protoplast division and colony development. Although MS based media were more satisfactory than KM8P/KM8 for initial culture of protoplasts (Table 1), KM8 was preferable to MSP1 for dilution of protoplasts cultured in both MS based media and in KM8P/KM8, since dilution with MSP1 resulted in protoplast browning and cessation of division. Cell colonies were visible to the naked eye within 4 weeks of protoplast isolation, and those transferred to MSD3 regenerated shoots within 15 to 20 days. Shoots rooted readily on MS0 and MSP2 agar media.

Medium	Plating efficiency (%) ± S.E.	
	Liquid	Liquid-over-agar
KM8P/KM8	2.5 ± 1.5	0.5
MSP19M	10.5 ± 3.1	5.5 ± 0.5
MSP19M-AA	26.6 ± 7.2	13.9 ± 1.6
MSP19M-1	9.9 ± 4.2	not
MSP19M-AA1	22.5 ± 0.5	tested

Table 1. Plating efficiency (number of dividing protoplasts expressed as a percentage of the total protoplast population) of *Rehmannia* mesophyll protoplasts after 7 days culture in various media (Data from 4 experiments).

Leaf explant culture - All leaf explants cultured on MSD3 formed multiple shoots after 10 days of culture, with very rapid growth of shoots (Fig 4). The latter rooted on MS0 or MSP2 agar medium. Explants developed callus on UM and MSP1-AA agar media, with more rapid callus induction on the latter medium. Similar results were obtained with stem and petiole explants. Callus of explant origin, like that derived from protoplasts, formed shoots on transfer to MSD3 medium.

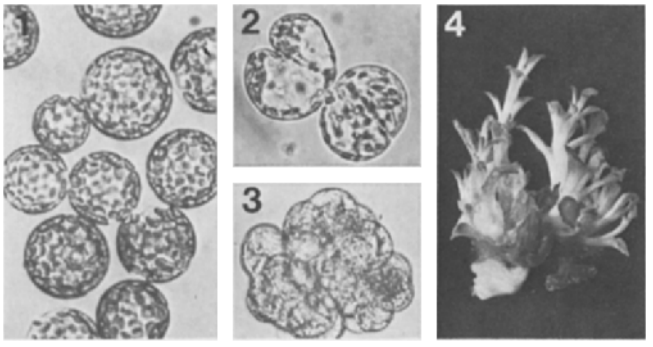

Fig 1. Freshly isolated *Rehmannia* mesophyll protoplasts. X 360.
Fig 2. First division in mesophyll protoplasts after 2 days in culture. X 340.
Fig 3. Cell colony after 10 days in culture. X 200.
Fig 4. Multiple shoot formation from a leaf explant. X 2.5.

DISCUSSION

Shoot cultures of *Rehmannia glutinosa* are a convenient source of experimental material for the isolation of totipotent mesophyll protoplasts. Plant regeneration occurred by shoot formation, unlike the situation in leaf protoplast-derived tissues of *Antirrhinum* which produced somatic embryoids (Poirier-Hamon et al. 1974). The stimulatory effect of the amino-acid mixture on division of *Rehmannia* mesophyll protoplasts was similar to that reported for mesophyll protoplasts of *Vicia narbonensis* (Donn 1978). However, m-inositol did not promote protoplast division when added to the culture medium, either alone or in combination with the amino-acid supplements. This result contrasts with that obtained for root protoplasts of *Phaseolus aureus* (Xu et al. 1981), *Glycine max* and *Brassica napus* (Xu et al. 1983) where m-inositol stimulated division. Such a result is not surprising, since differences in the metabolic requirements of various organs of the plant may be reflected in the nutritional and hormonal demands of protoplasts isolated from these organs. The results reported for *Rehmannia* now enable protoplast manipulation techniques, similar to those being used for potato (Shepard et al. 1980), to be applied to the breeding programme of this traditional Chinese herb.

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