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PLANT REGENERATION FROM SEEDLING EXPLANTS AND COTYLEDON PROTOPLASTS OF *GLYCINE ARGYREA* TIND.

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SUMMARY

Plants have been regenerated from nodular, green callus derived from cotyledon, petiole and leaf lamina explants of *G. argyrea*, a perennial relative of the soybean (*G. max*). The degree of response obtained was governed primarily by the genotype used, accession G1626 proving the most responsive. Shoots were also recovered from about 6.0% of cotyledon protoplasts of this genotype. The implications of these results are discussed in relation to genetic manipulations using this species.

Key words: Glycine argyrea; plant regeneration; cotyledon protoplasts; soybean.

INTRODUCTION

The transfer of agronomically useful traits (5,9,11) from perennial *Glycine* species to soybean is hindered by difficulties encountered using conventional crossing methods, in particular, low levels of plant recovery from cultured, rescued embryos (e.g. 13). Somatic hybridization, involving fusion of protoplasts, is a possible alternative technique to effect this gene flow, but relies upon previous knowledge of plant recovery from explants and protoplasts of at least one of the fusion partners. Plants have been obtained from hypocotyl (14) protoplasts of *G. canescens*, and cotyledon protoplasts of *G. canescens*, *G. clandestina* (6), and, more recently, soybean (*G. max*; 17). Extending the range of wild *Glycine* genotypes that produce plants from protoplasts will increase the germplasm that could be introgressed into the soybean genepool using somatic methods. This paper summarizes data on plant regeneration from seedling explants and protoplasts of *G. argyrea*, a recently described species (16).

MATERIALS AND METHODS

Seedling material. Seeds of *G. argyrea* accessions G1420, G1622, G1626, G1632, G2000, G2004, G2006 and G2010, obtained from Dr. A. H. D. Brown, CSIRO Division of Plant Industry, P. O. Box 1600, Canberra, Australia, were sterilized (20 min.) in a 10% (v/v) "Domestos" commercial bleach solution (Lever Bros., London, UK), rinsed four times in sterile water, and scarified with a scalpel. They were soaked in sterile water (48 h with a change after 24 h), prior to sowing at a rate of 6 seeds per 30 ml of hormone-free, agar-solidified (0.6% w/v; Sigma) B5 medium (1) contained in 175 ml screw-capped powder round glass jars (Beatson Clark, Rotherham, UK). Germination occurred at 27° C and 1.6 Wm^{-2} continuous irradiance produced by Thorn Pluslux daylight fluorescent tubes.

Preparation of explants. Fourteen-20 d after seed sowing, cotyledons and whole trifoliate leaves were removed from the seedlings. The cotyledons were cut transversely into halves; leaflets were removed from the leaves, and the petiole cut transversely into four equal lengths. The explants were placed onto 125 ml of semi-solid SC2 medium (4; B5 salts and organics, 30 gl^{-1} sucrose, 1.1 mgl^{-1} benzylaminopurine, 0.005 mgl^{-1} indole butyric acid and 6.0 gl^{-1} agar, pH 5.8), contained in 14 cm plastic Petri dishes at the rate of 30–35 explants per dish. These cultures were maintained under the same conditions as described for seed germination, and transferred to fresh SC2 at regular, 2-week intervals. Callus tissues that developed shoots were transferred to 30 ml of HB50 medium (5; half-strength, hormone-free B5 solidified with 6.0 gl^{-1} agar, pH 5.8), contained in 175 ml jars, and regularly transferred to fresh medium every two weeks. Once 3 cm or more in length, shoots were excised from the callus, and transferred to fresh HB50 for rooting.

Rooted plantlets were transferred to a 1:1 mixture of Levington Compost (Fisons, Ipswich, UK) and sharp sand, in 7 cm diameter, plastic pots, and placed in polythene bags within a growth chamber. Plants were exposed to a 12 h photoperiod provided by four Compton warm white, fluorescent tubes, 30 cm overhead, fitted with light diffusers (0.35 Wm^{-2}), and day and night temperatures of 24° C and 19° C respectively. At daily intervals, a hole made in each bag was gradually increased in size to acclimatize the plantlets to ambient humidity. The bags were removed after 7 d.

Protoplast isolation and culture. Cotyledons were excised from 10–12 d old seedlings, sliced transversely, and preplasmolysed by immersion in 4.0 ml CPW9M solution (6), contained in a 9 cm plastic Petri dish. The CPW9M was removed, replaced by 10 ml of EM2 enzyme mixture (12), and the dish transferred to the dark for 16 h

at 27° C. The resulting protoplast preparation was passed through a 45 μm nylon mesh, and centrifuged (55 x g, 7 min.). The pellet was twice resuspended and centrifuged in CPW9M solution. Protoplasts were suspended, at a density of 6.0×10^3 ml^{-1}, in K8P medium (3) containing 6.0 gl^{-1} agarose (Sigma Type VII), cooled to 40° C. Protoplasts were dispensed as 55 μl drops in the bottom of a 9 cm plastic Petri dish (3.0 ml total per dish). Ten ml liquid K8P medium were added to each dish following agarose gelling. Cultures were maintained at 27° C in the dark. After 7 d the dishes were transferred to the light conditions used for seed germination. At weekly intervals, the liquid bathing medium was removed from the dishes and replaced, progressively, with 5.0 ml volumes of 1:2, 1:1 and 1:2 mixtures of K8P and liquid SC2, followed by SC2 alone. After 6–8 weeks of culture, protoplast-derived colonies were released from the agarose by applying pressure with a spatula; regular replacement of the SC2 liquid with fresh medium was continued. Once 2-3 cm in diameter, dark-green, nodular colonies were transferred to 30 ml of agar-solidified SC2 medium, overlaid with 2.0 ml of liquid SC2 medium, in 9 cm Petri dishes. Subsequently, callus was transferred to fresh, semi-solid SC2 medium every two weeks until shoot buds developed. The resulting protoplast-derived tissues were treated as described for complex explant-derived material.

RESULTS

Seedling explants. Varying numbers of seedling explants from accessions G1622, G1626, G2000, G2004, and G2006, but not those of G1420, G1632 or G2010, produced green, nodular callus following 3 2-week periods on SC2 medium. The development of this tissue was accompanied by the accumulation of brown, phenolic material around the explants. At this stage, growth of the cultures ceased, and the tissue turned chlorotic. However, callus development was promoted by regular subculture to fresh medium. During their third passage on SC2 medium, cultures ceased to produce phenolic compounds, and callus of accessions G1622, G1626, G2000 and G2006 developed shoot buds (Table 1). The highest rate of bud production was obtained from explants of accession G1626, with 50% of the cultures showing a response. Buds of this accession developed into shoots following one to six regular subculture periods on HB50 medium. Fifty-nine percent of the regenerated shoots produced roots following excision from the parental callus, and insertion into fresh HB50 medium. All of the 30 rooted shoots which were tested developed into morphologically normal, fertile plants when transferred to soil, and grown under glasshouse conditions (Fig. 1a). No attempts were made to recover whole plants from shoot buds produced by other genotypes.

Cotyledon protoplasts. Each cotyledon, weighing on average 8.4 mg, produced approx. 3.0×10^4 protoplasts when incubated in EM2 enzyme solution (Fig. 1b). Protoplasts plated in agarose-solidified K8P medium began to divide after 4 d of culture (Fig. 1c), giving a division frequency of 60-70% at day 7. The developing protoplast-derived colonies (Fig. 1d) became nodular and green following 3-4 weeks of osmoticum reduction using liquid SC2. However, further colony growth was associated with a browning of the culture medium. Release of developing colonies from the agarose drops into liquid medium, with weekly replenishment of the SC2 medium was essential to maintain colony growth. Using the procedure described, 43% of the plated protoplasts produced viable cell colonies. Growth of the green, nodular tissue initially declined following transfer to agar SC2 medium, but resumed after 3–4 weeks. Fifteen percent of the green, nodular tissues produced shoot buds after 3 further subculture periods on the same medium (Fig. 1e). Subsequently, these buds developed into shoots when the parental callus tissues were regularly transferred to HB50 medium. In total, 6.4% of the protoplasts originally plated developed into tissues which produced shoots.

DISCUSSION

The methods described represent an efficient and reproducible procedure to recover whole plants from explants and protoplasts of *Glycine argyrea*. However, tissues of this species produced large quantities of oxidized phenolic compounds, necessitating regular subculture to fresh SC2 medium in order to sustain callus growth and shoot formation.

In common with *G. canescens*, *G. clandestina* and *G. latrobeana* (4,5), shoots of *G. argyrea* developed only from green, nodular callus, this tissue not having been observed in either *G. cyrtoloba* or *G. falcata*, which both produce shoots on SC2 medium, or in other, non-regenerating *Glycine* species (5,10). This suggests that regeneration via green, nodular tissue is specific within the Genus *Glycine* to species with an AA genome designation, since *G. argyrea*, *G. canescens* and *G. clandestina* belong to this group of species (8), and preliminary evidence from meiotic pairing studies suggests that *G. latrobeana* is probably allied to these taxa (2).

TABLE 1

INCIDENCE OF SHOOT BUD FORMATION FROM SEEDLING EXPLANTS OF FOUR DIFFERENT ACCESSIONS OF *G. ARGYREA* FOLLOWING THREE 2 WEEK PERIODS ON SC2 MEDIUM

	Accession							
	G1622		G1626		G2000		G2006	
Explant	t	s	t	s	t	s	t	s
Cotyledon	46	13	50	70	17	24	12	4
Leaf lamina	40	10	118	61	36	17	39	10
Petiole	18	0	104	28	28	29	43	1
Total	104	9	272	50	81	70	94	16

t, total number of explants examined; s, percentage of explants producing shoot buds.

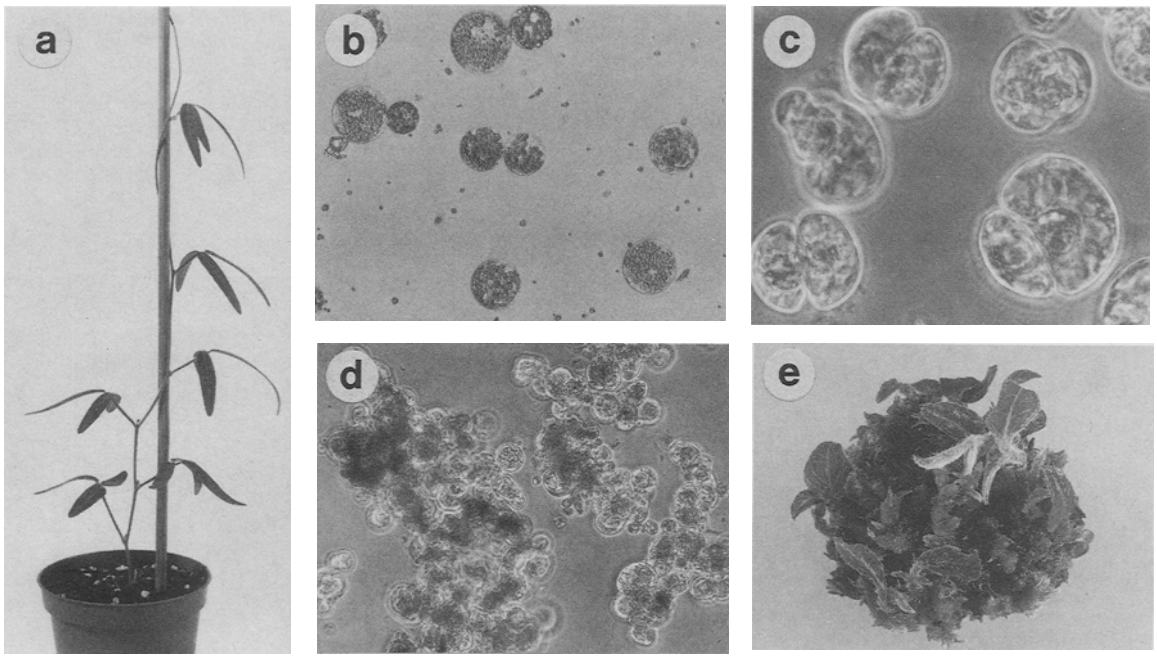

FIG. 1. Plant regeneration in *Glycine argyrea*. a, an established, leaf explant-derived plant of *G. argyrea* G1626 (×0.35). b, protoplasts freshly isolated from seedling cotyledons of accession G1626 (×200). c, first division in cotyledon protoplast-derived cells of G1626 after 8 d of culture in agarose-solidified K8P medium (×450). d, protoplast-derived cell colonies after 21 d of culture in agarose-solidified K8P medium (×140). e, shoot bud formation following three 2 week subcultures of protoplast-derived nodular green tissues on SC2 medium (×4).

The response to SC2 medium within *G. argyrea* was dependent on the genotype, with G1626 exhibiting the greatest rate of shoot production. This accession was chosen for studies with protoplast-derived tissue, since in previous investigations, a correlation has been observed between the high regeneration potential of complex seedling explants of *G. canescens* G1171 and *G. clandestina* G1231 (4,5), and the ability to recover shoots from protoplasts of these accessions (6). The results presented in this report for *G. argyrea* G1626 substantiate the view that perennial *Glycine* genotypes carrying the AA genome, and exhibiting high rates of regeneration from seedling explants (>50% of the explants tested) are also likely to produce shoots from protoplast-derived tissues. Thus, investigations into shoot production by seedling explants and protoplast-derived tissues from other accessions, showing less efficient regeneration, were discontinued in the present study.

The superior nature of the plant regeneration response of *G. canescens* G1171 over other perennial *Glycine* accessions (5,6), has enabled the recovery of transformed plants of this genotype from roots following inoculation of seedlings with *Agrobacterium rhizogenes* (15). It is likely that the high rates of regeneration obtained in the present study from seedling tissue of *G. argyrea* G1626, will also enable transgenic plants to be recovered from transformed roots of this taxon. Furthermore, the observation that shoots can be recovered from a higher proportion (>6%) of protoplasts in *G. argyrea* G1626 than in *G. canescens* G1171 or *G. clandestina* G1231 (0.6-1.2% and 0.2-0.4% respectively; 6), suggests that somatic hybrid plants might be produced more efficiently from tissues derived from heterokaryons resulting from fusion of soybean protoplasts with those of *G. argyrea*, rather than from *G. max* with either *G. canescens* (7) or *G. clandestina*.

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Book Review

Pathology of Cell Receptors and Tumor Markers. Application of Immunocytochemistry and Hybridization in Tumor Diagnosis.

G. Seifert and K. Hübner, Editors

New York: Gustav Fischer Verlag; 1987

This volume is a collection of the main lectures delivered at the 70th Meeting of the German Society of Pathology in 1986. Its aim is to present new techniques for the improvement of tumor diagnosis and prognosis. Most of the techniques utilize immunocytochemistry to identify cell type-specific markers in tumor samples. The intended audience appears to be clinical pathologists who are unfamiliar with immunological techniques for identifying proteins. Because of this, each of the 15 chapters is a rather basic introduction to the concepts and the techniques used for identifying marker proteins of a particular type. The first 2 chapters spell out the basic principles and procedures in a straightforward fashion. The following chapters consist of meticulous descriptions and comparisons of (mostly) immunocytochemical markers for epithelial and mesenchymal tumors, viruses and hormones produced by tumors, and a variety of tumors in specific organs. The English usage is unusual in places, but understandable. (For example, the subtitle more accurately reflects the book's contents than does the primary title.) Most chapters are brief (10 pages or so), and consist of a condensed, workmanlike description of how each marker can be used for tumor identification. Many chapters contain representative photographs of sections of tumors to illustrate their appearance when stained with reagents specific for each of the markers. Most chapters also end with numerous references, but they are run together in a compressed format that makes them difficult to use. In sum, this volume is not aimed at the basic scientist seeking information on recent advances in tumor protein identification. Rather, it is designed to introduce the basic concepts to pathologists who should find them of use for more accurate identification of tumor specimens. I believe that more such attempts are needed to bridge the gap between basic research and clinical applications. This volume should help fill that gap.

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