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Isolation, culture and callus regeneration of protoplasts of wild and cultivated *Helianthus* species

N.E. Bohorova [1], E.C. Cocking [2], and J.B. Power [2]

[1] Institute of Genetics, Bulgarian Academy of Sciences, 113 Sofia, Bulgaria
[2] Plant Genetic Manipulation Group, Department of Botany, University of Nottingham, Nottingham NG7 2RD, UK

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ABSTRACT

Protoplasts were isolated from seedling roots, hypocotyls, and cotyledons of four cultivars of Helianthus annuus and from leaves of axenic shoot cultures of the wild species H. praecox, H. scaberimus and H. rigidus. Optimal culture conditions were established for the respective protoplast systems, using the agarose bead method of culture. Protoplast division was induced for all the species examined. In the case of the cultivars of H. annuus, hypocotyl and cotyledon protoplast division was sustained leading to callus formation, which in turn, could be induced to produce roots and organised meristematic regions in the presence of NAA and 6-BAP.

ABBREVIATIONS

6-BAP, 6-benzylaminopurine; NAA, α-naphthalene acetic acid; IAA, indole-3-acetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; MS, Murashige and Skoog.

INTRODUCTION

The genus Helianthus comprises some 67 species, many of which are of potential value through hybridisation with the cultivated sunflower (H. annuus) (Schilling and Heiser 1981). Traits such as disease resistance (Georgieva-Todorova, 1976), superior oil quality (Carter, 1978), elevated protein content (Thompson et al., 1981) and sources of cytoplasmic male sterility (Leclecq, 1969) have been identified in wild Helianthus species. Although limited success has been achieved using conventional hybridisation techniques many of the wild species are reproductively isolated (Thompson et al., 1981).

Tissue culture technology and somatic hybridisation in particular offer new options that could complement conventional breeding approaches. The number of studies on sunflower tissue culture are rather limited (Sadhu, 1974; Greco et al., 1984; Bohorova et al., 1985; Paterson and Everett, 1985). The first very brief report of sunflower protoplast isolation and culture was described by Binding et al., 1981, using an unnamed genotype. Recently Lenee and Chupeau (1986) have tested the division capability of sunflower protoplasts from mesophyll tissues, stems, cotyledons and hypocotyls, but only those from hypocotyls underwent any division.

This report describes the conditions for the reproducible isolation and division of sunflower protoplasts from roots, cotyledons and hypocotyls and from leaves of wild Helianthus species protoplasts, thereby providing a greatly improved foundation for further studies on the genetic manipulation of sunflower through cell fusion techniques.

MATERIALS AND METHODS

Plant Material

Seeds of H. annuus (2n = 2x = 34) cultivars Peredovik, Stadion and accession line NS-26 were obtained from the Institute of Wheat and Sunflower, General Toshevo, Bulgaria and the commercially available cultivar Tall Single from Asmer Seeds Ltd., Leicester, U.K. Seed of H. praecox E. and Gray (2n = 2x = 34), H. scaberimus A. Gray (2n = 4x = 68) and H. rigidus Dest. (2n = 6x = 102) was from the Institute of Genetics, Sofia, Bulgaria. All seed material was surface sterilised in 15% Domestos solution (Lever Bros., U.K.) (30 min) followed by six changes of sterile tap water, and germinated on the surface of Murashige and Skoog (1962) (MS) medium lacking phytohormones but with 3% w/v sucrose or in the same medium with 0.01 mg/1 IAA, 0.03 mg/1 kinetin and 0.001 mg/1 folic acid (BGS medium). Both media were solidified with 0.8% w/v agar (Sigma), pH 5.8. Seeds were maintained at 25°C and in the dark for the cultivars of H. annuus and in the light (2,000 lux, daylight fluorescent tubes, continuous illumination) for the wild species. Shoot cultures of the latter group were maintained on BGS medium with the same cultural regime.

Protoplast isolation and culture

H. annuus

Dark grown seedling material was the preferred source and all were handled similarily. Apical root pieces (1.0 cm) cut transversely, of 3 day-old seedlings: cotyledons (1 mm wide pieces) and hypocotyl sections, cut longitudinally, both of 7 day-old seedlings were placed in CPW salt solution with 13% w/v mannitol (Frearson et al., 1973) (CPW 13M medium) for 1-2 hours. The plant tissues were

Offprint requests to: J.B. Power

transferred to a filter-sterilised enzyme mixture which consisted of 2% w/v Rhozyme HP 150 (Rohm and Haas Ltd., Philadelphia, U.S.A.), 2% w/v Meicelase (Meiji Seika Kaisha Ltd., Tokyo, Japan), 0.03% w/v Macerozyme R10 (Yakult Biochemicals Ltd., Tokyo, Japan), in CPW 13M medium, pH 5.8, and incubated at 25°C for 16 hours on a rotary shaker (30 cycles/min). After incubation the digested tissue was passed through a nylon sieve (64μ pore size) with gentle washing in small volumes of CPW 13M medium.

Root protoplasts were collected by centrifugation (120 x g, 10 min) and freed of cellular debris by flotation in CPW salts medium with 21% w/v (CPW 21S medium) coupled with centrifugation (150 x g, 15 min). Hypocotyl and cotyledon protoplasts were harvested in a similar way with an extended period (18 min) of centrifugation in CPW 21S medium.

Protoplasts were washed twice in CPW 13M medium, resuspended in the appropriate culture medium and plated in a range of densities (5×10^4 - 2.5×10^5 protoplasts/ml).

Wild Helianthus species

Leaves of axenically grown shoots (4 weeks old) were cut into 1 mm wide strips and protoplasts isolated as described for H. annuus but with the following modifications. The enzyme mixture consisted of 2% w/v Cellulase R10 (Kinki Yakult Manuf. Co. Ltd., Japan) and 0.05% w/v Macerozyme R10 in CPW 13M medium, pH 5.8. Following incubation leaf protoplasts were pelleted at 120 x g (7 min) and floated at 150 x g (10 min).

Media and handling protocol

Three media were ultimately found to support protoplast division for the Helianthus species using agarose bead culture (Shillito et al., 1983). Molten agarose (F.M.C. Corp., Marine Colloids Div., Rockland, U.S.A.) was prepared at double strength (0.8% w/v) in distilled water, mixed 1:1 with double strength medium and this in turn mixed 1:1 with the protoplasts (at twice their required density) in single strength medium. The protoplast/agarose mixture (5.0 ml) was dispensed as small beads (0.25 ml) into a 9 cm petri dish which in turn contained 6.0 ml of liquid culture medium. Dishes were sealed with Nescofilm and cultured either in the dark or in the light (700 lux, daylight fluorescent tubes, continuous illumination).

As an alternative, protoplasts were plated directly in liquid medium (4.0 ml) in 5.0 cm petri dishes at the appropriate density.

The media used for initial protoplast culture and subsequent reduction of the osmotic pressure were:- K8P medium or K8P medium mixed with K8 medium (Kao, 1977) in the following ratios: 3:1, 2:1, 1:1, 1:2; KM8P medium or KM8P medium mixed with KM8 medium (Kao and Michayluk, 1975) in the same ratios; MS medium with 2.0 mg/l NAA, 0.5 mg/l 6-BAP with 9% mannitol (MSP1 9M medium).

For the agarose bead culture, fresh medium (0.5 ml per dish) of progressively lower osmotic pressure was added at 7-10 day intervals coupled with a prior withdrawal of a similar volume from the dish.

Plating efficiency, defined as the percentage of the original protoplasts giving colonies (10-60 cells) was measured at 20 days.

Following the final dilution (7-10 weeks) three cultures yielding clearly visible colonies/callus were transferred to a range of agar-solidified (0.5% w/v) MS based media with differing growth regulators:- 2.0 mg/l NAA, 0.5 mg/l NAA, 0.5 mg/l 6-BAP (MS3); 0.05 mg/l NAA, 0.25 mg/l 6-BAP (MS4); 0.1 mg/l NAA alone (MS5) and 1.0 mg/l zeatin alone (MS6).

All cultures were kept at 25°C with a continuous illumination of 700 lux.

RESULTS AND DISCUSSION

Protoplast yields are given in Table 1. For all the protoplast systems and species examined protoplast division was not sustained in liquid media (all three) and at densities below 1×10^5 protoplasts/ml for both agarose and liquid cultures. MSP1 9M medium did not support continued division (H. annuus only) much beyond 20 days. On the basis of these experiments agarose appears to be an essential gelling agent for Helianthus protoplast culture.

For Helianthus annuus, hypocotyl protoplasts clearly gave the highest plating efficiencies as compared to root or cotyledon systems, with K8P medium being superior (Table 1). Dark culture conditions reduced the plating efficiency for all non-leaf cultured protoplasts. Leaf protoplasts of the wild Helianthus species responded in the reverse manner exhibiting superior plating efficiencies when maintained for an initial period of 1-4 weeks in the dark (Table 1).

First division was after 2 days (root, hypocotyl) or 5 days (leaf, cotyledon) with visible colonies (8-60 cells) after 14-21 days in K8P/KM8P media; thus emphasising the need for rich media to support Helianthus protoplast division. Protoplasts of only two varieties of H. annuus, Peredovik and Tall Single, divided in MSP1 9M medium but to a limited extent.

Perhaps the most critical factor for Helianthus protoplast culture is the procedure for lowering the osmotic pressure of the media in order to sustain division and minimise browning of cells. Dilution was most effective when initiated after 14 days and thereafter at 10 day intervals.

By 7-8 weeks colonies of the H. annuus cultivars could be transferred, using fine forceps, to the surface of the MS regeneration media. Calluses of hypocotyl or cotyledon protoplast origin proliferated on MS1, MS2 and MS6 media. Most calluses were white, friable and grew vigorously.

Calluses on MS3 medium produced masses of meristem-like structures and upon transfer to MS5 medium, roots were proliferated. It would appear therefore, that NAA and 6-BAP are important in stimulating callus formation and organogenesis in sunflower. This is in agreement with earlier observations on Helianthus callus initiation and shoot regeneration (Bohorova et al., 1985).

This study forms the basis for the development of selection procedures, following induced protoplast fusion and utilising the in-built visual identification of interspecific fusion products between leaf protoplasts of wild Helianthus species (H. praecox, H. scaberimus, H. rigidus) and

TABLE 1. Yield, plating efficiency and media response of protoplasts of cultivated sunflower and wild Helianthus species

Species/cultivar r = root; hyp = hypocotyl; c = cotyledon; lf = leaf	Yield x 10^5 per g fresh wt.	Medium/Plating Efficiency (%) l = light; d = dark; * division not sustained					
		K8P		Km8P		MSP1 9M	
		l	d	l	d	l	d
Helianthus annuus							
Peredovik r	2	40	0	-	-	4*	8*
hyp	5	53	0	23	19	3*	6*
c	1	24	0	16	11	0	0
Tall Single r	2	27	23	-	-	6*	7*
hyp	6	50	-	43	39	11*	14*
c	2	25	-	-	-	0	0
Stadion hyp	4.5	51	2	5	0	0	0
c	1	18	2	7	10	0	0
NS-26 hyp	4	35	33	49	36	0	0
c	2	14	12	21	19	0	0
H. praecox lf	2	10	40	13	23	0	0
H. scaberimus lf	4	12	50	11	25	0	0
H. rigidus lf	3	0	44	0	0	0	0

colourless, etiolated hypocotyl or cotyledon protoplasts of cultivated sunflower, H. annuus. Plant regenerative capacity in such somatic hybrid combinations is likely to be provided by the wild Helianthus parent species since callus, of the three species described here, obtained directly from explants, can be induced to undergo plant regeneration on MS3 medium (unpublished data).

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