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Increase of callus and embryoid production from hypocotyl protoplasts of sunflower (*Helianthus annuus* L.) by culture in microdrops

J. ŠAMAJ* , A. OKOLOT** , M. BOBÁK * and Yu. GLEBA**

*Department of Plant Physiology, Comenius University, Mlynská dolina B-2, 842 15 Bratislava, Slovak Republic**
*Institute of Cell Biology and Engineering, Lebedeva Street 1, 252 650 Kiev GSP 22, Ukraine***

Abstract

Hypocotyls of seedling plants of the sunflower inbred line HNK-81 were used as a source of protoplasts. Optimal conditions of isolation and culture of protoplasts were established. Using the method of individual culture in microdrops, a 77 % final plating efficiency was achieved. Cytokinins, especially zeatin, showed a significant influence on the formation of globular embryo-like structures, but these failed to develop into mature embryos. Calli obtained in liquid media containing auxins and cytokinins grew on agar solidified media without growth regulators.

Introduction

Stabilized high yield protoplast culture with an optimized system of regeneration of intact plants of cultivated sunflower (*Helianthus annuus* L.) is a basic assumption for effective improvement of direct transformation techniques (PEG method, electro-transfection) and somatic hybridization method in biotechnological work with this important oil crop.

Various tissue sources has been used for isolation of sunflower protoplasts, giving different results. Some authors have tested the dividing capacity of isolated protoplasts and production of calli from root, hypocotyl, cotyledon, stem and mesophyll derived protoplasts of sunflower (Bohorova *et al.* 1986, Lenee and Chupeau 1986), but only hypocotyl protoplasts divided with a high efficiency. Other tissue types are thought to be relatively recalcitrant for further regeneration.

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Abbreviations: BAP - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; KIN - kinetin; MS-Murashige-Skoog medium; NAA - 1-naphthaleneacetic acid; ZEA - zeatin.

Later, Guiley and Hahne (1989) reported the formation of green calli from mesophyl protoplasts. Schmitz and Schnabl (1989) showed that petiolar protoplasts had been demonstrated as a very good system for regeneration of calli and rhizoids. Fischer and Hahne (1991) described a development of cotyledon-derived protoplasts and routine regeneration of calli from them.

Calli derived from protoplasts can produce meristem-like structures or proliferate to roots (Bohorova *et al.* 1986). Some authors have reported embryoids or embryo-like structures obtained from protoplasts. But these embryoids failed to develop into mature embryos and gave rise to calli or produced roots (Moyne *et al.* 1988), hairy roots (Dupuis *et al.* 1988) and some rhizoids (Schmitz and Schnabl 1989).

Krasnyanski and Menczel (1991) have reported the development of abnormal shoots from protoplast-derived embryoids. Recently, the regeneration of shoots and complete plants from hypocotyl or cotyledon protoplasts was successful (Burrus *et al.* 1991, Fischer *et al.* 1992). Thus it seems, that genotype and the culture conditions of protoplast cultures play the most important roles in increasing the regeneration capability of sunflower protoplasts.

We evaluated and optimized conditions for production and culture of protoplasts of an economically important genotype of sunflower (inbred line HNK-81) using new methodological approaches.

Materials and methods

Plant material: Sunflower seeds were obtained from State Examination Station Bratislava, Slovak Republic. All seed material was sterilized for 30 s in 70 % ethanol and 15 min in 10 % calcium hypochlorite solution, and then rinsed four times with sterile distilled water. Sterile seeds were germinated on agar-solidified MS basal medium (Murashige and Skoog 1962) and maintained for 9 d in the light/dark cycle [16/8 h, irradiance of 11.2 μmol m^{-2} s^{-1} (400-700nm), temperature of 23 - 25 °C].

Sunflower inbred line HNK-81 which had shown the highest regeneration potential on meristem culture level (Šamaj *et al.* in press) was used for protoplast isolation and culture.

Protoplast isolation: The best results were achieved when protoplasts were isolated from small fragments of hypocotyls in the saline medium W-5 (Medgyesy *et al.* 1980) containing a mixture of enzymes: Cellulase-Onozuka R.10 (0.2 %), Dricelase (0.2 %), Cellulysin (0.1 %) and Macerozyme (0.1 %) at pH=5.8. After 28 h of incubation in the dark (temperature 23 °C), the digested tissue was passed through a 120 μm Kapron sieve.

Protoplasts were freed from cellular debris in 0.5 M sucrose using centrifugation (130 *g*, 10 min). Then they were washed twice with W-5 solution (100 *g*, 8 min), plated into plastic Petri dishes (40 × 10 mm) and resuspended by appropriate culture media to densities of 1 - 10 × 10^4 protoplasts per cm^3. Plates were sealed with parafilm and maintained under diffuse light in cultivation chambers [irradiance 6.4

μmol m^{-2} s^{-1} (400-700nm), photoperiod 12/12 h, temperature 23 - 25°C, relative air humidity 98 - 99.5 %].

Results and discussion

The capacity of various young seedling organs - hypocotyls, cotyledons, stems and leaves for their maximum initial yield of protoplasts was evaluated (Table 1), using various combinations of four enzymatic compounds - Cellulase, Dricelase, Cellulysin and Macerozyme (Table 2).

Table 1. Yields of protoplasts [$\times 10^5$(protoplasts) g^{-1}(f.m.)]. Composition of enzymes: Cellulase (0.2 %), Dricelase (0.2 %), Cellulysin (0.1 %) and Macerozyme (0.1 %) at pH 5.8.

Explants	Yields of protoplasts	
	medium NT_{mod}	solution W-5
Cotyledon	3	4
Stem	2	5
Hypocotyl	10	25
Leaf	20	8

Modified NT media (Nagata and Takebe 1971) were used (Table 3) at early stages of protoplast culture. The contents of osmotics, nitrogen sources and growth regulators were varied in media. The best development of cell colonies was observed in the media with 0.4 M mannitol (Fig. 2*A-C*).

Table 2. Composition of enzymes in the W-5 solution and protoplast yields obtained from hypocotyl explants [$\times 10^6$(protoplasts) g^{-1}(f.m.)]. (Means from five independent experiments).

Composition of enzyme in W-5 solution [%(m/v)]	1	2	3	4	5	6
Cellulase	0.1	0.2	0.3	0.4	0.5	1.0
Dricelase	0.1	0.2	0.5	0.1	0.2	0.5
Cellulyzin	0.3	0.1	0.2	0.3	0.1	0.2
Macerozyme	0.2	0.1	0.05	0.2	0.1	0.05
Yields of protoplasts, pH 5.6	1.2 ± 0.2	2.5 ± 0.3	0.9 ± 0.2	0.6 ± 0.1	1.5 ± 0.3	0.2 ± 0.2

Four sources of nitrogen were tested: KNO_3, NH_4NO_3, glutamine and ammonium jantare (Table 3). Only glutamine (680 mg l^{-1}) together with small amounts of ammonium nitrate (200 mg l^{-1}) had favourable effects on protoplast viability and division. Protoplasts were not able to divide in the media containing potassium nitrate only (Lenee and Chupeau 1986, 1989). Similar effects of inorganic nitrogen sources was also observed using the NT medium (Table 3).

Table 3. Composition of sunflower protoplast culture media. Composition of NT_1, NT_2 and NT_3 media was identical to NT_{mod} except for the nitrogen sources (Nagata and Takebe 1971).

Concentration [mg l^{-1}]	NT	NT_1	NT_2	NT_3	NT_{mod}
$MgSO_4$ $7H_2O$	1233	1233	1233	1233	1233
KH_2PO_4	680	680	680	680	680
$CaCl_2$. $2H_2O$	220	220	220	220	220
Nitrogen sources:					
KNO_3	950	-	500	300	-
NH_4NO_3	825	300	-	-	200
Ammonium jantare	-	800	720	-	-
Glutamine	-	-	-	750	680
$MnSO_4$ $4H_2O$	111.5	13.2	13.2	13.2	13.2
$ZnSO_4$ $7H_2O$	43	2	2	2	2
H_3BO_3	31	3	3	3	3
KI	4.15	0.75	0.75	0.75	0.75
$Na_2MoO_4.2H_2O$	1.25	0.25	0.25	0.25	0.25
$CuSO_4$ $2H_2O$	0.125	0.025	0.025	0.025	0.025
$CoCl_2.6H_2O$	0.125	-	-	-	-
$CoSO_4.6H_2O$	-	0.025	0.025	0.025	0.025
$FeSO_4$. $7H_2O$	27.85	27.85	27.85	27.85	27.85
Na_2EDTA. $2H_2O$	37.25	37.25	37.25	37.25	37.25
Inositol	100	100	100	100	100
Casamino acid	-	150	150	150	150
Nicotinic acid	-	1	1	1	1
Pyridoxine	-	1	1	1	1
Thiamine-HCl	1	1	1	1	1
2,4-D	1	1	1	1	1
NAA	-	0.5	0.5	0.5	0.5
BAP	3	1	1	1	1
MES	-	700	700	700	700
Sucrose	10 000	10 000	10 000	10 000	10 000
Mannitol	127 500	72 800	72 800	72 800	72 800
			pH = 5.6		

MES - morpholinoethanesulfonic acid

Five combinations of growth regulators were chosen (following previous testing) and their effect evaluated on the dividing activity of protoplasts and cell colonies (Table 4). The best results were observed in the presence of 1 mg l^{-1} BAP, 0.5 mg l^{-1} NAA and 1 mg l^{-1} 2,4-D.

The other very important prerequisite for further dividing and growing activity of protoplasts and cell colonies is suitable plating density (Lenee and Chupeau 1986, Moyne *et al.* 1988). The most effective initial plating density was 5×10^4 protoplasts per cm^3 (Fig. 1). The protoplast culture was diluted on the 3th day (to a plating density 2 - 3 $\times$ 10^4 dividing cells per cm^3) and on the 7th day (to a plating density 1 - 1.5 $\times$ 10^4 microcolonies per cm^3) with appropriate volumes of optimal medium NT_{mod} (Fig. 2*D*).

Table 4. Composition of growth regulators and percentage of dividing cell colonies.

Var.	Concentration [mg l^{-1}]			Dividing cell colonies	
				A	B
	BAP	NAA	2,4-D	at 8th day [%]	in microdrops at 18th day [%]
1	1	3	-	20	80
2	1	2	-	5	65
3	1	1	-	5	41
4	1	0.5	1	60	95
5	0.1	0.1	0.05	10	24

For further development and growth of cell colonies two approaches were used at the stage of 14-d-old culture. Some of the cultures had been solidified by the agarose (0.3 %) bead culture method (Shillito *et al.* 1983). The plating density was 1 - 10 × 10^3 colonies per cm^3. The following three combinations of growth regulators were used:

1. 1.0 mg l^{-1} BAP + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} 2,4-D
2. 1.0 mg l^{-1} BAP + 3.0 mg l^{-1} NAA
3. 0.1 mg l^{-1} BAP + 0.1 mg l^{-1} NAA + 0.05 mg l^{-1} 2,4-D

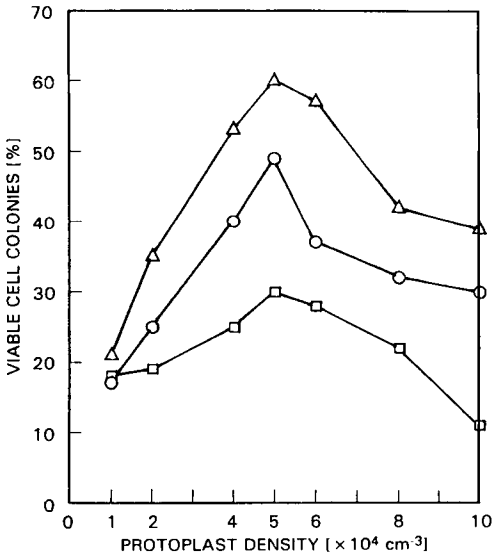

Fig. 1. Percentage of viable colonies from various starting protoplast densities in media NT_{mod} (*triangles*), L (*circles*, Lenee and Chupeau 1986) and K8P (*squares*, Kao and Michayluk 1975)

The number of growing microcalli was very low on each occasion (4 - 6 microcalli per cm^3) and it was independent of initial colony density or content of growth regulators.

The method of individual culture of cell colonies was used with more effective results. The microcalli were isolated by micropipette (Gleba 1978) and individually cultured in microdrops (volume 15 μl). When media containing cytokinins only were used (BAP, KIN or ZEA in concentrations from 0.05 to 5 mg l^{-1}) cell colonies assumed a compact embryo-like structure (Fig. 2*F*).

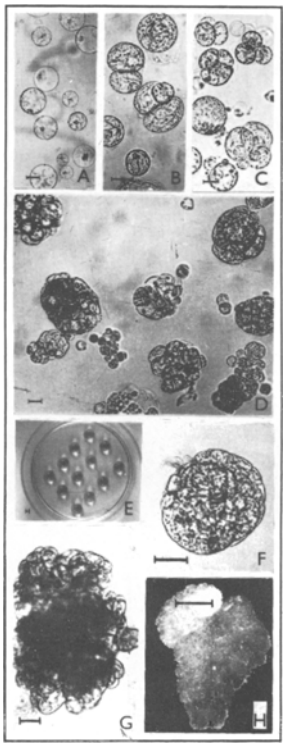

Fig. 2. Differentiation of sunflower hypocotyl protoplasts.
A. Freshly isolated and diluted hypocotyl protoplasts with initial plating density of 5×10^4 protoplast per cm^3. Bar: 50 μm.
B. The first division of protoplasts after 48 h of culture in the media with mannitol. Bar: 50 μm.
C. Development of cell microcolonies at 4^{th} day of culture. Bar: 50 μm.
D. Formation of cell colonies in the optimal nutrient media at 9^{th} day of culture. Bar: 50 μm.
E. Individual culture of colonies in microdrops. Bar: 50 μm.
F. Compact embryo-like structure in the microdrop containing medium with zeatin (0.25 mg l^{-1}) at 15^{th} day of culture. Bar: 100 μm.
G. Formation of microcalli in media with cytokinins and auxins at 20^{th} day of culture. Bar: 100 μm.
H. Friable callus growing on the solidified agar medium without growth regulators. Bar: 1 cm.

Development and growth of such embryoids was best in the medium with zeatin (0.25 or 0.5 mg l^{-1}). Long cultivation in these media led to a decrease in cell division activity, but cell colonies were able to live for a long time. Embryoids failed to develop into mature embryos and their growth became slower. If cell colonies or globular embryoids were transferred to microdrops with a medium containing auxins and cytokinins (Table 4*B*) microcalli were formed (Fig. 2*G*). We were able to reach a greater than 77 % total plating efficiency using subcultivation in microdrops.

After 25 d of cultivation microcalli were transferred to the solidified media with 7 g l^{-1} agar, where they could grow as a hormone-independent tissue. Calli were white and friable (Fig. 2*H*), and after transfer to the media containing cytokinins they became more compact and turned green.

Dupuis *et al.* (1988) reported the morphogenic influence of 2,4-D in the formation of embryo-like structures. Other authors observed the formation of embryoids on media which were supplemented only by BAP and NAA (Moyne *et al.* 1988). However, we have demonstrated thr creation of compact embryoid-like structures in the culture media in the presence of cytokinins only.

We showed that using agarose or the alginate bead culture method (Lenee and Chupeau 1986, Schmitz and Schnabl 1989) is not absolutely necessary. We can obtain similar results by the method of individual cultivation in microdrops. The most important thing is to avoid the lysis effect from the surrounding crumbling colonies. A significant increase of the yield of colonies can be achieved by optimizing the procedures of production and culture of protoplasts (Chanabe *et al.* 1986).

We expect that culture method of individual sunflower protoplast-derived colonies in microdrops will help evaluate the regeneration potential of various genotypes at a protoplast level. It is very important for further fusion experiments with wild species from the genus *Helianthus*. Further experiments in evaluating the regeneration potential at different levels of regeneration from protoplast cultures and calli as well as transformation experiments are in progress.

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