CALLUS FORMATION AND PLANT REGENERATION FROM PROTOPLASTS OF SUNFLOWER CALLI AND HYPOCOTYLS

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ABSTRACT

Sunflower (cv. Girapac SH222) protoplasts were obtained from 4-7 day-old hypocotyls and cotyledons and from two-month old calli. Higher yields of protoplasts were achieved with medium E1 ($\text{KCl } 25 \text{ g} \cdot \text{dm}^{-3}$, $\text{CaCl}_2 \text{ } 2 \text{ g} \cdot \text{dm}^{-3}$, MES $0.7 \text{ g} \cdot \text{dm}^{-3}$, pH 5.5) and the combination of Driselase Fluka 0.2%, Macerozyme Onozuka 0.2% and Cellulase Onozuka R10 0.2%. Hypocotyls gave the highest yields of protoplasts, followed by cotyledons and calli. Protoplasts were cultivated in liquid and on solid media using both L4M (Burrus et al., 1991) and V-KM (Bokelman and Roest, 1983) media. Culture on solid M1 medium (L4M medium supplemented with NAA $3.0 \text{ mg} \cdot \text{dm}^{-3}$, 2,4-D $0.1 \text{ mg} \cdot \text{dm}^{-3}$ and BA $1.0 \text{ mg} \cdot \text{dm}^{-3}$) gave a good planting efficiency with the development of many white-green colonies. These colonies gave rise to small calli which were transferred to MSmod medium (MS medium supplemented with $\text{KCl } 5 \text{ g} \cdot \text{dm}^{-3}$, and polyvinylpyrrolidone (PVP, $4 \text{ g} \cdot \text{dm}^{-3}$) containing benziladenine (BA, $0.5 \text{ mg} \cdot \text{dm}^{-3}$), naphthaleneacetic acid (NAA, $0.5 \text{ mg} \cdot \text{dm}^{-3}$) and gibberelic acid (GA_3 $0.1 \text{ mg} \cdot \text{dm}^{-3}$). After two weeks, calli were transferred to MSmod medium containing BA $1.0 \text{ mg} \cdot \text{dm}^{-3}$, NAA $0.1 \text{ mg} \cdot \text{dm}^{-3}$, and GA_3 $0.1 \text{ mg} \cdot \text{dm}^{-3}$ for shoot formation. Shoots were excised and induced to root in MSmod supplemented with BA $0.1 \text{ mg} \cdot \text{dm}^{-3}$, NAA $1.0 \text{ mg} \cdot \text{dm}^{-3}$, and GA_3 $0.1 \text{ mg} \cdot \text{dm}^{-3}$. Plantlets were then transferred to sterilised vermiculite for greenhouse acclimation.

KEY WORDS: *Helianthus annuus*, protoplast, plant regeneration.

INTRODUCTION

Genetic improvement of sunflower has been a constant necessity due to the importance of this crop in oil and food industry. Protoplasts are essential for breeding techniques involving somatic hybridisation and direct transformation involving either traditional genotypes and new cell lines obtained by somaclonal variation. Sunflower protoplasts have been isolated previously from different organs such as hypocotyls (Chanabe et al., 1991; Burrus et al., 1991), leaves (Guilley and Hahne, 1989; Samaj et al., 1994) and cotyledons (Bohrova et al., 1986; Samaj et al., 1994) or stem (Samaj et al., 1994). However, successful isolation from callus has not been reported yet in this species. Nevertheless, callus can be used for selection of tolerant cells to physical stresses (Santos and Caldeira, 1995, 1997), and these variant cells can be used, later, as a source for hybridisation or transformation.

Regeneration of plants from sunflower protoplasts is a very difficult task. Chanabe et al. (1991) succeeded in regeneration of plants from *H. petiolaris* protoplasts and, later, Burrus et al. (1991) reported the regeneration of fertile plants from cv. Pioneer PT024.

From literature, two assumptions may be made for regeneration of plants from protoplasts in sunflower: 1) solid me-

dium is better for cell division (Burrus et al., 1991; Chanabe et al., 1991; Alibert, Chanabe and Burrus, 1994), and 2) protoplast yield and division and plant regeneration is extremely dependent on genotypes (Chanabe et al., 1991; Alibert et al., 1994). Even in the same genotype and with the same laboratory conditions it may be observed great variability of division frequencies between experiments (Guilley and Hahne, 1989).

We describe here a simple and efficient method for routine callus formation from calli protoplasts and for plant regeneration from hypocotyl protoplasts of a sunflower commercial cultivar Girapac SH222.

MATERIALS AND METHODS

Plant material

Seeds of *Helianthus annuus* cv. Girapac SH 222 were obtained from SCLEPAL (Portugal). Seeds were soaked for 8–12 hours and surface sterilised in 20% commercial bleach for 30 min., followed by six changes of sterile water. Seeds were germinated on MS1/2 medium (Murashige and Skoog, 1962) lacking phytohormones and with 1% sucrose, 0.7% w/v Phytigel and pH 5.7. Cultures were kept in a 16 hour day light cycle at $73 \mu\text{Em}^{-2}\text{s}^{-1}$.

Initiation and maintenance of callus culture

In vitro cultures were initiated from young cotyledons and maintained in MSmod medium (MS medium supplemented with KNO_3 5 g · dm⁻³, PVP 4%, BA 0.5 mg · dm⁻³, NAA 0.5 mg · dm⁻³, and GA₃ 0.1 mg · dm⁻³). Cultures were sub-cultured at 3 week intervals and kept in a light/dark cycle (16/8h, 53 $\mu\text{Em}^{-2}\text{s}^{-1}$) at 23 ± 1°C.

Protoplast isolation

Two month-old calli, cotyledons and hypocotyls fragments from 3-7 day-old plantlets were incubated overnight at 26°C, using four different digestion media (E1, E2, E3 and E4) according to the following table (Table 1):

TABLE 1. Composition of the different enzymatic solutions used in the isolation of protoplasts from cotyledons, hypocotyls and calli of *Helianthus annuus* cv. Girapac SH 222.

Media	E1	E2	E3	E4
Cellulase R10 (%)	0.2	0.2	0.5	0.50
Macerozyme R10 (%)	0.2	0.2	0.02	0.02
Driselase (%)	0.2	0.2	0.02	0.02
KCl (g · dm ⁻³)	25.0	—	25.0	—
CaCl ₂ (g · dm ⁻³)	2.0	—	2.0	—
MES (g · dm ⁻³)	0.7	—	0.7	—
Manitol (M)	—	0.6	—	0.6
pH	5.5	5.5	5.5	5.5
mOsmKg ⁻¹				
hypocot and cotyledon	500±10	500±10	500±10	500±10
calli	620±10	620±10	620±10	620±10

In all media osmolality was adjusted to 620 mOsm · Kg⁻¹ for calli and 500 mOsm · Kg⁻¹ for hypocotyls and cotyledons (Table 1). The digested tissue was gently shaken to release the protoplasts and filtered through a nylon sieve (100 μm pore size). The filtrate was centrifuged at 75xg. Cell debris were removed by centrifuging protoplasts in salt or manitol solution containing 12% Ficoll for 15 min. at 250xg (Burrus et al., 1991). Floated protoplasts were washed by two other centrifugations in S medium (Burrus et al. 1991) and diluted to a final density of 10×10^4 protoplasts per cm³ in L4M medium (Burrus et al., 1991) or V-KM medium (Bokelman and Roest, 1983).

Culture in liquid medium

Protoplasts were diluted to different densities (2.5×10^4 and 5×10^4 protoplasts per cm³) in Petri dishes (60 × 15 mm) containing 3 cm³ of liquid medium. Four different media were tested: **M1** (L4M medium supplemented with NAA 3.0 mg · dm⁻³, 2,4-D 0.1 mg · dm⁻³ and BA 1.0 mg · dm⁻³); **M2** (L4M medium supplemented with NAA 0.1 mg · dm⁻³, and BA 2.2 mg · dm⁻³); **M3** (V-KM medium supplemented with NAA 3.0 mg · dm⁻³, 2,4-D 0.1 mg · dm⁻³ and BA 1.0 mg · dm⁻³); **M4** (V-KM medium supplemented with NAA 0.1 mg · dm⁻³ and BA 2.2 mg · dm⁻³). Protoplasts were incubated at 23°C in the dark. After seven days of culture, cultures were diluted with the sa-

me media but with reduced osmolality (350 mOsm · Kg⁻¹) and reduced NAA concentration (0.1mg · dm⁻³).

Culture on solid medium

Protoplasts were mixed, to final densities of 5×10^4 protoplasts per cm³ and 2.5×10^4 protoplasts per cm³, with 0.6% (w/v) low gelling agarose (Sigma) melted either in L4M or V-KM media. Twenty droplets of 0.25 cm³ were dispensed in Petri dishes (100 × 15 mm), allowed to solidify and surrounded by, respectively, L4M (Burrus et al., 1991) or V-KM liquid media (5 cm³/dish). Protoplasts were incubated in dark for 2 days at 23°C. After seven days of culture, protoplasts were transferred to light. Every 8 days, 2.5 cm³ of old surrounding medium was replaced by 2.5 cm³ of new medium with reduced osmolality (350 mOsm · Kg⁻¹) and reduced NAA (0.1 mg · dm⁻³). Microcalli regenerated from protoplasts were transferred to MSmod medium containing BA 0.5 mg · dm⁻³, NAA 0.5 mg · dm⁻³, and GA₃ 0.1 mg · dm⁻³, for growth. After two weeks, calli were transferred to MSmod medium containing BA 0.5 mg · dm⁻³, NAA 0.5 mg · dm⁻³, and GA₃ 0.1 mg · dm⁻³ for shoot formation. Shoots were excised and induced to root in MSmod supplemented with BA 0.5 mg · dm⁻³, NAA 0.5 mg · dm⁻³, and GA₃ 0.1 mg · dm⁻³. Rooted plants were potted in vermiculite and adapted to greenhouse.

RESULTS AND DISCUSSION

Isolation of protoplasts

We evaluated the capacity of different organs (hypocotyls and cotyledons) and callus for their maximum yield of protoplast, using two different enzymatic combinations (Cellulase Onozuka R10, Macerozyme Onozuka R10 and Driselase Fluka) and osmotica (KCl and CaCl₂ or manitol) (Table 2). The di-

TABLE 2. Protoplast yields from hypocotyls, cotyledons and calli of *Helianthus annuus* cv. Girapac SH222 after 12 hours of digestion in different enzymatic solutions (values are expressed in $\times 10^5$ protoplasts per gram of fresh weight).

Tissue	Yield of prot ($\times 10^5$ /gfw)			
	E1	E2	E3	E4
cotyledons	6.6	1.6	1.2	0.7
hypocotyls	9.8	4.8	1.8	1.3
callus	3.2	0.9	0.9	0.4

gestion medium E1 (Cellulase R10 0.2%, Macerozyme R10 0.2%, Driselase 0.2%, KCl 25 g · dm⁻³, CaCl₂ 2 g · dm⁻³ and MES 0.7 g · dm⁻³) gave the best yields in all tissues. Some authors have already observed that adjusting osmolality of the enzyme mixture with mineral salts instead of manitol markedly improved protoplasts yield in sunflower (Chanabe, Burrus and Alibert, 1989; Burrus et al., 1991). Comparison of different enzymatic combinations showed that the mixtures used is this report and by other authors (Lenée and Chupeau, 1986; Bohorova, Cocking and Power, 1989) were more efficient than other enzymes (Cellulase Sigma, Pectolyase Sigma, or

TABLE 3. Comparative rates of cell division (expressed in %) and colony formation (expressed in %) from protoplasts isolated from hypocotyls, cotyledons and calli of *Helianthus annuus* cv. Girapac SH222 in different media. (SM: solid medium; LM: liquid medium).

Tissue origin	Number (%) of protoplasts that suffered at least one division					Number (%) of colonies with more than 8 cells				
		M1	M2	M3	M4		M1	M2	M3	M4
Hypocotyl	SM:	39	23	17	0.08	SM:	22	9	2	0.5
	LM:	41	40	2	0.1	LM:	10	13	0.5	< 0.01
cotyledon	SM:	55	9	4	< 0.01	SM:	7	2	0.06	< 0.01
	LM:	43	0.1	< 0.01	0.05	LM:	15	< 0.01	< 0.01	< 0.01
callus	SM:	13	0.5	2	0.01	SM:	5	0.03	0.08	< 0.01
	LM	5	< 0.0	< 0.01	< 0.01	LM:	0.6	< 0.01	< 0.01	< 0.01

only Macerozyme and Cellulase Onozuka) used previously by Santos and Caldeira (1993). Hypocotyls were the best source of protoplasts followed by cotyledons and calli (Fig. 1.1). Protoplasts from seedling material have been reported to give higher and more stable plating efficiencies than leaves (Lenée and Chupeau, 1986). Also, protoplasts from hypocotyls gave the best rates of cell division and colony formation (Figs 1.2, 1.4 and 1.5), while only few colonies were observed in cotyledon and calli protoplasts (Table 3). Protoplasts from calli frequently enlarged and developed evaginated structures (Fig. 1.3). Some authors have also described cell divisions only in hypocotyl derived protoplasts (Dupuis et al., 1990; Lenée and Chupeau, 1986; Moyene et al., 1988). On the other hand, Chanabe et al., (1989) reported the division at low frequency of cotyledon protoplasts, and Fisher and Hahne (1992) described colony formation from cotyledon protoplasts. Using the cultivar Girapac SH222, and using the method described in this report, we have achieved colony formation in all types of protoplasts. To our knowledge this is the first report of successful cell division and microcalli formation from sunflower calli protoplasts. Although with low rates of cell division, calli must be regarded as important sources of protoplasts, with particular interest when dealing with calli stably adapted to physical stresses such as salt and osmotic stress (Santos and Caldeira, 1995).

Good rates of cell division and colony formation were only obtained with the density of 5×10^4 protoplasts per cm^3 . Sustained growth and microcalli formation was only achieved in M1 medium (L4M medium supplemented with NAA $3.0 \text{ mg} \cdot \text{dm}^{-3}$, 2,4-D $0.1 \text{ mg} \cdot \text{dm}^{-3}$ and BA $1.0 \text{ mg} \cdot \text{dm}^{-3}$) (Fig. 1.4). Most of the cells growing in M3 and M4 media (both media have the composition of V-KM medium) became brown and died after some weeks. In M3 and M4 media only hypocotyl derived protoplasts regenerated into colonies, although without reproducibility.

Embedding protoplasts in agarose increased colony formation by more than 50% fold (M1) compared with protoplasts cultured in liquid medium. Colonies formed in both solid and liquid systems were unorganised and friable. Colony formation in solidified medium was slower but more colonies originated calli. Burrus et al. (1991) reported the lack of callus formation in liquid medium but in *Helianthus annuus* cv. Girapac SH222 we found that it is possible to regenerate calli in both culture systems. Cell division and calli formation seem highly dependent on the presence of 2,4-D in combination

with NAA and BA. Similar needs for 2,4-D, NAA and BA have already been reported for other sunflower genotypes (Burrus et al., 1991; Samaj et al., 1994).

By 5-7 weeks colonies were transferred to MSmod medium (MS medium containing KCl $5 \text{ g} \cdot \text{dm}^{-3}$, PVP $4 \text{ g} \cdot \text{dm}^{-3}$ and supplemented with BA $0.5 \text{ mg} \cdot \text{dm}^{-3}$, NAA $0.5 \text{ mg} \cdot \text{dm}^{-3}$, and GA₃ $0.1 \text{ mg} \cdot \text{dm}^{-3}$). Colonies from hypocotyls, cotyledons and calli protoplasts originated microcalli. However, the number of microcalli regenerated from cotyledons and calli protoplasts was low and with great variability between experiments. Microcalli derived from hypocotyl and callus protoplasts became green and sustained growth on MSmod. However, calli derived from cotyledon protoplasts became brown and died 1 to 2 weeks after incubation in MSmod medium. This fact indicates that the origin of protoplasts is crucial not only during the first weeks of protoplast culture as stated by other authors (Lenée and Chupeau, 1986; Guilley and Hahne, 1989) but also in later stages of regeneration. Calli derived from hypocotyl protoplasts were transferred to MSmod medium supplemented with BA $1.0 \text{ mg} \cdot \text{dm}^{-3}$, NAA $0.1 \text{ mg} \cdot \text{dm}^{-3}$, and GA₃ $0.1 \text{ mg} \cdot \text{dm}^{-3}$ (Fig. 1.7). These calli regenerated structures which we identified as shoots (Fig. 1.7) by optical microscopy. In fact these structures did not present the typical individuality and bipolar organisation of embryoids. Although most authors refer the regeneration of sunflower protoplasts by embryogenesis (Dupuis et al., 1988; 1990; Moyene et al., 1988; Chanabe et al., 1991; Kransnyanky and Menczel, 1993), Bohorova, Cocking and Power (1986) described the regeneration of plants from sunflower protoplasts by organogenesis using MS medium supplemented with NAA and BA. These authors state that NAA and BA are important for stimulating callus formation and organogenesis (Bohorova, Cocking and Power (1986). Also, protoplasts from *Helianthus tuberosus* originated calli with roots (Binding et al., 1981).

Up to this moment, no shoot formation was observed on calli originated from callus protoplasts. Root formation was achieved on MSmod medium supplemented with BA $0.1 \text{ mg} \cdot \text{dm}^{-3}$, NAA $1.0 \text{ mg} \cdot \text{dm}^{-3}$, and GA₃ $0.1 \text{ mg} \cdot \text{dm}^{-3}$. Most of the plants regenerated from protoplasts were etiolated, with small leaves (Fig. 1.9) and some of them showed premature flowering and vitrification. Vitrification is a physiological disorder that affects numerous genotypes of *Helianthus* in vitro (Burrus et al., 1991; Chraïbi et al., 1992; Santos and Caldeira, 1997). Also premature flowering is a frequent problem in sunflower (Paterson, 1984; Greco et al., 1984; Lupi et al., 1987;

Fig. 1. Plant regeneration from *Helianthus annuus* cv. Girapac SH222 protoplasts: 1) freshly isolated hypocotyl derived protoplasts; 2) protoplast first division; 3) evagination; 4) protoplast derived second division; 5) colony of cells derived from protoplasts; 6) microcallus derived from protoplasts; 7) calli derived from protoplasts; 8) shoot emerging from callus; 9) rooting of shoot.

Knittel et al., 1990; Santos and Caldeira, 1997). To reduce flowering *in vitro*, Wirtzens et al. (1988) suggested the addition of phenolic glycosides, while Lupi et al. (1987) suggested the use of Nitsch medium supplemented with IBA or the addition of GA₃ to MS medium.

The protocol we report here is easy and efficient for plant regeneration from hypocotyls of sunflower while calli protoplasts divide forming calli. This is the first report of plant regeneration from protoplasts of *Helianthus annuus* cv. Girapac SH222 and of callus formation from callus protoplasts. Studies are undertaken to reach the regeneration of plants from callus protoplasts.

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POWSTAWANIE KALUSA A REGENERACJA ROŚLIN Z PROTOPLASTÓW KALUSA SŁONECZNIKA I HYPOKOTYLI

STRESZCZENIE

Protoplasty słonecznika (cv. Girapac SH222) uzyskiwano z 4-7 dniowych hypokotyli i liścieni oraz z dwumiesięcznych kalusów. Większą wydajność protoplastów uzyskiwano stosując roztwór trawiący EI ($\text{KCl } 1 \text{ g} \cdot \text{dm}^{-3}$, $\text{CaCl}_2 \text{ } 2 \text{ g} \cdot \text{dm}^{-3}$, $\text{MES } 0,7 \text{ g} \cdot \text{dm}^{-3}$, pH 5,5) zawierający 0,2% Driselase (Fluka), 0,2% mace-razę R10 (Ozonuka) i 0,2% celulazę R10 (Ozonuka). Największą ilość protoplastów uzyskiwano z hypokotyli, następnie z liścieni i kalusów. Protoplasty hodowano na pożywkach płynnych i stałych, w obu przypadkach stosując pożywki L4M (Burrus et al. 1991) i V-KM (Bokelman i Roest, 1993).

Kultury na stałej pożywce M1 (pożywka L4M uzupełniona o NAA $3,0 \text{ mg} \cdot \text{dm}^{-3}$, 2,4-D $0,1 \text{ mg} \cdot \text{dm}^{-3}$ i BA $1,0 \text{ mg} \cdot \text{dm}^{-3}$) dały dobrą efektywność wysiewu z rozwojem licznych białozielonych kolonii. Kolonie te zapoczątkowały rozwój drobnych kalusów, które przenoszono do pożywki Msmod (pożywka MS uzupełniona o $\text{KCl } 5 \text{ g} \cdot \text{dm}^{-3}$ oraz poliwinylpirolidynę (PVP, $4 \text{ g} \cdot \text{dm}^{-3}$) zawierającej benzyloadeninę (BA, $0,5 \text{ mg} \cdot \text{dm}^{-3}$), kwas naftalenoctowy (NAA, $0,5 \text{ mg} \cdot \text{dm}^{-3}$) i kwas giberelinowy (GA_3 , $0,1 \text{ mg} \cdot \text{dm}^{-3}$). Po dwóch tygodniach kalusy przenoszono do pożywki Msmod zawierającej $1,0 \text{ mg} \cdot \text{dm}^{-3}$ BA, $1,0 \text{ mg} \cdot \text{dm}^{-3}$ NAA i $1,0 \text{ mg} \cdot \text{dm}^{-3}$ GA_3 w celu tworzenia pędów. Pędy odcinano i indukowano ukorzenianie w pożywce Msmod uzupełnionej o $1,0 \text{ mg} \cdot \text{dm}^{-3}$ BA, $1,0 \text{ mg} \cdot \text{dm}^{-3}$ NAA oraz $1,0 \text{ mg} \cdot \text{dm}^{-3}$ GA_3 . Następnie rośliny przenoszono do szklarni.

SŁOWA KLUCZOWE: *Helianthus annuus*, protoplast, regeneracja roślin.