Plant Cell Reports (1991) 10:161–166

Plant Cell Reports

Regeneration of fertile plants from protoplasts of sunflower (*Helianthus annuus* L.)

Monique Burrus[1,2], **Christel Chanabe**[1], **Gilbert Alibert**[1], **and Dennis Bidney**[2]

[1] Pioneer Hi-Bred International, Inc., Biotechnology Research, 7300 NW 62nd Avenue, Johnston, IA 50131, USA
[2] Laboratoire de Biotechnologie et Amélioration des plantes ENSA, 145 Av. de Muret 31076 Toulouse, France

Received December 11, 1990/Revised version received March 14, 1991 – Communicated by R. B. Horsch

Summary. Sunflower hypocotyl protoplasts (*Helianthus annuus* L.) from 5 PIONEER genotypes (PT024, SMF3, EMIL, HA300*PT024, VK5F) and 1 public line (RHa 274) formed colonies at frequencies of up to 60% when plated in 0.25ml agarose beads in a modified L4 medium (Lenée and Chupeau 1986) containing 3mg/l NAA, 1mg/l BA and 0.1mg/l 2,4-D, and 1000mg/l casamino acids. Protoplast-derived colonies grew slowly into calli. Organogenesis was obtained from callus of PT024 on a MS medium containing NAA and BA at 1mg/l and GA at 0.1mg/l. Freshly excised shoots were induced to root by an IAA treatment. Regenerated plants were transferred to the greenhouse and seed was harvested within 7 months of the initial protoplast isolation.

Abbreviations. BA, 6-benzylaminopurine; NAA, α-naphtaleneacetic acid; GA, gibberellic acid; IAA, indole-3-acetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; MS, Murashige and Skoog mineral elements; B5, Gamborg mineral elements

Introduction

The transformation of isolated plant protoplasts by naked DNA has become a routine procedure. One major limitation in the application of protoplast transformation technology to crop plant improvement effort is the ability to recover fertile plants from transformed protoplasts. While a number of plants have been regenerated from protoplasts including representatives of the genera *Chicorum, Chrysanthemum, Crepis, Dimorphotheca, Lactuca, Petasites, Rudbeckia, Senecio* and *Gaillardia* (Roest and Gilissen 1989), plants have not been regenerated from sunflower (*Helianthus annuus* L.) callus derived from protoplasts.

Reports have been published describing plant regeneration from sunflower callus of hypocotyl explants (Paterson and Everett 1986; Greco et al. 1984), immature embryos (Finer 1987; Power 1987; Freyssinet and Freyssinet 1988), shoot-tips (Paterson 1984; Bohorova et al. 1985; Lupi et al. 1987), cotyledons (Nataraja and Ganapathi 1989; Knittel et al. 1991) and stem parenchyma (Bohorova et al. 1985). However, the literature dealing with regeneration from isolated sunflower protoplasts is more limited. The first report of the shoot regeneration from protoplasts was by Binding et al. (1981). Organization occurred in calli of shoot apex protoplasts on a B5 medium supplemented with 15μM BA. No information was presented concerning the reproducibility of the system or whether it was possible to recover fertile plants. Subsequent attempts to develop a protoplast to plant regeneration system in sunflower were unsuccessful for hypocotyl (Lenée and Chupeau 1986; Lenée and Chupeau 1989; Bohorova et al. 1986; Dupuis et al. 1988; Moyne et al. 1988), mesophyll or cotyledonary protoplasts (Bohorova et al. 1986; Schmitz and Schnabl 1989; Guilley and Hahne 1989). Recently, Chanabé et al. (1991) succeeded inregenerating plants from protoplasts of *H. petiolaris*. In this study, we report protocols for the regeneration of plants from callus of *H. annuus* hypocotyl protoplasts.

Materials and methods

Plant growth, protoplast isolation, culture, and plant regeneration. Six genotypes were used in this study: PT024, a tissue culture selection of USDA germplasm release SFM-3 (cms/H. petiolaris Nuttall//cms HA89 backcross); SMF-3, a Pioneer selection of SFM-3; RHa 274 a public restorer line; VK5F; and 2 Pioneer hybrids (Emil and HA300*PT024). These genotypes were chosen because of the variation in their tissue culture response. PTO24 has a high capacity to regenerate plants from callus of hypocotyl explants while SFM-3 and RHa 274 are less regenerable than PT024 and Emil is regeneration negative (C. Garnaat, personal communication).

Seeds were sterilized in a 20% (v/v) Chlorox solution for 30 minutes and rinsed in sterile water. The seeds were germinated and grown for 8 days in Flow boxes on a MS basal medium containing 0.5% sucrose (Chanabé et al. 1990) and 0.3% Gelrite, pH 5.6, in a light/dark cycle (16-8

Offprint requests to: D. Bidney

hours) at 26°C. Shredded hypocotyls were incubated for 10-12 hours at 25°C in a mixed enzyme solution containing 0.2% Cellulase Onozuka R10, 0.2% Macerozyme Onozuka R10 (both from Yakult Pharmaceutical, Japan) and 0.2% Driselase (Sigma) in S medium (Table 1)(Lenée and Chupeau 1986) without bromocresol purple. The macerated hypocotyls were filtered through a 125µm mesh Nitex sieve and the protoplasts were pelleted by centrifugation (10 min, 100g). Cell debris was removed by cenrtifuging the resuspended pellet in S medium containing 12% Ficoll (400,000 FW) for 15 minutes at 250g. The floated protoplasts were rinsed twice in 50 ml of S medium and resuspended at twice the required final plating density in a modified L4 medium(Table 1) (Lenée and Chupeau 1986) containing 1000 mg/l casamino acids and 0.1mg/l 2-4 D. Protoplasts cultured in liquid medium were diluted in L4M at a final density of 50,000 protoplasts/ml and plated in Nunclon petri dishes (55 x 10mm).

Protoplasts in L4M liquid medium were mixed 1:1 (v/v) with 0.6% (w/v) agarose ('Sea-plaque' FMC, Rockland, USA) melted in L4M medium. The final protoplast density was 50,000 protoplasts/ml and the suspension was dispensed as 0.25ml beads into 100x15mm chilled petri dishes (20 beads/dish). The dishes were incubated for one hour at 4°C. The solidified beads were surrounded with liquid L4M medium (5ml/dish). Dishes were sealed with Nescofilm and maintained in the dark at 25°C. Cultures were transferred 10 days later to the light (30 µmEinstein/m^2/s). Every 8 to 10 days, 2.5ml of the old medium surrounding the beads was removed and replaced by 2.5ml of fresh L4M medium modified by the adjustment of NAA to 0.1mg/l, mannitol to 3% and sucrose to 0.1% (L'4M). Calli (1-2mm diameter) from protoplasts embedded in agarose were transferred to various media in an attempt to induce plant regeneration (Table 2). About 150-200 calli were used in each treatment. Cultures were maintained under the same conditions of light and temperature.

Regenerated shoots were elongated by weekly subculture on R539 medium (Table 1). Rooting was induced in excised shoots by dipping in 1mg/ml IAA in 0.1M KOH followed by continued culture on R539 medium. Well rooted plantlets were potted in a soil mixture and hardened for a week in a mist chamber prior to transfer to the greenhouse.

Results and Discussion

Protoplast culture

Protoplast yields from hypocotyls digested for 10-12 hours ranged from 1.9 to 2.3x10^6/gram fresh weight for the genotype Emil. Extended periods of digestion (over 14 hours) decreased protoplast yields to less than 1x10^6/gram fresh weight (Figure 1). Protoplasts were heterogeneous in size ranging from 10 to 90µm in diameter (Figure 2a). Protoplasts purified by flotation in Ficoll, as suggested by Chanabé et al. (1989), showed a very low level of contamination by undigested cells. Approximately 1 cell per 50,000 protoplasts was observed in the final suspension. Freshly isolated protoplasts were largely devoid of remnant cell wall as suggested by the lack of fluorescent staining when preparations were treated with the cell wall indicator Cellofluor (Nagata and Takebe 1970). Protoplasts divided in all 6 genotypes tested. First divisions occurred within 72 hours when protoplasts were embedded in agarose and within 48 hours when cultured in liquid L4M medium. By day 7, 20-25% of the protoplasts platted in agarose divided while 35-40% divided in liquid medium. Division frequencies as high as 60% (Emil) were observed by day 15 (Table 3).

Table 1. Media composition for protoplast isolation, culture and plant regeneration

Component	S	L4M	L'4M	R501	R502	R539
Elements (mg/l)						
$CaCl_2$-$2H_2O$	2000	440	440	440	440	75.5
KCl	25000	1177	1177	-	-	-
KH_2PO_4	-	68	68	170	170	1250
KNO_3	-	-	-	1900	1900	-
$MgSO_4$-$7H_2O$	-	738	738	370	370	97.5
NH_4NO_3	-	-	-	1650	1650	-
$(NH_4)2PO_4$	-	-	-	-	-	150
$CoCl_2$-$6H_2O$	-	0.024	0.024	0.025	0.025	0.05
$CuSO_4$-$5H_2O$	-	.0025	.0025	.0025	.0025	0.156
Na_2EDTA	-	37.25	37.25	37.25	37.25	10
$FeSO_4$-$7H_2O$	-	27.85	27.85	27.85	27.85	7.5
H_3BO_3	-	6.2	6.2	6.2	6.2	2.5
KI	-	-	-	0.83	0.83	0.5
$MnSO_4$-H_2O	-	0.17	0.17	16.9	16.9	5
Na_2MoO_4-$2H_2O$	-	0.024	0.024	0.025	0.025	0.05
$ZnSO_4$-$7H_20$	-	0.28	0.28	8.6	8.6	0.5
Vitamins (mg/l)						
Biotin	-	0.01	0.01	-	-	-
i-Inositol	-	100	100	100	100	100
Glycine	-	-	-	2	2	2
Nicotinic acid	-	1	1	0.5	0.5	0.5
Ca-panthotenate	-	1	1	-	-	-
Pyridoxine-HCl	-	1	1	0.5	0.5	0.5
Thiamine-HCl	-	1	1	0.4	0.4	0.4
Amino acids (mg/l)						
L-Glutamine	-	1095	1095	-	-	-
Casamino acids	-	1000	1000	500	500	-
Sugars (g/l)						
Sucrose	-	20	0.1	30	30	-
Mannitol	-	80	40	-	-	-
Hormones (mg/l)						
NAA	-	3	0.1	1	0.5	-
2,4-D	-	0.1	0.1	-	-	-
BA	-	1	1	1	0.5	0
GA	-	-	-	0.1	0.1	-
Other						
Gel-Rite (g/l)	-	-	-	-	-	2
Phytoagar (g/l)	-	-	-	8	8	-
MES (mg/l)	700	700	700	-	-	-
pH	5.6	5.7	5.7	5.6	5.6	5.5

Plating densities in the 25,000 to 250,000 protoplasts/ml range would support initial cell division, but would not necessarily sustain growth for colony formation. Liquid or agarose culture conditions were compared at a protoplast density at 50,000/ml. Embedding protoplasts in agarose increased the efficiency of macrocolony production by 20 to 40 fold compared to protoplasts cultured in liquid. Colonies from all genotypes that formed in agarose were either unorganized or dense, bipolar structures while those formed in liquid

medium were loose and unorganized. The proliferation of bipolar structures in agarose varied from over 70% for Emil to 35% for VK5F. Colony formation in liquid medium was slow and microcalli produced were friable and did not exhibit bipolar organization. Bipolar structures in agarose could lead to what appeared to be early heart shaped embryos within 21 to 28 days of protoplast culture (Figure 2b). Previously described embryo maturation media (Chandler et al. 1983; Ying-hong et al. 1988; Table 2) did not support the further development of the heart shaped colonies. Heart shaped embryos maintained in L'4M medium also failed to develop further and gradually lost their organized appearance.

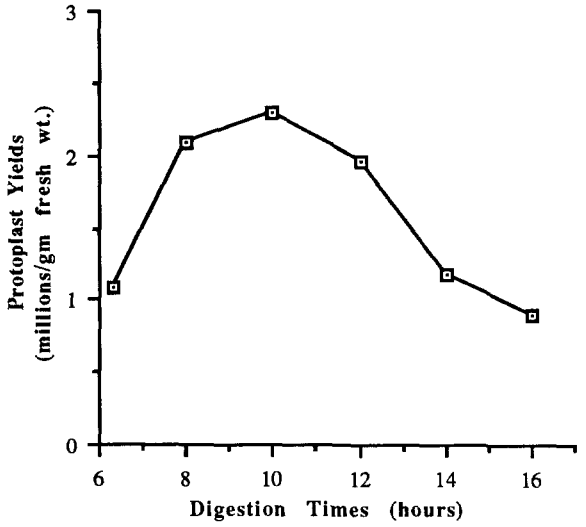

Fig. 1. Time course of protoplast yields from hypocotyls of Emil

Brown et al. (1987) found that culturing lettuce protoplasts in agar prevented the accumulation of growth inhibitor pigments. In our system, however, replacement of the liquid medium surrounding the embedded protoplasts was also required to avoid the accumulation brown pigment and the subsequent death of the colonies. This response is frequent in sunflower protoplast culture (Chanabé et al. 1991) and has also been described in *Cychorium inthybus* (Crepy et al. 1982; Saski et al. 1986). Pigment accumulation has been related to an excess of sugars in the culture medium. Decreasing the sucrose concentration from 2g/l to 0.5g/l, Sasky et al. were able to increase the callus production by 4 fold. We did not observe, however, such an increase in the recovery of calli by reducing sucrose levels to as low as 0.1g/l. Medium changes were sufficient to prevent pigment accumulation at the colony stage.

Protoplasts in agarose usually formed macroscopic colonies at frequencies between 21% to 45% after 4 weeks of culture (Table 3). Protoplast plating efficiencies were enhanced by using 2,4-D at 0.1mg/l in conjunction with NAA and BAP. The addition of casamino acids aided macrocolony formation by 5 fold but had little effect on initial protoplast division. Addition of bovine serum albumin, amino acid mixture (Chandler and Beard 1983) or sugar mixture (sorbitol, cellobiose, fructose, mannose, sucrose, ribose, each at 0.25g/l) or substituting mannitol for sucrose had no discernable effect on early protoplast division or colony formation. Acetyl-salicylic acid (0-300mg/l), known to stimulate divisions in maize protoplasts (Shillito et al. 1989), had an inhibitory effect on the division of the sunflower protoplasts.

Table 2. Media variations tested for **PTO24** colonies

Medium base or Reference	Hormones (mg/l) NAA	BA	GA	Callus Growth	Response
	0.1	0.1	0.1	++	-
	0.5	0.1	0.1	+	roots
	1.0	0.1	0.1	++	roots
	0.1	0.5	0.1	+	-
	0.5	0.5	0.1	+	- a
	1.0	0.5	0.1	+	roots
MS base	0.1	1.0	0.1	++	-
0.5 g/l casamino acids	0.5	1.0	0.1	+	-
3% sucrose	1.0	1.0	0.1	+	shoots[b]
0.8% Phytagar	0.1	0.1		-/+	-
	0.5	0.1		-	roots
	1.0	0.1		-	-
	0.1	0.5		-	-
	0.5	0.5		-	-
	1.0	0.5		-	-
	0.1	1.0		-	-
	0.5	1.0		-	-
	1.0	1.0		-	-
Paterson et al. (1987)	1.0	1.0	0.1	+	+c
	0.1			-	-
	0.5			-	-
Binding et al. (1981)	1.0			+	roots
	2.0			+	roots
	3.4			++	-
MS + 100ml/l cocowater	1.0	1.0	1.0	+++	-
MS+40mg/l AdSO4				+	-
MS+40mg/l AdSO4 +.5mg/l IAA		0.5		+	-
MS+40mg/l AdSO4 +.1mg/l IAA		0.5		++	-
MS+40mg/l AdSO4	0.1	0.5		++	-
MS+40mg/l AdSO4	0.5	0.5		++	-
R539 (Table 1)				+	roots
Chandler et al. (1983)				-/+	-
Ying-hong et al. (1988)				-/+	-

a shoots, cv. Emil on medium R502, Table 1
b medium R501, Table 1
c green spots

Transition from colonies to calli occurred at a very low frequency. In experiments with PT024, only 0.3% of the protoplasts grew into calli that could be transferred to regeneration media (Table 3). Most of the colonies ceased dividing and turned brown once they reached the 0.2-0.4mm size . The surviving calli were of 3 types: nodulated, green and dense; a friable, whitish callus; or a yellowish callus producing purple to deep red pigments.

Shoot regeneration

A range of media (Table 2) were compared for the ability to support plant regeneration from protoplast-derived calli (Figure 2c). One medium, R501 (Table 1) modified from Paterson and Everett (1985), supported shoot development from protoplast derived colonies of PTO24 and leafy, abnormal structures from the hybrid HA300*PT024 (Table 3). Calli typically assumed a brownish appearance on R501. Small green compact areas would appear around the brown callus after 4 weeks of light incubation. Some of these compact areas developed into meristematic nodules that differentiated into leafy structures or shoots (Figure 2d). Differentiation was not observed in calli incubated in the dark. Another medium, R502 (Table 1), induced the formation of shoots from colonies of Emil in a single experiment. R502 was ineffectual for shoot formation in PTO24 callus. The NAA/BAP ratio (wt./vol.) of R501 and R502 media, was 1 and each medium contained GA. No regeneration was observed in absence of GA. Fast growing roots developed from calli incubated on MS media with a ratio NAA/BA>1. Calli cultured on B5 based media were unable to regenerate shoots.

Table 3. Cultural response of hypocotyl protoplasts and resulting colonies and callus

Genotype	Division[a]	Colonies[b]	Calli[c]	Shoots[d]	Roots[e]
Emil	60%	45%	0.08%	0	-
PTO24	34%	25%	0.05%	1.8%	14.5%
HA300*PTO24	54%	33%	0.08%	3.5%[f]	0
SMF-3	45%	40%	0.06%	0	-
RHa274	41%	28%	<0.01%	0	-
VK5F	45%	21%	0	-	-

a based on starting number of protoplasts in agaraose/L4M starting conditions, day 15
b based on starting protoplasts in agarose/L4M, day 30
c following transfer to R501, day 60
d based on number of calli transferred to R501
e based on number of regenerated shoots treated
f vitreous, leafy shoots

Regenerating calli continued to producing new shoots (up to 10/callus in some cases) when the calli were transferred to a no hormone containing medium R539 (Table 1). Well-developed shoots of PT024 (Figure 2e) were rooted by dipping the freshly cut stem in IAA (1mg/ml). An additional IAA treatment was required for 65% of the shoots that formed roots. To date, 215 normal and abnormal shoots have been obtained from PTO24 calli with 31 forming one or more roots. Fourteen plants with well developed root systems were transferred to the greenhouse (Figure 2f). The plants were stunted (25 to 30 cm tall) and tended to form a branched growth habit. Ten formed flowers, and pollen was produced on 7 plants. Viable seeds were recovered from 6 plants. The time required for the protoplast to seed progeny cycle was approximately 7 months.

The influence of genotype had an impact on the ability to regenerate plants from protoplasts. Only 1 line out of 6 gave rise to shoots capable of normal development. PT024 produced the best response with 4.4% of the calli producing green spots and 1.8% forming shoots in 10 independent experiments while Emil produced a few shoots in only one experiment. We scored the hybrid HA300*PTO24 as regeneration positive in Table 3, however, the regenerating structures were aberrant and we were unable to recover functional plants. One of the more difficult problems we encountered in handling regenerated shoots was the tendency of the system to produce vitreous plants (water soaked, translucent leaves and stems). This occurred in about 50% of the PTO24 shoots. Vitrification is a physiological disorder that affects numerous plants propagated in vitro. Ziv et al. (1990) have suggested that an increased degree of vitrification can be correlated with lower and disoriented cellulose synthesis and increased synthesis of callose leading to a decrease in cell rigidity, increased water uptake, cell disintegration, non-functioning guard cells and defective cuticle formation. These conditions cause increased transpiration and desiccation of in vitro formed leaves. Zimmermann and Cobb (1989) found that leaves of vitrified plants had significantly lower levels of inositol (that is thought to be involved in osmoregulation) but higher levels of reducing sugars than normal plants. Vitrification may be limited by controlling carbohydrate concentrations and sources (Rugini 1986; Orlikowska 1987), by increasing agar (Debergh 1983; Ziv et al. 1983) or Gelrite concentrations (Zimmermann and Cobb 1989), combining agar with Gelrite (Pasqualetto et al. 1986) or by controlling gas exchange (Dillen and Buysens 1989). No significant reduction in vitrification occurred in our system by increasing Phytoagar concentration up to 1.2%, switching Phytoagar to Gelrite, or using different types of culture vessels (Magenta boxes, culture tubes, Flow boxes).

Flowering in vitro occurred in 20% of the protoplast-derived shoots. Typically, such plants were unable to produce seed. However, those shoots with good root systems and in the very early stages of flowering in vitro could sustain a transfer to greenhouse conditions and produce seed. Premature flowering of shoots is a frequent problem in sunflower (Paterson 1984; Greco et al. 1984; Lupi et al. 1987; Wirtzens et al. 1988; Knittel et al. 1990). Wirtzens et al. (1988) recommend the inclusion of phenolic glycosides such as phloridzin, naringin or esculinin in the regeneration medium to reduce flowering without suppressing regeneration. Inclusion of phloridzin in the regeneration medium, even at low concentrations (30mM), inhibited sunflower shoot regeneration and could not be tested in this system.

This paper presents the first report of fertile plant regeneration from protoplast cultures of cultivated sunflower. These results should encourage continued efforts to develop a system suitable for the transfer agronomically useful genes into sunflower or to produce somatic hybrids between cultivated and wild sunflower

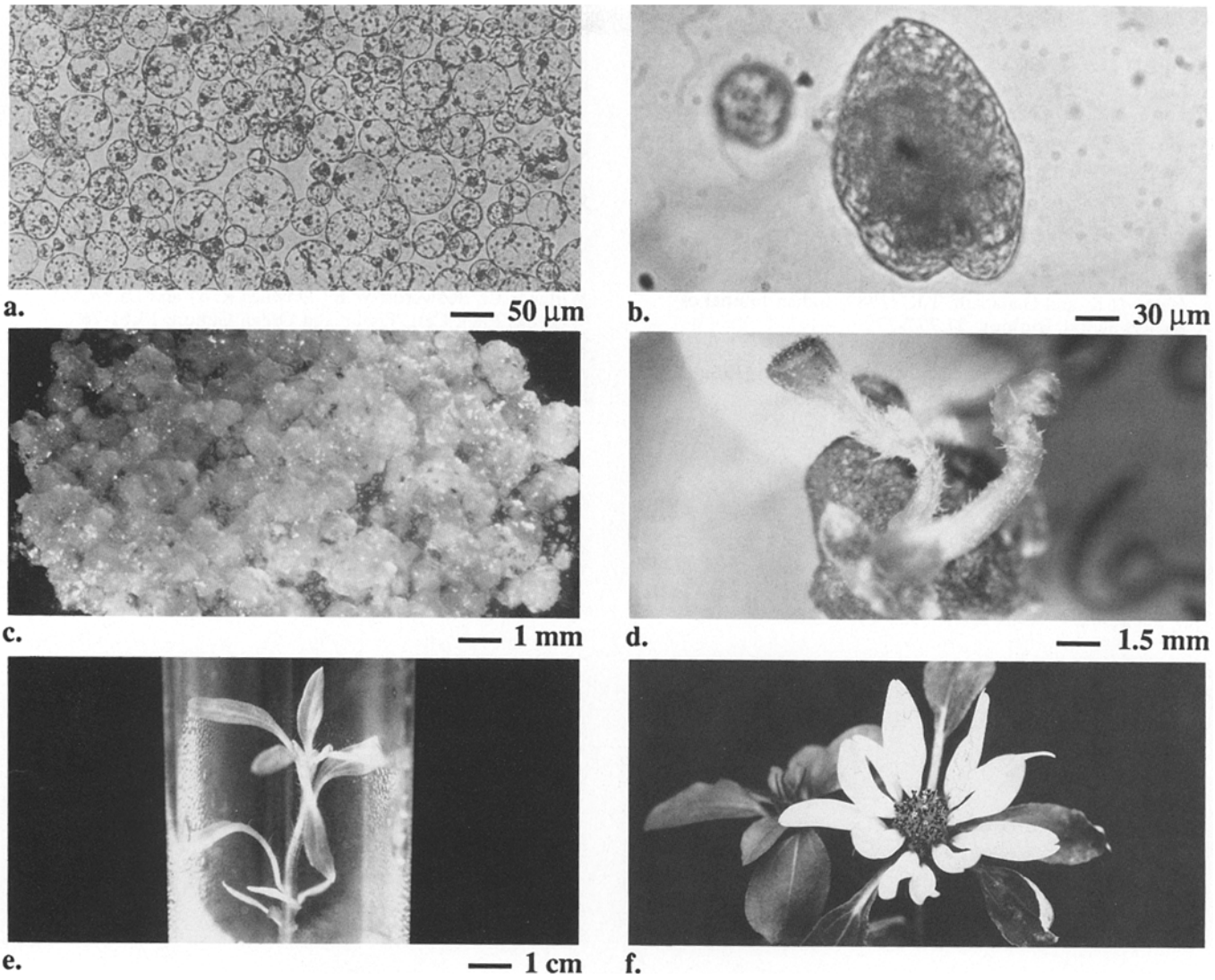

Fig. 2. Plant regeneration from *Helianthus annuus* L. protoplasts, genotype PT024. a. freshly isolated hypocotyl protoplasts; b. protoplast-derived colony (day 28); c. protoplast-derived microcalli; d. shoot emerging from callus e. rooting of protoplast-derived shoot; f. plant in the greenhouse.

species. We are aware, however, that the current system efficiencies of several steps are low enough to make it difficult to use this protocol in lines of research as those mentioned above. Specifically, the system described will need improvement in the areas of colony to callus conversion, shoot regeneration quality and frequency, and rooting frequency. Numerous attempts to vary media composition and incubation conditions to correct these problems resulted in limited success. Additional screening to identify cultivars competent for regeneration within our cultural conditions will be required and may yield the most significant gains in system optimization.

References

Binding H., Nehls R., Kock R., Finger J. and Mordhorst G. (1981) Z. Pflanzenphysiol. 101:119-130.

Bohorova N.E., Atanassof A. and Georgieva-Todorova J. (1985) Z. Planzenphysiol. 95:37-44.

Bohorova N.E., Cocking E.C. and Power J.B. (1986) Plant Cell Reports 5:256-258.

Brown C., Lucas J.A. and Power J.B. (1987) Plant Cell Rep 6, 180-182.

Chanabé C., Burrus M. and Alibert G. (1989) Plant Science 64:125-132.

Chanabé C., Burrus M., Bidney D.and Alibert G.(1991) Plant Cell Reports 11:635-638.

Chanabé C. (1991) PhD thesis ENSA Toulouse France

Chandler J.H. and Beard B.H. (1983) Crop Science 23:1004-1007.

Crepy L., Chupeau M.C. and Chupeau Y. (1982) Z. Pflanzenphysiol., 107, 123-131.

Debergh P.C. (1983) Physiol Plant 59:270-276.

Dillen W. and Buysens S. (1989) Plant Cell Tissue and Organ Culture 19:181-188.

Dupuis J.M., Gomiero M., Plantevin C.and Chagvardieff P. (1988) C. R. Acad. Sci. Paris 307:465-468.

Dupuis J.M., Péan M.and Chagvardieff P. (1990) Plant Cell Tissue and Organ Culture 22: 183-189.

Finer J.J. (1987) Plant Cell Reports 6: 372-374.

Freyssinet M. and Freyssinet G. (1988) Plant Science 56:177-181.

Greco B., Tanzarella O.A., Carrozzo G. and Blanco A. (1984) Plant Science Letters 36:73-77

Guilley E. and Hahne G. (1989) Plant Cell Reports 8:226-229.
Knittel N., Escandon A.S. and Hahne G. (1991) Plant Science 73:219-226.
Lenée P. and Chupeau Y. (1986) Plant Science 43:69-75.
Lenée P. and Chupeau Y. (1989) Plant Science 59:109-117.
Lupi M.C., Bennici A., Locci F. and Gennai D. (1987) Plant Cell Tissue and Organ Culture 11:47-55.
Moyne A.L., Thor V., Pelissier B., Bergounioux C., Freyssinet G. and Gadal P. (1988) Plant Cell Reports 7:437-440.
Nagata T. and Takebe I. (1970) Planta 92:301-308.
Nataraja K. and Ganapathi T.R. (1989) Indian Journal of Experimental Biology 27;777-779.
Orlikowska T (1987) Acta Hort 212:237-244.
Pasqualetto P.L., Zimmerman R.H. and Fordham I (1986) J. Amer Sci Hort Sci 111:976-980.
Paterson K.E. (1984) Amer.J. Bot 71(7): 925-931.
Paterson K.E., Everett N.P. (1985) Plant Science 42:125-132.
Power C. (1987) Amer.J.Bot. 74: 497-503.
Roest S. and Gilissen L.J.W. (1989) Acta Bot. Neerl 38(1):1-23.
Rugini E (1986) Almonds. In: Handbook of Plant Cell Culture, Vol 4 (DA Evans et al.) Macmillan Pub. Co, London, pp 574-611.
Saski N., Dubois M., Millecamps J.L. and Vasseur J. (1986) C.R. Acad. Sc. Paris 302, 165-170.
Schmitz P. and Schnabl H. (1989) J. Plant Physiol. 135:223-227.
Shillito R.D., Carswell G., Johnson C.M., DiMaio J.J., Harms C.T. (1989) Bio/Technology 7:581-587.
Wirtzens B., Scowcroft W.R., Downes R.W. and Larkin P.J . (1988) Plant Cell, Tissue and Organ Culture 13:61-76.
Zimmermann T.W. and Cobb B.G. (1989) Plant Cell Reports 8:358-360.
Ziv M., Meir G. and Haley A.H. (1983) Plant Cell Tissue Organ Culture 2:55-65.