HELIA, **22**, Nr. 30, p.p. 61-70, (1999) UDC 631.524.8:633.854.78

AN OPTIMIZED PROCEDURE FOR SUNFLOWER PROTOPLAST (*Helianthus* ssp.) CULTIVATION IN LIQUID CULTURE

P.C. Binsfeld[1,2], R. Wingender[1], H. Schnabl[1]

[1] *Institut für Landwirtschafliche Botanik der Universität Bonn, Meckenheimer Allee, 176, D-53115 Bonn, Germany*
[2] *Centro de Biotecnologia, Universidade Federal de Pelotas, C.P. 354, CEP 96010-900, Pelotas-RS, Brazil*

Received: July 02, 1998
Accepted: September 28, 1998

SUMMARY

Mesophyll protoplasts isolated from four sunflower species: *H. annuus*, *H. giganteus*, *H. maximiliani*, *H. nuttallii* were subjected to cultivation in liquid media. Various factors were analyzed and their optimization resulted in a high and reproducible percentage of viable and dividing cells in liquid culture. Donor plant pre-cultivation in the dark for two days, usage of 8-12 day-old leaves, selection of undifferentiated protoplasts, a cultivation density of 6×10^4 protoplasts ml^{-1} and cultivation in day/night cycle were found to be essential for a high and synchronous division rate of more than 70% dividing cells. Cell division was obtained for all four species at different rates reflecting their cultivation potential.

Key words: Protoplast growth, *Helianthus*, cell division, cell suspension

INTRODUCTION

Protoplasts are considered as one of the most important vehicles for genetic improvement of plants (Bekkaoui *et al.*, 1987) and provide the start point for many transformation and manipulating techniques. Species of the *Helianthus* genus provide important genetic variability like stable stress tolerance or pathogen resistance thought to contribute to improvement of sunflower production (Korel *et al.*, 1996). On the other hand protoplast culture of these species is known to present major difficulties (Fischer *et al.*, 1992). In the last years many efforts were made to use protoplast biotechnology such as somatic hybridization (Krasnyanski and Menczel, 1995; Henn *et al.*, 1998), *A. tumefaciens* transformation (Knittel *et al.*, 1994; Schonenberg *et al.*, 1994) and direct DNA transfer (Machlab, 1996) in sunflower. A new aspect is represented by the microprotoplast fusion technique enabling the intergeneric transfer of partial genome and alien genes from sexually incompatible

donor species (Ramulu *et al.*, 1995). A prerequisite for this method is the establishment of synchronously dividing cell cultures.

This technique has so far not been applied in sunflower where protoplast cultivation was mostly optimized for plant regeneration (Chanabe *et al.*, 1991; Burrus *et al.*, 1991; Polgar and Krasniansky, 1992; Fischer *et al.*, 1992; Krasnyanski and Menczel, 1993; Wingender *et al.*, 1996). Since organogenic or embryogenic responses were only obtained with agarose droplet or Ca-alginate embedded protoplasts (Fischer *et al.*, 1996) reports on protoplast culture in liquid media are rare and without much success. Bohorova (1986) reported that using three different media and different *Helianthus* species no cell divisions were induced in liquid media. Lenné and Chupeau (1986) succeeded in liquid culture of protoplasts from various sources but only hypocotyl protoplasts were found to divide under the conditions tested. Liquid culture of mesophyll protoplasts was first achieved by Guilley and Hahne (1989) who obtained 70% dividing cells after 13 days in culture.

In this report we present an efficient procedure for inducing cell divisions in a high percentage of cells in liquid culture from different *Helianthus* species. This technique will allow to obtain synchronized cell divisions and thus enable genome manipulations, like transformation, direct gene transfer, induction of micronuclei or metaphase chromosome isolation from the dividing cells.

MATERIAL AND METHODS

Plant material

Plants of the cultivar Florom-328 (*H. annuus*) (Schmitz and Schnabl, 1989) from the Institute of Cereal and Industrial Plant Research, Fundulea, Romania and *in vitro* propagated (Imhoff *et al.*, 1996) plants of three wild sunflower species (*H. giganteus, H. maximiliani, H. nuttallii*) were cultivated for 0, 12, 22, 48 and 72 hours in the dark before protoplast isolation. Leaves (1 g) were placed intact in 20 ml enzyme solution which was prepared as described by Henn *et al.* (1998) for mesophyll protoplasts. The leaves were incubated at 20°C for 14^h and protoplasts liberated by subsequent rotation at 45 rpm at 27°C for 2^h. All steps were performed in the dark. The enzyme-protoplast mixture was filtered through 50 and 40 µm nylon sieves and protoplasts purified as described by Henn *et al.* (1998).

Cultivation

Prior to cultivation the protoplast suspension was enriched with undifferentiated protoplasts by making use of their slow sedimentation. The protoplasts obtained from 3 g material (3 ml in salt buffer) were transferred into 6 ml KMAR liquid medium (Table 1) in a test tube. After 0, 5, 10, 20 and 40 min the floating protoplasts were removed with a pipette and adjusted to densities of 2-15 x 10^4 protoplasts ml^{-1} with medium. Subsequently they were cultivated in Petri dishes

(ϕ=9 cm) in 10 ml liquid KMAR supplemented with different combinations of plant growth regulators (IAA 5.7 µM, NAA 5.4 µM, BAP 4.4 µM and zeatine 4.6 µM) and ascorbic acid (0, 10, 20 and 40 mg l^{-1}) in the dark or day/night cycle (14^h at 30 µmol $m^{-2}s^{-1}$) at 26°C. After 8 days the cells were transferred to fresh medium.

Table 1: Composition of KMAR medium

Inorganic compound		**Sugar**		**Vitamine**			
KNO_3	15.0 mM	Cellobiose	250 mg l^{-1}	Ascorbic acid	2.0 mg l^{-1}	Histidine	0.1 mg l^{-1}
$CaCl_2$ $2H_2O$	10.0 mM	Fructose	250 mg l^{-1}	Pantothenic acid	1.0 mg l^{-1}	Lysine	0.1 mg l^{-1}
$MgSO_4$ $7H_2O$	4.0 mM	Mannose	250 mg l^{-1}	Choline Chloride	1.0 mg l^{-1}	Proline	0.1 mg l^{-1}
NH_4NO_3	4.0 mM	Rhamnose	250 mg l^{-1}	Nicotinamide	1.0 mg l^{-1}	Tyrosine	0.1 mg l^{-1}
$NH_4H_2PO_4$	0.5 mM	Ribose	250 mg l^{-1}	Pyridoxine-HCl	1.0 mg l^{-1}	Tryptophan	0.1 mg l^{-1}
FeNa EDTA	0.1 mM	Sucrose	250 mg l^{-1}	Thiamine	1.0 mg l^{-1}	Cysteine	0.1 mg l^{-1}
$MnCl_2$ $4H_2O$	23.0 µM	Sorbitol	250 mg l^{-1}	Folic acid	0.4 mg l^{-1}	Glycine	0.1 mg l^{-1}
KI	15.0 µM	Xylose	250 mg l^{-1}	Aminobenz. Acid	10 µg l^{-1}	Asparagine	0.1 mg l^{-1}
$ZnSO_4$	5.0 µM	Inositol	100 mg l^{-1}	Vitamine B_{12}	20 µg l^{-1}	Arginine	0.1 mg l^{-1}
Na_2MoO_4 H_2O	0.4 µM	Mannitol	21.0 g l^{-1}	Biotin	10 µg l^{-1}	Leucine	0.1 mg l^{-1}
$CuSO_4$	0.06 µM	Glucose	68.4 g l^{-1}	Vitamine A	10 µg l^{-1}	Serine	0.1 mg l^{-1}
H_3BO_3	0.05 µM			Vitamine D_3	10 µg l^{-1}	Isoleucine	0.1 mg l^{-1}
$CoCl_2$ $6H_2O$	0.04 µM	**Nucleic acid**				Methionine	0.1 mg l^{-1}
		Adenine	0.10 mg l^{-1}	**Amino acid**		Valine	0.1 mg l^{-1}
Organic acid		Uracil	0.03 mg l^{-1}	Glutamine	1.0 mg l^{-1}	Threonine	0.1 mg l^{-1}
Malic acid	40 mg l^{-1}	Xanthine	0.03 mg l^{-1}	Glutamic acid	0.6 mg l^{-1}		
Fumaric acid	40 mg l^{-1}	Guanine	0.03 mg l^{-1}	Cysteine	0.2 mg l^{-1}		
Citric acid	40 mg l^{-1}	Thymine	0.03 mg l^{-1}	Aspartic acid	0.1 mg l^{-1}		
Na- Pyruvate	40 mg l^{-1}	Hypoxanthine	0.03 mg l^{-1}	Phenylalanine	0.1 mg l^{-1}		
Other addition							
Casein hydrolysate		250 mg l^{-1}		MES	600 mg l^{-1}		
Osmolarity		600 mOsmol/kg	pH	5.7			

Determination of cell viability

One volume of cell suspension was mixed with one volume of 50 mg l^{-1} fluorescein diacetate which was prepared fresh by diluting a 4 g l^{-1} stock in growth medium. The percentage of viability was calculated as the number of cells showing green fluorescence under UV illumination, in relation to the total number of cells (100%), counted under white light.

Statistical analysis

All experiments were repeated three times. The analysis of variance was according to completely random design and multiple comparison test of the mean according to Duncan's test at $P<0.05$ (using MS-SANEST software).

RESULTS AND DISCUSSION

Effects of plant growth regulators

The highest yield of 4.5 x 10^6 mesophyll protoplasts per gram of fresh weight was obtained with *H. giganteus* followed by *H. nuttallii* 2.4 x 10^6, *H. annuus* 2 x 10^6 and *H. maximiliani* 1.5 x 10^6. Incubation of the leaves for over 16^h in enzyme solution increased the yield but decreased the viability of the isolated protoplasts. Preliminary tests showed that the best relation auxine/cytokinine was 1.2, independent of the type and the combination of growth regulators which is in agreement with Wingender *et al.* (1996). All tested combinations of NAA, IAA, BAP and zeatine were appropriate for induction of cell division (Table 2).

Table 2: Effect of different combinations of plant growth regulators in the medium on protoplast division (%) after 5 days in culture

	Helianthus				
growth regulator	*annuus*	*giganteus*	*maximiliani*	*nuttallii*	mean
IAA/zeatine	62.3 a	64.3 a	45.4 a	46.7 b	54.6
NAA/BAP	63.3 b	61.1 a	40.8 b	49.8 a	53.7
IAA/ BAP	58.1 b	54.6 c	39.8 b	45.4 b	49.5
NAA/zeatine	56.0 b	57.4 b	37.6 c	41.3 c	48.0
mean	59.9	59.4	40.9	45.7	

Values followed by the letters are significantly different in each column ($P<0.05$).

The overall best results were obtained with IAA/zeatine where also earlier (2 days) onset of mitosis was observed. This combination was therefore chosen for all subsequent experiments. Regardless of the growth regulator supplementation species specific differences of the division rates were obtained reflecting probably genetical control of regeneration competence. Similar responses in sunflower genotypes were reported by Chanabe *et al.* (1991) and Wingender *et al.* (1996). For the rapid recovery from isolation stress and a synchronous cell division it was important to use a rich and complete culture medium. In preliminary tests KMAR was compared with mKM (Binding and Nehls, 1977) and MS (Murashige and Skoog, 1962) medium and found to give the best results.

Effect of protoplast density, ascorbic acid and plant pre-cultivation in the dark

For most plant cells a minimum effective cell density of about 10^4-10^5 cells ml^{-1} (Sakurai and Mori, 1996) is required in order to initiate cell division. We tested densities of 2-15 x 10^4 protoplasts ml^{-1}. Only the lowest density was found to promote cell division rates of about 54% while with the highest density only 29% could be achieved (Figure 1). In addition, many protoplasts became brown and died after four days. In order to reduce the mortality rate of protoplasts we added ascorbic acid to the culture medium and in contrast to the results reported by Xu and Jia (1996) we observed a decreasing viability and division ability of the cells in the pres-

ence of more than 10 mg l^{-1} of ascorbic acid. As shown in Figure 2, plant pre-cultivation in the dark resulted in an increase of cell viability and division rate. The pre-cultivation for 48^h in the dark was the most effective leading to a 7% increase in viability and to 1% higher division rate.

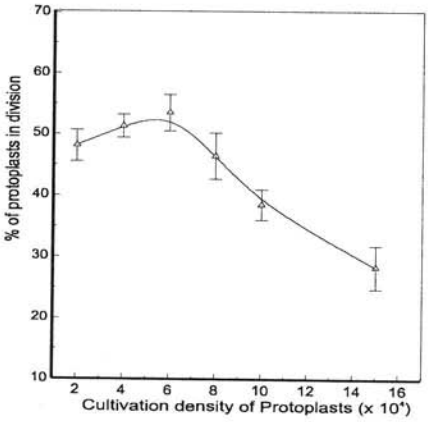

Figure 1: Effect of cell density on division rates of cells derived from mesophyll protoplasts cultivated for 5 days in KMAR liquid medium in the presence of 5.7 µM IAA and 4.6 µM zeatine, mean values for the four Helianthus species.

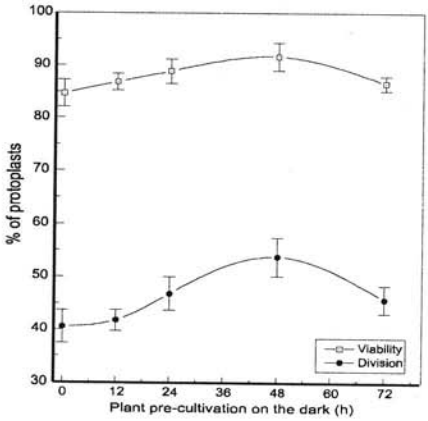

Figure 2: Effect of dark pre-cultivation on the viability and division rates of cells derived from mesophyll protoplasts of H. giganteus cultivated in liquid culture KMAR supplemented with 5.7 µM of IAA and 4.6 µM zeatine, after 5 days in culture.

Selection of the protoplasts with the highest growth potential

Another factor that contributed to a high protoplast division rate in liquid culture was to select small protoplasts. As show in Figure 3, both of the evaluated parameters, cell viability and division ability, increased with a cell selection. We could verify that protoplasts that quickly sedimented had low division ability, in contrast to those that floated (not shown). After 10 min sedimentation, we could select protoplasts with higher division rates (between 14-17%) in relation to control protoplasts. It can be speculated that this effect might be due to the differentiation state of the cells and size of vacuole. Cells with a large vacuole are supposed to sediment quicker than cells with small vacuoles. Xu and Jia (1996) described a poor division ability of cells from *A. sphaerocephala* when the cells had a large vacuole and only those protoplasts with a dense cytoplasm could continuously divide, accordingly Schmitz and Schnabl (1989) obtained protoplast division after removal of the vacuole by centrifugation.

Age of the leaves for protoplast isolation

Protoplasts isolated from 8-12 days-old leaves performed superior to those from older material for the analyzed parameters. After five days in culture the cells

showed more than 90% viability and over 60% of dividing protoplasts. Cells from older leaves showed a drastic inhibition of the viability and division ability (Figure 4).

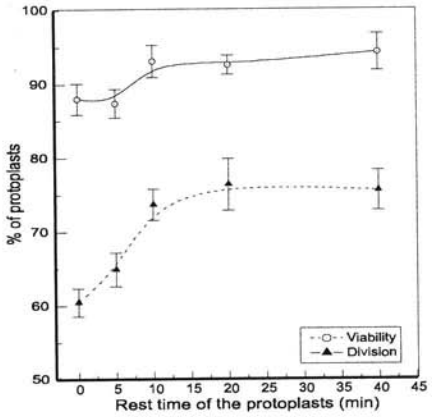

Figure 3: Effect of the sedimentation time on the selection of H. maximiliani protoplasts with higher viability and division ability cultivated in KMAR liquid medium with 5.7 µM IAA and 4.6 µM zeatine, after 5 days in culture.

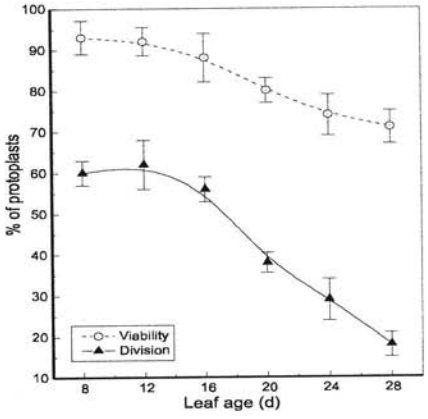

Figure 4: Effect of the leaf age on viability and division ability of H. giganteus cells cultivated at $5x10^4$ protoplasts ml^{-1} in KMAR liquid medium with 5.7 µM IAA and 4.6 µM zeatine.

Leaves older than 20 days were difficult to digest leading to a reduced yield and to a high percentage of protoplasts that quickly sedimented. Young fully developed leaves were easily digested and gave a higher percentage of floating undifferentiated protoplasts, these protoplasts show a satisfactory viability and division ability, accordingly Barth *et al.* (1994) reported that the viability of the protoplasts of *H. annuus*, *H. laetiflorus* and *H. pauciflorus* were age dependent. In young leaves, most of the cells exhibited a round shape and a small vacuolar system. Our observations agree with results obtained by Ochatt and Power (1992) who reported that growth ability of protoplasts is proportional to their dedifferentiation. Older mesophyll protoplasts are more differentiated and had also a large developed vacuole resulting in a low growth ability.

Effect of the light

A high protoplasts division rate was observed in both (light and dark) cultivation regimes (Figure 5). Light regime stimulated the protoplast division during the exponential phase. In this phase the division was approximately 20% higher than in the dark. No negative effects could be observed like protoplast browning or premature death of cells. During the exponential division phase (3 to 6 days), most cells exhibited a round shape and a small size concomitant with a relatively small vacuolar system. The cells became larger during the stationary phase and at this point the medium had to be changed. For the light regime this phase was reached after 7

or 8 days and in the dark after 11 or 12 days. Exchange of the medium after early stationary phase lead to an increase in dead cells.

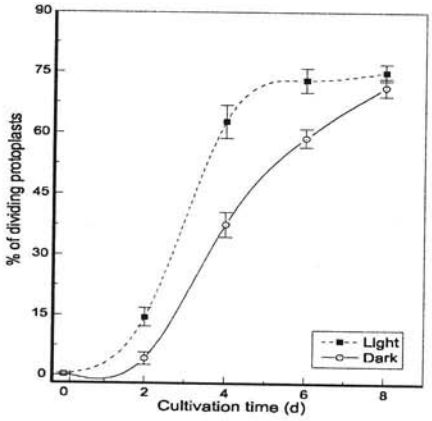

Figure 5: Effect of the cultivation regime on the division ability of H. giganteus cells during 8 days cultivated in KMAR liquid medium with 5.7 µM IAA and 4.6 µM zeatine.

The high division ability of cells in liquid culture obtained with the optimization described here provides the first step towards a rapid and homogeneous growth rate of cells, especially for synchronization of the culture for micronuclei induction or metaphasic chromosome isolation.

CONCLUSION

Five factors appear to be essential for liquid culture of *Helianthus* protoplasts: a rich medium with an adequate growth regulator combination supplying all required elements for growth induction and for the rapid stabilization of the stress caused by the isolation, rather young not fully differentiated donor tissue, selection of floating undifferentiated protoplasts, pre-cultivation of the donor plants in the dark and cultivation in day night cycle.

ACKNOWLEDGMENT

The authors would like to thank the DFG for financial support.

REFERENCES

Barth, S., Voeste, D., Wingender, R. and Schnabl, H., 1994. Selection and enrichment of differentially labeled plant protoplasts. J. Biochem. Biophys., 106: 220-222.

Bekkaoui, F., Saxena, P.K., Attree, S.M., Fowke, L.C. and Dustan, D.I., 1987. The isolation and culture of protoplasts from an embryogenic cell suspension culture of *Picea glauca* (Moench). Voss. Plant Cell Rep., 6: 476-479.

Binding, H. and Nehls, R., 1977. Regeneration of isolated protoplast to plants in *Solanum duleamara*. Z. Pflanzenphysiol., 85: 279-280.

Bohorova, N., Cocking, E.C. and Power, D., 1986. Isolation, culture and callus regeneration of protoplasts of wild and cultivated *Helianthus* species. Plant Cell Rep., 5: 256-258.

Burrus, M., Chanabe, C., Alibert, G. and Bidney, D., 1991. Regeneration of fertile plants from protoplasts of sunflower (*Helianthus annuus* L.). Plant Cell Rep., 10: 161-166.

Chanabe, C., Burrus, M., Bidney, D. and Alibert, G., 1991. Studies on plant regeneration from protoplasts in the genus *Helianthus*. Plant Cell Rep., 9: 635-638.

Fischer, C., Klethi, P. and Hahne, G., 1992. Protoplasts from cotyledon and hypocotyl of sunflower (*Helianthus annuus* L.): shoot regeneration and seed production. Plant Cell Rep., 11: 632-636.

Fischer, C., Larrapa, H., Crarriere, F., Jung, J.L. and Hahne G., 1996. Regeneration of plant from protoplast of *Helianthus annuus* L. (sunflower). In: Bajaj, Y.P.S. (eds.), Biotechnology in Agriculture and Forestry, Vol. 38. Plant Protoplast and Genetic Engineering VII. Heidelberg, Spring-Verlag, pp. 49-63.

Guilley, E. and Hahne, G., 1989. Callus formation from isolated sunflower (*Helianthus annuus*) mesophyll protoplasts. Plant Cell Rep., 8: 226-229.

Henn, H.J., Wingender, R. and Schnabl, H., 1998. Regeneration of fertile plants from *Helianthus nuttallii* T&G and *Helianthus giganteus* L. mesophyll protoplasts. Plant Cell Rep. (in press).

Imhoff, U., Wingender, R., Dresen, B. and Schnabl, H., 1996. Micropropagation of three *Helianthus* wild Perennial species. Angew. Bot., 70: 137-139.

Knittel, N., Gruber, V., Hahne, G. and Lenee, P., 1994. Transformation of sunflower (*Helianthus annuus* L.): a reliable protocol. Plant Cell Rep., 14: 81-86.

Korell, M., Brahm, L., Horn, R. and Friedt, W., 1996. Interspecific and intergeneric hybridization in sunflower breeding. I. General breeding aspects. Plant Breeding Abst., 66: 925-931.

Krasnyanski, S. and Menczel, L., 1993. Somatic embryogenesis and plant regeneration from hypocotyl protoplast of sunflower (*Helianthus annuus* L.). Plant Cell Rep., 12: 260-263.

Krasnyanski, S. and Menczel, L., 1995. Production of fertile somatic hybrid plants of sunflower and *Helianthus giganteus* L. by protoplast fusion. Plant Cell Rep., 11: 7-10.

Lenee, P. and Chapeau, Y., 1986. Isolation and culture of sunflower protoplasts (*Helianthus annuus* L.). Factors influencing the viability of cell colonies derived from protoplasts. Plant Sci., 43: 69-75.

Machlab, H., 1996. Genetic transformation of sunflower (*Helianthus annuus* L.): *Agrobacterium* mediated and direct gene transfer into embryo axes and hypocotyl protoplasts. PhD. Thesis, University of Bonn, Germany, pp. 91.

Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant, 15: 473-497.

Ochatt, S.J. and Power, J.B., 1992. Plant regeneration from cultured protoplasts of higher plants. In: Fowler, M.W., Warren, G.S. and Moo-Young, M. (eds). Plant Biotechnology. Comprehensive Biotechnology, Second supplement. Pergamon Press, Oxford, pp. 99-127.

Pólgar, Z. and Krasnyansky, S., 1992. Plant regeneration from cell suspension and mesophyll protoplasts of *Helianthus maximiliani* (Schrad.). Plant Sci., 87: 191-197.

Ramulu, K.S., Dijkhuis, P., Rutgers, E., Blass, J., Verbeek, W.H.J., Verhoeven, H.A. and Colijn-Hooymans, C.M., 1995. Microprotoplast fusion technique: a new tool for gene transfer between sexually-incongruent plant species. Euphytica, 85: 255-268.

Sakurai, M. and Mori, T., 1996. Stimulation of anthocyanin synthesis by conditioned medium produced by strawberry suspension cultures. J. Plant Physiol., 149: 599-604.

Schmitz, P. and Schnabl, H., 1989. Regeneration and evacuolation of protoplasts from mesophyll, hypocotyl and petioles from *Helianthus annuus* L. J. Plant Physiol., 155: 223-227.

Schonenberg, J.M., Scelonge, C.J., Burrus, M. and Bidney, D.L., 1994. Stable transformation of sunflower using *Agrobacterium* and split embryonic explants. Plant Sci., 103: 199-207.

Wingender, R., Henn, H.J., Barth, S., Voeste, D., Machlab, H. and Schnabl, H., 1996. Regeneration protocol for sunflower (*Helianthus annuus* L.) protoplasts. Plant Cell Rep., 15: 742-745.

Xu, Z.Q. and Jia, J.F., 1996. Callus formation from protoplasts of *Artemisia sphaerocephala* Krasch and some factors influencing protoplast division. Plant Sci., 44: 129-134.

OPTIMIZACION DE CULTIVO DE PROTOPLASTOS DE GIRASOL (*Helianthus* ssp.) EN MEDIO DE CULTIVO LÍQUIDO

RESUMEN

Protoplastos de células del mesófilo fueron aislados de cuatro especies de girasol (*H. annuus, H. giganteus, H. maximiliani , H. nuttallii*). Los cultivos de protoplastos fueron sometidos en medio de cultivo líquido. Varios factores fueron analizados para la optimizacion y obtencion de un alto y reproducible porcentaje de viabilidad y division celular en medio de cultivo liquido. Densidad de cultivo de 6 x 10^4 protoplastos ml^{-1}, seleccion de protoplastos no diferenciados, precultivo de la planta donadora en la oscuridad por dos dias, utilizacion de hojas entre 8-12 dias y ciclos de cultivos dia/noche, fueron determinates para la obtención de una alta y sincronisada tasa de división celular mayor de 70% de division celular. Division celular fue obtenida en las cuatro especies en diferentes tasas, evidenciando el potencial del protocolo de cultivo.

UNE TECHNIQUE OPTIMISÉE DE CULTURE DES PROTOPLASTES AU MILIEU LIQUIDE CHEZ LE TOURNESOL (*Helianthus* ssp.)

RESUMÉ

Des protoplastes du mesophylle de quatre espèces d'*Helianthus* (*H. annuus, H. giganteus, H. maximiliani, H. nuttallii)* ont été isolés et mis en culture en milieu liquide. De nombreux facteurs ont été analysés et un pourcentage élevé et reproductible de cellules vivantes et en division a été obtenu. Pour ce résultat les paramètres suivants étaient importants: culture des plantes pendant deux jours à l`obscurité, isolement des protoplasts à partir des feuilles âgées de 8 à 12 jours, sélection des protoplasts peu differentiés, culture des protoplasts à raison de 6 x 10^4 protoplasts ml^{-1} et à un régime jour/nuit. Un pourcentage de division jusqu'à 70% a été obtenue quoique le nombre de divisions étaient variable parmi les espèces selon leurs aptitude à la culture au milieu liquide.