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INDUCTION OF SUSPENSION CULTURES OF SUNFLOWER PROTOPLASTS

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SUMMARY

Protoplasts were isolated from hypocotyls, petiols and leaves of cultivated and wild *Helianthus* species. Highest number of protoplasts were obtained from petiols of cultivar Euroflor i.e. 9.0 x 10^5 PP g^{-1} fw while lowest numbers were obtained from mesophylls of both cultivated as well as from wild *Helianthus sps.* The isolated protoplasts were cultured as liquid suspensions cultures at densities ranging from 1 x 10^4 to 2 x 10^5 PP ml^{-1} with two concentrations of BAP and NAA ie at 0.5 and 1.0 mg/l. Hypocotyl protoplasts plated at 1 x 10^5 PP ml^{-1} from cultivars Apisol, Cerflor and Euroflor showed good plating efficiencies i.e., above 75% with 1.0 mg/l of BAP and NAA followed by petiol protoplasts which showed 40-47%. In case of mesophyll protoplasts divisions were observed only in cultivar Cerflor but not significantly whereas mesophyll protoplasts derived from the wild species of *Helianthus*, ie *H. grosseserratus* and *H. maximillianii* did not undergo any division even after 10 days and remained recalcitrant.

Key words **: *Helianthus annuus*, mitotic index, plating efficiency protoplasts yield.**

INTRODUCTION

The worldwide production of sunflower (*Helianthus sps*) in the recent years has increased over 70%. Out of the existing 67 species in this genus only a few species have been identified with disease resistance, superior oil quality, elevated protein quantities and source of cytoplasmic male sterility (Georgieva-Todorova 1976, Carter 1978, Thompson *et al.* 1981). Limited success has been achieved in the conventional breeding programmesin this species. Biotechnological programmes involving tissue culture, gene cloning, etc. were used recently in *Helianthus sps.* to introduce genetically improved plants. However, this genus is highly susceptible to diseases caused by *Phomopsis* or *Sclerotinia.* At this juncture modern methods like chromosomal mediated gene transfer and microcell hybridization which helps introducing characters like disease resistance and high yield that are controlled by multiplegenes. For the last few decades protoplasts isolated from various plant parts including cell suspensions have been the primary target for the genetic manipulations. Many efforts were made to use the protoplasts from *Helianthus sps.* (Binding *et al.* 1981, Krasnyanski *et al.* 1992).The regeneration from the protoplasts from genus *Helianthus* is genotype dependent and yet it has also been mentioned that the regeneration may be due to the presence of partial wild species genome that has been acquired during the process of classical breeding. However, Gilbert *et al.* (1994) reported that protoplasts showed regeneration under agarose gel matrix embedded conditions. Modern techniques like microcell hybridization and chromosomal mediated gene transfer may help bringing the desired characters from the wild *Helianthus* species to their corresponding cultivated *Helianthus* species which in turn depends on the regeneration capacities.

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Experiments were conducted in this laboratory in *Helianthus sps.* on the induction of micronuclei. The formation of a micronuclei though reported from meristematic regions, it is found often difficult to induce and isolate in high quantities for microcell mediated gene transfer. Development of suspension cultures will greatly ease not only the formation of micronunclei but also for their isolation in good numbers in most of the species including sunflower. It is a prerequisite to develop suspension cultures with a high frequency of mitotic index (MI). Accordingly a programme was designed for isolation and induction of suspension cultures, optimize conditions for developing liquid cultures in three cultivars of *H. annuus*, and in two wild species viz. *H. gigantiles* and *H. grosseserratus* at the level of growth regulator supplementation as well as initial densities allowing the direct cultivation of protoplasts for initiation of suspension cultures.

MATERIALS AND METHODS

For the present study, three different cultivars of *H. annuus* L cv., Apisol, Cerflor and Euroflor (Seeds from Rustica Semenes, Pflanzenzucht GmbH, Hamburg, Germany) and two wild species cv. *H. grosseserratus* and *H. maximillianii* (from the axenic cultures) were selected. The seeds were surface sterilized for one hour in 4.5% sodium hypochlorite solution, washed three to four times with sterile deionized water and were germinated in dark (hypocotyls) or under light conditions (plantlets) on a hormone free MS medium (murashige and Skoog 1962), 0.8% agar and pH 5.6-5.7. The newly formed shoots from the axillary buds were used for the experiments and further subcultures.

The isolation, purification and counting of protoplasts from various plant parts were carried as per the method of Wingender *et al.* (1996) unless otherwise stated. The protoplasts isolated through the above methods were cultured in modified Kao and Michayluk (1974) with an osmolarity of 600 mOsmol/kg as liquid suspension cultures either on (i) mKM media for cultivars of *H. annuus* or (ii) KMAR media for *H. grosseserratus* and *H. maxmilliani* (Wingender *et al.* 1996) with BAP and NAA each at 0.5mg/l or 1.0 mg/l concentrations. Plating density, protoplast viability, protoplasts regeneration and plating efficiency were estimated as per standard protocols. Subcultures were carried out either by drastic change of media or gradual change of media. Both BAP and NAA concentrations were maintained at the same levels except that the osmolarity of the media has been reduced from 600 to 500 mOsmol/kg in the subsequent subcultures.

RESULTS AND DISCUSSION

Seedlings with 12-14 cm long and 2 mm diameter hypocotyls yielded good quantities of protoplasts. The

Table 1. Yield of protoplasts, viability and Mitotic indices from cultivated and wild *Helianthus sps.*

Species	Plant part	Protoplasts yield**	Viability***		Hrs. of first division		Mitotic index on 5th day***	
			0.05 mg/l*	1.0mg/l*	0.5mg/l*	1.0 mg/l*	0.5 mg/l*	1.0 mg/l*
H. annuus	Hypocotyl	1.4×10^6	70 ±5.52	76 ± 6.16	72	42	23 ± 3.67	53 ± 5.61
Apisol	Petiol	9.0×10^5	60 ± 3.80	70 ± 5.47	96	72	15 ± 1.0	28 ± 4.30
	Mesophyll	1.4×10^6	54 ± 4.89	51 ± 5.24	120	120	-	-
Cerflor	Hypocotyl	1.2×10^6	72 ± 3.16	79 ± 6.74	72	36	28 ± 3.53	47 ± 4.63
	Petiol	8.1×10^5	68 ± 4.52	60 ± 4.47	72	72	30 ± 2.91	23 ± 4.63
	Mesophyll	1.3×10^6	42 ± 3.16	44 ± 4.84	96	96	7 ± 1.58	10 ± 3.16
Euroflor	Hypocotyl	1.5×10^6	76 ± 5.78	81 ± 2.12	36	28	26 ± 4.24	46 ± 3.67
	Petiol	9.4×10^5	61 ± 3.39	66 ± 4.74	72	72	17 ± 1.87	21 ± 4.30
	Mesophyll	1.1×10^6	44 ± 3.16	47 ± 4.30	120	96	-	4 ± 1.22
H. grosseserratus	Mesophyll	1.3×10^6	42 ± 5.87	51 ± 3.80	120	120	-	-
H. maximillianii	Mesophyll	1.2×10^6	41 ± 3.24	53 ± 5.33	120	120	-	-

*BAP and NAA concentrations
**Protoplasts g^{-1} fw
***Results expressed as percentage ±SD

yields of protoplasts from hypocotyls are presented in Table 1. Highest yield of protoplasts was found in cultivar Euroflor in all the extractions which ranged from 1.7×10^6 to 2.0×10^6 PP g^{-1} fw followed by Apisol from 1.5×10^6 to 1.8×10^6 PP g^{-1} fw. Lowest yield of protoplasts was found in Cerflor variety from five different extractions where it ranged from 1.0×10^6 to 1.3×10^6 PPg^{-1} fw. Considerable variations were observed in the size of the protoplasts which ranged from 30 μm to 90 μm in diameter.

Seedlings grown under the light conditions attained a 4-leaf stage in 21 to 25 days of growth. Petioles ranging from 1.0 to 1.4 cm long but mostly from the first pair of leaves were used in the present study. Care was taken to avoid the leaf lamina while segmenting the petiols. The highest yield was found in Euroflor variety with an average of 9.4×10^5 PPg^{-1} fw from five isolations followed by Apisol petiols where the yield was 9×10^5 PPg^{-1}fw. In cultivar Cerflor the yield was low with 8.1×10^5 PPg^{-1} fw. The size of the protoplasts varied from 20-30 μm. The yield of protoplasts varied considerably between isolations from the same cultivars even after maintaining the same conditions. The highest yield of mesophyll protoplasts was from the cultivar Apisol with 1.4×10^6 PPg^{-1} fw followed by Cerflor and Euroflor cultivars where the yields were 1.3×10^6 and 1.1×10^6 PPg^{-1} fw. In both *H. grosseserratus* and *H. maximillianii* six successive mesophyll protoplasts isolations were carried out. Young and newly emerging leaves yielded good quantities of viable protoplasts when compared to older leaves where the yield is very low. The size of the protoplasts varied from 30 to 35 μm in diameter. The yields ranged from 1.0×10^6 to 1.0×10^6 PPg^{-1} fw in *H. grosseserratus* and 1.5×10^6 to 2.0×10^6 PPg^{-1} fw in *H. maximillianii*.

The mesophyll protoplasts isolated from the cultivated species of *H. annuus* cv. Apisol, Cerflor and Euroflor yielded higher quantities of viable protoplasts compared to protoplasts isolated from the mesophylls of *H. grosseserratus* and *H. maximillianii* grown under similar conditions. In a few isolations the protoplasts were observed lysed. The yields of protoplasts from the mesophyll cells of *H. annuus* and wild *Helianthus sps* was reported to be influenced by various physical factors such as humidity (Phileppe *et al.* 1986) gaseous phases surrounding the donor plants (Elconore and Gunther 1989) and the relative age of the donor plants (Barth 1994). In the present study the lower yields of viable protoplasts from the wild species of *Helianthus* may be due to the above factors. Besides an increase in the osmolarity of the isolation media may enhance the yields of protoplasts.

Protoplasts from hypocotyls, petiole and mesophyll cells were cultured at a density of 5.0×10^4 PP ml^{-1} in respective media. They were observed under the light microscopy after 48 h of initial cultures. Cells which were round and enlarged in volume with respect to normal freshly isolated protoplasts were accounted for the viability determinations. The observations made on the rates of viability are presented in Table 1. In hypocotyl protoplasts highest percentage viability was observed in cultivar Euroflor (81%) with BAP and NAA at 1.0 mg/l concentrations when compared with cultivars Cerflor and Apisol where the per cent viabilities were 79% and 76% respectively. Petiole protoplasts from cultivar Apisol showed 70% viability followed by cultivar Euroflor (64%) at 1.0 mg/l each of BAP and NAA while in cultivar Cerflor lower concentrations of BAP and NAA at 0.5 mg/l favoured more viability of protoplasts (68%). The mesophyll protoplasts from the cultivated *Helianthus* species showed upto 54% viability in cultivar Cerflor with 0.5 mg/l of BAP and NAA but on the other hand BAP and NAA at 1.0 mg/l in Euroflor showed 47% followed by 44% viability in cultivar Cerflor. In the previous studies from different authors (Lenee and Chupeau 1986, Chanable *et al.* 1989, Samaj *et al.* 1994) it has been shown that the embedding techniques results in good plating efficiencies. In the present study we have optimized a liquid culture method at the level of growth regulator supplementation and initial densities allowing the direct cultivation of protoplasts for initiation of suspension cultures.

Growth regulators like 2,4-D, BAP and NAA are reported to enhance the cell divisions in various plant species including *H. annuus* (Dupuis *et al.* 1988, Moyne *et al.* 1988, Paterson and Adams 1987) at varied compositions. In the present study protoplasts from the hypocotyls of cultivars Apisol, Cerflor and Euroflor showed more than 70% viability in mKM media supplemented with BAP and NAA of 1 mg/l. Mesophyll protoplasts from the cultivars Apisol, Cerflor and Euroflor showed 40% viability while those from *H. grosseserratus* and *H. maximillianii* were very fragile and did not survive

for more than 48 h in KMAR medium supplemented with 0.5 and 1 mg/l of BAP and NAA. Frequent trails for their survivability in liquid cultures did not yield any positive results as was observed in the present study. Protoplasts from the wild *Helianthus* species *H. nutalli, H. giganteus* showed good plating efficiencies when they are embedded in agarose cultures supplemented with BAP and NAA. Barth (1994) observed no divisions from the mesophyll protoplasts of *H. laetifilorus* and *H. pauciflorus*. On the other hand it has been observed in the present study that the mesophyll protoplasts from the cultivars Cerflor and Euroflor divided in liquid cultures with 1.0 mg/l of BAP and NAA. Elconore and Gunther (1989) while working with *H. annuus* inbred line HA 300, reported that 68% of the living protoplasts showed mitosis after 5-7 days of cultures. Mesophyll protoplasts from the cultivar Apisol did not show any divisions even after 10 day of cultures but they have been increased in their volume and settled at the bottom of the petriplates and became mitotically inactive. The results from the present experiments indicate that a change in the growth regulators composition may improve the cell division rates in Apisol mesophyll protoplasts. The regeneration of protoplasts into calli has also been reported to be genotype dependent in *Helianthus* species. Barth (1994) reported that the vitality of the protoplasts is age dependent in *H. annuus* and *H. laetiflorus* and *H. pauciflorus*. It has also been observed that the lower rates of cell divisions in cultures when the protoplasts isolated and cultured during the winter periods in *Petunia* and *Pisum* species (Binding *et al.* 1974, Lehminger-Mertnes and Jacobsen 1989).

Culturing of protoplasts at ideal densities plays a major role in inducing higher Mitotic Index rates and the regeneration of calli. In the present study it has been observed that protoplasts derived from the hypocotyls from the cultivars Apisol, Cerflor and Euroflor when cultured at various densities ranging from $5x10^4$ to $1x10^5$ PP ml^{-1} in a modified mKM media the cells divided with high frequencies. Schmitz and Schnabl (1991) while working on *H. annuus* suggested that protoplast densities between 2-5 x 10^4 PP ml^{-1} are required for maintaining sustainable cell divisions and colony formations. In the present study protoplasts plated at $1x10^4$ PP ml^{-1} proved to be very low as compared to the densities ranging from 5 x 10^4 to 1 x 10^5 PP ml^{-1}. Bohorova *et al.* (1986) suggested that protoplasts densities below 1 x 10^5 PP ml^{-1} in both the liquid and agarose embedded cultures did not support the sustainable cell divisions in *H. annuus*. In the present experiment it has been shown that protoplasts plated at densities above 5 x 10^4 PP ml^{-1} have efficiently divided but for higher yields of microcolonies it may be useful to culture the protoplasts at densities of 1 x 10^5 PP ml^{-1} for micronuclei induction studies.

Cells plated with BAP and NAA with 0.5 or 1.0 mg/l in either KMAR or in mKM for five days were observed for their divisibility. In each treatment all cell suspensions were made uniform and samples equivalent to 100 µl were analysed. The cells undergoing division were counted from 500 to 1000 cells and the results are summarized in Table 1. Hypocotyl protoplasts from all the cultivars of *Helianthus* species showed good divisibility at both the levels of BAP and NAA. The first division of protoplasts was observed within 36 h after the initial culture in cultivars of Cerflor and Euroflor with 1.0 mg/l of BAP and NAA while with 0.5 mg/l concentrations the cell division was observed between 60 and 72 h. Highest percentage plating efficiency was observed in cultivar Apisol (53%) with 1.0 mg/l of BAP and NAA where the first division was observed after 48 h followed by cultivar Cerflor (47%) and Euroflor (42%). On day 10, the cultures composed of single and small aggregates of cells. They were of different size and shape in all the cultivars of *H. annuus* studied. There were large and small cells in colonies besides free cells and cells were found dividing asymmetrically. BAP and NAA at 0.5 mg/l concentrations resulted in the slow growth of the cells in all the cultivars where the initial plating efficiency on the 5th day ranged between 23 to 28%.

A slow growth was noticed in petiole protoplasts from all the cultivars tested. The first cell divsion in all these cultivars was observed after 72 h of initial cultures in all treatments except in cultivar Apisol where the first division was observed after 96 h with 0.5 mg/l of BAP and NAA. In Cerflor both BAP and NAA at 1.0 mg/l showed 30% of cells dividing followed by Apisol and Euroflor with 28% and 17% respectively. Protoplasts from all the cultivars maintained consistent divsions with 1 mg/l of BAP and NAA. The microcolonies were further subcultured in the mKM media with a reduced osmolarity i.e. 500 mOsmol/kg with 1.0 mg/l BAP and NAA. However, further growth of the cells of microcolonies

was not consistent in Eurolfor. Petiole derived protoplasts though developed into colonies in Apisol and Cerflor cultivars but with increase in time in suspension conditions the cells turned brown (necrosis) and a large portion of cells lost their viability.

Mesophyll protoplasts on the other hand showed a different response in the suspension cultures. Divisions were observed only from the protoplasts of cultivated *Helianthus* species. The cells have increased in their volume and in many cells irregular divisions were noticed. The first divisions from all the cultivars showed divisibility after 96 h of initial cultures. In cultivar Cerflor, BAP and NAA supported the cell division at both the levels i.e. 7% with 0.5 mg/l and 10% with 1.0 mg/l. In cultivars Apisol and Euroflor lower concentrations of BAP and NAA did not support the cell divsion and at 1 mg/l levels, only a very less number of cells showed divisibility i.e. Apisol with 5% and Euroflor with 4% respectively. The mesophyll cells of Apisol and Euroflor did not show any further divisions while divided cells of Cerflor though forming microcolonies did not develop further.

The cell suspensions when subcultured by exchanging the media completely (drastic change of media) showed strong stress symptoms. In another method where the cell suspensions were reduced to 2 ml and 6 ml fresh media was added, supported good growth of cells in all the cultivars. It was observed from the present study that hypocotyl derived protoplasts from all the cultivars of *Helianthus* species cv. Apisol, Cerflor and Euroflor showed high rates of cell divisions. Other tissue types were observed to be recalcitrant for further regeneration into calli.

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