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Regeneration of fertile plants from *Helianthus nuttallii* T&G and *Helianthus giganteus* L. mesophyll protoplasts

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Abstract Interspecific hybridisation in the genus *Helianthus* via somatic cell fusion is thought to play an important role in future sunflower breeding programs. The establishment of this technique requires, however, the development of single-cell-regeneration protocols. For this purpose, we applied a regeneration protocol recently developed for *Helianthus annuus* L. to mesophyll protoplasts of two wild sunflowers (*H. nuttallii* T&G, *H. giganteus* L). Protoplasts of both species were embedded in agarose droplets and covered by liquid mKM medium. After 4–5 weeks, callus was transferred onto solid differentiation medium yielding plating efficencies of 1.5% (*H. nuttallii*) and 2.5% (*H. giganteus*). Emerging shoots were elongated on hormone-free medium, and root formation was induced by an NAA treatment. Regenerated plants were transferred to the greenhouse where they grew up to a height of 2 m and flowered after 3 months. Seeds were harvested from regenerated plants of both species.

Key words *Helianthus nuttallii* T&G · *Helianthus giganteus* L. · Organogenesis · Plant regeneration · Protoplasts

Abbreviations *BAP* 6-Benzylaminopurine · *2,4-D* 2,4-Dichlorophenoxyacetic acid · *NAA* 1-Naphthaleneacetic acid

Introduction

Wild *Helianthus* species present a wide spectrum of characteristics such as cytoplasmic male sterility, drought resistance, superior oil quality and disease resistance (Seiler 1992 a; Skoric 1992). Therefore, they offer an attractive tool for increasing the narrow genetic base of *Helianthus annuus* L. and for enlarging sunflower breeding programs. However, the use of wild species in breeding protocols is limited by poor crossability and sterility of interspecific hybrids (Vannozzi 1994). Biotechnological methods such as embryo rescue (Espinasse et al. 1985; Kräuter and Friedt 1989) or interspecific cell fusion are required to overcome this barrier (Friedt 1992; Seiler 1992 b). A prerequisite for somatic hybridisation is the amenability of the fusates to tissue culture, their regeneration capacity being either provided by both parents or by only one. *H. annuus* can be regenerated via embryogenesis (Krasnyanski and Menzel 1993) or organogenesis (Burrus et al. 1991; Fischer et al. 1992; Wingender et al. 1996) and both regeneration routes have also been reported for various wild sunflowers. Organogenesis of protoplast-derived callus was obtained for *H. petiolaris*, at a low yield (Chanabe et al. 1991) as well as for *H. praecox*, *H. scaberimus* and *H. rigidus* (Bohorova et al. 1986), the latter authors providing no details on plant regeneration. A high percentage of embryogenic callus and plants were obtained in the case of *H. giganteus* (Krasnyanski et al. 1992) and *H. maximiliani* (Polgar and Krasnyanski 1992).

The main objective of this work was to test whether wild sunflower protoplasts exhibit a similar regeneration potential as those from cultivated sunflower by submitting them to a *H. annuus* regeneration protocol. We selected the two wild species mentioned since they are possible donors for disease resistance (Christov and Venkow 1994) to cultivated sunflower. We report the regeneration of fertile plants from *H. giganteus* and for the first time also from *H. nuttallii* mesophyll protoplasts via organogenesis. The results are compared with respect to *H. annuus* cv. Florom-328.

Materials and methods

Plant material

H. nuttalli T&G (botanical garden, Bonn) and *H. giganteus* L. (botanical garden, Mainz) plants originated from an in vitro propagation system.

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Protoplast isolation

Leaves were cut into small pieces and incubated in an enzyme mixture containing 0.15% cellulase (Onozuka R10; Serva), 0.05% pectolyase (Serva), 0.75% macerozyme (R10; Serva), 0.005% driselase (Sigma) and 1.0% bovine serum albumin. The enzymes were dissolved in a salt solution containing 340 mM KCl, 1.4 mM $CaCl_2 \cdot 2H_2O$ and 3 mM MES, pH 5.6. The digestion was carried out at 18 °C for 18 h followed by slow shaking (20 rpm) at 26 °C for 1.5 h in the dark. The resulting suspension was filtered through a 50 µm steel sieve, and protoplasts were collected by centrifugation at 50 *g* for 5 min. The pellet was resuspended in sucrose solution (0.5 M sucrose, 0.14 mM $CaCl_2 \cdot 2H_2O$, 3 mM MES, pH 5.6), covered with salt solution and centrifuged again as above. The purified protoplasts were removed from the interphase.

Protoplast culture

The protoplasts were cultured in 50-µl agarose solidified droplets of culture medium (Shillito et al. 1983) at a density of 5×10^4 ml^{-1}. The droplets were covered with liquid mKM medium (Wingender et al. 1996) and cultured at 26 °C in the dark. For the first 8–10 days the medium was supplemented with 4 µM BAP and 5 µM NAA to initiate cell division. The osmolarity was adjusted to 0.6 osmol kg^{-1} H_2O with mannitol and the pH to 5.6. When 60–70% of the protoplasts showed cell division the entire medium was replaced by medium containing 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D), and the osmolarity was reduced to 0.5 osmol kg^{-1} H_2O. Five days later, this medium was replaced by a medium supplemented with 4 µM 6-benzylaminopurine (BAP) and 0.5 µM 1-naphthaleneacetic acid (NAA), and the osmolarity was reduced to 0.4 osmol kg^{-1} H_2O. After calli reached a size up to 0.3 mm, the osmolarity of the medium was reduced again to 0.3 osmol kg^{-1} H_2O and the cultures were transferred to a 12-h light period (50 µmol s^{-1} m^{-2}) at 26 °C, which was maintained in all further cultivation steps.

Shoot differentiation

After 8–10 days, agarose droplets were transferred to solid differentiation medium (D) based on MS salts (Murashige and Skoog 1962) with the following additions: 87.6 mM sucrose, 2.7 mM myo-inositol, 3 mM MES, 7.4 µM thiamine-HCl, 2 nM nicotinic acid, 1.2 nM pyridoxine-HCl, 13 µM glycine, 5.8 µM silver nitrate and 4 g l^{-1} phytagel, pH 5.6. BAP and NAA were added to final concentrations of 4.4 and 0.1 µM respectively.

Small shoots were transferred to hormone free shoot elongation (SE20) medium (1/2 Murashige and Skoog salts, 58.43 mM sucrose, 2.7 mM myo-inositol, 3 mM MES, 7.4 µM thiamine-HCl, 2 nM nicotinic acid, 1.2 nM pyridoxine-HCl, 5.8 µM silver nitrate and 4 g l^{-1} phytagel, pH 5.6).

Transfer of plants into soil

Shoots were cut, dipped into 5.3 M NAA solution for one second and cultured on modified SE_{20} medium supplemented with 2 g l^{-1} casein hydrolysate and 13 µM glycine. Plants with well-developed roots were transferred to a 50/50 mixture of vermiculite and garden soil and cultured in the greenhouse. Flowering plants were crossed with regenerants of the same species.

Results and discussion

Protoplast isolation and agarose droplet culture

The average yield of isolated protoplasts was 2×10^6 g^{-1} fresh weight for *H. nuttallii* and 3×10^6 g^{-1} fresh weight for *H. giganteus*. Plants grown in vitro for 3 weeks were the best source for protoplasts with respect to yield and vitality for both species. The wild sunflower protoplasts were found to be rather fragile, resulting in low survival rates after embedding. This was overcome by incubating them in salt solution for 1 h at room temperature in the dark. Longer precultivation in salt solution as well as in culture medium (Chanabe et al. 1991) resulted in a high percentage of dead protoplasts. For both species, an initial embedding density of 5×10^4 protoplasts was found to be optimal. Higher densities led to brownish callus after the 2,4-D treatment, the cells being inhibited in their growth, while lower plating densities resulted in poor division rates. In agreement with other authors (Bohorova et al. 1986; Krasnyanski et al. 1992) the use of young in-vitro-grown plants and agarose bead culture were crucial for further development of the protoplasts.

After 8–10 days of culture, 50–60% *H. nuttallii* and 60–70% *H. giganteus* protoplasts showed cell divisions. The high initial number of dividing cells was probably due to the plant growth regulator supplementation (4 µM BAP, 5 µM NAA) since lower yields were obtained for *H. giganteus* using 8.8 µM BA and 0.5 µM NAA (Krasnyanski et al. 1992). An important factor for callus and in particular shoot development was the duration of the 2,4-D treatment applied during the 2nd week of bead culture. In contrast to Krasnyanski et al. (1992), who regenerated *H. giganteus* without an auxin teatment, we found the use of 2,4-D to be essential. Callus stopped growing when the auxin treatment was longer than 6 days and neither organogenesis nor shoot differentiation was observed when 2,4-D was present for less than 4 days. For *H. nuttallii* in particular, this timing had to be followed strictly. Although high auxin concentrations are thought to favour embryogenesis (Finer 1987; Prado and Berville 1990), sunflower reacted by organogenesis. The high-auxin medium was replaced by a medium containing BAP and NAA at a ratio of 8/1, and after calli reached 0.3 mm (8–10 days), the cultures were transferred to the light. Shifting cultures with smaller calli to a 12-h light period led to lower plating efficiencies and reduced shoot formation.

Callus cultivation and shoot differentiation

The duration of bead culture depended on the growth rate of the colonies which differed between experiments. The timing given in Table 1 reflects therefore an average, since the colonies had to reach a size of 0.5 mm prior to their transfer onto solid D medium. No differentiation processes were observed with smaller and bigger callus, suggesting that a given developmental stage of the callus was needed for successful induction of organogenesis. The plating efficiency calculated from the total number of protoplasts was 1.5% with *H. nuttalli* and 2.5% with *H. giganteus* (Table 1). The first shoots appeared after 3 (*H. giganteus*) and 4 weeks (*H. nuttallii*) and were cut off 1 week later (Fig. 1 A). Their elongation was carried out on SE_{20} medium lacking growth regulators in order to avoid vitrifica-

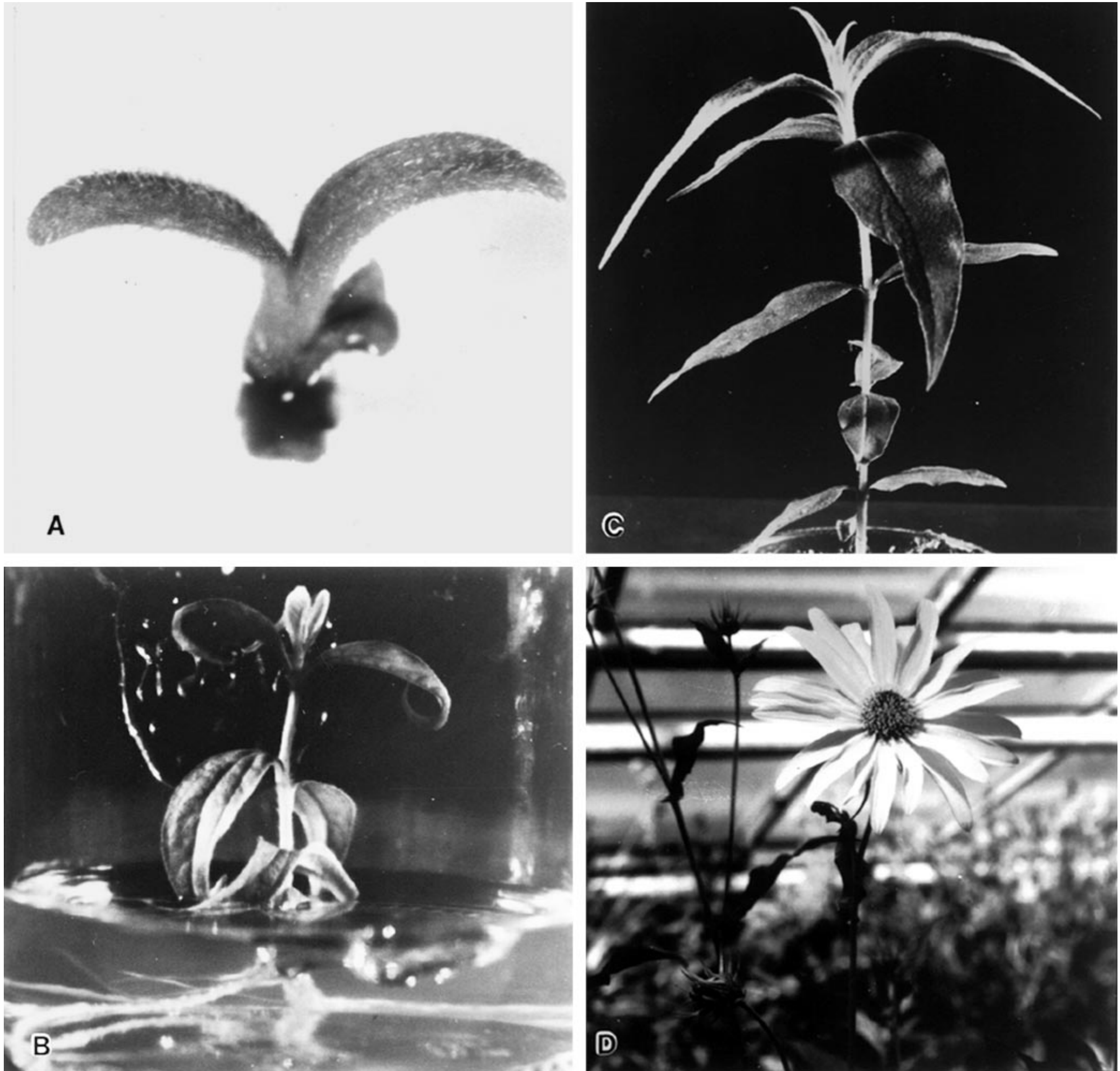

Fig. 1 *Helianthus nuttallii* shoot with well-developed primary leaves (× 5), (**A**) rooted shoot after NAA treatment (× 2), (**B**) and greenhouse plant (× 0.5) (**C**) which flowered (× 0.5) 3 months after transfer

tion. New callus appearing at the cutting had to be removed carefully, otherwise shoot development was inhibited. In 10–12 days the explants reached a length of 1.0–1.5 cm and rooting was induced by dipping their bases into 5.3 M NAA solution, followed by cultivation on modified SE_{20} medium. Most of the shoots from both species formed branched roots after 8–10 days (Fig. 1 B). However, callus formed in a few cases which had to be removed, and the NAA treatment could be repeated after 1 week. In total, 80% of the shoots from both species could be planted into a 50/50 mixture of vermiculite and garden soil 1 month later (Fig. 1 C). Per 1×10^6 embedded protoplasts, we obtained 20 (±3) fertile plants from *H. nuttallii* in three independent experiments, and only 4 (±2) fertile plants from *H. giganteus* (four independent experiments). Over about 3 months the plants grew to a height of 2 m and showed normal morphology. All plants were branched in the upper third and formed up to 20 flower buds (Fig. 1 D) which flowered in succession. Heads were small and from each eight to ten seeds were harvested.

From these results, it can be concluded that the culture regime applicable for *H. annuus* will also yield fertile plants from protoplasts of *H. giganteus* and *H. nuttallii*. Much lower efficiencies and differences in subculture tim-

Table 1 Yield and cultivation of *Helianthus nuttallii* T&G and *H. giganteus* L. mesophyll protoplasts in comparison with *H. annuus* Florom-328 hypocotyl protoplasts (from Wingender et al. 1996). *Plating efficiency* is calculated from the total number of protoplasts. Values for *plants in the greenhouse* are based on 1×10^6 embedded protoplasts (in *parentheses*, the mean deviation of independent experiments

	H. nuttalii	*H. giganteus*	*H. annuus* Florom
Culture efficiency			
Yield of protoplasts g^{-1} fresh weight	2×10^6	3×10^6	2×10^6
Dividing cells after 8–10 days (%)	50–60	60–70	60–70
Plating efficiency (%)	1.5	2.5	5.0
Rooting frequency (%)	80	80	90
Plants in the greenhouse	20 (±3)	4 (±2)	350 (±40)
Number of seeds/head	6–8	6–8	5.25
Plant height (m)	2.5	2.0	0.3
Cultivation timing			
Duration of 2,4-D treatment (days)	5	5	7
Duration of bead culture (days)	30	30	21
Shoots appearance (weeks)	4	3	1
Shoot elongation (days)	10–12	10–12	14–21
Time to flowering (months)	3	3	1

ing were observed compared to *H. annuus* cv Florom-328 (Table 1). Nevertheless, it seems highly likely that interspecific hybrids could be regenerated by this protocol and further experiments will be conducted to demonstrate this assumption.

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