H.-J. Henn · R. Wingender · H. Schnabl

Regeneration of fertile interspecific hybrids from protoplast fusions between *Helianthus annuus* L. and wild *Helianthus* species

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Abstract The use of interesting characteristics from wild *Helianthus* species in sunflower breeding is limited by poor crossability or sterility of interspecific hybrids. To overcome this barrier, mesophyll protoplasts of *Sclerotinia sclerotiorum*-resistant clones of *Helianthus maximiliani*, *H. giganteus* and *H. nuttallii* were fused with hypocotyl protoplasts of *H. annuus* in the presence of polyethyleneglycol and dimethylsulfoxide. Fusion products were embedded in agarose and subjected to a regeneration protocol developed for sunflower protoplasts. Organogenic calli were transferred onto solid medium and emerging shoots were elongated in the absence of plant growth regulators. Rooting of shoots was induced by a 1-naphthaleneacetic acid treatment and putative hybrid plants from fusions between *H. annuus* + *H. maximiliani* and *H. annuus* + *H. giganteus* were transferred into the greenhouse. All of them exhibited a hybrid phenotype with a high percentage of rhizome producing plants. Their hybrid origin was confirmed by random amplified polymorphic DNA analysis. Plants flowered after 3–4 months and set seeds, of which 70–80% germinated.

Key words *Helianthus* · Somatic hybridisation · Organogenesis · Plant regeneration · RAPD analysis

Abbreviations *BAP* 6-Benzylaminopurine · *DMSO* Dimethylsulphoxide · *NAA* 1-Naphthaleneacetic acid · *PEG* Polyethyleneglycol · *RAPD* Random amplified polymorphic DNA

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H.-J. Henn · R. Wingender (✉) · H. Schnabl
Institut für Landwirtschaftliche Botanik der Universität Bonn, Meckenheimer Allee 176, D-53115 Bonn, Germany
Fax: +49-228-695168

Introduction

In recent years, the increased production of sunflower (*Helianthus annuus* L.) has revealed several problems linked with the narrow genetic base of the cultivars. Pathogens, for example *Sclerotinia sclerotiorum* and *Botrytis cinerea*, are major limiting factors for sunflower production in northern regions (Masirevic and Gulya 1992), since resistance against these fungi is lacking among sunflower cultivars (Bazzalo et al. 1991). Wild *Helianthus* species are regarded as important sources for disease resistance (Seiler 1992; Skoric 1992; Henn et al. 1997) but their use in sunflower breeding is limited by poor crossability and sterility of interspecific hybrids (Atlagic et al. 1993; Vannozzi 1994; Atlagic et al. 1995). This barrier in classical breeding protocols can be overcome using biotechnological methods such as embryo rescue (Espinasse et al. 1985) or somatic hybridisation via protoplast fusion. Interspecific hybrids have been obtained with several wild *Helianthus* species using embryo rescue (Kräuter et al. 1991), whereas protoplast fusion followed by regeneration of hybrid plants has only been reported with *H. giganteus* as wild species (Krasnyanski and Menczel 1995). Successful application of the fusion technique demands a protocol for plant regeneration from protoplasts. Regeneration of plants from hypocotyl protoplasts of *H. annuus* (Wingender et al. 1996) and from mesophyll protoplasts of *H. nuttallii* and *H. giganteus* (Henn et al. 1998) was achieved by similar culture regimes which differed mostly with respect to cultivation timing. That the media and growth regulator supplementation were identical in both protocols should render them suitable for heterokaryons.

In this paper we describe (1) the fusion of *H. annuus* hypocotyl protoplasts with mesophyll protoplasts from *Sclerotinia*-resistant clones of *H. maximiliani*, *H. giganteus* and *H. nuttallii* (Henn et al. 1997), (2) the regeneration and morphology of fertile *H. annuus* + *H. maximiliani* and *H. annuus* + *H. giganteus* hybrid plants and (3) random amplified polymorphic DNA (RAPD) analysis revealing the presence of the parental genomes.

Materials and methods

Plant material and protoplast isolation

Hypocotyl protoplasts from *H. annuus* cv. Florom-328 (Institute of Cereal and Industrial Plant Research, Fundulea, Romania) were isolated as described by Wingender et al. (1996). Mesophyll protoplasts from the wild sunflower species, *H. maximiliani* Schrader (botanical garden, Lisbon), *H. nuttallii* T&G (botanical garden, Bonn) and *H. giganteus* L. (botanical garden, Mainz) were isolated according to Henn et al. (1998).

Protoplast fusion

Freshly prepared hypocotyl protoplasts from *H. annuus* were mixed at a ratio of 1/2 with mesophyll protoplasts from the wild species which had been stored for 1 h at room temperature in the dark in salt solution. The protoplast density was adjusted to 2×10^6 ml^{-1} with salt solution (340 mM KCl, 1.4 mM $CaCl_2 \cdot 2H_2O$ and 3 mM MES, pH 5.6). The protoplasts were fused by mixing 250 µl each of protoplast suspension and fusion solution [15% polyethyleneglycol (PEG), 5% dimethylsulfoxide (DMSO), 90 mM mannitol, 60 mM $CaCl_2 \cdot 2H_2O$, 25 mM glycin] in a petri dish (10 cm diameter). Using *H. giganteus* as the donor of mesophyll protoplasts, the PEG and DMSO concentrations were raised to 20% and 10%, respectively. After incubation for 20 min in the light, protoplasts were washed with salt solution and collected by centrifugation at 120 *g* for 5 min. The percentage of fused protoplasts was determined microscopically on the basis of the different chloroplast contents. Hypocotyl protoplasts exhibit extensive cytoplasmic streaming while mesophyll protoplasts are densely packed with chloroplasts. Fusion products, therefore, showed cytoplasmic streaming combined with the presence of chloroplasts.

Protoplast culture and plant regeneration

Protoplasts were cultured in 50-µl agarose-solidified droplets of culture medium (Shillito et al. 1983) at a density of 8×10^4 ml^{-1} and cultured as described by Henn et al. (1998).

Calli with diameters of 1 mm were transferred to solid differentiation (D) medium based on Murashige and Skoog (1962) salts with the following additions: 87.6 mM sucrose, 2.7 mM myo-inositiol, 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. 6-Benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA) were added to final concentrations of 4.4 µM and 0.1 µM respectively.

Organogenic calli were transferred onto hormone-free shoot elongation (SE_{20}) medium (1/2 Murashige and Skoog salts, 58.43 mM sucrose, 2.7 mM myo-inositiol, 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).

Rooting of shoots occurred on modified SE_{20} medium (supplemented with 2 g l^{-1} casein-hydrolysate and 13 µM glycine) after dipping the stems into 5.3 M NAA solution. Plants with well-developed roots were transferred to a 50/50 mixture of vermiculite and garden soil and cultured in the greenhouse.

DNA isolation

DNA was isolated from plants 2 weeks after transfer to the greenhouse. Fifty milligrams of fresh leaf material was ground in liquid nitrogen and lysis was carried out using 4 ml CTAB buffer (2% CTAB, 1% PEG, 1.4 M NaCl, 100 mM Tris-HCl, 20 mM EDTA). After 20 min at 74 °C, 4 ml chloroform/isoamylalcohol (24/1) was added and cell debris was pelleted by centrifugation for 10 min (5000 *g*, 4 °C). The supernatant was removed and DNA was precipitated with isopropanol. After centrifugation (5000 *g*, 20 min, 4 °C), the DNA pellet was resuspended in 1 ml 1 M NaCl, and 400 µg RNase was added followed by incubation at 37 °C for 30 min. DNA was further purified using anion-exchange columns (Genomic-tip 20, Qiagen). DNA concentration and purity were estimated by measuring the absorbance at 260 nm and 280 nm.

RAPD

DNA polymorphisms were investigated using RAPD analysis with ten base primers from Pharmacia. Of the five primers screened, P1 (5′-CGGCCACTG-3′) and P2 (5′-GGACTGGCG-3′) were selected on the basis of maximum differences in polymorphisms between the parents. PCR was carried out in a 25-µl reaction volume containing 50 ng DNA, 200 µM each of dATP, dCTP, dGTP, dTTP, 0,3 µM primer, 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$ and 1 unit Tag polymerase. DNA was amplified in a thermocycler (Autogene II, Grant Instruments Ltd.) at 94 °C for 5 min followed by 38 cycles each with 36 °C for 0.8 min, 72 °C for 1.5 min, 94 °C for 0.8 min and finally 36 °C for 0.8 min, 72 °C for 10 min. The PCR products were separated on 1.5% agarose gels containing 0.005% ethidium bromide. Gels were analysed under UV light (302 nm).

Germination of hybrid seeds

Seeds from hybrid plants were surface sterilised with 4.5% NaOCl (35 min), washed three times in sterile water and germinated in a 12-h light period on a medium containing 1/2-strenght Murashige and Skoog salts, 30 g l^{-1} sucrose and 4 g l^{-1} phytagel, pH 5.6.

Results and discussion

Protoplast isolation, fusion and agarose bead culture

The sensitivity of *Helianthus* protoplasts, especially mesophyll protoplasts of wild species, to PEG treatment was very high, as reported for leaf protoplasts of other species (Haydu et al. 1977; Menczel and Wolfe 1984). On the other hand, electrofusion, known to enhance the regeneration potential (Barth et al. 1993; Belarmino et al. 1996) was not performed due to the differences in size between the protoplasts, resulting in different sedimentation velocities. The diameter of mesophyll protoplasts from the wild species was about 26 µm whereas hypocotyl protoplasts of *H. annuus* varied between 20–92 µm with most of them being larger than 36 µm. To reduce extensive damage of protoplasts, lower PEG concentrations than those of Krasnyanski and Menczel (1995) were used in combination with DMSO which is known to enhance PEG-mediated cell fusion (Klebe and Mancuso 1982; Menczel and Wolfe 1984). The average yield of fusion products was dependent on the wild *Helianthus* species used as mesophyll protoplast donor. About 8–10% fusion products were recovered with *H. giganteus* and *H. nuttallii*, whereas fusion rates of 5–6% were obtained with *H. maximiliani*. The fusion yields and survival rates were also affected by the vitality of protoplasts which was increased by storage for about 1 h.

In the presence of 4 µM BAP and 5 µM NAA, 40–50% of the cells showed divisions after 6–8 days, whereas Krasnyanski et al. (1995) obtained lower yields for *H. annuus* + *H. giganteus* fusions using 8.8 µM BAP and 0.5 µM NAA.

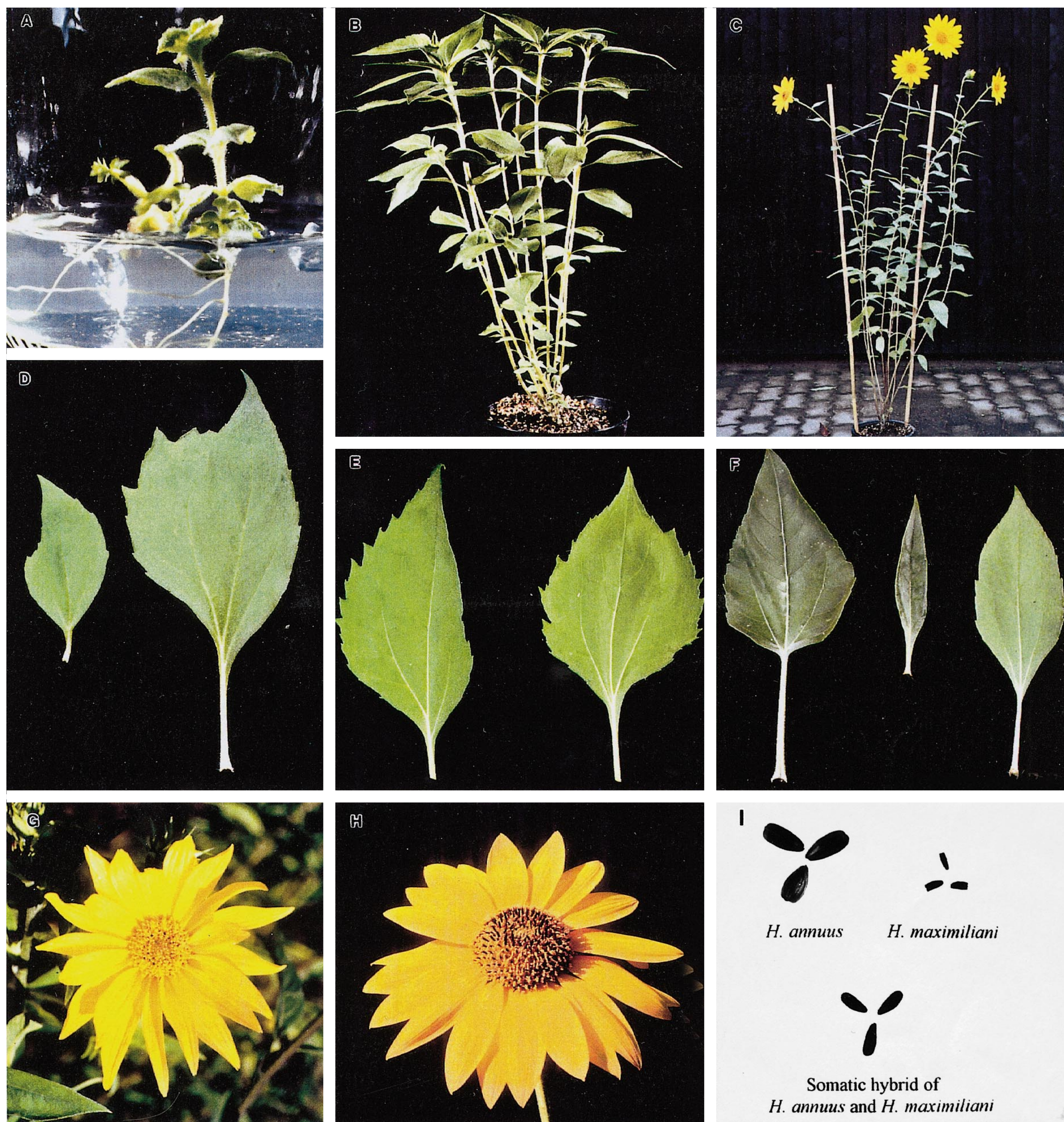

Fig. 1 **A** *Helianthus annuus + H. maximiliani* rooted shoot after NAA treatment (height 4 cm). **B** Plant in the greenhouse (height 45 cm). **C** Flowering *H. annuus + H. maximiliani* hybrid plant (height 1.5 m), **D, E** Leaves of *H. annuus + H. maximiliani* hybrids (from left to right: 4 cm, 10 cm, 7 cm, 7 cm). **F** From left to right, leaf of *H. annuus* (8 cm), *H. maximiliani* (4 cm), *H. annuus + H. maximiliani* hybrid (6 cm). **G** Flower of *H. maximiliani* (7 cm diameter). **H** Flower of *H. annuus + H. maximiliani* hybrid (12 cm diameter). **I** Seeds of *H. annuus* (8 mm in length), *H. maximiliani* (4 mm in length), *H. annuus + H. maximiliani* (6 mm in length)

With respect to cultivation timing, the tissue culture behavior of the fusions resembled wild sunflower (Henn et al. 1998) more closely than *H. annuus* cells (Wingender et al. 1996). The 2,4-dichlorophenoxyacetic acid treatment during the 2nd week of bead culture was therefore only applied for 5 days (Henn et al. 1999). Subsequently, calli with *H. giganteus* and *H. maximiliani* as fusion partners showed vigorous growth; in contrast, most fusions with *H. nuttallii* mostly stopped growing.

Table 1 Results from regeneration of fertile somatic hybrid plants of *Helianthus annuus* + *H. maximiliani* (four independent experiments) and of *H. annuus* + *H. giganteus* (ten independent experiments)

	H. annuus + *H. maximiliani*	*H. annuus* + *H. giganteus*
Plating efficiency (%)	2.5	3.0
Organogenic calli (% of all calli)	0.5	0.1
Rooting frequency (%)	43	21
Hybrid plants in the greenhouse	125	47
Flowering hybrid plants	28	3
Number of seeds/plant	5–10	25–30
Regeneration time (months)	8–14	10–15

Callus cultivation and plant regeneration

The plating efficiency calculated from the total number of protoplasts was 3.0% for *H. annuus* + *H. giganteus* and 2.5% for *H. annuus* + *H. maximiliani* fusions (Table 1). During the next 2–4 weeks 0.1% *H. annuus* + *H. giganteus* and 0.5% *H. annuus* + *H. maximiliani* calli produced shoots. Since the latter exhibited extensive branching on SE_{20} medium, the axillary shoots had to be cut off to promote shoot elongation. Over 2–3 months, the explants reached a length of 1.0–1.5 cm and rooting was induced by a NAA treatment. Most of the shoots formed callus at their base which had to be cut off, and the treatment was repeated. Finally, 43% of *H. annuus* + *H. maximiliani* and 21% of *H. annuus* + *H. giganteus* shoots rooted (Fig. 1 A) and 3–5 weeks later plants could be transferred into the greenhouse (Fig. 1 B). The results are summarized in Table 1.

Characterization of hybrid plants

To select hybrids, regenerated plants from the greenhouse were subjected to RAPD analysis. This technique is a tool for hybrid characterisation (Rieseberg and Ellstrand 1993) which proved to be ideal for *Helianthus* since knowledge of its genome is sparse and genetic markers were not available. Furthermore, it requires only very small amounts of tissue and can be applied at an early stage of plant develeopement. The band patterns of *H. annuus* + *H. maximiliani* hybrids (Fig. 2, lanes 4–7) showed all characteristic bands of *H. annuus* (lane 2) as well as those of *H. maximiliani* (lane 3), indicating that parts of the genomes from both parents were combined in the hybrids. Separation of amplification products of *H. annuus* + *H. giganteus* hybrid plants (Fig. 3, lanes 4–6) showed likewise that parts from different genomes (*H. annuus* and *H. giganteus*) were present in these plants. Mostly additive banding patterns were obtained, but rearrangements and loss of genetic material cannot be excluded.

In four independent experiments we obtained 125 hybrids of *H. annuus* + *H. maximiliani* and 47 hybrids of

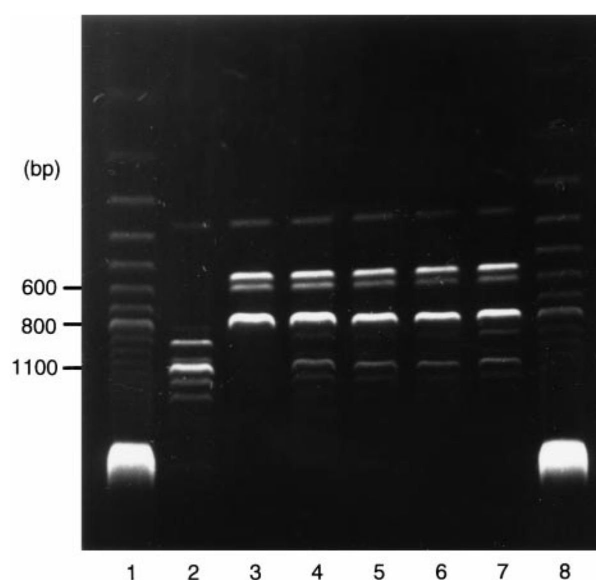

Fig. 2 RAPD patterns with primer P1 with DNA of *H. annuus* (*lane 2*), *H. maximiliani* (*lane 3*) and four different *H. annuus* + *H. maximiliani* hybrids (*lanes 4–7*). (*lanes 1 and 8* 100-base-pair ladder)

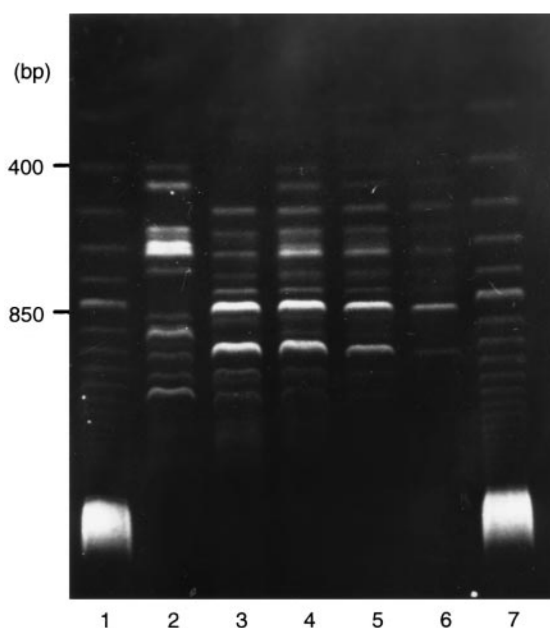

Fig. 3 RAPD patterns with primer P2 with DNA of *H. annuus* (*lane 2*), *H. giganteus* (*lane 3*) and three different *H. annuus* + *H. giganteus* hybrids (*lanes 4–6*). (*lanes 1 and 7* 100-base-pair ladder)

H. annuus + *H. giganteus* in 10 independent experiments (Table 1). The high number of regenerated plants argues for no inhibitory effect of PEG on tissue culture. None of the greenhouse plants showed a banding pattern typical for only one of the parents, either because the fusion solution is toxic to the parental protoplasts, or their growth is inhibited by hybrid regenerants, a phenomenon already de-

scribed for other genera like *Brassica* (Polgar et al. 1993) and *Dianthus* (Nakano and Mii 1993).

Morphology of somatic hybrid plants

Somatic hybrid plants showed high variability for most morphological traits, as described for *Medicago* (Nenz et al. 1996). Most plants were intermediate in leaf length and width between the parents (Fig. 1F); asymmetrical and serrated leaves were also observed (Fig. 1D, E). For 3–4 months, some plants grew unbranched up to a height of 1.5–1.8 m and formed one flower bud (mostly *H. annuus* + *H. giganteus*). Other plants were branched in the upper third and formed two to three flower buds which flowered in succession (mostly *H. annuus* + *H. maximiliani*) (Fig. 1C).

Heads were intermediate in size between the parents but ligulate flowers were similar to *H. annuus* (Fig. 1G, H). Six weeks after reciprocal pollination, 5–10 seeds were harvested from each *H. annuus* + *H. maximiliani* hybrid; hybrids of *H. annuus* + *H. giganteus* yielded 25–30 seeds per plant (Table 1). Seeds were similar in shape but were much smaller than *H. annuus* seeds (Fig. 1I). Numerous hybrid plants were perennial, a character typical for wild sunflowers, since they produced rhizomes from which shoots emerged after seed harvest. After 3 months storage, 70% of *H. annuus* + *H. maximiliani* and 80% of *H. annuus* + *H. giganteus* seeds germinated.

Fertile somatic hybrid plants of *H. annuus* + *H. giganteus* and for the first time of *H. annuus* + *H. maximiliani* were regenerated via organogenesis with a similar culture regime recently described for *H. annuus* (Wingender et al. 1996), *H. giganteus* and *H. nuttallii* (Henn et al. 1998). Regeneration of the hybrids required much more time, because shoot elongation and rooting were found to be difficult. Further experiments are underway to find out whether the *Sclerotinia* resistance from the wild *Helianthus* species (Henn et al. 1997) can be transferred to *H. annuus* via backcrossing.

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