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Regeneration of interspecific somatic hybrids between *Helianthus annuus* L. and *Helianthus maximiliani* (Schrader) *via* protoplast electrofusion

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Abstract *Helianthus maximiliani* is one of the wild *Helianthus* species with the genes for resistance to many pathogens including *Sclerotinia sclerotiorum*. Unfortunately, a transfer of disease resistance genes from this species into the cultivated sunflower is limited by its poor crossability with the cultivated sunflower and sterility of interspecific hybrids. To overcome this problem, mesophyll protoplasts of *Sclerotinia sclerotiorum*-resistant clone of *H. maximiliani* were electrically fused with etiolated hypocotyl protoplasts of the cultivated sunflower inbred line PH-BC_1-91A. Fusion products were embedded in agarose droplets and subjected to different regeneration protocols. Developed microcalluses were released from the agarose and transferred into solid media. Shoot regeneration was achieved by culture of calluses on regeneration medium containing 2.2 mg l^{-1} BAP and 0.01 mg l^{-1} NAA after the treatment with a high concentration of 2,4 D for a limited period of time. A morphological and RAPD analysis confirmed a hybrid nature of the regenerated plants.

Keywords Electrofusion · *Helianthus* · RAPD analysis · Regeneration · Somatic hybridization

Abbreviations 2,4 D: 2,4-Dichlorophenoxyacetic acid · ACO: Aconitase · ACP: Acid Phosphatase · BAP: 6-Benzylaminopurine · IAA: 3-Indolylacetic acid · IBA: 4-(3-Indolyl)butanoic acid · MDH: Malate Dehydrogenase · NAA: 1-Naphthylacetic acid · PCR: Polymerase Chain Reaction · PEG: Polyethylene Glycol · PGD: 6-Phosphogluconate Dehydrogenase · PGM: Phosphogluco Mutase · PHI: Phosphohexose Isomerase · RAPD: Random Amplified Polymorphic DNA

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Introduction

Wild sunflowers (*Helianthus* spp.) constitute an important source of resistance against several major sunflower (*Helianthus annuus* L.) diseases including white rot, caused by a fungus *Sclerotinia sclerotiorum* (Lib.) de Bary. White rot is the major disease of sunflower in countries with the humid climate, whereas in countries with the moderate climate it causes the yield loss in rainy years (Masirevic and Gulya 1992). There are no suitable cultural control methods against this disease and resistant genotypes of cultivated sunflower have not yet been found or developed. The breeding for *Sclerotinia* resistance is complicated task since the fungus attacks most parts of the plant: the root, stem, capitulum, leaf and terminal bud. *Sclerotinia* reaction is under polygenic control and different resistance mechanisms that generally appear independent can be involved in different plant organs (Robert et al. 1987; Castano et al. 1992; Roenicke et al. 2004). Beside genetic factors, accumulation of the soluble phenolic compounds, melanisation and lignification of tissues also play an important part in the plant resistance (Hemery-Tardin et al. 1998; Prats et al. 2003). The complexity of the resistance mechanisms prevented development of fully tolerant or resistant genotypes (Bazzalo et al. 1991; Vasic et al. 2002). One of the wild sunflower species with the highest resistance to *Sclerotinia* infections is *Helianthus maximiliani* (Schrader) (Skoric and Rajcan 1992; Henn et al. 1997). Several attempts have been made to cross this species with cultivated sunflower using conventional methods, but with limited success due to the poor crossability and sterility of interspecific hybrids (Atlagic et al. 1995).

Somatic hybridization by protoplast fusion is known as a mean for overcoming sexual incompatibility and production of hybrids of species that could not be crossed using conventional methods, as well as for the introduction of

desirable traits from the wild into the cultivated species of the same genus (Waara and Glimelius 1995). It has been successfully used for the introduction of resistance to viruses (Valkonen and Rokka 1998), bacterial (Collonier et al. 2003), and fungal pathogens (Liu et al. 1995; Furuta et al. 2004) from one plant species to another. Somatic hybridization via polyethylene glycol (PEG)-mediated fusion has been used to introduce cytoplasmic male sterility in the cultivated sunflower (Trabace et al. 1996) and for the production of interspecific hybrids between the cultivated sunflower and *Helianthus giganteus* L. (Henn et al. 1998a), as well as *H. maximiliani* (Henn et al. 1998a; Binsfeld and Schnabl 2002). Since the sensitivity of *Helianthus* protoplasts, especially mesophyll protoplasts of wild species, to PEG treatment is very high (Krasnyanski and Menczel 1995; Henn et al. 1998a), we tried to improve the existing fusion protocols and to produce somatic hybrid plants by using the electrofusion of protoplasts. Electrofusion is the most frequently used technique for generation of somatic hybrid plants between different species**,** as it helps in the maintenance of protoplast viability, reduces membrane damage, and protoplast distortion and disruption (Davey et al. 2005). Electrofusion has also been used for the fusion of sunflower protoplasts that resulted in formation of the calluses whose hybrid nature was subsequently confirmed by isozyme analysis (Barth et al. 1993). Also, it has been used for the fusion of sunflower and *H. maximiliani* protoplasts where formation of the hybrid calluses was confirmed by the random amplified polymorphic DNA (RAPD) analysis (Vasic 2003). However, plant regeneration from these calluses was not reported.

In this paper, we report the first successful regeneration of the somatic hybrid plants between the sunflower and *H. maximiliani* obtained by the electrofusion of protoplasts, and also provide a confirmation of their hybrid origin by morphological and RAPD analysis.

Materials and methods

Plant material

Helianthus annuus inbred line code number PH-BC_1-91A (obtained from the Institute of Field and Vegetable Crops in Novi Sad, Serbia and Montenegro) has been used for the protoplast isolation due to its high regeneration capacity, which was tested using the protocol of Paterson and Everett (1985) (unpublished results). The seeds were surface sterilized with 14% commercial bleach for 20 min, rinsed three times in the sterile distilled water, and dehulled. Dehulled seeds were sterilized again by soaking in 5% commercial bleach (for 60 min), rinsed three times in the sterile distilled water, and dry sterilized in thermostat at 45°C for 1 h (Taski and Vasic 2005). The seeds were germinated on MS medium (Murashige and Skoog 1962) supplemented with 2% sucrose and solidified with 0.8% agar. The seedlings were grown at 25°C in the dark.

H. maximiliani, accession 1631, was obtained from the wild *Helianthus* species collection of the Institute of Field and Vegetable Crops in Novi Sad, Serbia and Montenegro. This accession was found to be highly tolerant to the inoculation with *Sclerotinia* mycelium in field conditions (Skoric and Rajcan 1992). Apical shoots and nodal segments from a single plant were sterilized and propagated in vitro using a culture of apical shoots (Vasic et al. 2001b). Prior to transfer on propagation medium, the clones were dipped into 0.1% 4-(3-Indolyl)butanoic acid (IBA) solution for 4 min. The explants were grown on MS medium at 24°C and photoperiod 16 h (light)/8 h (dark). The same procedure was repeated every 2 weeks.

Protoplast isolation

Etiolated hypocotyls from the 7-day-old seedlings were cut longitudinally and plazmolysed in washing solution (M medium) (Vasic et al. 2001b). After 90 min, the M medium was replaced with a fresh one, supplemented with cell wall degrading enzymes according to Aslane-Chanabe (1991), and left for 17 h at 25°C. Protoplast mixture was filtered through 100 μm sieve and pelleted by centrifugation (70 g, 5 min). Pelleted protoplasts were purified by centrifugation in 10% Ficoll gradient according to Chanabe et al. (1989) at 1000 g for 20 min. Mesophyll protoplasts from *H. maximiliani* leaves were isolated according to the protocol of Vasic et al. (2001b). Protoplasts were purified by floating in 15% Ficoll gradient solution, and irradiated by UV rays (2 μmol m^{-2} s^{-1}) for 15 min.

Electrofusion

Protoplasts of sunflower and *H. maximiliani* were mixed at a ratio of 1:1. The protoplast density was adjusted to 6.7×10^5 ml^{-1} with TF solution (Aslane-Chanabe 1991). Electrofusion was carried out in Eppendorf Multiporator, in which protoplasts were fused with three pulses of 1250 V cm^{-1} for 30 μs. Electrofused protoplasts were incubated overnight in the dark at 25°C.

Protoplast culture and plant regeneration

Fusion products were embedded in 200 μl agarose solidified droplets (Shillito et al. 1983) at the final density of 10^5 ml^{-1} and incubated at 4°C for 1 h. The droplets were cultured according to the regeneration protocols of Krasnyanski and Menczel (1993), Wingender et al. (1996), and Trabace et al. (1995). The cultures were maintained in the dark at 25°C.

Microcalluses were released from the agarose droplets and transferred onto solid differentiation medium containing 2.2 mg l^{-1} 6-Benzylaminopurine (BAP) and 0.01 mg l^{-1} 1-Naphthylacetic acid (NAA) (Krasnyanski et al. 1992), but with the addition of silver nitrate (KR-R-Ag medium) (Vasic et al. 2001a), and incubated at 24°C with the photoperiod 16 h (light)/8 h (dark).

Well-developed calluses were transferred to the KR-R-Ag medium without BAP and NAA, but with 10 mg ml^{-1}

2,4-Dichlorophenoxyacetic acid (2,4 D) for 3 days and then transferred onto the KR-R-Ag medium. When the shoot elongation started, shoots were excised from the surrounding callus and placed onto the MS medium supplemented with 2% sucrose and solidified with 0.8% agar. The rooting of shoots occurred on the MS medium after the shoots were dipped into 0.1% IBA solution for 4 min.

Isozyme and RAPD analysis

The leaves of in vitro grown plants were homogenized in 0.5 M Tris-HCl buffer pH 6.8 with 1% 2-Mercaptoethanol and the crude extract was absorbed onto paper wicks (2 × 11 mm; Whatman 3MM). The samples were subjected to the starch electrophoresis. After electrophoresis, the gel was sliced horizontally into 1 mm slabs that were separately incubated in the specific staining solutions for visualization of the following enzymes: malate dehydrogenase (MDH; E.C. 1.1.1.37), acid phosphatase (ACP; E.C. 3.1.3.2), phosphohexose isomerase (PHI; E.C. 5.3.1.9), 6-phosphogluconate dehydrogenase (PGD; E.C. 1.1.1.44), phosphogluco mutase (PGM; E.C. 5.4.2.2), and aconitase (ACO; E.C. 4.2.1.3). Both electrophoresis and enzyme staining were performed according to Stuber et al. (1988).

DNA for RAPD analysis was isolated from the leaves of both parents and somatic hybrid, according to the protocol of Gentzbittel et al. (1994). The analysis was performed using ten 10-base primers from Operon Technologies, which were found previously to be unique markers for the wild *Helianthus* species (Sossey-Alaoui et al. 1998): A11 (5′-CAATCGCCGT-3′), A14 (5′-TCTGTGCTGG-3′), C02 (5′-GTGAGGCGTC-3′), C04 (5′-CCGCATCTAC-3′), C14 (5′-TGCGTGCTTG-3′), C15 (5′-GACGGATCAG-3′), C19 (5′-CCTCTAGACC-3′), E03 (5′-CCAGATGCAC-3′), E05 (5′-TCAGGGAGGT-3′), E09 (5′-CTTCACCCGA-3′), and E15 (5′-ACGCACAACC-3′).

All polymerase chain reactions (PCR) were carried out in a 25-µl reaction volume containing 2.5 µl buffer (Amersham Pharmacia Biotech); 0.2 mM dNTP; 0.5 µM primer; two units of *Taq* polymerase (Amersham Pharmacia Biotech) and 30 ng DNA. In the second PCR reaction, 2 µl of the PCR products were used instead of DNA. DNA was amplified in a termocycler (Biometra Tpersonal) at 94°C for 1 min followed by 35 cycles at 93°C for 1 min, 38°C for 30 s, 72°C for 30 s and final elongation at 72°C for 6 min. The PCR products were separated on 2% agarose gels containing 0.005% ethidium bromide. The gels were analysed under UV light.

Results and discussion

Protoplast isolation

The crude pellet containing isolated protoplasts obtained after the elimination of the enzyme solution was contaminated by debris from the broken cells. Moreover, *H.*

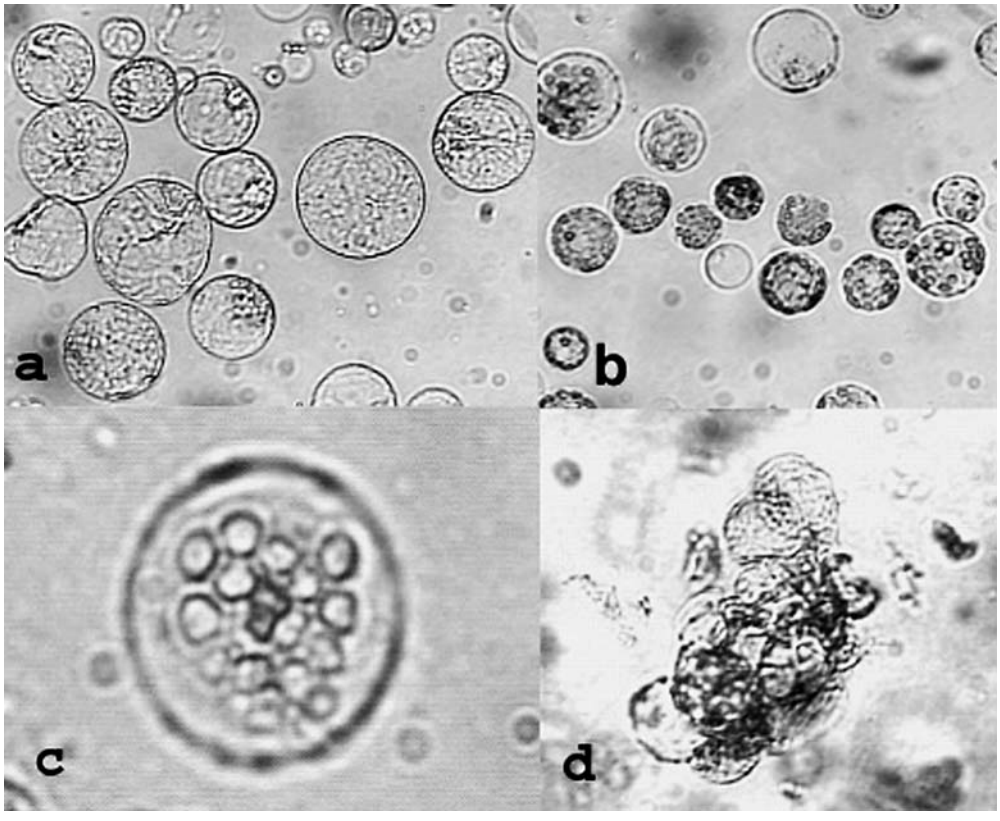

Fig. 1 Development of heterokaryons. **a** Hypocotyl protoplasts of *Helianthus annuus* inbred line. **b** Mesophyll protoplasts of *Helianthus maximiliani*. **c** Heterokaryons. **d** Protoplast-derived colonies

maximiliani protoplasts were very heterogeneous in size. Flotation in Ficoll gradient enabled the recovery of pure homogenous protoplast fractions in both species. The leaves of *H. maximiliani* grown in vitro gave high protoplast yield of about 5×10^6 g^{-1} of fresh weight, which is in accordance with the results obtained with the other wild *Helianthus* species: 3×10^6 g^{-1} of fresh weight from *H. giganteus*, 2×10^6 g^{-1} of fresh weight from *H. nuttallii* (Henn et al. 1998b), and 1.5×10^6 g^{-1} of fresh weight from *H. maximiliani* (Vasic et al. 2001b). *H. maximiliani* protoplasts were small with lots of chloroplasts (Fig. 1b). The yield of purified protoplasts isolated from the cultivated sunflower hypocotyls was $4–5 \times 10^5$ g^{-1} of fresh weight, which agrees with the results of Schmitz and Schnabl (1989), while> Wingender et al. (1996) and Krasnyanski and Menczel (1993) reported yield of $2–3 \times 10^6$ g^{-1} of fresh weight. The sunflower protoplasts were bigger then those of *H. maximiliani*, etiolated and with a large number of vacuoles (Fig. 1a).

Electrofusion and agarose droplets culture

Successful electrofusions resulted in the formation of heterokaryons (Fig. 1c). The fusion products were embedded in agarose droplets and subjected to different regeneration protocols. When regeneration protocols of Krasnyanski and Menczel (1993) and Wingender et al. (1996) were used, no callus formation was observed. Using the protocol of Trabace et al. (1995) during the first week of culture, symmetrical divisions of the protoplasts were observed and the protoplasts developed into small, macroscopic colonies (Fig. 1d). After 3–4 weeks in the culture, the number of white microcalluses became visible.

Callus cultivation and plant regeneration

Developed microcalluses that were transferred onto solid KR-R-Ag medium and exposed to the light continued to grow. Since it is known that the exposure of callus colonies to the high concentration of 2,4 D for a limited period of time induces subsequent development of somatic embryos (Krasnyanski and Menczel 1993), some of the vigorously growing calluses were treated with this hormone. The 2,4 D treatment improved the organogenic response of protoplast-derived calluses, since the shoot formation has been observed on two out of 350 calluses (regeneration frequency of 0.57%) when the calluses were returned onto the

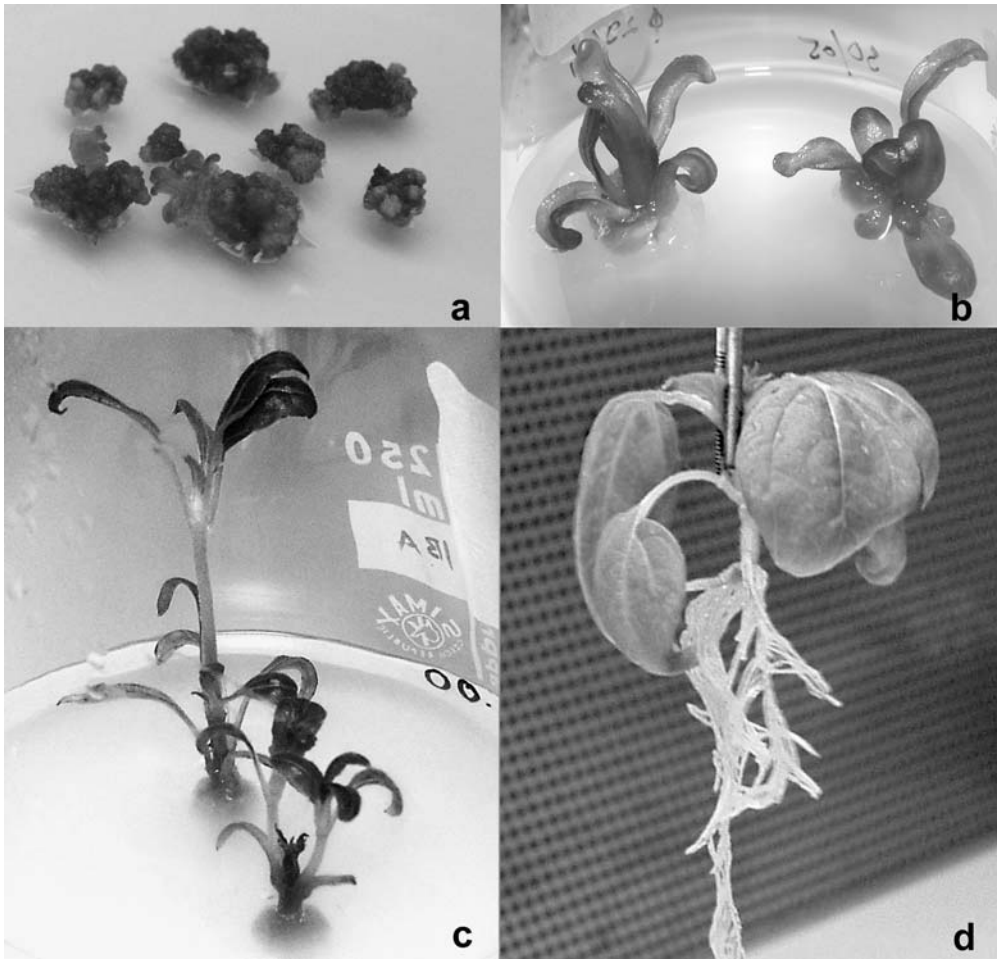

Fig. 2 Shoot regeneration and development from the fusion products. **a** Shoot regeneration. **b** Shoots with signs of hyperhydration. **c** Regenerated *Helianthus annuus* + *Helianthus maximiliani* hybrid plant. **d** Rooted hybrid plant

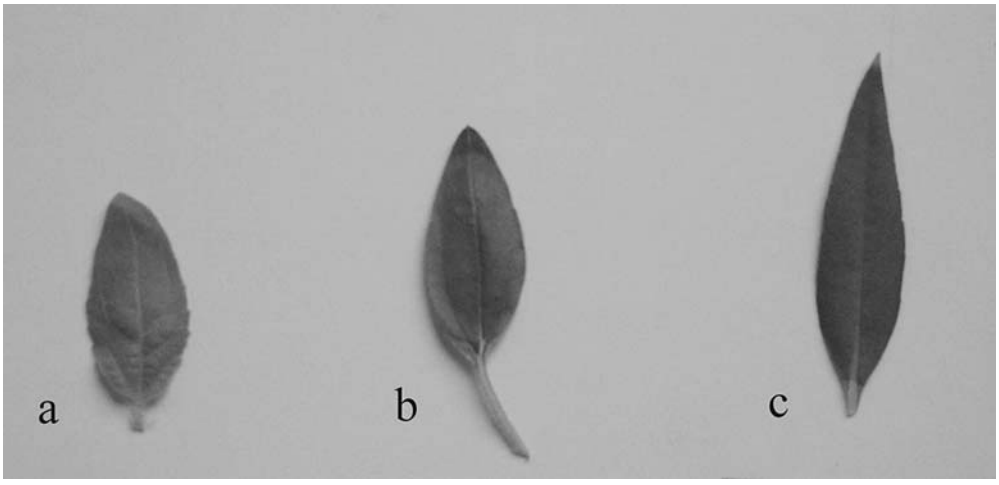

Fig. 3 Leaf shape of the hybrid plant and the parents. **a** PH-BC_1-91A. **b** PH-BC_1-91A + *H. maximiliani*. **c** *H. maximiliani*

KR-R-Ag medium (Fig. 2a). Beside 2,4 D treatment, genotype of the recipient parent could also be one of the factors that affected the shoot regeneration (Davey et al. 2005). Like Krasnyanski and Menczel (1993), we also used the cultivated sunflower genotype that was pre-screened for its regeneration capacity using the protocol of Paterson and Everett (1985). Trabace et al. (1995) also used a genotype of known regeneration capacity as a recipient in the fusion experiments.

In total, five shoots were obtained, three from one and two from the other regenerating callus. The shoots were further multiplied with varying success and fifteen clones were produced (Table 1). The clones showed signs of hyperhydration, although they were grown on KR-R-Ag medium that contained silver nitrate, which is known to be an inhibitor of ethylene action and agent that significantly reduces hyperhydration (Krasnyanski and Menczel 1993; Wingender et al. 1996). Krasnyanski and Menczel (1993) have reported that the shoots started to exhibit hyperhydration even in the presence of silver nitrate, if they were left on the regeneration medium for prolonged periods. In our study, the hyperhydration continued even when the shoots were excised from the surrounding callus and placed onto hormone-free MS medium containing silver nitrate (Fig. 2b). Since it is known that different factors can promote hyperhydration and silver nitrate is not always effective against it (Fischer et al. 1992), it was assumed that in our case hyperhydration was not the consequence of the gas exchange, but caused by an excess of cytokinins. Therefore, the shoots were transferred onto hormone-free MS medium without silver nitrate. It seems that time triggered the decrease of cytokinin level in the shoots, as hyperhydration was surpassed and the normal shoot elongation continued after the prolonged culture of shoots on the hormone-free medium (Fig. 2c). Well-developed shoots were rooted by dipping in a high-auxin solution, but rooting efficiency depended on the clone (Table 1). In contrast to Burrus et al. (1991) and Wingender et al. (1996), we used IBA instead of 3-Indolylacetic acid (IAA) or NAA. The root formation on auxin-treated shoots was observed after 3–4 weeks of culture on the hormone-free medium (Fig. 2d). The plantlets with well-developed roots were transferred to the growth chambers for acclimatization. In all the shoots in which a problem of hyperhydration was not completely solved, the treatment with IBA always resulted in callusing. According to the work of other authors, when regenerated shoots could not have been rooted they were grafted in order to be transferred in ex vitro conditions (Fischer et al. 1992; Krasnyanski and Menczel 1993).

Table 1 Regeneration and rooting of the shoots after somatic hybridization

	Regenerated shoots	No. of clones	No. of rooted clones
Regenerating calluses			
A	1	3	–
	1	1	–
	1	1	–
B	1	3	3
	1	7	–

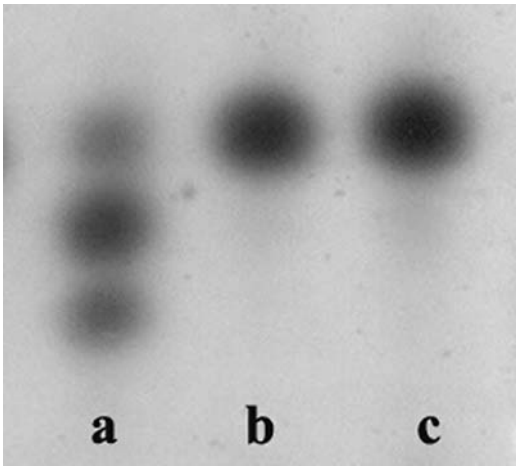

Fig. 4 Leaf PHI isoenzyme patterns. **a** *H. maximiliani*. **b** PH-BC_1-91A + *H. maximiliani*. **c** PH-BC_2-91A

Characterization of hybrid plants

Morphological comparisons between parents and R_1 plants were done. The R_1 plants were intermediate in the leaf length and width between the parents (Fig. 3). All plants were branched resembling those of the *H. maximiliani* parent. Zymograms of ACO, ACP and PGD did not reveal any polymorphism between the parents and regenerated plants. A polymorphism between the parents was observed on MDH, PGM and PHI (Fig. 4) zymograms, but the regenerated plants had the same allelic variant as the sunflower.

In order to prove the hybrid nature of regenerated plants, a molecular investigation of the regenerated plants was performed using RAPD markers. This type of markers is very useful in work with genus *Helianthus*, since the knowledge of the genome of the majority of wild species is quite sparse. The band patterns of the somatic hybrids obtained by primers A11, A14, C02, C04, C14 C15, C19, E03 (Fig. 5), E09, and E15 showed all characteristics of PH-BC_1-91A. The band characteristic for *H. maximiliani* was detected only on the RAPD profile generated with E05 primer, indicating the presence of a part of its genome in the hybrid plants (Fig. 6). The absence of bands characteristic for *H. maximiliani* in band patterns of the somatic hybrids generated by all, but one primer indicates asymmetric nature of these hybrids. It is probably the reason why there could not be detected any difference on isozyme level between the regenerated plants and sunflower. This is the result of the treatment with UV rays, which fragmentises the nuclear genome and has been successfully used for the production of asymmetric somatic hybrids in some other species (Vlahova et al. 1997; Forsberg et al. 1998).

In our work, we obtained the first somatic hybrid plants produced by electrofusion between the cultivated sunflower and its wild relative—*H. maximiliani*. The absence of bands characteristic for the wild parent in RAPD profiles generated by the majority of used primers indicates that produced hybrids are asymmetric, thus confirming the efficiency of UV light treatment. The studies are in progress in order to determine exact nature of the hybrids as well as their tolerance to *Sclerotinia sclerotiorum*. Since the introduction of tolerance to this pathogen into the cultivated sunflower is the ultimate goal of our work, in further experiments the fusion products will be cultured in the presence of oxalic acid, a known toxin of *S. sclerotiorum*, in order to increase the chances of regeneration of tolerant plants.

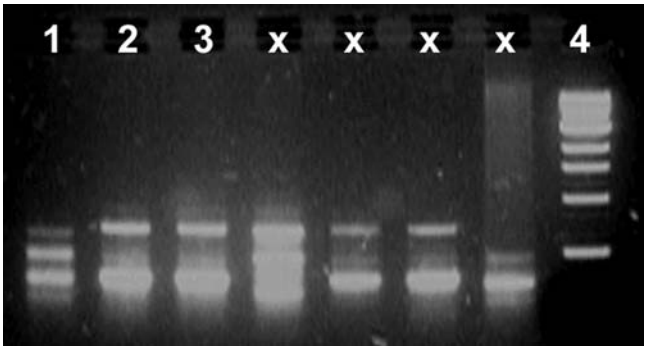

Fig. 5 RAPD profiles generated by the primer E03. 1 *H. maximiliani*, 2 PH-BC_1-91A + *H. maximiliani*, 3 PH-BC_1-91A, 4 100 bp extended DNA marker

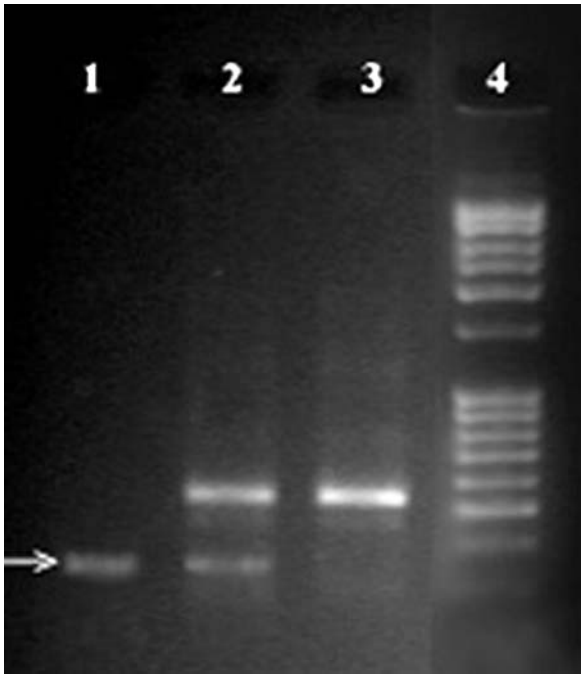

Fig. 6 RAPD profiles generated by the primer E05. 1 *H. maximiliani*, 2 PH-BC_1-91A + *H. maximiliani*, 3 PH-BC_1-91A, 4 100 bp extended DNA marker

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