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Electrofusion of protoplasts from *Solanum tuberosum, S. nigrum* and *S. bulbocastanum*

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Abstract

Leaf protoplasts of two wild species, *Solanum nigrum* var. *gigantea* (*S. ngr gig*) and *S. bulbocastanum* Dun. (*S. blb*), were electrofused with leaf protoplasts of two diploid potato clones, H-8105 and ZEL-1136, respectively, in order to confer the late blight-resistance from the wild species to the cultivated potato. The *S. ngr gig* mesophyll (+) H-8105 mesophyll combination resulted in regenerants of mostly normal *ngr* phenotype. Two regenerants from this combination were proved to be true hybrids by RAPD analysis but they rooted poorely *in vitro* and did not survive the transfer to soil. The *S. ngr gig* (+) H-8105 fusion combination was also performed with H-8105 cell suspension derived protoplasts enabling an easy identification of interspecific fusants on basis of their intermediate morphology. From the *S. ngr gig* mesophyll (+) H-8105 cultured cell combination, many abnormal shoots were regenerated. The two lines which survived had normal *ngr* phenotype but the presence of *tuberosum* (*tbr*) genome in those regenerants was not confirmed by RAPD analysis. No plants with *tbr* phenotype were obtained from both of *S. ngr gig* (+) H-8105 combinations. On the contrary, when *S. blb* mesophyll protoplasts were electrofused with ZEL-1136 mesophyll protoplasts, all regenerated plants had *tbr* phenotype, indicating much lower morphogenetic potential of *S. bulbocastanum* in comparison with that of *S. nigrum* var. *gigantea*. However, the hybridity of those regenerants has not been confirmed by RAPD analysis with two different primers. The efficiency of the applied fusion procedure and analysis of the regenerants is discussed.

Introduction

Wild *Solanum* species can be used as a source of valuable agronomic traits for introduction into cultivated potato through the method of somatic hybridization. Various disease resistance genes have been conferred to potato by somatic hybidization with wild species such as *Solanum acaule* (to potato virus X, Yamada *et al.* 1997), *S. brevidens* (to potato leaf roll virus, *e.g.* Helgeson *et al.* 1986; to late blight , Rokka *et al.* 1994), *S. circaeifolium* (to late blight, Matthej *et al.* 1992), *S. commersonii* (to bacterial wilt, Laferriere *et al.* 1999) and *S. etuberosum* (to potato virus Y, Novy & Helgeson 1994).

Our aim was to incorporate the genes of resistance to *Phytophthora infestans*, a fungal pathogen causing the late blight disease in potato, from the related wild species into potato genome. Several research groups succeeded in generating interspecific somatic hybrids with high resistance to late blight transferred from the wild fusion partner (Matthej *et al.* 1992; Rokka *et al.* 1994; Helgeson *et al.* 1998). However, an abundance as well as high variability and adaptability of *Phytophthora* races make the creation of durably resistant potato cultivars a permanent challenge for potato breeders.

We have chosen two wild species, *S. nigrum* var. *gigantea* and *S. bulbocastanum*, as sources of late blight resistance in an attempt to generate the interspecific *ngr* + *tbr* and *blb* + *tbr* somatic hybrids with an improved resistance to *P. infestans*. The potato fusion partners - diploid clones H-8105 and ZEL-1136 - are susceptible to the pathogen. The electrofusion procedure was applied as an alternative to chemical PEG-mediated fusion. This hybridization technique allowed simultaneous monitoring of the protoplast fusion in the electric field. For easier identification of the formed heterokaryons, the mesophyll protoplasts of *S. nigrum* species were also fused with the cell suspension derived protoplasts of H-8105 clone. In the last case, the heterokaryons were detected by the intermediate morphology: green chloroplasts from mesophyll protoplasts and cytoplasmic strands from suspension cell protoplasts.

Three electrofusion combinations were performed: (1) *S. nigrum* var. *gigantea* (+) H-8105 (both mesophyll protoplasts), (2) *S. nigrum* var. *gigantea* (+) H-8105 (mesophyll and cell suspension protoplasts, respectively), and (3) *S. bulbocastanum* (+) ZEL-1136 (both mesophyll protoplasts). The preselection of putative hybrids was based on their leaf and flower morphology. The hybridity was verified by RAPD analysis of the nuclear DNA. The ploidy level of regenerants was determined by flow cytometry method. In this article we report the results of morphological and molecular analysis of the products recovered as a result of three electrofusion combinations.

Material and methods

Genotypes

A. Wild Solanum species, resistant to P. infestans:

- *S. nigrum* var. *gigantea* (*S. ngr gig*, hexaploid, 2n = 6x = 72, non-tuberizing)

- *S. bulbocastanum* (*S. blb*, PI243512, clone 112/23, diploid, 2n = 2x = 24, tuber-bearing)

B. Cultivated potato:

- *S. tbr* clone H-8105 (dihaploid, 2n = 2x = 24) from Hettema, Holland; obtained from crosses of secondary dihaploid parental forms MH-73-17-1277 × SH-77-45-35; mid-early maturing; susceptible to *P. infestans*: field resistance scored 2 in 1-9 grade scale (9 = resistant in leaflet test)

- *S. tbr* × *S. chc* × *S. yun* interspecific hybrid ZEL-1136 (diploid, 2n = 2x = 24); ZEL-1136 = H8106 × 15/39-40 (H8106 = *S. tbr* dihaploid from Holland); clone 15/39-40 is the clone No. 52 from self-fertilization of OPS5; origin of self-compatible parental clone OPS5 (Pawełczak 1991): F_1 LI356 × (B16 x G609) P127; LI356 = *chc* CPC-3785 × *yun* GLK-s 67.107/3R; B16, G609 = dihaploids of *S. tbr* of Dutch origin; ZEL-1136 is considered very susceptible to *P. infestans*: field resistance scored 2 in 1-9 grade scale (9 = resistant in leaflet test) [*chc* = *S. chacoense*, *yun* = *S. yungasense*].

Plant material

The plants were propagated *in vitro* on hormone-free MS medium (Murashige & Skoog 1962) with 2 % sucrose and 0.6 % agar. The fully expanded leaves of 3-4 week old plants were excised and preconditioned according to Haberlach *et al.* (1985). Cell suspension of H-8105 clone was cultured in MS medium enriched with Gamborg vitamins (Gamborg *et al.* 1968), glycine 2 $mg \cdot l^{-1}$, casein hydrolysate 0.5 $mg \cdot l^{-1}$, NAA 5 $mg \cdot l^{-1}$, BAP 0.1 $mg \cdot l^{-1}$, and 3 % sucrose, on a rotary shaker (120 rpm) at 26 °C in the dark. The cell suspension was subcultured every 7 days. The cell samples were usually taken on the 5th day after subculturing.

Protoplast isolation

Cells from suspension culture and preconditioned leaves were digested overnight in the dark in K4 medium (Nagy & Maliga 1976) with 1.6 % cellulase Onozuka R-10 and 0.6 % macerozyme R-10 (cultured cells) or 0.4 % cellulase and 0.2 % macerozyme (leaves), at 28 °C (cultured cells) or 23 °C (leaves). Following the cell wall degradation, the protoplasts suspended in digestion medium were poured through steel filters of 74 µm (leaf protoplasts) or 100 µm (protoplasts from cultured cells) pore size, and centrifuged at 80g for 15 min in MPW-340 centrifuge with 1 ml of W5 medium applied to the top of suspension. Protoplasts were collected from K4/W5 interphase and suspended in W5 medium (Menczel *et al.* 1981).

Electrofusion procedure

The home-constructed apparatus (Pawłowski 1995) was used. The equipment linked an oscillator and a pulse generator connected with an electrode chamber. A 300 µl aliquot of a mixture (1:1) of protoplasts from each parent ($10^6 \cdot ml^{-1}$) in 0.5 M sorbitol with an addition of Biotoxym (= Cefotaximum, Institute of Biotechnology and Antibiotics, 1 ppm) was placed in fusion chamber. In order to align the protoplasts, an AC-field at 125 V/cm and 1 Mhz was applied for 15 s; subsequently, one square pulse of 1.2 kV/cm was applied for 40 s to achieve the protoplast fusion. Fusion was monitored by video-recording of the microscopic image of fusion chamber. Five to six fusion treatments were performed per each combination. Immediately after fusion, the protoplasts were transferred to the sterile W5 solution (Menczel *et al.* 1981) supplemented with 1 ppm of Biotoxym. After two hours, the sedimented protoplasts were suspended in liquid SKM medium and cultured as described below.

Protoplast culture

The procedure and media used for regeneration of shoots from leaf- and cell suspesion derived protoplasts were generally as those of Austin *et al.* (1993). Briefly, the fused and parental protoplasts were cultured for 3-5 days in liquid SKM medium without BSA, with two 50 % dilutions with fresh SKM medium on the 1st and 4th day after isolation. On the 4th or 5th day, the cultures were solidified with 0.5 % SeaPlaque agarose and further cultured on SKM+1 % BSA+0.8 % agar layer for at least 2 weeks at 26 °C (1st week in dark, 2nd week in dim light). Calli of about 1mm in diameter were transferred to culture (Cul) medium (Haberlach *et al.* 1985) for growth and greening for 2-3 weeks.

Plant regeneration

The green calli of 3-4 mm in diameter were transferred to MSR1 medium (SA4 medium of Austin *et al.* 1993) for shoot initiation. After 2 weeks, the calli were transferred to MSR2 medium with 2 $mg \cdot l^{-1}$ *t*-zeatin and 2 $mg \cdot l^{-1}$ GA_3, for shoot elongation. The differentiating calli were incubated at 22 °/19 °C and a 16h/8h day/night cycle with illumination of 150 $\mu E \, m^{-2} \cdot s^{-1}$. The detached shoots were rooted in hormone-free MS medium with 2 % sucrose and 0.6 % agar. To assess the morphology, the *in vitro* cultured regenerants were first transferred to the swelling peat pots (AgroWIT, Zielona Góra, Poland) and then, after a week, to 14-cm pots with autoclaved soil for growing in the greenhouse.

Flow cytometry and RAPD analysis

The ploidy level of the recovered regenerants was determined by flow cytometry method as described by Maciejewska *et al.* (1999).

For RAPD analysis, DNA was isolated from *in vitro* grown plants according to a modified method of Xu *et al.* (1993). Samples were collected by pinching out leaf discs with tube lids into sterile Eppendorf tubes (one disc per sample) and frozen in liqid nitrogen. The leaf discs were macerated with a teflon pestle in 400 µl of extraction buffer (100 mM Tris-HCl, pH 8.0, 500 mM EDTA, 0.7 $\mu l \cdot ml^{-1}$ 2-mercaptoethanol) for 15 s at room temperature. The extracts were centrifuged at 1300 g for 5 min at 4 °C and 350 µl portions of the supernatants were transferred to new Eppendorf tubes. The supernatants were incubated with 4 µl RNAse (stock 10 $mg \cdot ml^{-1}$) for 30 min at 37 °C. To precipitate the DNA, 870 µl of 96 % ethanol was gently mixed with the supernatant and left at -20 °C for 20 min. After centrifugation, the pellets were vacuum dried and dissolved in 100 µl of sterile distilled water. One µl of each sample was used as a template for DNA amplification.

Polymerase chain reaction (PCR) was performed in Gene Amp PCR System 2400 (Perkin Elmer) in 25 µl of reaction mixture containing: 0.5 U Dynazyme II DNA polymerase (Finnzymes OY), 1 x buffer and 1.5 mM $MgCl_2$ (supplied with the enzyme), 0.4 µM primer, 0.25 mM of each dNTP (Gibco-BRL) and the DNA template. The PCR cycling conditions: one cycle of 30 s at 94 °C followed by 45 cycles of 6 s at 94 °C, 18 s at 36 °C, 74 s at 72 °C, with a final extension of 5 min at 72 °C.

The amplification products (12.5 µl) were separated by electrophoresis in 1.5 % agarose in 0.5 xTBE buffer (Tris-borate-EDTA, Sambrook *et al.* 1989) at room temperature and stained with ethidium bromide. A 1 kb DNA ladder (Gibco-BRL) was used as a molecular marker. Decamer oligonucleotide primers from Operon Technologies Inc. were

preliminary tested to detect species specific amplification products. The RAPD patterns produced by primers OPA-16 (5'AGCCAGCGAA3') and OPH-04 (5'GGAAGTCGCC3') for each parental species were used to identify somatic hybrids. Negative control – the reaction mixture without genomic DNA – was run with each amplification. Since the primers are capable of forming complicated secondary structures, the production of amplification artifacts could be observed in the absence of genomic DNA. For that reason, the bands appearing in negative control were not taken into account as RAPD markers.

Results

Regeneration of the parental protoclones

Plant regeneration from the protoplasts of fusion partners was previously described in details (Szczerbakowa *et al.* 2000). The parental protoplasts responded to culture conditions differently: the regeneration (shoot formation) was achieved for mesophyll protoplasts of both potato protoclones and *S. nigrum* var. *gigantea* but failed in case of H-8105 cell suspension protoplasts and *S. bulbocastanum* mesophyll protoplasts. However, as shown by flow cytometry analysis (Table), the regenerants from protoplasts of diploid potato clones were tetraploids as a result of spontaneous fusion or polyploidization occurring during protoplast regeneration. This phenomenon was often reported in literature (*e.g.* Karp *et al.* 1982, Ramulu *et al.* 1983, Austin *et al.* 1985, Debnath and Wenzel 1987), being explained by the process of stabilization of the potato genome and its return to the most stable tetraploid level. Protoplasts isolated from H-8105 cell suspension culture did not regenerate shoots under the established culture conditions probably because of the partial loss of totipotency during the prolonged culture of cell suspensions.

Table. Ploidy level of the *in vitro* regenerants from the parental and electrofused protoplasts as determined by flow cytometry method

Regenerant number	Electrofusion combination	Ploidy level
S. ngr gig	protoclone	6x
H-8105	protoclone	4x
ZEL-1136	protoclone	4x
16	*S. ngr gig* $_{mes}$	10x±
17	(+) H-8105$_{mes}$	8x-
100		8x+
104		mixoploid
96	*S. ngr gig* $_{mes}$	7x+
138	(+) H-8105$_{sc}$	7x
22	*S. Blb*$_{mes}$ (+) ZEL-1136$_{mes}$	4x
76		4x
101		4x

The plants of the parental species established in soil are presented in Fig. 1 A,B and Fig. 4 A,B. After regeneration from the protoplasts, the plant vigor and appearance did not change for *S. nigrum* var. *gigantea* (Fig. 1C) and ZEL-1136 (not shown). As for H-8105 , the regenerants were much weaker, had creepy stems and mostly simple undissected leaves (Fig. 1D).

Combination S. ngr gig $_{mes}$ *(+) H-8105*$_{mes}$

There were 7 shooting and 4 non-shooting calli formed after electrofusion of mesophyll protoplasts of *S. ngr gig* and dihaploid potato line H-8105. Four shoots were detached for rooting. The largest shoot that was the most vigorous and the earliest to root, turned out to be a chimeric plant - as judged by variegated leaves - of *S. ngr gig* phenotype (Fig. 2A).The second shoot also produced a plant of *ngr* phenotype (Fig. 2B). Those plants were numbered 16 and 17, respectively. Flow cytometry analysis showed the regenerant 16 to be at decaploid (10x±) level while the regenerant 17 was a hypooctoploid (8x-) (Table). On the contrary, the third and the fourth shoots grew very slowly and produced calli instead of roots on MS medium (Fig. 2C). Those plants, numbered 100 and 104, remained dwarf even after many passages on MS propagation medium, and produced large calli and scare roots. Regenerants 100 and 104 had small dark green leaves settled close to each other, without internodes. Those clones, although maintained *in vitro*, did not survive the transfer to soil, while the regenerants 16 and 17 grew vigorously in pots, flowered profusely in the greenhouse and set berries spontaneously (without controlled pollination), with fully developed seeds. As shown by RAPD analysis, the regenerants 16 and 17 had only *ngr* genome (Fig. 5A, lanes 6,7) while the regenerants 100 (Fig. 5A, lane 5) and 104 (not shown) contained the combined ge-

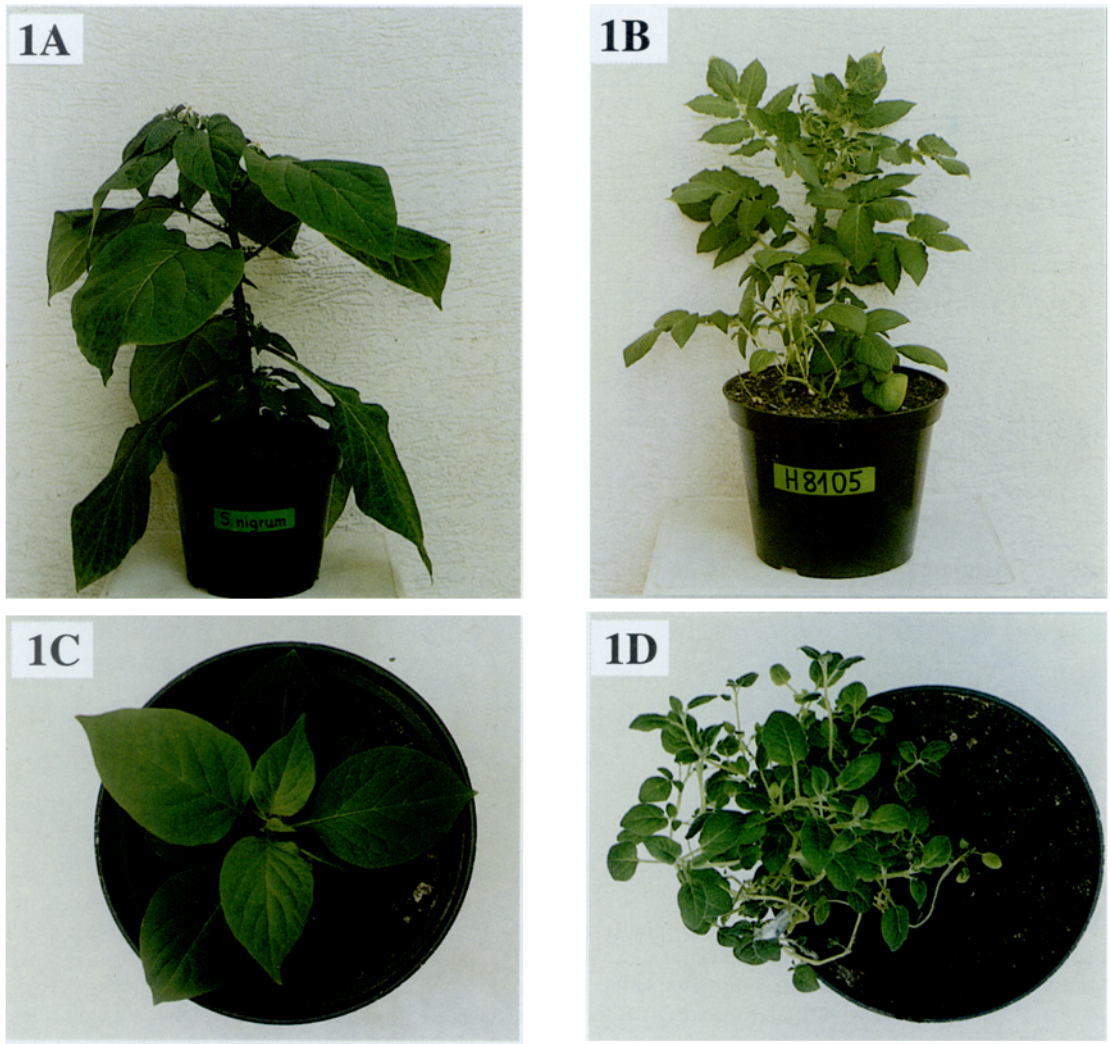

Fig. 1. Plants of the parental species, hexaploid *S. nigrum* var. *gigantea* (**A, C**) and dihaploid potato clone H-8105 (**B, D**), transferred to soil from *in vitro* culture (**A, B**) and after regeneration from mesophyll protoplasts (**C, D**). Plant age in weeks since the last *in vitro* passage: 7 (**A**), 8 (**B**), 4 (**C**), 21 (**D**).

nome. The regenerant 100 was found to be a hyperoctoploid, and the regenerant 104 was a mixoploid (Table).

Combination S. ngr gig $_{mes}$ *(+) H-8105*$_{sc}$

Nine shooting calli were recovered after electrofusion of *S. ngr gig* mesophyll protoplasts with cell suspension derived protoplasts of the potato dihaploid H-8105. Four of the calli were about 5 mm in diameter but only three of them produced abnormal shoots, too short for excision. The other five calli were larger (about 10 mm in diameter), four of them survived and six shoots were detached for rooting from the three of them. Two shoots formed large calli, distorted leaves and no stems, and were discarded. The other four rooted shoots were propagated. Finally, only two clones survived: number 96 from the dwarf plant that rooted poorly (not shown), and number 138 from the most vigorous shoot (Fig. 3). Both regenerants had *ngr* phenotype under the greenhouse conditions.The flow cytometry analysis showed the ploidy to be at 7x level for both regenerants in relation to the 6x level of the parental *S. ngr gig* (Table). However, the RAPD analysis did not reveal the presence of *tbr* genome in those regenerants (profiles not shown).

Combination S. blb$_{mes}$ *(+) ZEL-1136*$_{mes}$

Of 33 calli surviving three passages on elongation (MSR2) medium (five months of culture), only one

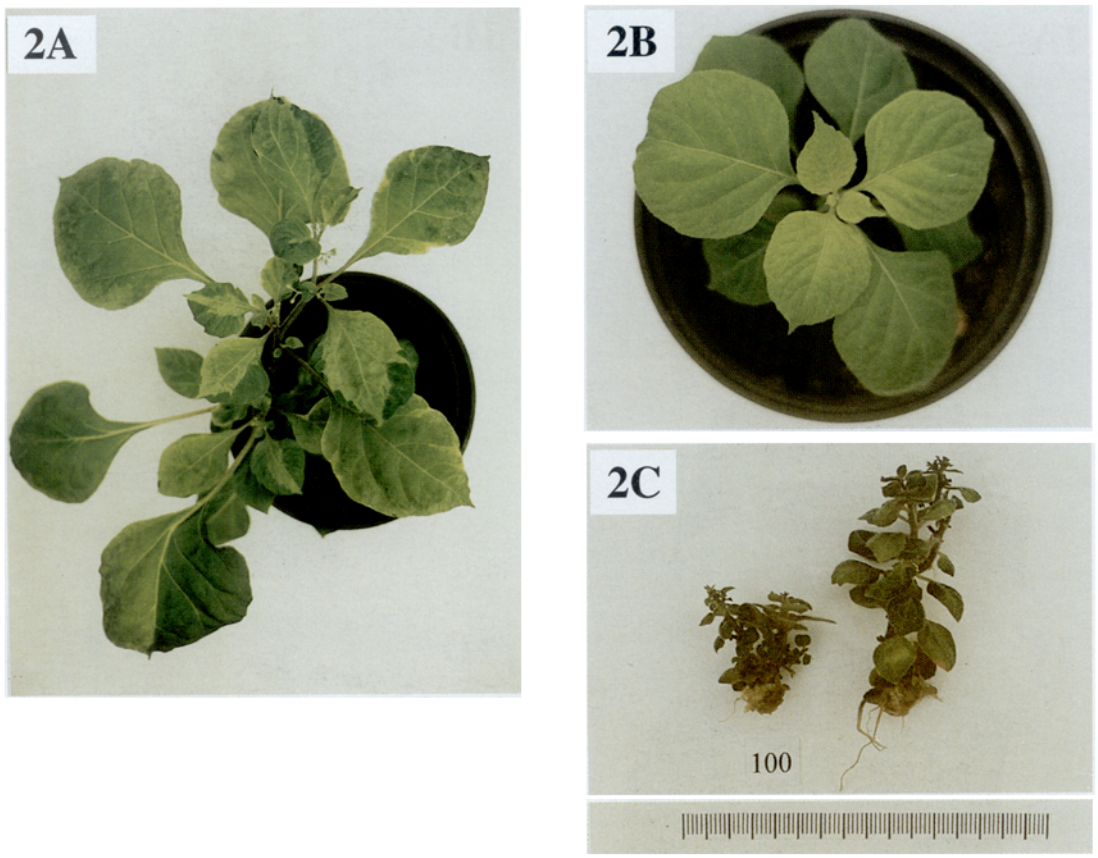

Fig. 2. Plants regenerated after electrofusion of mesophyll protoplasts of hexaploid *S. nigrum* var. *gigantea* with mesophyll protoplasts of dihaploid potato clone H-8105. **A**: leaf variegation indicates the chimeric nature of the regenerant 16 (10x). **B**: leaves of the regenerant 17 (8x-) are more rounded in comparison with a plant regenerated from the parental *S. ngr* protoplasts (Fig. 1C). **C**: dwarf *in vitro* plants of the regenerant 100 (8x+) are characterized by stunted growth, callus formation and scare roots. Plant age in

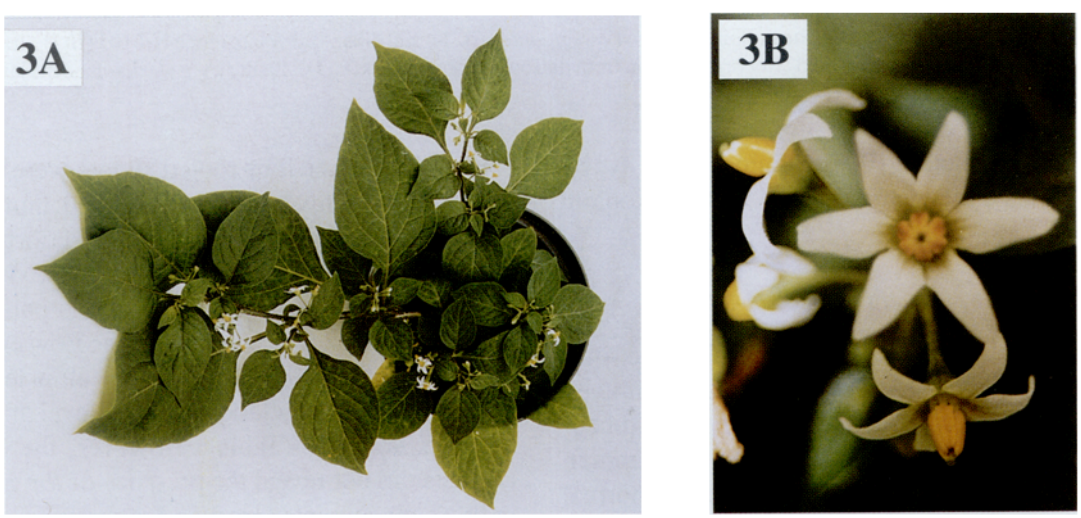

Fig. 3. Plant regenerated after electrofusion of mesophyll protoplasts of hexaploid *S. nigrum* var. *gigantea* with cell suspension derived protoplasts of dihaploid potato clone H-8105. The regenerant 138 (7x) has both leaves (**A**) and flowers (**B**) of *nigrum* type. Plant age: 7 weeks.

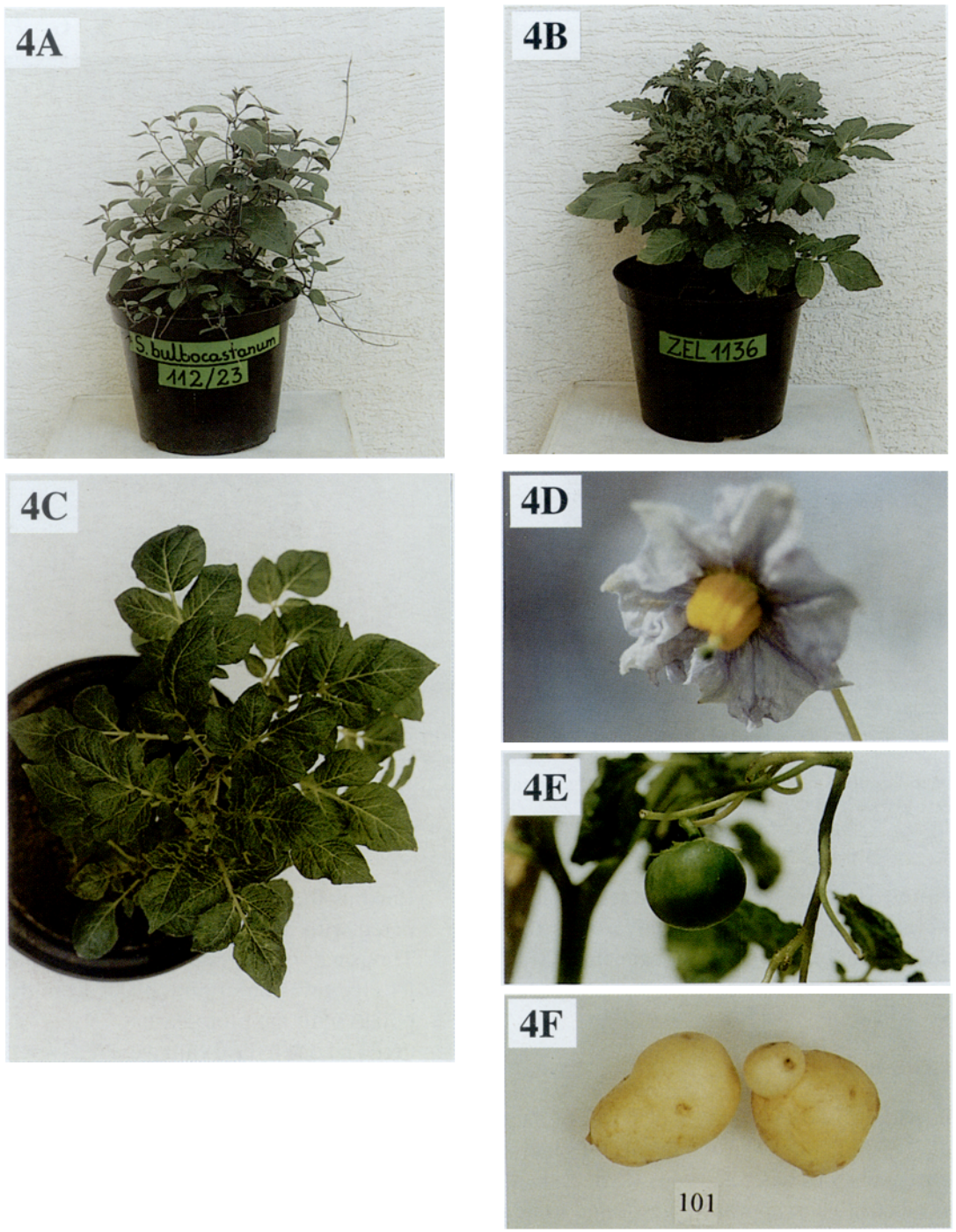

Fig. 4. Plants of the parental species, diploid *S. bulbocastanum* (**A**) and diploid interspecific *S. Tbr x S. Chc* x *S. yun* hybrid ZEL-1136 (**B**), as well as a typical tetraploid plant (101) regenerated after electrofusion of mesophyll protoplasts of *S. blb* and ZEL-1136 (**C**). Flower (**D**), parthenocarpic fruit (**E**) and tuber (**F**) of the regenerant 101 are characteristic for ZEL-1136. Plant age in weeks: 8 (**A**), 9 (**B**), 12 (**C**), 18 (**D**), 21 (**E**), 29 (**F**).

callus produced three shoots that were rooted and propagated. Some of the other calli also had shoot primordia but they did not develop into normal shoots. All the cultured calli turned brown and were discarded 10 months after electrofusion. Of the three lines which survived, one line (number 101) flowered in the greenhouse, set green seedless berries and tubers (Fig. 4 C-F). Flow cytometry analy-

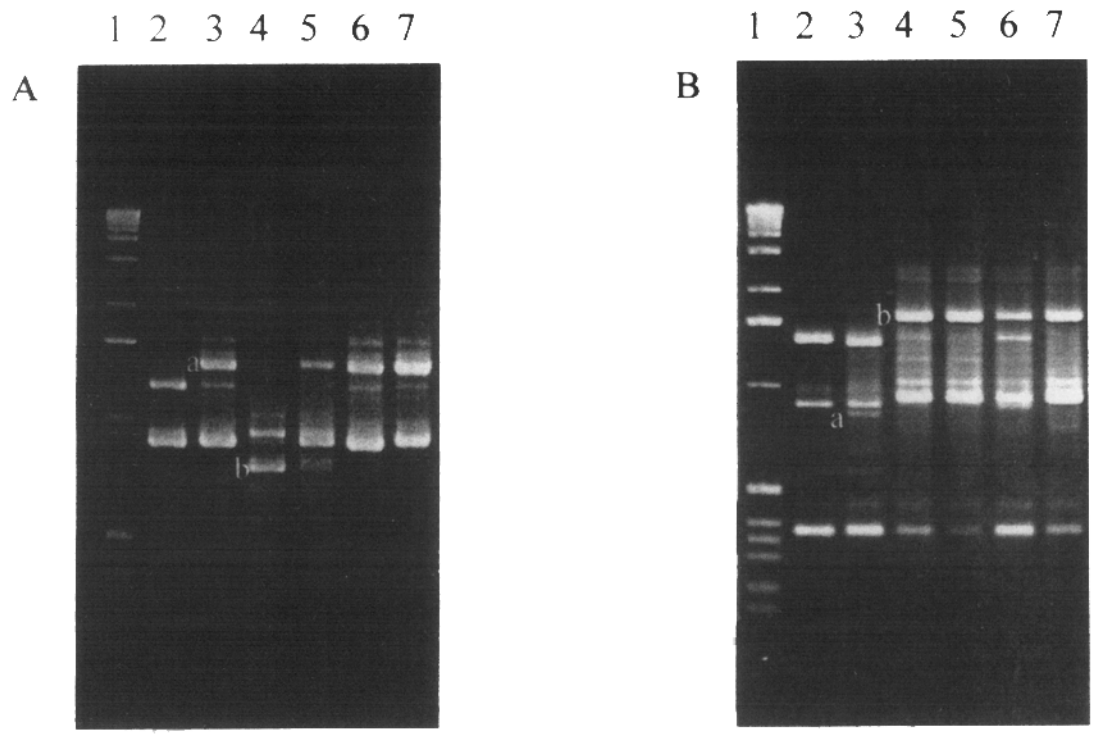

Fig. 5. RAPD profiles of the parental clones and electrofusion products from the combinations *S. ngr gig* $_{mes}$ (+) H-8105$_{mes}$ (**A**) and *S. blb*$_{mes}$ (+) ZEL-1136$_{mes}$ (**B**).
A: lane 3, *S. ngr*; lane 4, H-8105; lanes 5-7, regenerants 100, 17, 16, respectively; a, b, bands specific for *S. ngr* and H-8105, respectively.
B: lane 3, *S. blb*; lane 4, ZEL-1136; lanes 5-7, regenerants 22, 76, 101, respectively; a, b, bands specific for *S. blb* and ZEL-1136, respectively.
A, B: lane 1, a 1 kb DNA ladder (BRL); lane 2, negative control. Amplification was made with primer OPH-04.

sis proved those regenerants to be at tetraploid level (Table). Until now, RAPD analysis has not confirmed the hybridity of those regenerants (Fig. 5B, lanes 5-7).

Discussion

The widely used technique of electrofusion usually induces a high rate of fusion events thus increasing the frequency of heterokaryons (Fish *et al.* 1988). The electrofusion approach was found to be more effective than chemical fusion, though electrofusion yielded multiple fusion products more frequently (Pehu *et al.* 1989). The conditions employed for electrofusion (alignment field conditions, length of alignment time, fusion pulse voltage and duration, as well as protoplast density and fusion medium composition) had a strong effect on the genetic combinations found in generated hybrids (Jones 1988). Serraf *et al.* (1991) reported an average frequency of electrofusion of 30 %, defined as the percentage of fused protoplasts compared with the number of the aligned protoplasts. Thousands of calli were recovered in their study after electrofusion of protoplasts from the dihaploid potato with the protoplasts of *S. berthaultii.* Hundred and one hybrid calli were recovered after electrofusion between the diploid *S. tuberosum* and the diploid *S. circaeifolium* protoplasts (Mattheij *et al.* 1992). Plants were regenerated from 17 different calli but only nine of them rooted and could be transferred to soil, eight showing a hybrid and one a parental morphology. Sixteen calli were regenerated after electrofusion of the dihaploid potato protoplasts with the protoplasts from diploid *S. pinnatisectum,* while more than two hundred calli were regenerated from electrofusion combination of a sexual hybrid line *S. pinnatisectum x S. bulbocastanum* (diploid) with a triploid potato (Thieme *et al.* 1997). Cytophotometric analysis of the somatic hybrids revealed a wide variation in their ploidy level induced by multifusion or polyploidization events.

On the other hand, Yamada *et al.* (1997) obtained only 13 calli and recovered 18 regenerants, including 14 somatic hybrids, after electrofusion of mesophyll protoplasts of *S. tuberosum* tetraploid cv. Dejima and dihaploid *S. acaule*. The combining ability of different genotypes was found to be highly variable within the same species, as well as between the related species, and was presumably influenced by both the genotype and the ploidy level of fusion partners (Horsman *et al.* 1997).

In the current study, a low number of regenerants was recovered from all three fusion combinations. The observed bursting of mesophyll protoplasts in the electric field could limit the recovery of fusants in mesophyll (+) mesophyll protoplast combinations. Tempelaar *et al.* (1987) proposed an addition of 1 mM Ca^{2+} ions in order to improve the protoplast integrity and to enhance the fusion yield. In our case, however, the presence of divalent calcium cations caused the overheating of protoplast mixture and, consequently, the death of the protoplasts.

Though the protoplasts from suspension-cultured cells were found to be more stable in the electrical field than mesophyll protoplasts, the low totipotency of suspension-cultured cells could be a reason for low number of regenerating calli in mesophyll (+) cell suspension protoplast combination. Tempelaar & Jones (1985) reported a lower yield of electrofusion products for cell suspension derived protoplasts in comparison with mesophyll protoplasts, and recommended to increase the density of cell suspension derived protoplasts in the mixtures with leaf protoplasts to favor the heterofusion. In our experiments, however, single fusions between the mesophyll and cell suspension derived protoplasts were frequently observed and the heterokaryons were found to be viable and able to divide after three to four days in culture. As shown previously, the ploidy level of H-8105 suspension cells varied much and many of the cells contained two or three nuclei instead of one (Maciejewska *et al.* 1999) indicating the disturbances in cell division of the cultured cells. Thus, the various ploidy of H-8105 cell suspension derived protoplasts was probably responsible for the abnormalities in regeneration of this protoclone and its putative fusants with mesophyll protoplasts of *S. nigrum*.

Higher morphogenetic potential of a hexaploid *S. nigrum* var. *gigantea* in comparison with weak dihaploid potato clone could explain the preferable regeneration of *ngr* polyploids as the ones better surviving electrofusion. When the similar fusion was induced by PEG (data not shown), the autotetraploids of H-8105 were also found among the recovered regenerants in *S. ngr gig* $_{mes}$ (+) H-8105_{mes} combination, probably indicating an advantage of chemical fusion procedure in comparison with electrofusion. On the other hand, the large size of the expected octoploid hybrid genome in the *S. ngr gig* (+) *S. tbr* combinations could cause the abnormal growth of regenerating hybrids, as seen in the regenerants 100 (Fig. 2C) and 104 (not shown). An abundance of distorted leaves on the calli regenerating after electrofusion also reflected the growth disturbances. As only two regenerants from *S. ngr gig* (+) *S. tbr* combinations were proved by RAPD analysis with two different primers to be true hybrids, it might happen that a small part of dihaploid potato genome included into the nearly octoploid genome of the regenerants with dominant *ngr* phenotype was not revealed by this method.

The same could be true for the tetraploid regenerants obtained from *S. blb* (+) *S. tbr* combination: used primers could be inefficient in revealing the genome hybridity in case of disproportional combination of the parental genomes. Several primers from over one hundred tested, were used for revealing polymorphism between the tetraploid potato and *S. bulbocastanum* by Helgeson *et al.* (1998), who succeeded in generation of true *blb* + *tbr* somatic hybrids with *tbr* phenotype after PEG-mediated fusion. In our experiments, the parental ZEL-1136 clone regenerated from the protoplasts as vigorous tetraploid and thus also could be present among the products of electrofusion. In such a case, the after-fusion regenerants with *tbr* phenotype could represent the homozygous autotetraploids of ZEL-1136 (Fig. 4).

Though a tuber-bearing *S. bulbocastanum* is a preferable fusion partner for *S. tuberosum* in comparison with a non-tuberizing *S. nigrum,* the comparatively small size of *S. blb* diploid genome as well as low morphogenetic potential of *S blb* leaf protoplasts might limit the generation of its interspecific somatic hybrids with potato in the performed fusion

combination. After PEG-induced fusion between the same partners, only one tetraploid regenerant did have a modified potato phenotype but again the RAPD analysis did not reveal the presence of *blb* DNA in its genome (data not shown). On the other hand, it is known (Waara *et al.* 1992) that changes in chromosome number and structure occurring in potato somatic hybrid clones during a prolonged period of culture and regeneration, could be responsible for different morphology of regenerated shoots. However, the use of RAPD for routine screening of the putative somatic hybrids could be insufficient if the amount of DNA introgressed from one of the fusion partner is too low (*i.e.* below 8 %, Oberwalder *et al.* 1997). Further analysis of the putative hybrid DNA by *in situ* hybridization technique is planned for checking the genome origin of the obtained regenerants expressing the phenotype of a parent dominating in ploidy and vigor.

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