Plant Science, 86 (1992) 203–214
Elsevier Scientific Publishers Ireland Ltd.

Somatic hybridization between *Lycopersicon esculentum* and non-tuberous *Solanum* species of the *Etuberosa* series

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(Received April 3rd, 1992; accepted July 3rd, 1992)

Intergeneric somatic hybrid plants have been obtained by protoplast fusion between leaf mesophyll protoplasts from two cultivars of tomato (cv. Tamina and plastome mutant Pl-alb I) and mesophyll protoplasts from two representatives of the *Etuberosa* series of the genus *Solanum* (wild species *S. etuberosum* and sexual interspecific hybrid *S. brevidens* × *S. etuberosum*). A hybrid selection scheme was based on the faster growth of hybrid microcalli in comparison with parental calli and on the morphological characteristics of hybrid calli and hybrid regenerants. Hybridity was confirmed by morphological and cytological characteristics, esterase and peroxidase isozyme analyses. Somatic hybrids were also characterised by Southern hybridization of the 5,3 kb probe of the tomato meiotic gene mL-I. Twelve out of sixty hybrids produced fruits after selfing; however, their fruits had abortive seeds. Three progeny plants were obtained via embryo culture after selfpollination of intergeneric somatic hybrids.

Key words: Lycopersicon; Solanum; protoplast fusion; somatic hybrids; morphology; isozymes; cytogenetics

Introduction

Solanum etuberosum Lindl. and *S. brevidens* Phil. are wild diploid species of the *Etuberosa* series from the *Petota* section of the *Solanum* genus. The *Etuberosa* species are reproductively isolated [1,2] and they occupy a special position among the tuber-bearing species of the section *Petota*, differing from them in their morphological, biochemical and cytological characteristics. Along with typical potato traits (leaf and flower shape, purple corolla, not adnate anthers) the *Etuberosa* species however do not form tubers and stolones. According to a nuclear DNA restriction fragment length polymorphism *Etuberosa* species are most distantly related to the other *Petota* species [3]. This does not contradict cytological research: genome analysis showed a unique genome E in the *Etuberosa* series [4]. Also, *Etuberosa* species cytoplasmically are very far from all tuber-bearing *Solanum* species and according to chloroplast genome analyses the series *Etuberosa* occupies approximately intermediate position between the genus *Lycopersicon* and tuber-bearing *Solanum* species, but it is closer to the true potatoes [5]. This is not in conflict with crossability research data. If the sexual hybrids of *Etuberosa* species with wild tuber-bearing Solanum species can be obtained, though with considerable difficulty [6], crosses between *S. etuberosum* and *Lycopersicon esculentum* were absolutely unsuccessful [7].

Etuberosa species are rich sources of germplasm improvement for both cultivated tomato and potato. So, *Etuberosa* species are sources of frost resistance [8], general resistance to viruses [9,10] and some bacterial diseases [11].

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Abbreviations: MS, Murashige and Skoog; NAA, α-naphtalenacetic acid; GA_3, gibberellic acid; PEG, polyethylenglycol; (br × et), sexual hybrid *S. brevidens* × *S. etuberosum*.

0168-9452/92/$05.00

Although direct hybridization of *Etuberosa* species with cultivated potatoes by means of protoplast fusion are well known [12–14], data about similar experiments with tomatoes are not available. However such hybrids would be of interest not only for breeding programs but also for the study of phylogenetic inter-relationships between *Lycopersicon* and *Solanum*.

We report here on the regeneration and characteristics of somatic hybrids formed between cultivated tomato and the wild potato species of the series *Etuberosa*.

Materials and Methods

Plant material

Seeds of tomato, *Lycopersicon esculentum*, albino-plastome mutant-Pl-alb I [15,16] were obtained from the Bach Institute of Biochemistry, Moscow, Russia. Seeds of *L. esculentum* cv. Tamina (k 4505), seeds of diploid wild species of the series *Etuberosa*, *S. etuberosum* (k 9141) and sexual interspecific hybrid *S. brevidens* × *S. etuberosum* (br × et) (k II 671) were obtained from the N.I. Vavilov All Union Institute of Plant Industry, St. Petersburg, Russia. Seeds of these cultivars were sterilized with 10% hydrogen peroxide for 20 min and washed with sterile water three times. Then seeds were transferred in MS medium [17] without hormones. In vitro plants were grown under 5000 lux (16-h photo-period, 25 ± 1°C).

Protoplast isolation and fusion

Leaves from 30-day-old shoot cultures were sliced and incubated in the enzyme solutions (16 h in darkness, 23–25°C). For protoplast isolation from *Etuberosa* species, the enzyme solution contained 0.2% Cellulase Onozuka R-10 and 0.1% Driselase with 0.5 M sucrose and 5 mM $CaCl_2$ (pH 5.6). Pl-alb I and Tamina mesophyll protoplasts were isolated in the solution containing 0.6% Cellulysin and 0.1% Macerase and with the same concentration of sucrose and $CaCl_2$. The digested leaves were filtered through a 120-μm nylon screen and the filtrate was centrifuged at 100 × *g*. The floating protoplasts were resuspended in a wash medium W5 [18] and precipitated (1 or 2 times) for 2 min at 60 × *g*.

Parental protoplasts were fused with PEG-DMSO at a high pH according to Menczel et al. [19]. From three to eight fusion experiments were conducted for each combination; from 7×10^5 to 2×10^6 protoplasts were involved in each experiment. Control dishes were also prepared for both parental protoplasts.

Protoplast culture and shoot regeneration

Protoplasts were cultivated according to Shepard [20] with the modifications of Haberlach et al. [21]. After protoplast fusion treatments protoplasts were incorporated into agar layer CL medium over a reservoir of Res medium [21]. One month after initial plating the bilayer agar containing microcalli was cut into blocks and transplanted on Cul medium [21]. On Cul medium the potential hybrid calli were initially selected on the basis of differences in growth rate and morphological appearance as selection traits. The selected calli were transferred to fresh Cul medium; when calli had become green, they were transferred to Dif regeneration medium [21]. For elongtion of developing shoots calli were transferred to E-medium [22]. Morphologically normal regenerated shoots were transplanted to hormone-free MS medium for rooting. Morphologically abnormal regenerants were proliferated by transfer to MSGK-medium (MS + 0.5 mg l^{-1} GA_3 + 0.1 mg l^{-1} kinetin + 0.2 mg l^{-1} NAA) and rooted on auxin-containing MS medium (MS + 0.1 mg l^{-1} NAA). The rooted shoots were treated as shoot cultures and they were transferred to soil.

Morphological assessment

An assessment of plant morphology was carried out for parental plants: *L. esculentum* cv. Tamina, *S. etuberosum*, (br × et) and 60 putative somatic hybrids. All plants were grown under standard greenhouse conditions (10 000 lux, 12 h of light, 18–22°C).

Characteristics distinguishing the two parent species were species-specific and they were used for identification of somatic hybrids (leaf shape, inflorescence, flower colour and shape, position of pedicel articulation; size of calyx lobes; adnate or not adnate anthers; fruit morphology).

Isozyme analysis

Isozyme analysis was performed on young leaves of greenhouse-grown plants.

For peroxidase, sample extractions were carried out at 4°C. Leaves were homogenized in a 0.2 M acetate buffer (pH 5.4), containing 0.2% Triton-X-100 (1:2, w/v). Homogenate was centrifuged at 5000 × *g* for 15 min. Electrophoresis was carried out according to Davis [23]. Electrophoresis was carried out according to Davis [23]. Peroxidase was stained according to Scandalios [24] with some modifications.

For analysis of multiple molecular forms of esterase enzymes leaf tissue was homogenized in 0.05 M Tris–HCl buffer (pH 6.8) containing 12% glycerol and 0.2% mercaptoethanol (1:1, w/v). Homogenate was centrifuged at 10 000 × *g* for 15 min. Electrophoresis was carried out in a veronal buffer system [25]. Esterase were stained with α-naphtyl acetate and Fast Blue RR [26].

Analysis of the nuclear DNA

DNA was isolated from 3 g fresh weight of frozen young leaves. The leaf tissue was homogenized and lysed in buffer: 50 mM Tris (pH 8.0); 150 mM NaCl; 50 mM EDTA and 0.5% sarcosyl-Na. DNA was purified with standard phenol-chloroform extraction procedure. Total DNA was digested with *Eco*RI according to the manufacturer's instructions (Scientific Industrial Association 'Ferment', Vilnjus, USSR) and separated by agarose gel (0.8%) electrophoresis. Electrophoresis, Southern transfer to nitrocellulose filter and nick translation were performed using standard procedures [27].

DNA blots were hybridized with the nick-translated 5.3-kb fragment bearing tomato ml-1 gene [28,29]. We hybridized this probe to total plant DNA digests [30].

Fertility and cytogenetic analysis

The fertility of parent species and somatic hybrids was studied by the acetocarmine method. Meiosis was analysed on pollen mother cells according to Brown [31].

Embryo culture

Fruits were harvested 30–40 days after hand pollination and the embryos were excised under sterile conditions and cultured on the HLH medium [32].

Results

Selection of somatic hybrid lines and plant regeneration

Mesophyll protoplasts of *Etuberosa* genotypes were fused with mesophyll protoplasts of tomato in the following combinations: *L. esculentum* Pl-alb I + *S. etuberosum*; *L. esculentum* cv. Tamina + *S. etuberosum* and *L. esculentum* cv. Tamina + (br × et).

The hybrid plants were obtained from individual experiments and the results of protoplast culture from the different combinations are presented in Table I. The *L. esculentum* control showed that tomato protoplasts did not divide in cell plating medium CL and later died. In the control plates containing *Etuberosa* protoplasts intensive division was observed; the plating efficiencies (the percentage of plated protoplasts which underwent cell division on the 14th day after protoplast isolation) in CL/Res medium were 52 ± 5.7% for (br × et) and 42 ± 2.8% for *S. etuberosum*. So, bilayer agar of CL/Res medium played the role of selective factor for hybrid calli since tomato protoplasts were eliminated. The aim of the following study was to distinguish the hybrid calli from those of wild species.

Hybrid calli were selected at the microcalli stage using morphological peculiarities, differences in the growth rate and also according to the character of plant regeneration. Mainly the calli that arose in the fusion experiments were small, granular and yellow-green in colour; they were morphologically identical to control *Etuberosa* calli. However, calli with new characteristics appeared there very rarely. These calli had a colouring from light-brown to pale-green, they grew rapidly and formed large, dense colonies; unlike *Etuberosa* calli they were uniform in structure and 3–5-times bigger (Fig. 1). No calli with these characteristics were found either in plates of *Etuberosa* control protoplast cultures (homofusion experiments) or in mixtures of parental protoplasts

Table I. Morphogenetical characteristics of different fusion combinations.

Combination	No. of experiments	No. of selected calli	No. of calli regenerating (%)	Shoots from calli	Rooted shoots on MS media (%)	No. of anomal. shoots, (%)	No. of flowering (non flowering) plants
Pl-alb 1 + + *S. etuberosum*	3	55	29 (52.7)	101	19 (18.8)	80 (79.2)	18 (3)
Tamina + + *S. etuberosum*	8	332	18 (5.4)	91	54 (67.5)	44 (48.4)	22 (4)
Tamina + + (br × et)	3	58	4 (6.9)	21	12 (57.1)	7 (38.3)	14 (3)
Controls:							
S. etuberosum	8	57[a]	26 (45.6)	319	230 (72.1)	0	
(br × et)	3	59[a]	34 (57.6)	252	175 (69.4)	2 (0.63)	

[a]In control variants of homofusions the calli were selected randomly from populations of morphologically uniform protoclones.

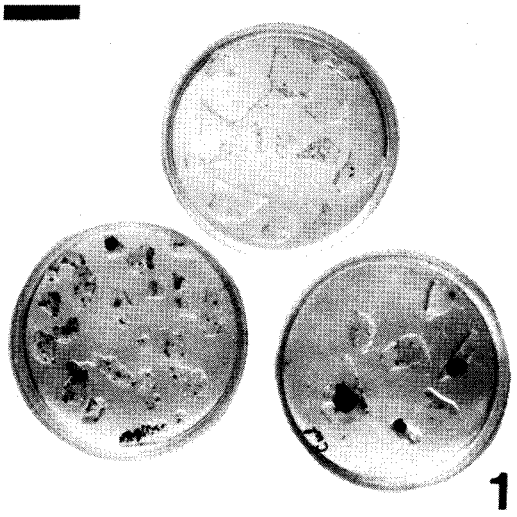

Fig. 1. Selection of the hybrid calli. Colonies plated into CL/Res medium and transferred on Cul medium. Upper plate — control *S. etuberosum*, two lower plates — Tamina + *S. etuberosum*. Bar = 30 mm.

(non-fusion treatment). Putative hybrid calli were selected and were cultured independently.

The selected calli had different characteristics of plant regeneration. Putative hybrid calli formed single shoots (Fig. 2) with thick stems and with dense and rounded leaves. Stimulation of supplemented shoot-production was achieved by removal of the first shoot and transferred to fresh medium. The majority of selected calli formed morphologically abnormal, distorted shoots with misshaped leaves; these plantlets did not develop internodes and root systems (Table I). In some cases, the development of morphologically abnormal shoots was achieved only by transfer to MSGK medium.

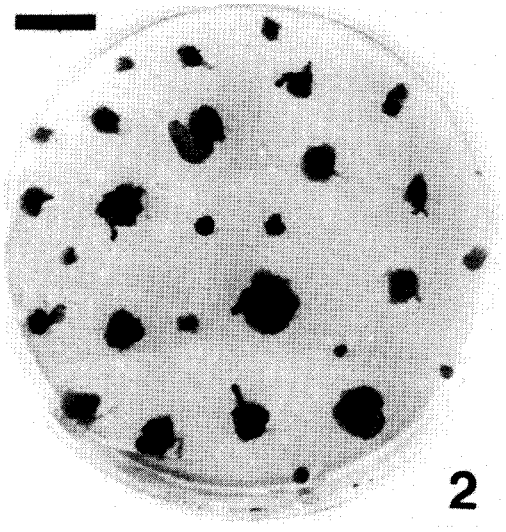

Fig. 2. Shoot regeneration of selected calli from combination Tamina + *S. etuberosum*. Bar = 14 mm.

S. etuberosum and (br × et) calli formed multiple shoots (8–10 shoots per clone) with thin stems and short internodes. A low frequency of anomalous types of regenerants was observed in control experiments (Table I).

Morphological comparison

The rooted shoots were planted into soil in a greenhouse. Plants of *S. etuberosum* and (br × et) with short, velvety pubescent leaves have pointed leaflets and many interjected leaflets; the terminal leaflet is oblong-elliptic. Pedicel articulate on or a little above the base. Flowers are purple, corolla rotate, calyx with small points. The anthers of *Etuberosa* species are not adnate as they are in all *Solanum* species and styles protrude.

Tomato plants are almost glabrous, without or sometimes with several small interstitial leaflets; terminal leaflet dissected. Pedicel articulate a little above the middle, corolla stellate, flowers yellow, calyx with long sepals and style as long as the stamens; anthers adnate in anthercolumn and their tips are sterile.

The comparative morphology of selected regenerants suggested that they were hybrid in nature (Figs. 3 and 4). They had morphology intermediate between the two parents and their hybrids showed much variation.

Isozyme analysis

Both parent species and the somatic hybrids from three combinations were analysed for esterase and peroxidase isozyme profiles.

Species-specific differences in the isoenzymes of peroxidase and esterase were observed between *L. esculentum* and *Etuberosa* species (Figs. 5 and 6). Polyacrylamide gel electrophoresis has confirmed the hybrid nature of the selected regenerants. All selected regenerants from the three fusion com-

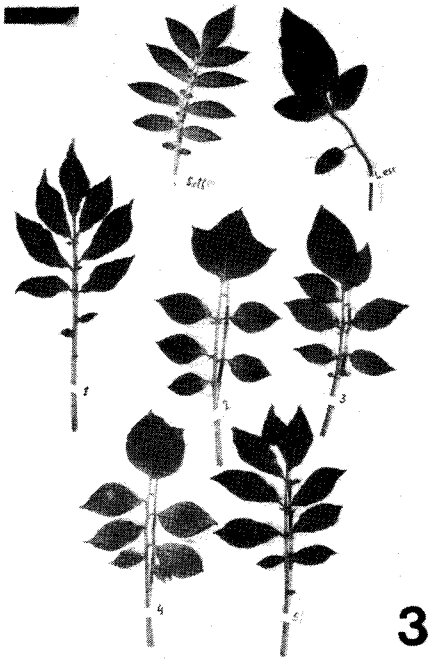

Fig. 3. Leaves of *L. esculentum* cv. Tamina (upper right), *S. etuberosum* (upper left) and their somatic hybrids (two lower rows). Bar = 50 mm.

binations tested, had patterns consisting of the bands from both parents and some of them had additional (hybrid) bands.

Analysis of the nuclear DNA

We have analysed DNA from parental species and somatic hybrids using Southern hybridization with tomato mL — 1 gene. This procedure revealed a distinction between the two parents: *Lycopersicon* and *Etuberosa* species. Seventeen hybrid-like plants from three combinations were analysed; data for eight hybrids are shown in Fig. 7. The examination of hybridization patterns exhibited some variations in band positions and relative band intensities, 15 hybrid plants exhibited all the ml-1 homologous bands presented in both original genomes (*Lycopersicon* and *Etuberosa*), while two hybrids had only the band from *L. esculentum*.

New bands have been also detected in some hybrids indicating possible DNA rearrangements.

Cytological characterization and fertility

Cytological studies are still incomplete. The chromosome numbers measured in several somatic hybrid plants from each combination are given in Table II.

Fig. 4. Flowers, inflorescense and calyx characteristics of Tamina (upper left, a), (br × et) (upper right, b) and their somatic hybrids (lower rows, c–e). Bar = 28 mm.

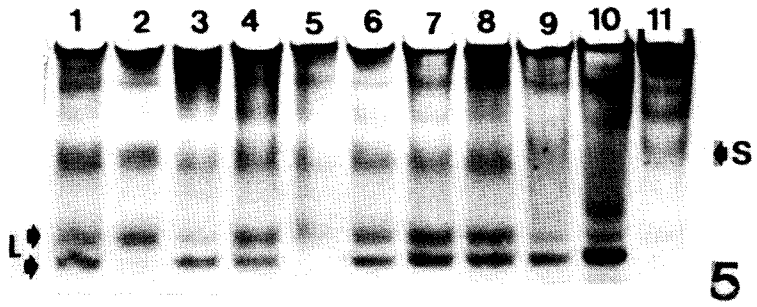

Fig. 5. Acrylamide gel electrophoresis of esterases of *L. esculentum* cv. Tamina (10); mixtures of extracts from leaves of parental species (1) and *S. etuberosum* (11) and their somatic hybrids (2–9). The species-specific forms are indicated by arrows (L — tomato, S — *S. etuberosum*).

Most of the hybrid plants from combination Tamina + *S. etuberosum* had the expected euploid chromosome number $2n = 4x$ $(2x + 2x) = 48$. Somatic hybrids from combination Pl-alb 1 + *S. etuberosum* were aneuploids at the tetraploid level and in Tamina + (br × et) combination hybrids were hexaploid and aneuploid at octo- and hexaploid levels. Most of the hybrids of this combination were aneuploids with chromosome numbers varying in different cells of an individual plant.

The frequency of chromosome association per cell at diakinesis in tetraploid somatic hybrids was 4.73 (range 0.25–8.61) for univalents, 21.23 (range 19.21–23.69) for bivalents, 0.21 (range 0.07–0.30) for trivalents and 0.18 (range 0.11–0.26) for quadrivalents. Random distribution of univalents between the poles resulted in formation of aneuploid gametes (Fig. 8).

Of the 60 hybrid plants grown in the greenhouse 46 flowered and their pollen had reduced viability (Table II). Most of the hybrids yielded no fruits, neither after selfing (hand pollination) nor after backcrossing with tomato parents. Only one plant could be crossed as male parent with *L. esculentum* but with a very low efficiency, the fruits had abortive seeds.

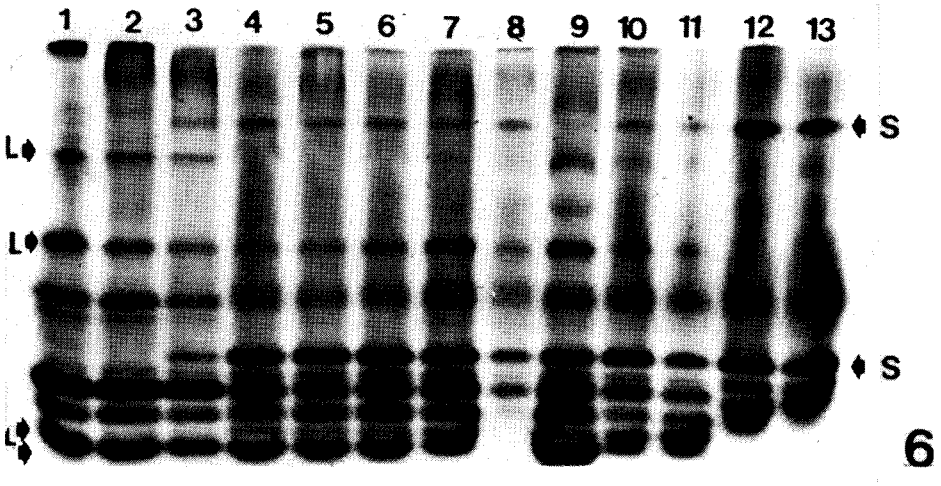

Fig. 6. Acrylamide gel electrophoresis of peroxidases of *L. esculentum* Pl-alb I (1,2), *S. etuberosum* (12,13), mixtures of extracts from leaves of parental species (3) and their somatic hybrids (4–11). The species-specific forms are indicated by arrows (L — tomato, S — *S. etuberosum*).

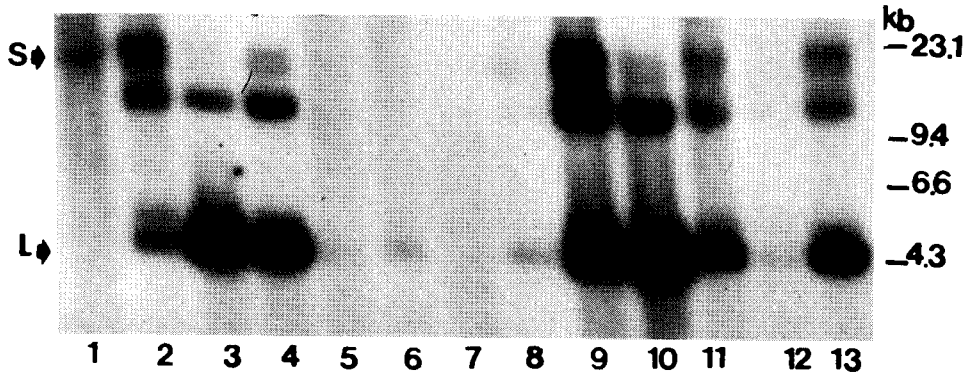

Fig. 7. Southern analysis of nuclear DNA from parental species (*L. esculentum* and *Etuberosa* species) and their somatic hybrids. Nuclear DNA was isolated from leaves of the plants, digested with *Eco*RI and hybridized with nick-translated probe of the tomato mL-I gene. Position of the *Hin*dIII fragments of phage lambda used as the molecular weight marker are indicated at the right. Tamina, lane 6; Pl-alb I, lane 5; somatic hybrids from combinations Tamina + *S. etuberosum*, lanes 2–4, 10–12; Tamina + (br × et), lanes 8,9 and Pl-alb I + *S. etuberosum*, lane 13; *S. etuberosum*, lane 1; (br × et), lane 7. The species-specific forms are indicated by arrows (L — tomato, S — *S. etuberosum*).

Twelve hybrids from three combinations set fruits after selfing. The colour of ripe fruits of these plants was yellow/orange whereas fruits of cv. Tamina were red. Out of twelve selfing hybrids seven formed only parthenocarpic fruits and in the fruits of five plants only a few seeds were found. In these fruits fertilization occured and seeds began to develop, but embryos began to die prior to their full differentiation. Rare globular, heart-shaped and torpedo-shaped embryos were cultured on HLH medium. In vitro embryo culture

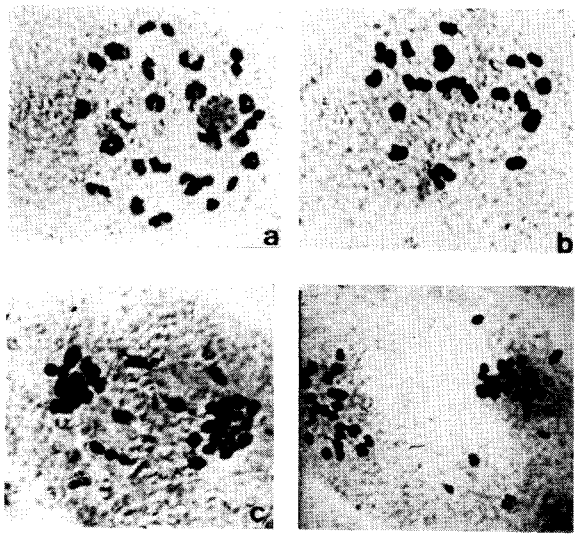

Fig. 8. Cytological analysis of the somatic hybrids between *L. esculentum* cv. Tamina and *S. etuberosum* ($x \approx 2000$). Chromosome associations at diakinesis (a) and metaphase I (b) in 5-u somatic hybrid ($2n = 4x = 48$). (a) $3_I + 2I_{II} + I_{III}$, (b) $22_{II} + I_{IV}$. Lagging chromosomes at anaphase-telophase I (c) and metaphase 2 (d) in a-40 somatic hybrid. Bar = 14 mm.

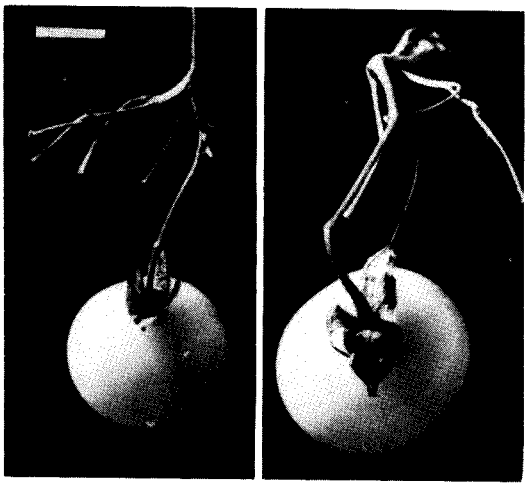

Fig. 9. Fruits of the 5a somatic hybrid.

Table II. An evaluation of chromosome number, pollen viability and results of selfing of intergenus somatic hybrids.

Sample	Chromosome number	Pollen stainability (%)	Selfed[a]			Embryos[b]			
			Flowers* pollinated	Fruits× formed	%	a	b	c	Germinated
Tamina	24	92.1	41	19°	46.3	Fully differentiated embryos			
S. etuberosum	24	68.2	28	8°	28.6				
(br × et)	24	18.6							
Pl-alb 1 + *S. etuberosum*									
a – 3	47	17.2	124	2	1.6	0	0	1	0
a – 7	46	5.9	283	1	0.4	0	0	0	
a – 8		9.1	116	0	0				
a – 9		21.5	330	4	1.2	0	0	0	
b – 3	48	18.1	81	0	0	0			
b – 5		14.2	80	11	1.3	0	0	0	
g – 3	72								
o – 2		11.5	52	0	0				
o – 8	48	50.5	159	0	0				
o – 10	48	26.1	85	0	0				
o – 11		0							
o – 15	47	26.0	212	2	0.9	0	0	0	
Tamina + *S. etuberosum*									
a – 1	48	52.3	186	30	16.1	0	1	1	1
a – 2	48	87.0	174	21	12.1	1	1	3	1
5 – a	48	61.9	199	16	8.3	2			0
5 – u	48	47.8	387	64	16.5	3	1	6	2
a – 40	45								
b – 40	47	13.4	48	6	12.5	0	0	0	
Tamina + (br × et)									
1 – a	71–84	8.5	159	0					
1 – g	70–82	45.0							
1 – e	68–72	10.5	77	0	0				
2 – a	72	16.1							
2 – b	78								
2 – v	62	8.1	112	4	3.6	0	0	0	
4 – a	63–72	7.6	63	0	0				
4 – b		18.4	35	2	5.7	0	0	0	

[a] *Hand pollination; ×Including parthenocarpic fruits; °Free pollination.

[b] a, globular; b, heart-shaped; c, torpedo-shaped embryos.

Individual calli were numbered (first symbol) and shoots taken from them were numbered too (second symbol).

of globular and heart-shaped embryos was not successful. Of the eleven torpedo-shaped embryos cultured on HLH medium four germinated and only three of them developed as normal plantlets (Table II).

Discussion

Four hundred-forty five presumably hybrid clones were selected from three fusion combinations. Selection based on the more active growth of hybrid fusion products in comparison with parent microcalli and on their morphological characteristics was quite efficient. All regenerants out of 51 regenerating protoclones were nuclear hybrids. This was proved by morphological, biochemical and cytological data. Hybridization of tomato with non-tuberous *Solanum* species of the *Etuberosa* series was carried out for the first time.

Potential possibilities of somatic hybridization between very distant species allowing the production of a hybrid cell are very wide but the formation of genetically stable, morphologically and functionally normal somatic hybrids is restricted only by fusion of closely related plant species. In our experiments the limited morphogenetical capability of hybrid fusion products resulted in the reduction of regeneration efficiency, in morphologically abnormal shoot formation and functionally imperfect plants (not capable of rooting and flowering) and in the sterility of hybrids as well.

Three intergeneric combinations differed in efficiency of regeneration ($P < 0.01$), frequency of morphological anomalities ($P < 0.05$), amount of rooting plants ($P < 0.01$), thus indicating the influence of parental genotypes on these morphogenetical characteristics. We suppose that the involvement of a larger genotypic variability of parental species into hybridization can extend the possibilities of distant somatic hybridization. So, in combination Pl-alb I + *S. etuberosum* practically all hybrid clones formed morphologically abnormal shoots, most of the hybrids of this combination were aneuploids and all plants were sterile. Fertile hybrids were obtained in combination Tamina + *S. etuberosum* and the frequency of morphologically normal euploid hybrids was relatively high in this combination.

The most important result of our research is obtaining of fertile forms of intergeneric somatic hybrids. Up to date fertile somatic hybrids were only obtained for intrageneric combinations of *Solanum* [33] and *Lycopersicon* [34]. It is also known that intertribal somatic hybrids of tomato and *Petunia hybrida* [35] as well as intergeneric hybrids with species of the most close genus *Solanum* (*S. nigrum* [36], *S. tuberosum* [37,38], *S. rickii* [39] and *S. lycopersicoides* [40]) obtained up to date were sterile. The hybrid sterility in fusion combination of *Lycopersicon* with the species of *Juglandifolia* series that are phylogenetically closer than *Etuberosa* can be explained by different facts. It may be the result of the low number of hybrid clones that don't allow the realization of the full genetic potential of the hybrid genome, the sterility is probably due to the aneuploidy and chromosome rearrangements of somatic hybrids.

In our research all fertile forms (a-I, a-2, 5-u) had euploid chromosome numbers and for 2 years their chromosome number remained stable. Isozyme analysis of the somatic hybrid progeny confirmed the presence of species-specific components from both *Lycopersicon* and *Solanum*. So, both parents transmit their genetical material to F_I generation of somatic hybrids.

Low frequency of univalent and multivalent formation and mostly regular meiosis were typical for fertile hybrids. Low frequency of multivalent associations indicates that there is no pairing between chromosomes from different genomes. However, it is difficult to draw a conclusion about the genome relationships between *Lycopersicon* and *Etuberosa* because chromosome pairing on the tetraploid and hexaploid levels may not always reflect their behavior on the diploid level. So, for example the sexual hybrid *L. esculentum* × *S. lycopersicoides* ($2n = 2x = 24$) exhibited nearly complete pairing at meiotic pachytene [41,43], however, mainly bivalents were formed in meiosis of allotetraploid *L. esculentum* × *S. lycopersicoides* ($2n = 4x = 48$) and nearly all pairing occurred between parental homologues [44].

In our opinion fertility of our hybrids *L. esculentum* cv. Tamina + *S. etuberosum* (though very

low), their genetic stability and formation of multivalents in meiosis (though with very low frequency) point to a certain level of relationship between *Lycopersicon* and *Etuberosa* species. Further study of our material will allow new data about the phylogenetical connection of *Solanum* and *Lycopersicon* to be obtained. Fertile somatic hybrids can be utilized in plant genetic breeding programs.

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