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Source of resistance against *Ralstonia solanacearum* in fertile somatic hybrids of eggplant (*Solanum melongena* L.) with *Solanum aethiopicum* L

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Abstract

Solanum aethiopicum is reported to carry resistance to bacterial wilt disease caused by *Ralstonia solanacearum*, which is one of the most important diseases of eggplant (*Solanum melongena*). These two species can sexually be crossed but the fertility of their progeny is very low. In order to transfer the resistance and improve the fertility, somatic hybrids between *S. melongena* cv. Dourga and two groups of *S. aethiopicum* were produced by electrical fusion of mesophyll protoplasts. Thirty hybrid plants were regenerated. When transferred to the greenhouse and transplanted in the field, they were vigorous and showed intermediate morphological traits. Their ploidy level was determined by DNA analysis through flow cytometry, and their hybrid nature was confirmed by examining isozymes and RAPDs patterns. Chloroplast DNA microsatellite analysis revealed that 18 hybrids had the chloroplasts of the eggplant and 12 those of the wild species. The parents and 16 hybrids were evaluated in the field for their fertility and resistance to bacterial wilt using a race 1, biovar 3 strain of *R. solanacearum*. All hybrids were fertile and set fruit with viable seeds. Their yield was either intermediate or as high as that of the cultivated eggplant. Both groups of *S. aethiopicum* were found tolerant to *R. solanacearum*, as about 50% of plants wilted after 8 weeks. The cultivated eggplant was susceptible with 100% of wilted plants 2 weeks after inoculation. All somatic hybrids tested were as tolerant as the wild species, except six hybrids showing a better level of resistance.

Keywords: Protoplast fusion; Flow cytometry; RAPDs; Chloroplast DNA microsatellite; Bacterial wilt

1. Introduction

Bacterial wilt, caused by *Ralstonia solanacearum*, is one of the most severe diseases of eggplant (*Solanum melongena*, $2n = 24$), especially in tropical regions. Perpetuated in soil, it enters the plant through the roots and progressively invades the stem vascular tissues, leading to a partial or complete wilting. It causes heavy yield losses ranging from 50 to 100% [1], thus seriously limiting the extensive development of eggplant cultivation. Since agro-chemicals are not effective and

Abbreviations: BAP, 6-benzylaminopurine; IAA, indole-3-acetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, α-naphthalenacetic acid; CPW, cell and protoplast washing solution; MES, 2-(*N*-morpholino) ethanesulfonic acid; KM, Koa and Michayluck basal medium; MS, Murashige and Skoog basal medium; PEG, polyethylene glycol 6000; DAPI, 4,6-diamidino-2-phenylindole; SSR, simple sequence repeat; I-SSR, inter-simple sequence repeat.

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sanitary cropping systems difficult to apply, control strategies of disease resistance have so far mainly consisted in plant breeding. Although screenings of eggplant accessions were conducted to find sources of resistance [1] and despite the fact that some resistant varieties have been developed [2–5], the level of resistance has become insufficient in hot planting season or poorly drained fields [6]. Traits of resistance against bacterial wilt have been identified in different wild relatives of eggplant, such as *Solanum torvum*, *S. sisymbrifolium* [3] and *Solanum aethiopicum* [3,7]. The latter species can sexually be crossed with eggplant, but the resulting F1 hybrids are either sterile or poorly fertile, limiting their further use in eggplant breeding programmes [8,9]. Consequently, these sexual F1 hybrids were used only as rootstocks for eggplant and tomato in naturally infected fields [8].

The ability of eggplant to regenerate easily from cultured protoplasts has led to the application of somatic hybridization to overcome sexual incompatibilities and introduce resistance traits from wild species into the cultivated eggplant [10]. Somatic hybrids of eggplant with *Solanum sisymbrifolium* were shown to be resistant to nematodes and tolerant to mites [11]. Somatic fusion was also successfully used to transfer resistance traits against *Verticillium* wilt from *S. torvum* into eggplant [10–13]. Highly fertile hybrids were recovered after somatic fusion between *S. melongena* cv. Dourga, and one accession of *S. aethiopicum* [9], but no information has so far been available about their resistance against bacterial wilt.

In this study, in order to further exploit the potential of bacterial resistance of *S. aethiopicum*, somatic hybridization was performed by using protoplasts fusion between *S. melongena* cv. Dourga and two accessions of this species. Somatic hybrids were morphologically and molecularly characterized, and evaluated for fertility and resistance to bacterial wilt in the field conditions in Indonesia.

2. Materials and methods

2.1. Plant materials

Seeds of eggplant, *S. melongena* L. cv. Dourga (white half-long fruit), and accessions of *S. aethiopicum*, groups *aculeatum* and *gilo*, were obtained from the collection of I.N.R.A.-Montfavet (France). They were initially sown in vitro, and the resulting plantlets micropropagated by subculture of leafy node cuttings on modified MS medium [14] containing 20 g l^{-1} sucrose and solidified with 7 g l^{-1} agar. Vitamins [15] were used at 1/2 and full strength for eggplant and its wild relatives respectively. Cultures were kept at 27°C, 60% relative humidity, and 12 h day^{-1} illumination at 62 μmol m^{-2} s^{-1}. Plants were subcultured at 4–5 week intervals.

2.2. Isolation, culture and fusion of protoplasts

About 500 mg of leaves taken from in vitro plants, aged 4 weeks, were scarified and incubated overnight at 27°C, in 5 ml enzyme solution composed of CPW salts [16], 0.5% (w/v) Cellulase RS, 0.5% (w/v) Macerozyme R10 (Yakult, Tokyo, Japan), 0.5 M mannitol and 0.05% (w/v) MES buffer, pH 5.5. After digestion in the dark, protoplasts were filtered through metallic sieves (100 μm mesh), and then purified and washed by centrifugation in 0.6 M sucrose and 0.5 M mannitol + 0.5 mM $CaCl_2$ solutions successively. Prior to fusion, the density of protoplasts from both species was adjusted to 3.5×10^5 protoplasts ml^{-1}.

Electrical fusion experiments were performed as described in Sihachakr et al. (1988) [17]. Briefly, the movable multi-electrodes were placed into a 15 × 50-mm Petri dish containing 600–800 μl of a mixture (1:1) of protoplasts from both fusion partners. Protoplasts were aligned for 15 s by the application of an AC-field at 230 V cm^{-1} and 1 MHz; subsequently, 2 DC pulses developing 1250 V cm^{-1} for 45 μs each were applied to induce protoplast fusion. The AC-field was then progressively reduced to zero. The whole fusion procedure was followed under an inverted microscope. After electrical treatments, electrodes were removed, and 6 ml of culture medium were added progressively to the Petri dish containing the mixture of fused protoplasts. The culture medium was KM medium [18] supplemented with 250 mg l^{-1} PEG, 0.2 mg l^{-1} 2,4-D, 0.5 mg l^{-1} zeatin, 1 mg l^{-1} NAA, 0.42 M glucose as osmoticum and 0.05% (w/v) MES. The pH of the medium was adjusted to 5.8 prior to sterilizing by filtration (0.22 μm filter, Millipore). Cultures were kept in darkness for 7 days, afterwards they were exposed to light. On day 15,

cultures were diluted eight times with fresh KM medium supplemented with 2 mg l^{-1} BAP and 0.1 mg l^{-1} 2,4-D. Calli (3–4 mm diameter) were then transferred to the regeneration medium, composed of MS medium supplemented with vitamins [15], 20 g l^{-1} sucrose, 2 mg l^{-1} zeatin, 0.1 mg l^{-1} IAA and solidified with 7 g l^{-1} agar.

Shoots were excised from callus and multiplied by subculturing leafy node cuttings on hormone-free MS medium. In vitro environmental conditions were 12 h day^{-1} illumination at 62 μmol m^{-2} s^{-1}, 27°C and 60% humidity.

2.3. Determination of ploidy level

The determination of the ploidy level of the hybrids was performed according to Sgorbati et al. [19] with modifications. About 1 cm^2 leaf material from in vitro plants was chopped with a razor blade in 1 ml buffer containing CPW salts [16], 0.5 M mannitol, 0.25% (w/v) PEG, 0.5% (v/v) Triton X-100, 0.25% (v/v) mercaptoethanol at pH 6.5–7.0. Crude samples were filtered with a nylon net (40-μm mesh) and stained with DAPI at 5 μg ml^{-1}. DNA analysis was performed on a PARTEC CA II flow cytometer (Chemunex, Maison — Alfort, France) equipped with a 100-W mercury lamp (type HBO). Blue fluorescence at 455 nm was recorded as a function of relative DNA content. About 10 000 nuclei were analyzed to generate each histogram. The DNA distribution was analyzed with DPAC software. The fluorescence scale was calibrated by using the diploid parental plants as external references.

Cytological analysis was done on root tips taken from greenhouse-grown plants as described in Sihachakr et al. [17].

2.4. Isozyme analysis

Samples of 100 mg of fresh leaves from in vitro-grown plants were ground at 4°C in 1.5 ml of Tris–HCl buffer (0.2 M, pH 8.5), containing 20% (w/v) sucrose, 0.03% (v/v) mercaptoethanol, 0.4% (w/v) sodium thioglycolate, 0.4% (w/v) PEG and 4% (w/v) polyvinylpyrrolidone (PVP). The mixtures were centrifuged twice at 15 000 × g for 15 min at 4°C and the supernatants stored at −80°C. Three systems of isozymes were examined: isocitrate dehydrogenase (Idh; EC.1.1.1.44), phosphoglucomutase (Pgm; EC.2.7.5.1), 6-phosphogluconate dehydrogenase (6-Pgd; EC.1.1.1.43). Isozymes patterns were obtained after electrophoresis on 10% starch gels and staining according to Shields et al. [20].

2.5. Molecular analysis

2.5.1. RAPDs

Total DNA was extracted from in vitro-grown plants using the DNeasy plant mini kit (Quiagen). Twenty decamer oligonucleotide primers from the kit AB-0320-1 (Fisher) and 14 primers previously used on potato by Xu et al. [21] and Baird et al. [22] were tested. PCR reactions contained 30 ng DNA in 25 μl containing buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X100, 0.2 mg ml^{-1} gelatin), 0.2 mM of each dNTP (Genaxis), 20 ng of primer and 1 U of Taq DNA polymerase (Appligene). Amplification was performed in a Techne Touchgene Thermocycler for 45 cycles. After initial denaturation for 5 min at 92°C, each cycle consisted of 1 min at 92°C, 1 min at 37°C and 1 min at 72°C. The 45 cycles were followed by a 8 min final extension at 72°C. Amplification products were resolved by electrophoresis in 1.4% agarose gel with TBE buffer for 3 h at 100 V and revealed by ethidium bromide staining. Gels were photographed on an UV box with Polaroid 665 films.

2.5.2. Chloroplast microsatellites

Chloroplast patterns were obtained with a pair of SSR primers designed from *Nicotiana tabacum* chloroplast sequences [23]: forward primer or SSR-ct1: CGT CGC CGT AGT AAA TAG GAG and reverse primer or SSR-ct1bis: GAA CGT GTC ACA AGC TTA CTC. PCR amplification of chloroplast microsatellites was performed with the reaction as described above, primers excepted. The thermal cycling profile was that of Brian et al. [24] including: 5 min at 92°C followed by 45 cycles of 92°C for 1 min, annealing temperature for 1 min, 72°C for 1 min and a final extension for 8 min at 72°C. Amplified microsatellites sequences were analyzed by electrophoresis, first on 1.8% (w/v) agarose gels containing ethidium bromide, for 3 h at 100 V, then on 6% polyacrylamide-8 M urea denaturing gels for 2 h at 40 W. Before being loaded on to polyacrylamide gels, the PCR products were denatured by incubation at 92°C for 5 min in presence of one volume of loading solution

containing 95% formamide. DNA bands on the polyacrylamide gels were visualized by silver staining with a silver-sequence DNA sequencing kit (Promega).

2.6. Morphological and fertility analysis

Fourteen somatic hybrid clones and their parental lines (*S. melongena* cv. Dourga, *S. aethiopicum* gr. *aculeatum* and *gilo*) were evaluated for morphology and fertility in the field conditions at Cipanas Experimental Station, located in the region of Bogor (Indonesia). The evaluation was made on 30 plants per clone, distributed in three replicates.

Pollen viability was evaluated by staining pollen grains with fluorescein diacetate (5 μg ml^{-1}). Samples of at least 250 pollen grains each were observed under UV light. Viability was expressed as the percentage of pollen grains with a fluorescent cytoplasm.

2.7. Tests for bacterial resistance

The tests for bacterial resistance were performed in the field conditions at Cipanas Experimental Station (Bogor, Indonesia). One strain of *R. solanacearum*, T 926 (race 1, biovar 3), isolated from *Solanum torvum*, and kindly provided by the Research Institute for Spice and Medicinal Crops (Bogor, W. Java, Indonesia), was used. Virulent colonies of *R. solanacearum* were selected after 2 days at 28°C on tetrazolium chloride medium (TTC) [25]. Bacteria were then routinely grown on sucrose-peptone medium (SPA) at 28°C [26]. Before inoculation, bacterial cells suspensions were prepared in sterile distilled water, using 1-day-old cultures, and their concentration was adjusted by spectrophotometry to 10^7 cfu (colony forming units) per ml ($OD_{650} = 0.1$).

Vitroplants were transplanted into individual plastic pots containing a sterilized mixture of soil and manure (1:1) and grown in the greenhouse for 4 weeks. Thirty healthy and uniform plants per clone, distributed in three replicates, were inoculated by stem pricking: after wiping the base of the stems with a tissue paper soaked in 70% ethanol and smearing it with Vaseline®, the calibrated bacterial suspension was pricked into the tissues with sterilized needles. Control plants were inoculated using sterile distilled water. One day later, all the plants were transplanted to the field.

Two, 4 and 8 weeks after inoculation, disease intensities were scored by using a foliar symptom scale ranging from 0 to 5: 0, healthy plants; 1, up to 25% wilted leaves; 2, up to 50% wilted leaves; 3, up to 75% wilted leaves; 4, up to 100% wilted leaves; and 5, dead plants. A disease index was calculated for each clone according to Winstead and Kelman [27]: $DI = [(\text{sum of scores})/(N \times \text{maximum score})] \times 100$, with N being the number of inoculated plants per clone.

Data on disease evaluation were subjected to statistical analysis using a fixed model of analysis of variance (ANOVA) with two criteria of cross classification: effects of genotype and period of assessments. Means separation was done by using Duncan's multiple-range test [28].

3. Results

3.1. Production of somatic hybrids

As described in Sihachakr et al. [17,29] and Daunay et al. [9], and in contrast to the results reported by Jarl et al. [13], leaves from in vitro plants were a competent source of viable protoplasts giving approximatly 4×10^6 cells g^{-1} fresh material. Several successful fusion experiments were conducted with fusion frequencies ranging from 10 to 20%. Two weeks after dilution of the fusioned protoplast suspension, hundreds of microcolonies appeared, and rapidly developped into calli when transferred onto a solid growth medium. Putative somatic hybrid calli were selected according to their ability to grow faster and to regenerate earlier than the parents [10]. Therefore, 2 weeks later, only those of at least 2–3 mm in size were transferred onto the regeneration medium. After 5 weeks, about 9% of the 950 selected calli produced shoots. One to three shoots were excised from each regenerating callus and subcultured on hormone-free MS medium. Finally, 83 calli gave rise to 120 plants, of which 82 from the fusions between Dourga and *S. aethiopicum* gr. *aculeatum*, and 38 from the fusions between Dourga and *S. aethiopicum* gr. *gilo*.

3.2. Ploidy level

The ploidy level of the regenerated plants was determined by using flow cytometry. The position

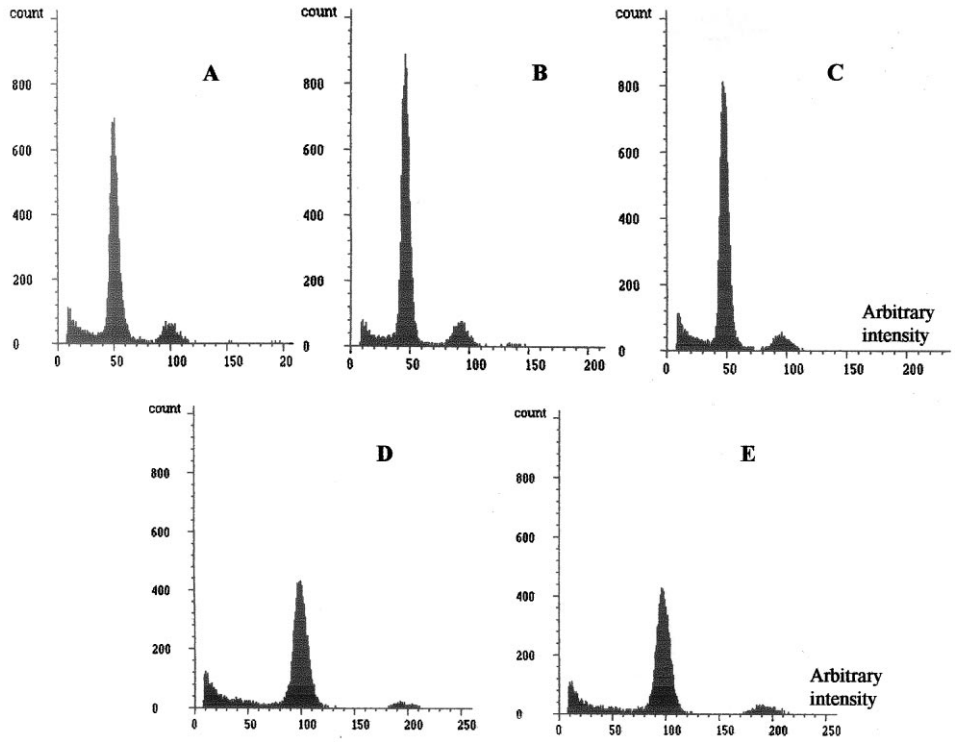

Fig. 1. Histograms of relative nuclear DNA contents obtained by flow cytometric analysis of 10 000 DAPI-stained nuclei isolated from leaves of the dihaploid parents: *S. melongena* cv Dourga (A), *S. aethiopicum* gr. *aculeatum* (B) and gr. *gilo* (C), and of two of their tetraploid somatic hybrids: DSa-18a (D) and DSa2-2 (E). Fluorescence intensity is proportional to nuclear DNA quantity and the position of the dominant peak reflects the ploidy level.

of dominant peaks corresponding to nuclei at G0–G1 phase of the cell cycle, was compared between putative hybrid and parental plants (Fig. 1). The analysis revealed that 23 out of 82 plants regenerated from the fusion between Dourga and *S. aethiopicum* group *aculeatum*, referred as to DSa, and seven out of 38 plants recovered from the fusion between Dourga and *S. aethiopicum* group *gilo*, referred as to DSa2, were at the expected tetraploid level. They were retained for further analyses to confirm their hybridity. The remaining plants, representing about 75% of the total, were diploids.

Chromosomal countings made on root tips of a random sample of hybrids confirmed the results obtained by flow cytometry, the tetraploid hybrids showing $2n = 4x = 48$ chromosomes per metaphasic cell (Fig. 2). Because of their morphological similarity, the two chromosome sets could not be distinguished from each other.

3.3. Isoenzyme analysis

Three isoenzyme systems, Pgm, 6-Pgd and Idh, were examined to confirm hybridity of the 30 selected tetraploid putative hybrids. They revealed polymorphism between the parental lines and distinguished the hybrids from the parents. For Pgm, the somatic hybrid patterns contained bands identical to the mixed extracts of the parents (Fig. 3B).

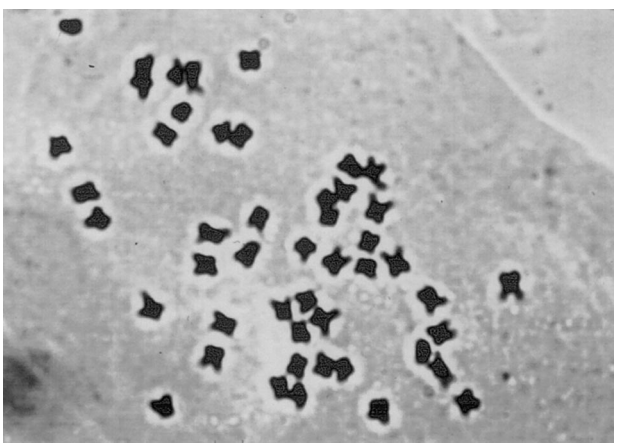

Fig. 2. Root-tip metaphasic cell of a tetraploid somatic hybrid (DSa 110) between *S. melongena* cv Dourga and *S. aethiopicum* gr. *aculeatum* ($2n = 4x = 48$ chromosomes).

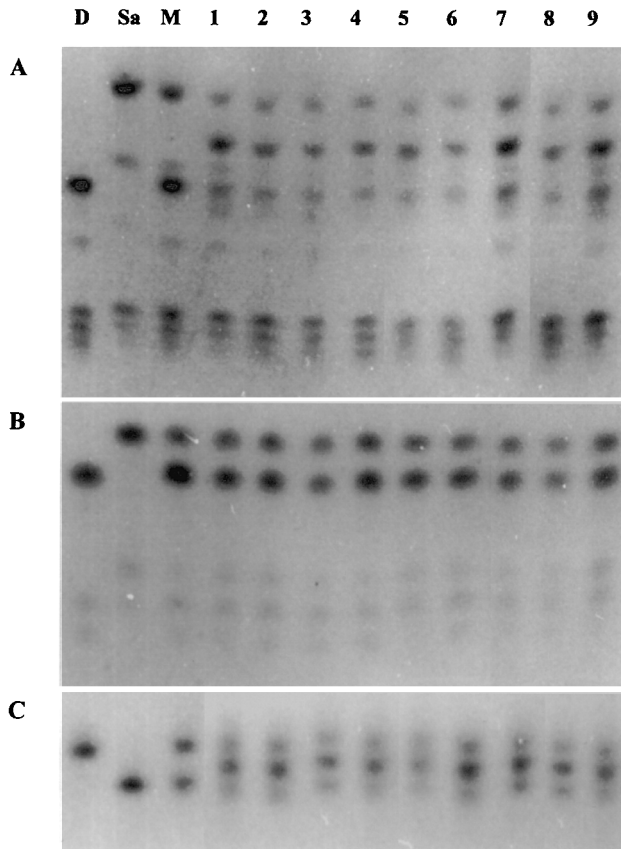

Fig. 3. Electrophoresis banding patterns of (A) 6-phosphogluconate dehydrogenase (6-Pgd), (B) phosphoglucomutase (Pgm) and (C) isocitrate dehydrogenase (Idh). Line D: *S. melongena* cv Dourga; line Sa: *S. aethiopicum* (both groups *aculeatum* and *gilo* had the same pattern); line M: mixture of DNA from *S. melongena* cv Dourga and *S. aethiopicum*; lines 1–7: hybrids DSa 1a, DSa 3a, DSa 4a, DSa 6a, DSa 17, DSa 20a, and DSa 26a; lines 8 and 9: hybrids DSa2-2 and DSa2-3.

For 6-Pgd and Idh, in addition to the sum of the parental bands, the hybrid pattern showed an additional band that was specifically relevant to the hybrid nature of the plants tested, and not found in the parental mixed extracts (Fig. 3A and C).

3.4. Nuclear genome analysis

The nuclear genome of the tetraploid putative hybrids was analyzed by PCR using 34 RAPDs, four SSR and two Inter-SSR primers. All the random primers used generated PCR products from the genomic DNA of both parents. They resulted in the amplification of two to 12 DNA fragments, from 0.1 to 1.2 kb. Fifteen primers of the kit (AB1-0320-1/1, 2, 4, 6, 7, 8, 10, 11, 12, 13, 14, 16, 17, 18, 20) and three primers previously used on potato (A10, A12, SC10-01) revealed polymorphism between the two pairs of parents. Five of them, AB1-0320-1/07, 08, 10, 12 and SC10-01, showing the best diagrams were chosen to analyze the hybrids.

AB1-0320-1/08 (Fig. 4A), AB1-0320-1/12 and SC10-01 (not shown) led to hybrid profiles with specific bands of both parents, thus confirming the hybridity of the 30 selected plants. For all these plants, the patterns obtained with AB1-0320-1/10

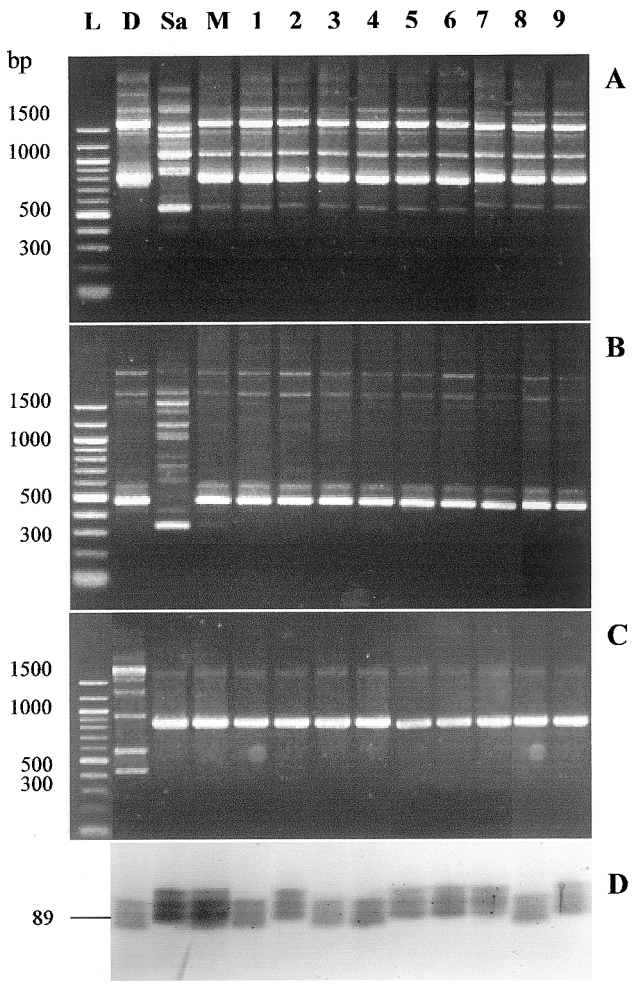

Fig. 4. Electrophoresis banding patterns of PCR amplification products. (A), (B), (C) RAPDs patterns obtained on 1.4% agarose gels using the primers AB1-0320-1/08, 10 and 07 respectively. (D) Chloroplast microsatellite patterns obtained on a 6% polyacrylamide gel using the couple of primers SSR-ct1/1bis. Line D: *S. melongena* cv Dourga; line Sa: *S. aethiopicum* (both groups *aculeatum* and *gilo* had the same pattern); line M: mixture of DNA from *S. melongena* cv Dourga and *S. aethiopicum*; lines 1–7: hybrids DSa 1a, DSa 3a, DSa 4a, DSa 6a, DSa 17, DSa 20a, and DSa 26a; lines 8 and 9: hybrids DSa2-2 and DSa2-3.

Table 1
Plant height, stem diameter, number and length of branches, length and width of leaves (Means of 30 plants ± S.D.)[a]

Lines	Plant height (cm)	Stem diameter (cm)	Branches		Leaves	
			Nb	length (cm)	length (cm)	width (cm)
S. melongena cv Dourga (D)	39.46 ± 1.37	0.86 ± 0.11	4.67 ± 0.17	23.71 ± 1.73	10.54 ± 0.24	6.79 ± 0.14
S. aethiopicum gr. *aculeatum* (Sa)	62.86 ± 1.55	1.35 ± 0.17	5.33 ± 0.15	32.26 ± 1.11	11.79 ± 0.21	8.00 ± 0.12
S. aethiopicum gr. *gilo* (Sa2)	65.70 ± 1.73	1.41 ± 0.17	5.56 ± 0.15	33.71 ± 1.12	12.32 ± 0.21	8.36 ± 0.13
DSa-18a	102.87 ± 0.82	0.97 ± 0.02	7.33 ± 0.08	49.96 ± 0.67	11.89 ± 0.07	8.04 ± 0.01
DSa-1a	76.60 ± 1.58	0.67 ± 0.03	5.20 ± 0.13	28.73 ± 1.00	11.75 ± 0.08	8.00 ± 0.03
DSa-4a	75.46 ± 0.79	0.58 ± 0.01	5.87 ± 0.15	36.44 ± 0.75	11.36 ± 0.10	7.97 ± 0.10
DSa-16	94.80 ± 1.47	1.07 ± 0.05	5.73 ± 0.17	42.20 ± 0.73	11.36 ± 0.10	8.10 ± 0.09
DSa-10	78.00 ± 1.70	0.68 ± 0.02	5.73 ± 0.15	31.53 ± 0.96	11.36 ± 0.07	7.57 ± 0.05
DSa-6a	98.67 ± 1.47	1.19 ± 0.10	7.87 ± 0.21	40.71 ± 2.11	11.98 ± 0.03	7.89 ± 0.07
DSa-4b	80.20 ± 1.80	1.05 ± 0.10	6.40 ± 0.24	35.46 ± 0.98	11.55 ± 0.02	7.87 ± 0.07
DSa-122	97.93 ± 1.18	1.05 ± 0.09	6.07 ± 0.13	41.25 ± 1.36	11.86 ± 0.04	8.12 ± 0.09
DSa-110	94.27 ± 1.31	1.05 ± 0.09	5.93 ± 0.16	40.75 ± 1.25	11.83 ± 0.10	8.00 ± 0.09
DSa-3a	97.80 ± 1.91	1.17 ± 0.10	6.53 ± 0.17	41.03 ± 0.81	11.62 ± 0.08	7.66 ± 0.06
DSa-20a	97.47 ± 0.69	0.80 ± 0.01	6.47 ± 0.21	43.42 ± 0.60	11.75 ± 0.06	7.95 ± 0.04
DSa-17	89.33 ± 2.56	0.79 ± 0.02	5.33 ± 0.19	37.14 ± 1.38	11.75 ± 0.08	8.13 ± 0.03
DSa-25b	Not evaluated					
DSa-26a	Not evaluated					
DSa2-3	96.86 ± 0.85	1.15 ± 0.03	7.00 ± 0.17	47.73 ± 1.06	12.10 ± 0.11	8.39 ± 0.04
DSa2-2	89.53 ± 0.93	0.91 ± 0.03	7.00 ± 0.11	42.15 ± 1.55	11.20 ± 0.14	7.78 ± 0.04

[a] The hybrids are designated by a number identifying the callus from which they are derived and a letter (a, b, c) when several plants are from the same callus.

(Fig. 4B) and AB1-0320-1/07 (Fig. 4C) showed only specific bands from Dourga, and from *S. aethiopicum*, respectively. When combined together, these results constitute a supplementary proof, though indirect, of the hybrid nature of the 30 tetraploid plants.

3.5. Chloroplast genome analysis

The ct genome type of the hybrids was determined by PCR using the couple of primers SSR-ct1/1bis. The amplification products were of about the expected length of 89 pb, as measured by comparison to the 100 bp DNA ladder (Biolabs) and allowed to distinguish the chloroplast genomes of the parents. All the hybrids showed the pattern of either one parent or the other (Fig. 4D). Among the 23 DSa hybrids, 14 possessed the *S. melongena* ct type and nine that of *S. aethiopicum* gr. *aculeatum*. The distribution of ct DNA was similar among the seven DSa2 hybrids: four and three with *S. melongena* and *S. aethiopicum* gr. *gilo* ct type respectively.

3.6. Morphological and fertility analysis

Fourteen somatic hybrid clones, including 12 DSa, two DSa2 and their respective parents, were evaluated for morphology and fertility in field conditions at the Cipanas Experimental Station (Bogor, Indonesia). As shown in Table 1, the hybrids grew vigorously and were taller than the parental lines. Their stem diameter was lower or intermediate between the parents. Their number and length of branches, as well as their length and width of leaves were rather close to those of the wild parents. The shape of hybrid leaves, flowers and fruits was relatively homogeneous and intermediate between the parents (Fig. 5). The somatic hybrids produced more flowers than the parents, but many of them aborted, and only few gave rise to fruit production (Table 2). Pollen viability,

measured by staining pollen grains with fluorescein diacetate, ranged from 28 to 50% for DSa hybrids and 37 to 40% for DSa2 hybrids, whereas the parental plants had 60–65% viable pollen. All hybrids set fruits with viable seeds. Traits of hybrid fertility, including the number, size and weight of fruits, was intermediate between the parents, except for two hybrid clones, DSa 6a and DSa 16 with less fruit production (Fig. 5, Table 2).

Taking into account the intermediate morphology, the ploidy level and the analysis of nuclear and chloroplast genomes of the selected plants by examining the isozymes and DNA markers, the 30 selected plants were confirmed to be somatic hybrids between *S. melongena* and *S. aethiopicum*.

3.7. Tests for resistance to bacterial wilt

For reasons of safety and limitation of pathogen spreading, the stem pricking inoculation method seemed to be the most appropriate for field trials. However, in order to determine the inoculation impact on evaluation of hybrid resistance to *R. solanacearum*, preliminary experiments were conducted in the greenhouse to compare different methods of bacterial inoculation: stem pricking, soil drenching and root immersing. Three sets of plants were inoculated at the same time. Stem pricking was carried out as described previously. Soil drenching consisted in adding 40 ml of bacterial suspension around the roots slightly wounded with a knife without digging up the plants. For root immersing, the plants were removed from their pots, and their roots were dipped into the bacterial suspension for 30 min before they were replanted. As for pepper [30] and unlike tomato [31], preliminary results showed that the wilting scores obtained for the three sets of plants were not significantly different (data not shown) and the inoculation methods did not seem to affect the evaluation of the resistance levels. Therefore, only the stem pricking technique was used for further bacterial tests.

Eighteen somatic hybrid clones, including 16 DSa and two DSa2, derived from separate calli, and their respective parental lines, were evaluated for resistance to *R. solanacearum* (race 1, biovar 3). The tests were performed from the end of the rainy season, in the fields of the Cipanas Experimental Station, located to the south of Bogor, at an altitude of 900 m above the sea level, in one of the main eggplant cultivation areas in Indonesia.

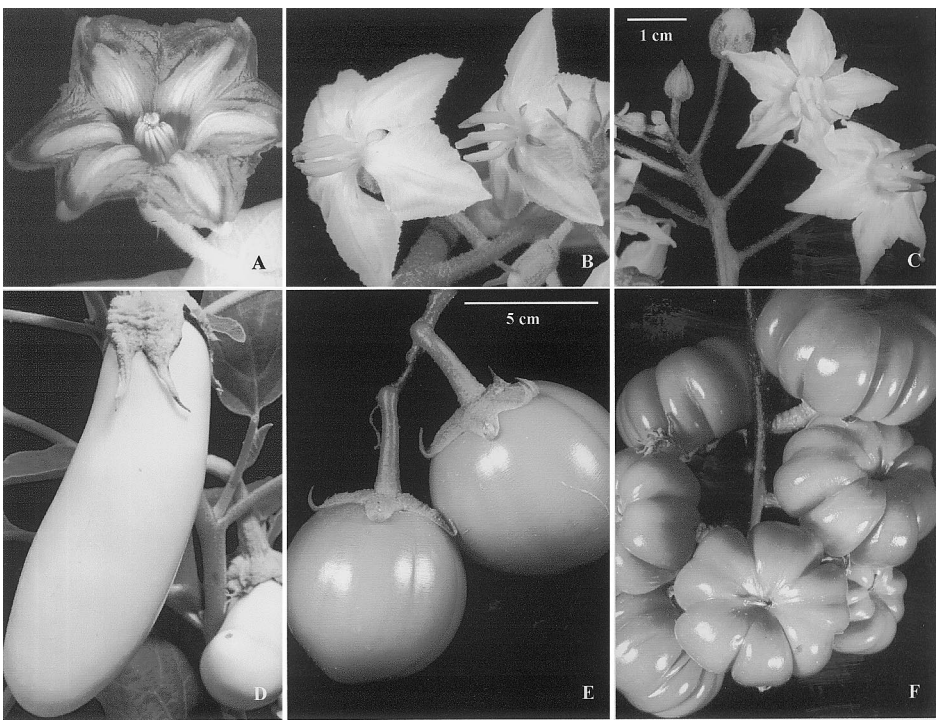

Fig. 5. Flowers from *S. melongena* cv Dourga (A), *S. aethiopicum* gr *aculeatum* (C), and their somatic hybrid (B). The white line in (C) is the scale for (A), (B) and (C). Fruits from *S. melongena* cv Dourga (D), *S. aethiopicum* gr *aculeatum* (F), and their somatic hybrid (E). The white line in (E) is the scale for (D), (E) and (F).

Table 2
Number of flowers per plant, number of fruits per plant, % of flowers setting fruit, fruit mean weight and fruit yield per plant (Means of 30 plants ± S.D.)[a]

Lines	ct DNA type	Nb flowers/plant	Nb fruits/plant	% flowers given rise to fruits	Weight of fruits	
					g fruit^{-1}	g plant^{-1}
S. melongena cv Dourga (D)	D	11.33 ± 0.22	5.80 ± 0.61	49.69 ± 4.43	254.38 ± 0.79	1468.10 ± 151.31
S. aethiopicum gr. *aculeatum* (Sa)	Sa	64.73 ± 5.26	23.27 ± 1.48	37.84 ± 1.40	21.76 ± 0.34	503.61 ± 30.77
S. aethiopicum gr. *gilo* (Sa2)	Sa2	127.75 ± 3.23	14.54 ± 0.66	8.44 ± 0.32	44.41 ± 0.34	493.54 ± 33.57
DSa-18a	D	192.67 ± 5.69	14.27 ± 1.83	10.94 ± 1.53	71.85 ± 2.27	990.28 ± 129.9
DSa-1a	D	126.53 ± 8.33	9.53 ± 0.96	7.27 ± 0.35	59.50 ± 6.47	561.23 ± 56.64
DSa-4a	D	103.40 ± 4.64	9.87 ± 0.18	9.99 ± 0.53	64.35 ± 0.45	633.67 ± 8.09
DSa-16	D	148.73 ± 7.91	6.27 ± 0.27	4.39 ± 0.27	55.16 ± 1.13	343.63 ± 14.52
DSa-10	Sa	146.60 ± 6.10	13.40 ± 0.67	9.07 ± 0.08	46.74 ± 5.44	586.63 ± 68.74
DSa-6a	D	255.67 ± 15.07	6.13 ± 1.45	3.46 ± 0.93	15.68 ± 2.86	163.20 ± 22.01
DSa-4b	D	140.27 ± 9.94	10.67 ± 0.48	8.19 ± 0.46	75.9 ± 0.64	813.84 ± 42.85
DSa-122	D	167.33 ± 8.16	15.27 ± 0.53	9.50 ± 0.52	63.70 ± 1.00	966.35 ± 29.09
DSa-110	D	162.93 ± 6.93	17.73 ± 0.26	11.11 ± 0.27	71.60 ± 0.78	1273.70 ± 33.03
DSa-3a	Sa	155.40 ± 5.15	14.87 ± 0.29	9.70 ± 0.28	73.16 ± 0.99	1092.90 ± 34.56
DSa-20a	Sa	135.13 ± 3.25	16.27 ± 0.71	11.97 ± 0.32	80.78 ± 0.35	1315.50 ± 59.08
DSa-17	Sa	130.73 ± 9.97	13.33 ± 0.98	10.51 ± 0.71	73.92 ± 0.44	982.92 ± 70.68
DSa-25b	D	Not evaluated				
DSa-26a	Sa	Not evaluated				
DSa2-3	Sa2	393.27 ± 8.56	5.80 ± 0.26	1.51 ± 0.09	97.99 ± 0.02	568.35 ± 25.74
DSa2-2	D	315.40 ± 3.79	8.80 ± 0.50	2.83 ± 0.19	98.91 ± 3.14	880.81 ± 59.92

[a] The hybrids are designated by a number identifying the callus from which they are derived and a letter (a, b, c) when several plants are from the same callus. Ct DNA type of *S. melongena* cv. Dourga (D), *S. aethiopicum* (Sa).

The cultivated eggplant, cv. Dourga, was susceptible, showing bacterial wilt symptoms (necrosis and wilting) on lower leaves 1 week after inoculation. All plants of eggplant died within 2 weeks, with disease indices of 100. Both groups *aculeatum* and *gilo* of the wild species, *S. aethiopicum*, displayed similar level of resistance against bacterial wilt, with only 50% wilted leaves on average (Table 3). The ANOVA of disease indices showed highly significant effects of the period of assessment and genotype on response to bacterial wilt at $P = 0.01$, but no significant effect of interaction between these two criteria was observed at $P = 0.05$. In order to compare the resistance levels of the hybrids and the parents, means of disease indices on the three periods of assessment were calculated and classified according to Duncan's multiple range test (Table 3). The level of resistance of the wild species was significantly higher than that of the susceptible cultivated eggplant, with 51.9 and 100% wilted leaves respectively. Eight DSa hybrids and two DSa2 hybrids appeared to have levels of resistance similar to those of *S. aethiopicum*, with means of disease indices ranging from 29.3 to 50.4 (Table 3). Interestingly, six DSa hybrids were significantly more resistant to race 1 strain than the wild parent, *S. aethiopicum*, with means ranging from 23.3 to 27.8 (Table 3). The disease indices of most hybrids and the wild species decreased with the period of assessment (Table 3), indicating that less leaves wilted 8 weeks after inoculation. In fact, those genotypes were tolerant to bacterial wilt, and leaves that had newly been formed did not wilt as the plants grew up.

4. Discussion

In this study, somatic hybrids have successfully been recovered after electrical fusion between protoplasts from *S. melongena* and two accessions of *S. aethiopicum*. Early selection of putative hybrids based on differences in cultural behavior of calli, was effective since 25% of selected plants were confirmed to be somatic hybrids. Similar methods, exploiting hybrid vigor as the only selection system of the hybrid calli, were previously used to obtain somatic hybrids of eggplant [10] and potato [32,33].

The early characterization of regenerated plants included determination of their ploidy level by flow cytometry, and confirmation of their hybrid status by isoenzymes and RAPDs analyses. Morphological observations and chromosome counting were conducted afterwards on plants grown to maturity in the greenhouse and the field, and confirmed the hybrid nature of the selected plants. RAPD markers were a powerful tool to characterize the nuclear genome of the hybrids. As they were expressed as dominant, the presence of at least one polymorphic amplification product from each parent in the patterns of the tested plants was enough to prove their hybridity. However, with a few of the 34 oligonucleotides tested, all the regenerated plants, and also the mixture of both parents DNA, showed the bands from only one parent. The same observations were previously reported in somatic hybrids between *S. tuberosum* and *S. brevidens* [34] and in several DNA mixing experiments reviewed in Reineke et al. [35]. The reasons of this phenomenon are unclear. It could be due to competition effects on primer-binding sites in the genome [36], especially if the RAPD products share extensive sequence homologies [35]. The presence of repetitive sequences in the RAPDs products could also lead to the suppression of the amplification by the formation of heteroduplexes between different copies of these repetitive se-

Table 3
Disease indices (DI) recorded 2, 4 and 8 weeks after root inoculation by race 1 strain of *R. solanacearum*[a]

Lines	Periods of assessment			Means
	2 weeks	4 weeks	8 weeks	
S. melongena cv Dourga (D)	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100 a
S. aethiopicum gr. *aculeatum* (Sa)	59.33 ± 4.56	49.33 ± 4.87	46.67 ± 5.57	51.8 b
S. aethiopicum gr. *gilo* (Sa2)	59.44 ± 2.13	49.41 ± 1.77	46.74 ± 1.67	51.9 b
DSa-18a	51.33 ± 0.91	46.67 ± 1.23	53.33 ± 4.59	50.4 b
DSa-1a	49.33 ± 3.37	45.33 ± 1.80	40.00 ± 1.82	44.9 bc
DSa-4a	48.00 ± 2.63	41.33 ± 2.34	20.00 ± 1.82	36.4 bc
DSa-16	34.67 ± 0.55	34.67 ± 4.76	36.67 ± 4.59	35.3 bc
DSa-26a	47.33 ± 2.69	31.33 ± 2.97	26.67 ± 4.21	35.1 bc
DSa-10	43.33 ± 2.64	32.00 ± 1.59	30.00 ± 3.16	35.1 bc
DSa-6a	42.00 ± 2.22	22.67 ± 1.80	26.67 ± 2.78	30.4 bc
DSa-4b	38.67 ± 0.91	22.67 ± 1.17	26.67 ± 2.78	29.3 bc
DSa-122	38.67 ± 1.52	24.67 ± 0.91	20.00 ± 3.65	27.8 c
DSa-110	33.33 ± 0.55	19.33 ± 0.55	26.67 ± 3.80	26.4 c
DSa-3a	38.67 ± 0.42	18.67 ± 1.28	16.67 ± 3.80	24.7 c
DSa-25b	37.33 ± 0.55	16.67 ± 0.55	20.00 ± 4.83	24.7 c
DSa-20a	39.33 ± 0.20	22.00 ± 1.26	10.00 ± 3.16	23.8 c
DSa-17	41.33 ± 1.72	15.33 ± 0.75	13.33 ± 4.21	23.3 c
DSa2-3	39.67 ± 1.32	34.00 ± 0.39	33.33 ± 2.10	35.7 bc
DSa2-2	41.33 ± 1.64	44.00 ± 0.36	33.33 ± 1.05	39.6 bc

[a] Disease intensities were scored using a 0–5 foliar symptom scale: 0, healthy plants; 1, up to 25% wilted leaves; 2, 26–50% wilted leaves; 3, 51–75% wilted leaves; 4, 75–100% wilted leaves; and 5, dead plant. DI = [(sum of scores)/($N \times$ maximum score)] $\times$ 100, where N is number of tested plants per clone. ANOVA was performed with two criteria of cross classification (genotype and assessment period): highly significant effects of the genotype ($F_{(df: 17; 108)} = 13.5$) and the assessment period ($F_{(df: 2; 108)} = 13.4$) at $P = 0.01$, and non significant effect of interaction ($F_{(df: 34; 108)} = 0.5$) at $P = 0.05$. Significant difference between genotypes at $P = 0.05$ is indicated by different small letters.

quences [35]. In these cases, it is not one primer, but the combination of a couple of primers, one, allowing the amplification of specific bands from one parent, and the other, the amplification of specific bands from the other parent, which constitutes a proof of the presence of both genomes in the hybrid.

In most somatic hybridization experiments dealing with different species, the initial mixture of the two parental cytoplasms was followed by the elimination of one of the two parental ctDNAs [37]. This was also the case for each hybrid recovered in this study. They all showed chloroplasts from only one parent. Biased chloroplast segregations were frequently reported in somatic hybrids [9,12]. The reason of this phenomenon could be a difference in organelle replication rate. But in this study, as reported in Pehu et al. [34] and in San et al. [37], the segregation of chloroplasts did not seem to be biased in favor of one or the other parent, since 14 out of the 23 DSa hybrids and four out of the 7 DSa2 had the eggplant ctDNA type, and the rest that of *S. aethiopicum*. In contrast to Daunay et al. [9], no correlation between the ctDNA type and any of the hybrid fertility criteria was observed in this study. The ctDNA type was not correlated either to the different resistance levels expressed by the hybrids.

Hybrid fertility is a prerequisite for further exploitation of somatic hybrids in breeding programs. Attempts at overcoming sexual barriers between distant relatives and eggplant through somatic hybridization often resulted in sterile hybrids [10]. This was observed with *S. sisymbrifolium* [11], *S. khasianum* [17], *S. nigrum* [38] and *S. torvum* [12,29]. This frequent sterility could be due to incompatibility between distantly related genomes. However, somatic hybrids between distant relatives may show reasonable levels of female fertility, as observed in backcrosses between potato and somatic hybrids of potato with *S. brevidens*, used as female parent. In the mean time, the hybrid pollen was ineffective in pollinations [39]. Likewise, fertility was recovered in highly asymmetric hybrids of eggplant with *S. torvum* [13]. When the fusion partners are phylogenetically closer, as *S. melongena* and *S. aethiopicum*, their progeny is more likely to be fertile. Our results on fertility of somatic hybrids of *S. melongena* with *S. aethiopicum* confirm those already obtained by Daunay et al. [9], and strongly support this hypothesis.

In the environmental conditions of our tests, all the somatic hybrids showed a similar or higher level of resistance to *R. solanacearum* race 1, biovar 3 than *S. aethiopicum*, both groups *gilo* and *aculeatum* being tolerant. From 2 to 8 weeks after inoculation, the disease indices of some of the hybrids progressively decreased, as new leaves were developing at the top of the plants. This apparent moving of the symptoms to the lower parts of the plants could be due to a limitation of spread of the bacteria. Tolerance to bacterial wilt in tomato was reported to be linked to a colonization restricted to vascular tissues [40], and was attributed to the induction of nonspecific physical barriers like the production of tyloses and other deposits [41].

In this study, interestingly, six hybrids were significantly more tolerant to bacterial wilt than the tolerant parent. If they did not undergo fluctuations of the experimental conditions in the field, these results could be linked to hybrid vigor and may be due to superdominance phenomenons resulting from genetic interactions induced by the combination of the two parental genomes. In fact, those somatic hybrids were among the most vigorous and productive clones that had been obtained in this study (Tables 1 and 3).

Results from this study are very encouraging and demonstrate that somatic hybridization is a rapide and effective way to introduce new sources of resistance into eggplant. The somatic hybrids obtained showed a similar or even higher level of resistance to *R. solanacearum* race 1 than the resistant parent. Nevertheless, further studies are needed to evaluate the stability of this resistance under different temperature and field conditions. Race 1 is a highly heterogeneous group common in the low-land tropics that can cause disease on many different species [42]. So far no data on strain specificity of the *S. aethiopicum* wilt resistance have been available and it would be interesting to test the resistance of the hybrids against some of the four other races of *R. solanacearum*, such as race 3, which is also very common in Asia.

By using anther and microspore culture, fertile dihaploid progenies have already been obtained from several somatic hybrids recovered in this study (data not shown). In order to produce new marketable varieties resistant to bacterial wilt, they are being used as breeding materials in backcrossing to the diploid recurrent eggplant includ-

ing resistance tests at each generation. Resistance to bacterial wilt in tomato was reported to be polygenic, and some QTL were detected on chromosomes 4 and 6 [43,44]. In eggplant, resistance seems to be controlled by a single dominant gene [45] and by a more complexe mixture of partially dominant or recessive genes [46]. Further analysis of selfed and backcrossed progenies of the dihaploids we obtained would contribute to elucidate the nature and inheritance of the resistance to *R. solanacearum*. These plants may also be useful to determine molecular markers linked to the resistance, through Bulk segregant analysis (B.S.A.) for example.

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