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Somatic hybrid plants produced by electrofusion between *Solanum melongena* L. and *Solanum torvum* Sw

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Summary. Somatic hybrid plants between eggplant (*Solanum melongena*) and *Solanum torvum* have been produced by the electrofusion of mesophyll protoplasts in a movable multi-electrode fusion chamber. Using hair structure as a selection criteria, we identified a total of 19 somatic hybrids, which represented an overall average of 15.3% of the 124 regenerated plants obtained in the two fusion experiments. Several morphological traits were intermediate to those of the parents, including trichome density and structure, height, leaf form and inflorescence. Cytological analyses revealed that the chromosome numbers of the somatic hybrids approximated the expected tetraploid level (2n = 4x = 48). Fifteen hybrid plants were homogeneous and had relatively stable chromosome numbers (46–48), while four other hybrids had variable chromosome numbers (35–48) and exhibited greater morphological variation. The hybridity of these 19 somatic hybrid plants was confirmed by analyses of phosphoglucomutase (Pgm) and esterase zymograms.

Key words: *Solanum melongena* – *Solanum torvum* – Protoplasts – Electrofusion – Somatic hybrids

Introduction

Eggplant (*Solanum melongena* L.) is susceptible to the soil-borne disease *Verticillium dahliae* Kleb. and to the root-knot nematode (*Meloidogyne incognita*), both of which are limiting factors in the production of eggplant crops. Several desirable agronomic traits, such as resistance to *Verticillium* wilt, *Fusarium* wilt (*Fusarium oxysporum*), bacterial wilt (*Pseudomonas solanacearum*) and root-knot nematodes have been identified in the wild species *Solanum torvum* Sw. (Yamakawa and Mochizuki 1978; McCammon and Honma 1983).

Attempts by McCammon and Honma (1983) to cross eggplant with *S. torvum*, the latter being used as the pollen parent, yielded only a few viable seeds which subsequently gave rise to highly sterile F_1 plants. Somatic hybridization by protoplast fusion is expected to provide a new possibility for increasing genetic variability, and also a means of transferring desirable traits into eggplant. Prerequisite protocols for regenerating plants from protoplast cultures in both eggplant (Bhatt and Fassuliotis 1981; Jia and Potrykus 1981; Saxena et al. 1981; Guri and Izhar 1984; Gleddie et al. 1986a; Sihachakr and Ducreux 1987a) and *S. torvum* (Guri et al. 1987) have already been developed. Somatic hybrids have also been obtained from PEG fusion between eggplant and *S. sisymbriifolium* (Gleddie et al. 1986b).

The present report describes the successful somatic hybridization between eggplant and *S. torvum* using electrofusion. This technique has also been used successfully for producing somatic hybrid plants of eggplant and *S. khasianum* (Sihachakr et al. 1988).

Materials and methods

Plant materials

Seeds of *Solanum melongena* (cv 'Dourga', white fruit) and *S. torvum*, kindly provided by Dr. B. Denoyes, IRAT, Guadeloupe, were aseptically sown on agar-solidified (7 g/l), hormone-free MS basal medium (Murashige and Skoog 1962) containing vitamins (Morel and Wetmore 1951) and 2% (w/v) sucrose. The plants were then propagated by subculturing leafy node cuttings on the same medium at 4-week intervals. Environmental conditions were 12 h/day illumination (62 $\mu E/m^2/s$), 27 °C and 60% humidity.

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Protoplast isolation

The sources of the protoplasts were leaves taken from 3 to 4-week-old plants. Protoplasts were prepared according to the methods of Sihachakr and Ducreux (1987a). Prior to fusion, protoplasts were first washed once in a 0.5 *M* mannitol solution, then suspended in this solution at a density of 3×10^5/ml and finally stored on ice.

Electrofusion apparatus and fusion procedure

The electric apparatus and the fusion procedure have previously been described by Sihachakr et al. (1988). Briefly, a movable fusion chamber (500–700 µl capacity) consisting of 12 parallel electrodes (2 mm apart) was connected to both a function generator (Enertec 4415) and a generator of D.C. square pulses (self-constructed unit). The movable multi-electrodes were placed into a 15 × 50 mm petri dish containing a 500–700 µl aliquot of a mixture (1:1) of mesophyll protoplasts of both species. In order to align the protoplasts, we applied 15 s of A.C.-field at 125 V/cm and 1 Mhz to the protoplast mixture; subsequently, two D.C. square pulses developing 1.2 Kv/cm for 20 µs each were applied to achieve protoplast fusion. During the electro-pulsing process, the A.C. field was automatically removed. After the fusion treatment was complete, the movable electrodes were transferred into the next petri dish. The fusion process was followed under an inverted microscope. Two independent fusion experiments, and control cultures of parental protoplasts without fusion were performed.

Protoplast culture

Immediately after the fusion process, 6 ml culture medium (Sihachakr and Ducreux 1987a) was progressively added to the fused protoplast mixture. The culture medium was KM8p (Kao and Michayluk 1975) supplemented with 0.2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg/l Zeatin, 1 mg/l α-naphthaleneacetic acid (NAA), 6.5% (w/v) glucose as osmoticum and 0.05% (w/v) 2-(*N*-morpholino)-ethanesulphonic acid (Mes). Protoplasts were initially cultured in the dark at 27 °C for 7 days. Afterwards, they were exposed to 12 h/day illumination at 62 µE/m²/s.

At day 20, the cultures were diluted 8 times with the same liquid medium, but the growth regulators were replaced with 0.1 mg/l zeatin (Sihachakr and Ducreux 1987b). For each fusion experiment, a random sample of 600–700 protoplast-derived calli 2–4 mm in diameter (6–8 weeks old) was taken from the culture dishes. The calli were transferred onto the regeneration medium which consisted of MS + vitamins (Morel and Wetmore 1951) + 2% (w/V) sucrose + 0.7% (w/v) agar and 2 mg/l zeatin. Shoots were excised from the callus and rooted on hormone-free MS medium; the rooted plants were then transferred into the greenhouse (16 h/day) illumination at 180 µE/m²/s, 20°–30 °C and 80% humidity).

Identification of somatic hybrids

Early identification of somatic hybrids was accomplished by screening regenerated plants for hair morphology: juvenile shoots of eggplant were covered with very small glandular hairs and branching hairs (Fig. 1 A), while those of *S. torvum* were covered with non-branching glandular hairs (Fig. 1 C). These selection criteria were based on the observations that (1) very long-stalked glandular hairs were densely scattered over the shoots of the somatic hybrids (Fig. 1 B) and (2) under the culture conditions described here, we were unable to obtain plant regeneration from *S. torvum* protoplasts.

Chromosome counting

Root tips were pretreated with a saturated solution of α-chloronaphthalene for 2–3 h at room temperature, fixed in ethanol glacial acetic acid (3:1, v/v) for 24 h and hydrolysed in 5 N HCl for 30 min. The preparation was then stained with acetocarmine.

Isoenzyme analysis

Isoenzyme analysis was performed either on young leaves of greenhouse-grown plants or on shoots from in vitro cultures. Samples of plant materials were homogenized in a 0.2 *M* TRIS-HCl buffer, pH 8.5, containing 0.03% (v/v) 2-mercaptoethanol, 20% (w/v) sucrose, 0.32% (w/v) sodium thiosulfate and 0.32% (w/v) PEG. Phosphoglucomutases (Pgm) (E.C. 2.7.5.1) were separated by electrophoresis on 13% starch gels (Smithies 1955) and esterases (E.C. 3.1.1.2), on polyacrylamide gels (Ornstein 1964). Stein formulations were as described by Vallejos (1983).

Results and discussion

Following protoplast alignment, the application of 2 D.C. square pulses of 1.2 Kv/cm for 20 µs each resulted in an average fusion rate of 35%. Heterokaryon frequency was not determined since the protoplasts of the fusion partners were of the same type. However, 20% of the fusion products were estimated to be binary fusions. In a previous fusion experiment using the same electric apparatus (unpublished results) with green mesophyll protoplasts of *Solanum tuberosum* and colourless cell suspension protoplasts of *Nicotiana sylvestris,* heterokaryons formed one quarter of the fusion products (50% fusion rate overall). Puite et al. (1986) estimated that heterokaryons formed 6% of the fusion products obtained when *S. tuberosum* and *S. phureja* were subjected to electrofusion using a movable multi-electrode system.

One week after the electric treatment, the frequency of protoplast division averaged 15%, which was slightly lower than the 17.5% reported by Sihachakr et al. (1988) for the electrofusion between eggplant and *S. khasianum* protoplasts. The highly diluted state of the cultures on a low zeatin medium resulted in a rapid growth of calli: they reached a diameter of 2–4 mm within 6–8 weeks. Plant regeneration, generally one shoot per callus (Fig. 1 D), occurred 4–6 weeks after the calli were transferred onto regeneration medium, its frequency averaged 9.2% (Table 1).

Using hair structure as selection criteria (Fig. 1 B), we were able to select 19 putative somatic hybrid plants derived from 19 individual calli. When grown to maturity in a greenhouse, most of these were relatively homogeneous in their morphology (Fig. 1 I). The exceptions, four plants, showed variations in leaf morphology and shoot height: they had irregular leaf forms, retarded development and difficulties in rooting; consequently, their height was considerably reduced. Several traits of most of the putative somatic hybrids were intermediate to

those of the parents (Table 2). The putative hybrids closely resembled eggplant in their height in maturity, but were only one-fourth of the height of *S. torvum*. The leaves were either lobed or slightly divided and light-green in colour (Fig. 1 H). The shoot tips were densely covered with a very thick white pubescence, as in eggplant, but the leaf veins and the stems had long spines, and the stems exhibited the presence of anthocyanin, as in *S. torvum*.

During this study, five hybrid plants recently flowered: they took 3 months to initiate flowers; eggplant normally requires 2 months and *S. torvum* at least 6 months under the conditions described here. Novel characteristics relating to precocious flowering have also been observed in the somatic hybrids of eggplant and *S. sisymbriifolium* (Gleddie et al. 1986b), those of eggplant and *S. khasianum* (Sihachakr et al. 1988) and in those of *Moricandia arvensis* and *Brassica oleracea*, where flowering no longer required vernalization (Toriyama et al. 1987).

The inflorescence of the putative somatic hybrids was a cyme as in *S. torvum*, but had a long spike, as in eggplant (Fig. 1 F). The number of flowers per inflorescence was lower and the flower size was larger than that of *S. torvum* (Table 2). Somatic hybrid flowers and those of *S. torvum* parental plants failed to mature under our greenhouse conditions, although they did develop sufficiently for some detailed morphological observations. The flowers with a long calyx (Fig. 1 F) closely resembled those of eggplant (Fig. 1 E); those of *S. torvum* had a short calyx and were minuscule in size (Fig. 1 G).

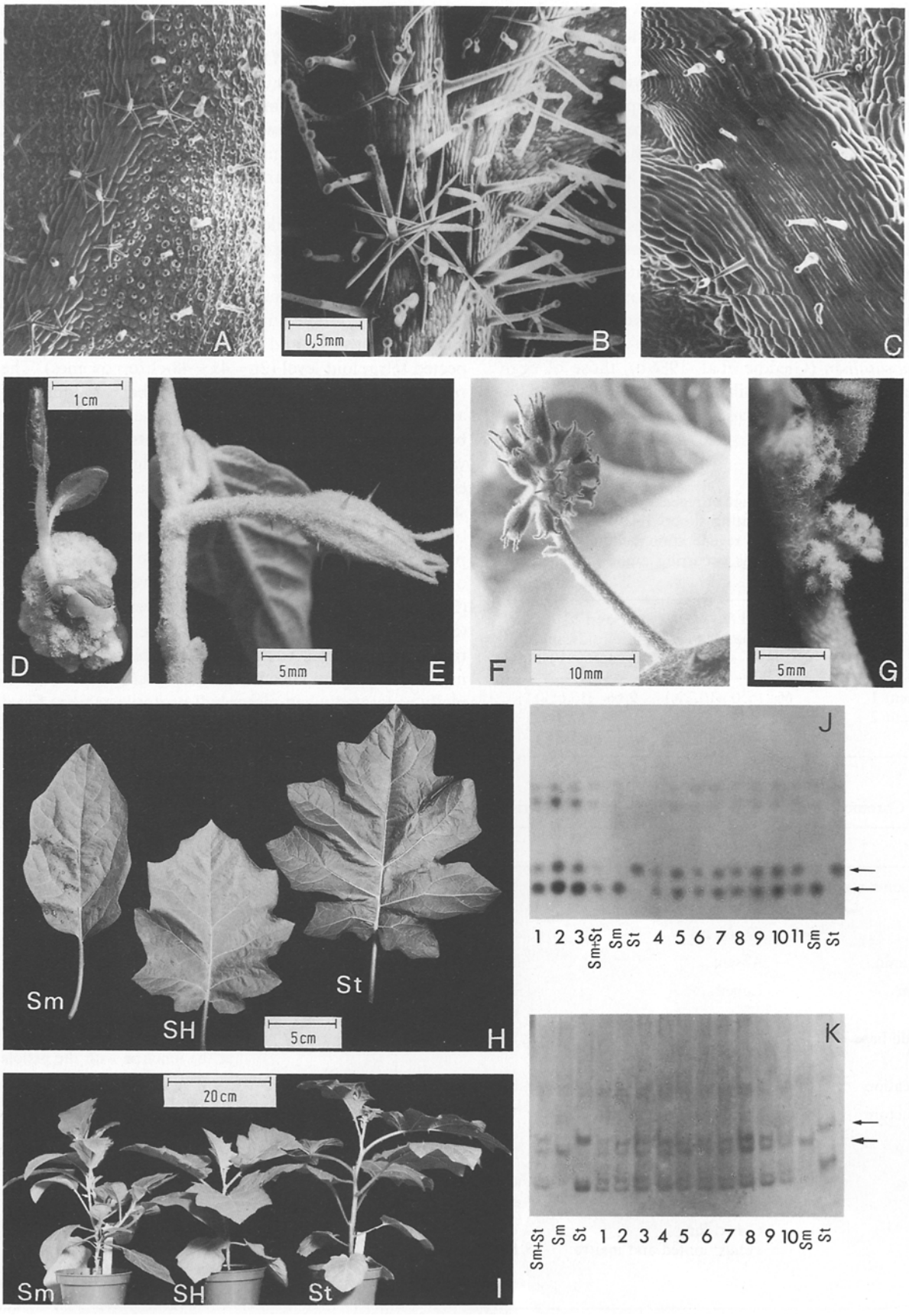
The chromosome numbers of the 15 putative hybrids varied between 46 and 48: this approximated the expected tetraploid level (2n = 4x = 48 chromosomes). The relative stability of chromosome numbers undoubtedly accounted for the homogeneity of these 15 putative hybrid plants. The remaining four plants which showed variable chromosome numbers ranging from 35 to 48 exhibited greater morphological variation. Relatively stable chromosome numbers in the majority of the putative hybrids may be related to the use of mesophyll protoplasts. This was also the situation of the somatic hybrids of eggplant and *S. khasianum*, which were all tetraploid (2n = 4x = 48 chromosomes) and relatively homogeneous (Sihachakr et al. 1988). In contrast, the somatic hybrids recovered from cell suspension protoplasts exhibited a higher variation in chromosome numbers than was normally generated when mesophyll protoplasts were fused (Schieder and Kohn 1986). That was also the case eggplant and *S. sisymbriifolium* were fused using cell suspen-

Table 1. Number and percentage of regenerated plants (defined as percentage of calli regenerating shoots relative to the total number of calli subcultured on regeneration medium) and number and percentage of hybrids occurring among regenerated plants

	No. and % of regenerated plants	No. and % of hybrids among regenerated plants
Experiment 1	66/ 650 (10.2%)	9/66 (13.6%)
Experiment 2	58/ 700 (8.3%)	10/58 (17.2%)
Total	124/1,350 (9.2%)	19/124 (15.3%)

Table 2. Chromosome number and morphological characteristics of *Solanum melongena*, *S. torvum* and their hybrids

	S. melongena	Hybrids	*S. torvum*
Chromosome number	24	15 plants: 46–48 4 plants: 35–48	24
Height	50–60 cm	50–60 cm	200–250 cm
Anthocyanin	Absent	Present	Present
Leaf form	Lobed	Lobed at juvenile stage, slightly divided at adult stage	Deeply divided
Leaf-blade base	United	Slightly divided	Divided, partially superposed at the junction with the petiole
Spine location	Calyx, leaf veins	Stem, petiole leaf veins	Stem, petiole, leaf veins
Hair structure	Very small glandular hairs and branching hairs	Densely covered with a mixture of long branching hairs and very long-stalked, glandular hairs	Non-branching glandular hairs at juvenile stage, but branching hairs at adult stage
Flowering	Solitary flower	Cyme (4–15 flowers) with a long spike	Cyme (30–40 flowers)
Flower	*Calyx*: long *Petals*: united and mauve	*Calyx*: long flowers aborted prematurely	*Calyx*: short *Petals*: united and white according to McCammon and Honma (1983)


A
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J
1 2 3 Sm+St Sm St 4 5 6 7 8 9 10 11 Sm St
K
Sm+St Sm St 1 2 3 4 5 6 7 8 9 10 Sm St

sion protoplasts: the majority of the somatic hybrids had 38–48 chromosomes and were phenotypically heterogeneous (Gleddi et al. 1986b).

Species-specific differences in the isoenzymes of phosphoglucomutase (Pgm) and esterases were observed between eggplant and *S. torvum* (Fig. 1J, K). Evidence of hybridity of the 19 selected regenerants was further provided by the banding patterns of both Pgm (Fig. 1J) and esterase (Fig. 1K) activities which were composed of bands found in both parents.

The fact that the 19 confirmed hybrid plants accounted for an average of 15.3% (Table 1) of the regenerated plants suggested that they certainly exhibited hybrid vigour which was expressed, in this case, by a higher capacity for earlier regeneration. Similar results were obtained for somatic hybrid plants of eggplant and *S. khasianum* which exhibited stronger hybrid vigour in their ability to regenerate plants, since at least 50% of the 173 regenerated plants were identified as somatic hybrids. Consequently, no particular selection methods have been used to recover somatic hybrid plants (Sihachakr et al. 1988). The complementary action of the parental genomes resulted in an exhibition of high regenerative capacity. In the somatic hybridization between *Moricandia arvensis* and *Brassica oleracea,* somatic hybrids were also successfully recovered without any selection methods (Toriyama et al. 1987). Similar results were reported for the fusion between *Citrus sinensis* and *Poncirus trifoliata* protoplasts (Grosser et al. 1988).

The great resemblance between somatic and F_1 sexual hybrids, described by McCammon and Honma (1983), especially in leaf form, spin-ness, inflorescence type and number of flowers per inflorescence, provides further evidence for the hybridity of somatic hybrids of eggplant and *S. torvum*. Similar observations have been reported for hybrids of eggplant and *S. khasianum* (Sharma et al. 1980; Sihachakr et al. 1988), and those of *Lotus* species (Wright et al. 1987).

Fig. 1. **A–C** The lower leaf surface of juvenile shoots of **A** eggplant note the very small glandular and branching hairs), **B** a somatic hybrid note the high density of very long-stalked glandular hairs and branching hairs and **C** *S. torvum* (glandular hairs only). Photographs were all reproduced on the same scale by scanning electron microscopy. **D** Regenerated shoot of a somatic hybrid. **E–G** An inflorescence of **E** eggplant with a solitary flower, **F** somatic hybrid with a long spike having 4–15 flowers and **G** *S. torvum* with 30–40 minute flowers. **H** Leaves of eggplant (*Sm*), somatic hybrid (*SH*) and *S. torvum* (*St*); **I** parental (*Sm* and *St*) and somatic hybrid (*SH*) plants. **J** and **K** electrophoresis banding patterns of **J** Pgm and **K** esterases from a sample of somatic hybrids (*lanes* 1–11 for Pgm, and *lanes* 1–10 for esterases), eggplant (*Sm*), *S torvum* (*St*), and a mixture of both parents (*Sm* + *St*)

This study has shown that somatic hybrid plants of *S. melongena* and *S. torvum* can be produced by protoplast electrofusion. Future efforts will be focussed on the determination of the environmental conditions required to obtain flower maturity in somatic hybrids. However, eggplant somatic hybrids were sterile, those of eggplant and *S. sisymbriifolium* produced anthers without pollen (Gleddie et al. 1986b) and those of eggplant and *S. khasianum* had a pollen viability of 12% (Sihachakr et al. 1988). Further research is needed to elucidate hybrid sterility and to restore fertility. Moreover, somatic hybrid plants must be evaluated for the inheritance of desirable agronomic traits.

The results of this study together those of our previous work (Sihachakr et al. 1988) demonstrate the effectiveness of the electrofusion technique, which can be used as a routine fusion procedure for somatic hybridization.

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References

Bhatt DP, Fassuliotis G (1981) Plant regeneration from mesophyll protoplasts of eggplant. Z Pflanzenphysiol 104:481–489

Gleddi S, Keller WA, Setterfield G (1986a) Somatic embryogenesis and plant regeneration from cell suspension derived-protoplasts of *Solanum melongena* (eggplant). Can J Bot 64:355–361

Gleddie S, Keller WA, Setterfield G (1986b) Production and characterization of somatic hybrids between *Solanum melongena* L. and *S. sisymbriifolium*. Theor Appl Genet 71:613–621

Grosser JW, Gmitter Jr FG, Chandler JL (1988) Intergeneric somatic hybrid plants of *Citrus sinensis* cv. 'Hamlin' and *Poncirus trifoliata* cv. 'Flying Dragon'. Plant Cell Rep 7:5–8

Guri A, Izhar S (1984) Improved efficiency of plant regeneration from protoplasts of eggplant (*Solanum melongena* L.). Plant Cell Rep 3:247–249

Guri A, Volokita M, Sink KC (1987) Plant regeneration from leaf protoplasts of *Solanum torvum*. Plant Cell Rep 6:302–304

Jia J, Potrykus I (1981) Mesophyll protoplasts from *Solanum melongena* var. depressum Bailey regenerate to fertile plants. Plant Cell Rep 1:71–72

Kao KN, Michayluk MR (1975) Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at very low population density in liquid media. Plant 126:105–110

McCammon KR, Honma S (1983) Morphological and cytological analyses of an interspecific hybrid eggplant, *Solanum melongena* × *Solanum torvum*. Hort Science 18:894–895

Morel G, Wetmore RH (1951) Fern callus tissue culture. Am J Bot 38:141–143

Murashige T, Skoog F (1963) A revised medium for rapid growth bioassays with tobacco tissue cultures. Physiol Plant 15:473–479

Ornstein L (1964) Disc electrophoresis. Ann NY Acad Sci 121:321–347

Puite KJ, Roest S, Pijnacker LP (1986) Somatic hybrid potato plants after electrofusion of diploid *Solanum tuberosum* and *Solanum phureja*. Plant Cell Rep 5:262–265

Saxena PK, Gill R, Maheshwari SC (1981) Plantlet formation from isolated protoplasts of *Solanum melongena* L. Protoplasma 106:355–359

Schieder O, Kohn H (1986) Protoplast fusion and generation of somatic hybrids. In: Vasil IK (ed) Cell culture, somatic cell, genetics of plants. Academic Press, Orlando San Diego New York Austin Boston London Sydney Tokyo Toronto, pp 569–588

Sharma DR, Chowdhury JB, Uma Ahuja, Dhankhar BS (1980) Interspecific hybridization in genus *Solanum*, a cross between *S. melongena* and *S. khasianum* through embryo culture. Z Pflanzenzücht 85:248–253

Sihachakr D, Ducreux G (1987a) Cultural behavior of protoplasts from different organs of eggplant (*Solanum melongena* L.), and plant regeneration. Plant Cell Tissue Organ Cult 11:179–188

Sihachakr D, Ducreux G (1987b) Variation of morphogenetic behavior and plant regeneration in cultured protoplasts of *Solanum nigrum*. Plant Sci 52:117–126

Sihachakr D, Haicour R, Serraf I, Barrientos E, Herbreteau C, Ducreux G, Rossignol L, Souvannavong V (1988) Electrofusion for the production of somatic hybrid plants of *Solanum melongena* L. and *Solanum khasianum* C. B. Clark. Plant Sci 57:215–223

Smithies O (1955) Zone electrophoresis in starch gels: group variation in the serum proteins of normal human adults. Biochem J 61:629–641

Toriyama K, Hinata K, Kameya T (1987) Production of somatic hybrid plants *"Brassicomoricandia"*, through protoplast fusion between *Moricandia arvensis* and *Brassica oleracea*. Plant Sci 48:123–128

Vallejos CE (1983) Enzyme activity staining. In: Tanksley SD, Orton TJ (eds) Isozymes in plant genetics and breeding. Elsevier, Amsterdam, pp 469–516

Wright RL, Somers DA, McGraw RL (1987) Somatic hybridization between birsdfoot trefoil (*Lotus corniculatus* L.) and *L. conimbricensis* Willd. Theor Appl Genet 75:151–156

Yamakawa K, Mochizuki H (1978) Studies on the use of disease resistance of wild non-tuberous *Solanum* species in the breeding of eggplants. Abnstract no. 1674, 20th Int Cong Hortic Meeting, Sydney, Australia