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# Somatic hybrids obtained by protoplast fusion between *Solanum tuberosum* L. subsp. *tuberosum* and the wild species *Solanum circaeifolium* Bitter

# Híbridos somáticos obtenidos por fusión de protoplastos entre *Solanum tuberosum* L. subsp. *tuberosum* y la especie silvestre *Solanum circaeifolium* Bitter

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## Abstract

Interspecific somatic hybrids were obtained by polyethylene glycol fusion of protoplasts from tetraploid *Solanum tuberosum* L. and the diploid wild species *S. circaeifolium*. Fusion-treated protoplasts were cultured in V-KM medium supplemented with bovine serum albumin. First cell divisions occurred within 3—4 days. A rapid calli proliferation was observed after colonies developed. Nineteen somatic hybrid plants were obtained and confirmed by RAPD analysis. Chromosome observations indicated that all hybrids were aneuploids. The morphology of fusion-derived regenerants was intermediate between the donor parents. This study shows that somatic hybrid potato plants can be obtained by the fusion method presented.

**Keywords:** *Solanum*, protoplast fusion, polyethylene glycol, somatic hybrid, RAPD analysis.

## Resumen

Con la finalidad de obtener híbridos somáticos interespecíficos, se fusionaron protoplastos de la especie tetraploide *Solanum tuberosum* y de la especie silvestre diploide *Solanum circaeifolium* utilizando polietilenglicol. Los productos de fusión fueron cultivados en el medio V-KM suplementado con albúmina de suero bovino. Las primeras divisiones celulares ocurrieron a los 3 a 4 días de cultivo. Después de la formación de colonias se observó una rápida proliferación de callos, a partir de los cuales se regeneraron 19 plantas. El análisis molecular usando RAPD, confirmó que los regenerantes presentaban segmentos de ADN de ambos parentales, sugiriendo su posible naturaleza de híbridos somáticos. Las observaciones del número de cromosomas indicaron que todos los híbridos fueron aneuploides. En condiciones de invernadero, los regenerantes derivados de la fusión de protoplastos, mostraron características morfológicas intermedias entre las líneas parentales. Este estudio muestra la producción de híbridos somáticos de papa con el método de fusión presentado.

**Palabras claves:** *Solanum*, fusión de protoplastos, polietilenglicol, híbridos somáticos, RAPD.

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## Introduction

Potato, *Solanum tuberosum* L. (2n=4x=48, 4 EBN: endosperm balance number) is an economically important crop species. The wild species *S. circaeifolium* (2n=2x=24, 1 EBN) is considered to be a source of agronomic traits such as resistance to *Phytophthora infestans* (Mont.) de Bary (Mattheij et al., 1992; Colon, 1994). Many wild *Solanum* species are regarded as important sources for disease resistance and tolerance to many abiotic stresses (Hawkes, 1994) but their use in potato breeding is limited due to poor crossability and sterility of interspecific hybrids. These barriers in classical breeding can be overcome using biotechnological methods such as somatic hybridization by protoplast fusion (Carputo et al., 1995; 1998; Millam et al., 1997; Davey et al., 2005). Using chemical or electrical procedures (Jones, 1988) protoplasts from different donor plants can be fused together and somatic hybrids regenerated from the fusion products. This technique has created novel cellular genome configurations by combining sexually incongruent species.

Somatic potato hybrids have been produced by fusion of diploid wild species with tetraploid *S. tuberosum* (Barsby et al., 1984; Austin et al., 1993; Cardi et al., 1993;) or with dihaploid *S. tuberosum* lines (Austin et al., 1985; Rokka et al., 1994). Successful application of the fusion technique demands a protocol for plant regeneration from protoplasts.

An analysis of putative fusion products is essential to confirm hybrid status (Masuelli et al., 1995; Penner et al., 1996; Matthews et al., 1997) and expression of the desirable traits. This can be done via morphological, biochemical, cytological and molecular markers (Pinto et al., 1995), and increasingly sophisticated molecular discrimination methods have recently been reported (Provan et al., 1996; Matthews et al., 1999; Harding and Millam, 2000; Trabelsi et al., 2005; Guo et al., 2007).

In this paper we describe the fusion of leaf protoplasts of *S. tuberosum* L. with *S. circaeifolium* Bitter in order to obtain somatic hybrids. RAPD and chromosomal analysis of the hybrids are also presented.

## Materials and methods

### Plant material and protoplast isolation.

In vitro 4—6 week-old leaves of tetraploid *Solanum tuberosum* L. cv. Désirée plants and wild species diploid *S. circaeifolium* plants (accession 2.1) were used as explants sources. The latter has a natural resistance to late blight, *Phytophthora infestans* (Mont.) de Bary (Mattheij et al., 1992; Colon, 1994).

These plants were grown on a lacking hormones Murashige and Skoog (1962) medium, and supplemented with 25% sucrose and 3,5% phytagel, pH 5,6.Plants were maintained in a controlled environmental cabinet with 18—22 °C, 16 h photoperiod and 80% relative humidity.

For protoplast isolation, 1 g of plant material was placed in petri dishes containing a plasmolyzing solution of sorbitol 0,5 M for 1—2 hours. Then, leaves were cut into small pieces and incubated with 10 mL of an enzymatic solution mixture containing 0,25% macerozyme R-10, 1% cellulase R-10, 700 mg calcium chloride, 1% 2-N-morpholinoethane sulfonic acid (MES) in sorbitol 0,5 M, pH 5,6.

The enzyme treatment was carried out overnight with gentle agitation. The protoplast suspension was filtered through 50 µm nylon screens. Protoplasts were precipitated by centrifugation

at 1000 rpm during 10 minutes. The pellets were mixed with 13% mannitol and protoplasts were purified by flotation over 21% sucrose solution. The protoplast band was collected and washed with 13% mannitol. Final concentration was $10^5$-$10^6$ protoplasts per millilitre.

**Protoplast fusion, culture and regeneration**

Prior to fusion, protoplasts from *S. tuberosum* cv. Desiree and *S. circaeifolium* were mixed in a 1:1 ratio in a tube with 0,2% calcium chloride and 2,5% potassium chloride solution, pH 6,9. Protoplasts were precipitated by centrifugation at 800 rpm during five minutes.

Some drops of fusion solution containing 25% polyethylene glycol, (PEG, MW 1500) were placed on a plastic petri dish. Then 100 µl from a mixture of partner protoplasts were placed over each fusion solution drop. After that, protoplasts settled down. During 20—30 minutes protoplasts were incubated. Fusion frequency (F.F.) was defined as Fish et al. (1988):

$$\text{F.F.} = \frac{\text{N.º of fused protoplasts x 100}}{\text{N.° of protoplasts}}$$

After fusion time, PEG was diluted by addition of a volume of washing solution (7,2% mannitol, pH 5,8). The mixture was incubated for thirty minutes and then protoplasts were collected by centrifugation at 800 rpm during five minutes.

The fusion-treated protoplasts were mixed with an equal volume of 2,8% sodium alginate and the suspension dropped onto a polymerization solution of 50 mM calcium chloride in sorbitol 0,4 M. The plates with alginate beads were stored during twelve hours at 4 ºC. After this time, the solution was replaced by protoplasts culture V-KM medium (Binding and Nehls, 1977) supplemented with 1% serum bovine albumin. The osmolality of this medium was fixed at 500 mOsm. Plates were incubated at 25—28 ºC under dark conditions. After seven days, the culture medium was diluted 1:1 with V-KM medium of 300 mOsm. Then, at fifty days, all culture media were replaced by a 300 mOsm V-KM medium.

When colonies had developed, alginate beads were despolimeryzed using 20 mM sodium citrate solution in sorbitol 0,3 M, pH 7,4 and gently shaking. Then the suspension was centrifugated at 1000 rpm during ten minutes. After that, colonies were dispersed into plates with solid MS13K regeneration medium (Benke, 1975). The cultures were maintained in an incubation room at 22—24 ºC under indirect light conditions. Regenerants from the fusion experiments were propagated in vitro using the Murashige and Skoog (1962) medium.

**RAPD analysis**

The hybridity of the fusion regenerants was confirmed by RAPD markers. For this analysis, DNA extraction was performed according to Doyle and Doyle (1990) with some modifications. Random decameric oligonucleotides from GENSET were used for PCR amplification.

PCR was carried out in a 10 µL reaction volume containing: 4,76 µL mili-Q water; 1,5 µL 10X reaction buffer; 50 mM MgCl2; 2,5 mM dNTPs; 10 ng/µL primer and 0,09 µL Taq polymerase (isolated at the International Potato Center laboratories). To this reaction mixture, 5 µL of genomic DNA and 50 µL mineral oil were added. PCR amplification was realized in a PTC-100 thermal cycler (Programmable Thermal Cycler) programmed for one cycle of 3 minutes at 94 ºC followed by 40 cycles of 1 minute at 94 ºC, 1 minute at 35 ºC, 2 minutes at 72 ºC and one cycle of 7 minutes. Amplification products were analyzed by gel electrophoresis in 1,4% agarose gels containing ethidium bromide. Gels were analyzed and photographed under UV light.

**Chromosome analysis**

Root tips from the regenerants and the fusion parents were pretreated with 100 ppm of Ambush pirethroid insecticide, pH 5 for 24 hours at 4 ºC. These samples were obtained from rooted plants maintained in a greenhouse. Hydrolysis was carried out with 1 N HCl at 60 ºC during 8 minutes. Root tips were stained by lacto-propionic orcein.

## Results and discussion

**Protoplasts fusion and plant regeneration**

Protoplasts fusion from *S. tuberosum* cv. Désirée and *S. circaeifolium* were induced with the fusion conditions described (Fig. 1a). Fusion frequencies of 20—30% were obtained. Aggregations of more than two protoplasts were also observed. Heterokaryons can not be identified because protoplasts of the parents were of the same type and shape. Due to that, fused and unfused protoplasts were cultured together.

The first cellular divisions happened within 3—6 days of culture in V-KM medium (Fig. 1b) further forming several colonies (Fig. 1c), indicating that the survival after fusion treatment was still high. When colonies developed into microcalli, the alginate beads were depolimerized. Microcalli were transferred onto MS-13 medium to induce shoot regeneration under indirect light conditions. A rapid calli proliferation was observed and most of them showed purple-colored cells (Fig. 1d). Shoot regeneration started twelve weeks after calli were transferred to MS-13 medium. Shoots also showed purple-colored meristematic zones. Nineteen plants were regenerated from calli (Fig. 1e). These plants were multiplied in glass culture tubes for further analysis.

From the two fusion experiments, the regeneration frequency was 26,8% (Table 1). This value is different from that obtained by Mattheij et al. (1992), who, after protoplasts electrofusion, reported a regeneration frequency of 14%.

This difference in the regeneration frequency could be explained due to the fact that one of the parents, the tetraploid cultivar Désirée, has a very good morphogenetic capacity (Espejo et al., 1999). The other parent, the wild species *S. circaeifolium*,

**Table 1.** Shoot regeneration from calli derived from protoplasts fusion with PEG.

| Fusion combination | Number of calli | Shoot regeneration | | Number of shoots | Regeneration frequency [a] (%) |
|---|---|---|---|---|---|
| | | N.º | % | | |
| Désirée + *S. circaeifolium* | 71 | 14 | 19,7 | 19 | 26,8 |

[a] shoots from calli produced (Waara et al., 1991)

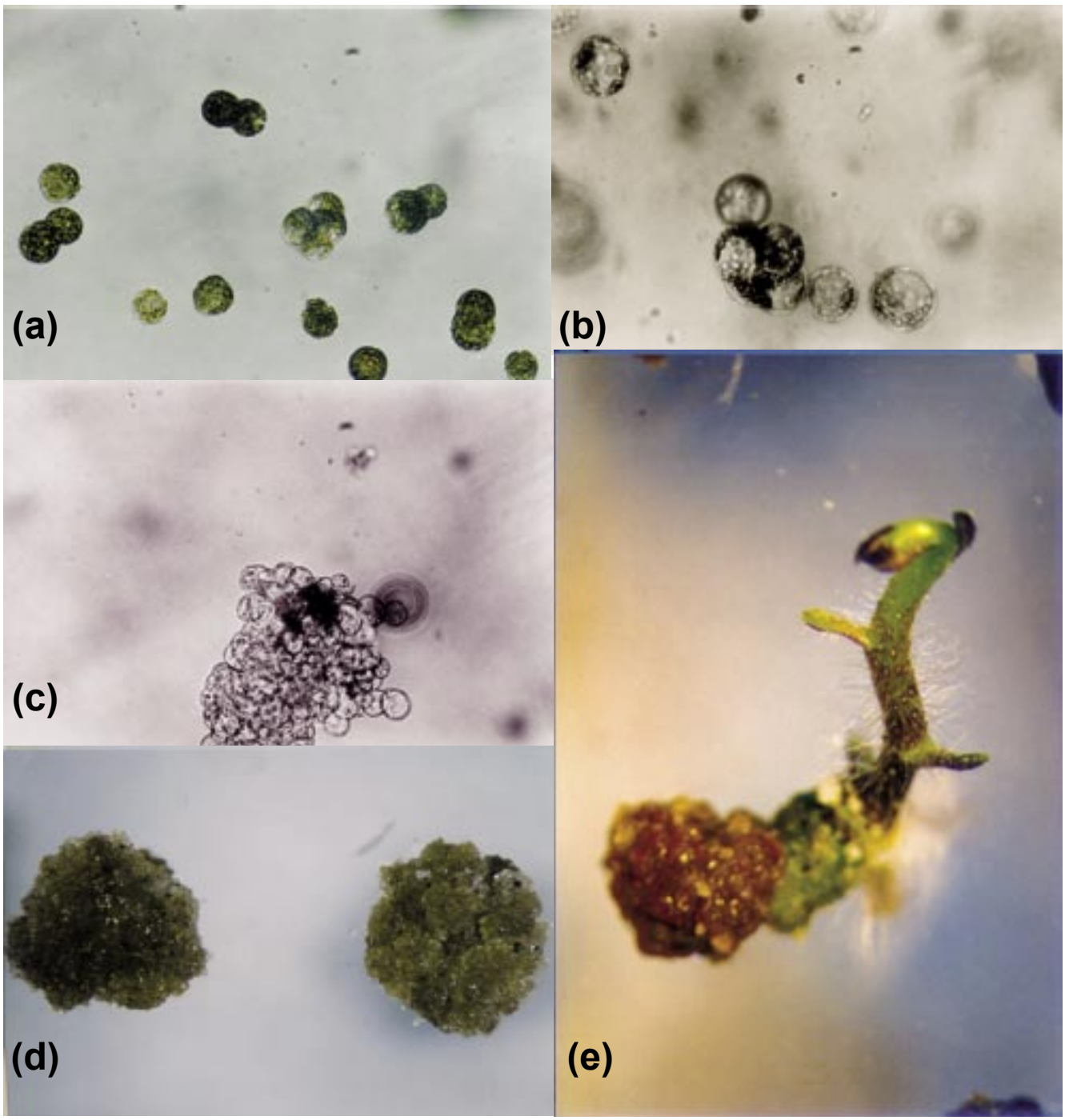


**Figure 1.** (a) Protoplast fusion PEG –induced. (b) Cell divisions with 3-6 days. (c) Colony divided from fused protoplasts at 20 and 25 days, respectively, after fusion.(d) Calli derived from fused protoplasts showed purple-colored cells.(e) Plant regeneration.

also has response for regeneration but its morphogenic capacity is lower than the Désirée cultivar (Espejo, 2000). Under these conditions, calli hybrids follow the developmental pathway of the morphogenic parents.

**RAPD analysis**

To select hybrids, regeneration of in vitro plants was subjected to RAPD analysis. This technique is a tool for somatic hybrid characterization (Baird et al., 1992; Yong-Sheng, 1993; Takemori, 1994; Rasmussen and Rasmussen 1995; Penner et al., 1996; Henn et al., 1998; Rokka et al., 1998).

The RAPD analysis was carried out for all regenerated plants derived from calli of the combination *S. tuberosum* cv. Désirée + *S. circaeifolium*. The RAPD markers showed that all of the regenerants were somatic hybrids between *S. tuberosum* and *S. circaeifolium*. The primer OPM10 (5'-TCTGGCGCAC-3') amplified a *S. tuberosum* specific band of 805 bp which appeared in the fusion-derived regenerants. The same primer amplified four specific bands of 740, 1000, 1270 and 1750 bp in *S. circaeifolium*. These bands also appeared in the fusion regenerants (Figs. 2a, b), indicating that parts of the genomes from both parents were combined in the hybrids.

With the primer OPM11 (5'-GTCCACTGTG-3'), these two parental lines exhibited clear differential banding patterns which also confirmed the hybrid nature of regenerants (Figs. 2c, d). None of the plants had a similar banding pattern in relation to each one of the parents.

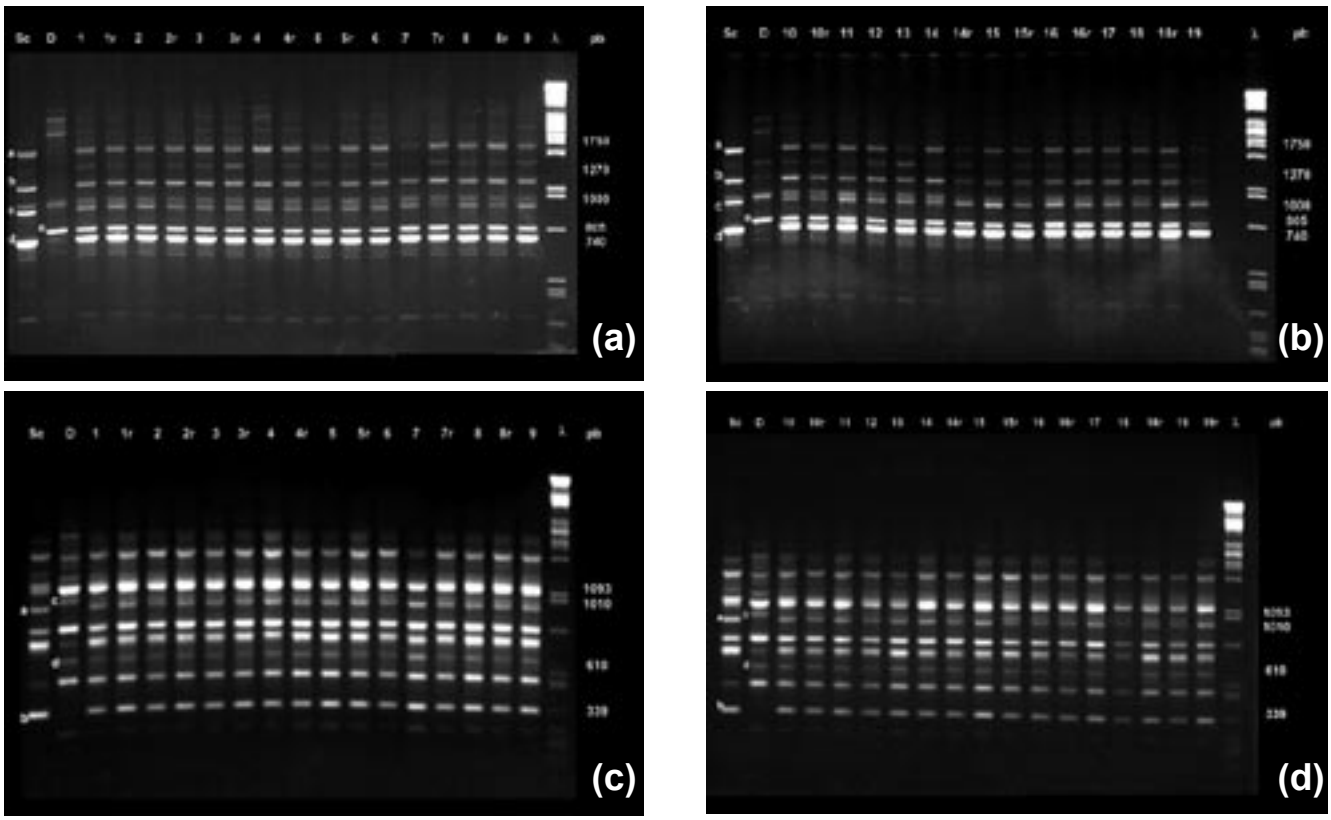


**Figure 2. (a)** RAPD patterns with primer OPM10. From left to right: Sc (*S. circaeifolium*), D (*S. tuberosum* cv. Désirée); 1 to 9 (somatic hybrids of Sc+D), r (repetition); λ (Lambda Pst I). a, b, c and d (specific bands for Sc); e (specific bands for D). **(b)** RAPD patterns with primer OPM10. From left to right: Sc (*S. circaeifolium*), D (*S. tuberosum* cv. Désirée); 10 to 19 (somatic hybrids of Sc+D), r (repetition); λ (Lambda Pst I). a, b, c and d (specific bands for Sc); e (specific bands for D). **(c)** RAPD patterns with primer OPM11. From left to right: Sc (*S. circaeifolium*), D (*S. tuberosum* cv. Désirée); 1 to 9 (somatic hybrids of Sc+D), r (repetition); λ (Lambda Pst I). a, b (specific bands for Sc); c, d (specific bands for D). **(d)** RAPD patterns with primer OPM11. From left to right: Sc (*S. circaeifolium*), D (*S. tuberosum* cv. Désirée); 10 to 19 (somatic hybrids of Sc+D), r (repetition); λ (Lambda Pst I). a, b (specific bands for Sc); c, d (specific bands for D).

Identification of somatic hybrids is a requisite for the effective exploitation of the protoplasts fusion in potato improvement. In this study, confirmation of fusion-derived regenerants by the use of RAPD markers represented an effective system for the detection of somatic hybrids from *S. tuberosum* cv. Désirée and *S. circaeifolium*.

**Chromosome analysis**

The numbers of chromosome of *S. tuberosum* was 2n=2X=48 and *S. circaeifolium* was 2X=2X=24. For the obtained somatic hybrid plants, the chromosome number varied from 80-94 (Table 2). These results showed that all hybrids were aneuploids (Fig. 3). None of the regenerants had 72 chromosomes, which results from the protoplasts fusion of a tetraploid with a diploid genome.

The production of somatic hybrid plants with a chromosome number deviating from the expected is well documented in many fusion combinations where potato protoplasts have been used as one or both fusion parents (Austin et al., 1985; Fish and Karp, 1986; Fish et al., 1988; Perl et al., 1990; Waara et al., 1991; Masuelli et al., 1995; Pinto et al., 1995).

**Table 2.** Chromosome numbers and obtained somatic hybrid plants

| Chromosome number | 80 | 82 | 84 | 86 | 87 | 88 | 90 | 94 |
|---|---|---|---|---|---|---|---|---|
| Number of plants | 7 | 1 | 3 | 2 | 1 | 2 | 2 | 1 |

After protoplasts fusion and during plant regeneration it frequently happens that genetic changes such as variations in chromosome number occur. The origin of this chromosome instability has been studied elsewhere in potato (Kumar, 1994).

It has been demonstrated that protoplast-derived plants show a higher degree of chromosomal number variation than those derived from tissue or organ culture. It has been suggested that protoplasts are more prone to chromosome instability because they undergo a long period of greater stress during initial stages of cell division and dedifferentiation (Pijnaker and Sree Ramulu 1990; Carrasco et al., 1998).

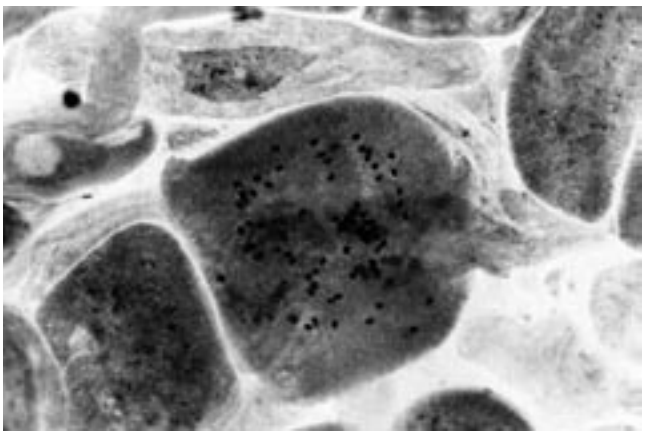

**Figure 3.** Chromosomes in a root-tip cell of an aneuploid somatic hybrid plant (80 chromosomes).

The ploidy of the genotype used in plant regeneration has been shown to profoundly affect the type of variation observed in morphological characters and chromosome numbers among regenerant plants. Plant regeneration from mono- and dihaploid potato genotypes often results in ploidy changes, but little aneuploidy; on the other hand, regenerated plants from tetraploid genotypes produce a wide range of aneuploidy (Kumar, 1994). For these reasons and considering the obtained results, it can be suggested that there is a high probability of fusion-derived regenerants which are aneuploids with an increase or loss of some chromosomes when one or both fusion partners have a high ploidy level.

Somatic hybrids were confirmed morphologically under greenhouse conditions, when intermediate characteristics between parent lines, such as shoot coloration, leaf shape and pubescence were observed.

Potato is an example of a crop in which advantages can be obtained by the use of alternative techniques which exchange and introgress 'new' genetic information, conferring beneficial traits from wild species into conventional cultivars (Millam et al., 1997). Tissue culture procedures to produce somatic hybrids and the application of molecular biological tools for their analysis are well established.

In conclusion, somatic fusions between the cultivated potato *Solanum tuberosum* and the wild species *S. circaeifolium* were produced in order to incorporate desirable traits into the potato gene pool. Nineteen somatic hybrid plants were obtained from fusion experiments using PEG in an effort to combine elite traits from both parents. Cytological and RAPD marker analysis confirm their hybrid nature. Further work is needed to evaluate their resistance level to *Phytophthora infestans* (Mont.) de Bary.

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