

An Efficient Method of Protoplast Isolation and Plant Regeneration in the Wild Species *Solanum papita* Rydberg

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ABSTRACT

Several workers have achieved the isolation of potato protoplasts and their subsequent regeneration. However, many wild species have not been tested.

An improved procedure of protoplast isolation has been developed. Regeneration of *Solanum papita* protoplasts and their response to established culture media were observed. More than 75% of protoplasts divided within the first two weeks. The protoplast-derived cell colonies developed calli, which formed shoots. Regenerated shoots were able to develop into whole plants.

Key words: protoplast culture, somatic hybridization organogenesis, regeneration, *Solanum papita*

RESUMEN

En este trabajo se ha desarrollado un procedimiento de regeneración de plántulas a partir de protoplastos de la especie silvestre *Solanum papita* Ridberg. Durante las dos primeras semanas más del 75% de los protoplastos aislados se dividieron permitiendo la formación de colonias celulares a partir de las cuales se desarrollaron callos. Cuando los callos fueron transferidos a un medio de regeneración se indujo en ellos la formación de múltiples brotes en el medio de cultivo establecido. Los brotes fueron capaces de regenerar plantas completas.

Palabras clave: Cultivo de protoplastos, hibridación somática, organogénesis, regeneración, *Solanum papita*

INTRODUCTION

Information on necessary conditions for *in vitro* culture and totipotency of cultured tissues, cells, and protoplasts is essential for somatic hybridization studies. Plant cells without cell walls are called protoplasts and

they are appropriate receptors for transferring genes. In the *Solanum* genus, protoplast culture and plant regeneration procedures have been established for *S. tuberosum* (Shepard & Totten, 1977), *S. dulcamara* (Binding & Nehls, 1977), *S. nigrum* (Nehls,

1978), *S. phureja* (Schumann *et al.*, 1980), *S. aviculare* (Gleddie *et al.*, 1985), and *S. tuberosum* (Wenzel *et al.*, 1979; Tavazza & Ancora, 1986).

Optimization for large-scale isolation of viable protoplasts and subsequent *in vitro* plant regeneration are pre-requisites for producing plants with desired traits through somatic hybridization and genetic engineering techniques (Saxena *et al.*, 1987; Kaur *et al.*, 2006).

The potato (*Solanum tuberosum* L., $2n=4X=48$; 4 EBN: endosperm balance number) is a tuber-bearing crop, which has a remarkable economic, agricultural, and nutritional importance, and is characteristic from cold and temperate regions. It has good adaptation ability to different world climates; although it is sensible to freezing and drought. In addition it is affected by pests and diseases, which can decrease average yields. For these reasons its genetic improvement is important in order to obtain cultivars with appropriate resistance levels to abiotic and biotic stresses, good yields and agronomic quality.

Solanum papita Rydberg ($2n=4X=48$, 2 EBN) is a Mexican wild relative of *S. tuberosum*, (Luna-Cavazos *et al.*, 2007) resistant to several stresses including virus and drought (Hannemann & Bamberg, 1986; Hannemann, 1989; Vayda, 1994; Estrada, 2000; Contreras, 2005). However, due to post-zigotic barriers, their use in potato breeding is limited due to poor crossability and sterility of interspecific hybrids.

The use of protoplasts is an alternative, because through their manipulation it is possible to achieve somatic hybridization by protoplasts fusion among sexually incompatible cultivated species and their wild

relatives which have natural resistance to abiotic and biotic factors. However, to be able to apply protoplasts technology in potato plant breeding it is necessary to develop efficient protoplast isolation, culture and plant regeneration systems from protoplasts of the genotypes of interest (Foulger & Jones, 1986; Mollers & Wenzel, 1992; Davey *et al.*, 2005; Winkelmann *et al.*, 2006).

Considering the importance of protoplasts and plant regeneration within a potato breeding program, in the present study we propose the following objectives: To isolate protoplasts at large scale and achieve a high rate of colony formation with subsequent plant regeneration. The protocol described allows reproducible regeneration of plants from protoplasts of the wild species *Solanum papita*.

MATERIALS AND METHODS

Plant material

Leaves of 4-6 weeks-old *in vitro* plants of the wild species *Solanum papita* and *Solanum tuberosum* L. cv. Désirée (plant considered as a model for plant regeneration (Foulger & Jones 1986) were used as an explant source.

Protoplast isolation and culture

To optimize the conditions for protoplasts 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 (Kinki Yakult MFG. Co., Ltd.), 1% cellulase (Onozuka R-10), 700 m/L calcium chloride, 1% 2-N-morpholinoethane sulfonic acid (MES) in sorbitol 0.5 M. The pH was adjusted to 5.6. At

the end of the incubation period (16-18 hours at 25 °C, darkness conditions and slow shaking at 60 rpm), the resulting suspension was filtered through 50 µm nylon screens and transferred into 10 ml centrifuge tubes. Protoplasts were collected by centrifugation at 1,000 rpm (in a Gyrozen centrifuge, model Gyro416G with fixed angle rotor, GRA-15-16) during 10 minutes. The pellet was mixed with 13% mannitol and protoplasts were purified by flotation over a 21% sucrose solution and centrifuged at 700 rpm for 5 minutes. Protoplasts were carefully collected and washed with 13% mannitol. After, the protoplast solution was mixed with an equal volume of sodium alginate and the suspension dropped into a polymerization solution (50 mM calcium chloride in 0.4 M sorbitol). The plates with alginate beads were stored during 12 hours at 4 °C. After this time the polymerization solution was replaced by a V-KM culture medium (Binding & Nehls, 1977) supplemented with 1% Serum Bovine Albumin. The osmolality of this medium was fixed at 500 mOsm. After 10 days the culture medium was diluted 1:1 with V-KM medium of 300 mOsm. Plates were incubated at 22 °C under dark conditions.

Plant regeneration

Two or three weeks later multicellular colonies developed and alginate beads were despolimerized using a 20 mM sodium citrate solution in 0.3 M sorbitol, pH 7.4 and gently shaken. Then the suspension was centrifuged at 1,000 rpm during 10 minutes and the pellets were washed with sorbitol 0.5 M and resuspended in V-KM medium. Colonies were carefully dispersed in plates with a solid MS13K regeneration medium (Benhke, 1975).

The cultures were maintained in an incubation room at 22-24 °C under low light conditions.

Regeneration data were statistically analyzed with the t test. The number of shoots and the number of regenerated plantlets were compared between both species. For this analysis the transformation of the original data (root square + 1) was necessary. Also, regression coefficient b was calculated for each species.

RESULTS AND DISCUSSION

The isolation procedure proposed, based on preliminary experiments, was effective in the releasing of protoplasts from the two evaluated genotypes (Fig.1a). High quantity of protoplasts was obtained at levels of 10^5 - 10^6 cel ml⁻¹. The first cell division occurred after four days of culture (Fig. 1b). Further divisions took place after six days (Fig. 1c), and were followed by successive divisions resulting in the formation of masses of cells. More than 75% of protoplasts divided during the first two weeks. After 15-20 days of culture, cell divisions developed into colonies (Fig.1d), which were transferred to a regeneration medium. During the initial four weeks of culture, *Solanum papita* and Désirée colonies developed calli in approximately 80%. These calli were light-brown and then became green. Regeneration of shoots started 35 - 40 days after transferring to regeneration medium.

Calli developed multiple shoots (Fig. 1e), which elongated up to 2-3 cm within three weeks and produced roots when were transferred to propagation medium. Désirée formed an average number of 1.77 shoots/callus, and *Solanum papita*, average 3.06 (Table 1) (**annexed**). The t test showed highly significant differences between these

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data. Average regeneration was 0.89 and 2.29 plantlets/callus respectively, which were statistically different ($p=0.01$). The proportion of regenerated plantlets taking into account the initial number of shoots was calculated

with the regression coefficient b , which showed that for each shoot formed by Désirée, 0.59 plantlets were developed while in *Solanum papita* 0.62 plantlets (Fig. 1f) were developed ($p<0.001$).

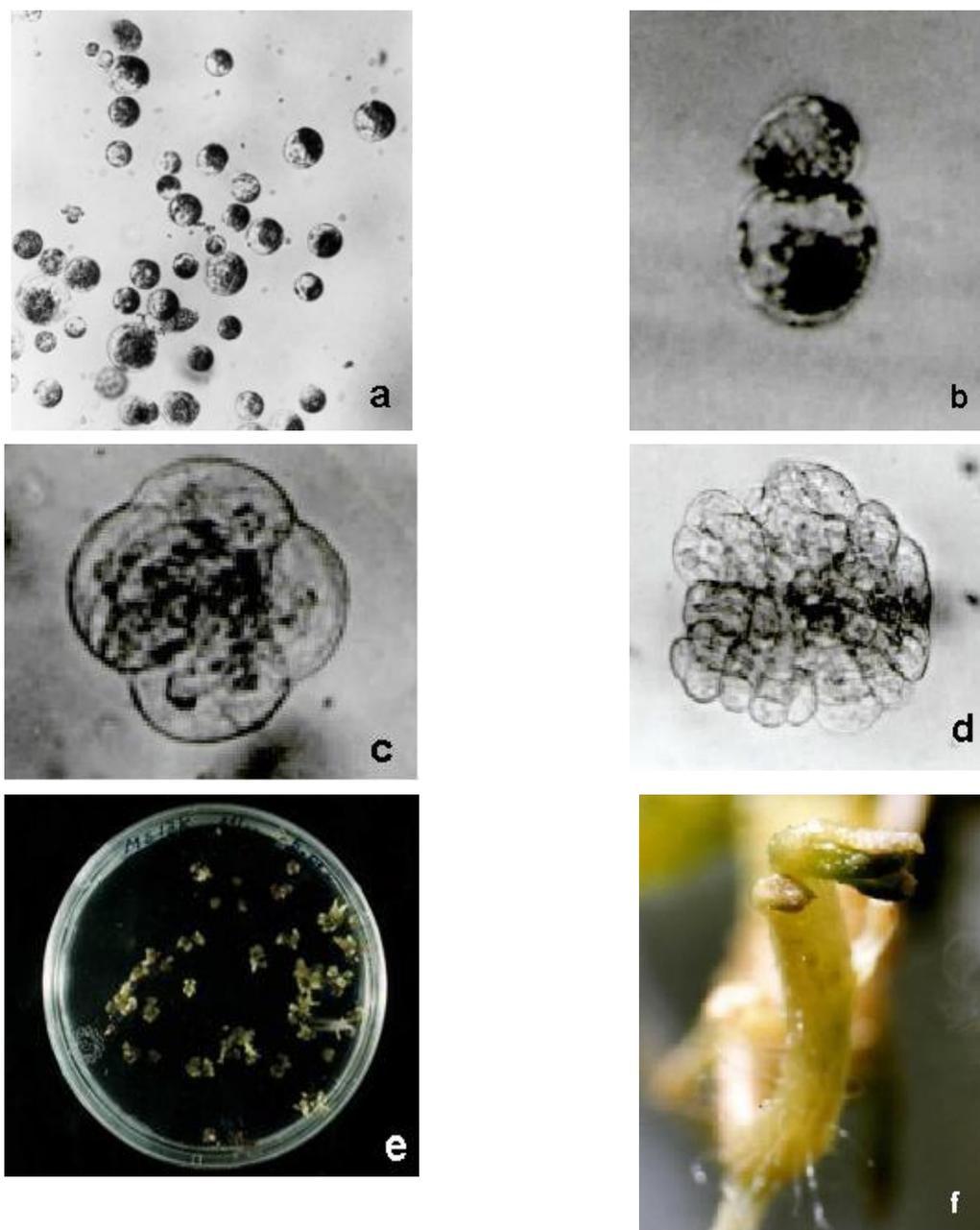


Fig. 1. Steps in plant regeneration from protoplasts in the *Solanum papita* wild species. a) isolated protoplasts, b) first division after four days of protoplast culture, c) second cell division after six days in culture, d) multicellular colony 15-20 days after culture, e) early stage of shoot formation form calli, f) regenerated plantlet

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Table 1. Average number of shoots and regenerated plants for protoplast-derived calli^a

Species	Average number of shoots per callus ^b	Average number of regenerated plants per callus ^b
<i>S. tuberosum</i> cv. Désiree	1.77	0.89
<i>S. papita</i>	3.06	2.29

^a Analysis performed on 79 calli for each species.

^b Calli have not been divided.

Several parameters, particularly the source tissue, culture medium, and environmental factors, influence the ability of protoplasts to express their totipotency and to develop into plants, although it has been shown that the responsiveness of plants to protoplasts isolation, culture and regeneration is genotype dependent (Radke & Grun, 1986; Fish *et al.*, 1988; Karp, 1991). In the present experiment the proportion of the two enzymes: cellulose R-10 (1%) and macerozyme (0.25%) was appropriate for protoplast isolation of *Solanum papita* and cultivar Désirée during darkness conditions (16-18 horas). Tavazza & Ancora (1986) found out that low concentrations of macerozyme avoided the toxic effects due to long exposures to this enzyme. Conversely high concentrations of the same enzyme yielded more protoplasts but there was more debris and non viable protoplasts. Protoplasts are fragile and sensitive to digesting enzymes.

Plasmolysis prior to enzymatic digestion of source tissues in sugar alcohol solutions, such as sorbitol 0.5 M causes the cell to draw back from the cell wall and reduces cytoplasmic damage and spontaneous fusion of protoplasts from adjacent cells. Protoplast yield and viability can be further enhanced by slicing source (preplasmolysed) tissues in a suitable osmotic solution (Power *et al.*, 2004). Also, the effect of the plasmolyzing solution (mannitol 13%) in the recovery of viable

protoplasts was positive, which is similar to the results observed by Belarmino *et al.* (1994) working with sweetpotato. It has been proposed that this solution reduces the osmotic shock during protoplast isolation by decreasing leakage of electrolytes (Winkelmann *et al.*, 2006).

Most protoplasts have an optimum plating density related to their potential division. At this density (usually 10^4 - 10^5 cel ml⁻¹) plated protoplasts produce cell colonies after 2-3 weeks of culture (Davey *et al.*, 2005). In our experiments *Solanum papita* and Désirée yielded 10^5 - 10^6 cel ml⁻¹ protoplasts with the isolation method proposed. With these densities the cell colonies arising from individual protoplasts and tend to grow at a fairly early stage in culture. The nutritional components of the most commonly used culture media are not sufficient to induce divisions in protoplasts plated at low density.

Generally high light intensity inhibits protoplasts growth when applied from the beginning of culture. It is better to initiate protoplasts culturing in darkness conditions. There are reports of better protoplasts growth when the cultures are kept in continuous darkness (Chawla, 2002).

Growth and shoot initiation of potato protoplasts also depends on the composition of the culture media and genotype (Foulger & Jones, 1986). Protoplasts cultured on V-KM

medium supplemented with 1% serum bovine albumin (Binding & Nehls, 1977) divided many times at a relatively high frequency leading to a high rate of colony formation in both species. The same pattern of cell division was observed by Fock *et al.* (2001) when they cultured protoplasts of *Solanum stenotomum* and *S. tuberosum* in V-KM medium. Protoplasts from *S. papita* and cultivar Désirée showed a higher division frequency when they cultured in alginate beads and V-KM medium. Likewise nutritional and physical factors affect colony formation from protoplasts, and dark conditions were suitable for culturing protoplasts and to induce colony development (Ehsanpour & Jones, 2001; Davey *et al.*, 2005; Winkelmann *et al.*, 2006). Low light conditions were beneficial for calli formation and regeneration (not shown). This result was similar to that mentioned by Shepard & Totten (1977) working with potato, who found out that high light intensities decreased the callus ability to form shoot buds. Shoots arose from non-embryogenic calli.

It has been reported that shoot formation from calli derived from protoplasts is genotype dependent (Thomas, 1981). In our experiments statistical analysis of calli the English version of this article.

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regeneration considering the number of shoots and the number of regenerated plantlets showed significant differences between *Solanum papita* and Désirée. The wild species *Solanum papita* showed a higher ability for shoot induction than Désirée plants (considered as a model for plant regeneration (Tavazza & Ancora, 1986). Although, regression coefficient b showed the same behavior for shoot conversion into plants for both species.

Regenerants from *S. papita* and *S. tuberosum* cv. Désirée, were successfully produced using the improved culture procedure. The plant regeneration pathway from protoplasts opens the possibility for somatic hybridization and thereby new breeding strategies in the generation of biotechnological applications for the introgression of resistance to abiotic and biotic factors in potato plants.

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