

## Conservation of potato (*Solanum tuberosum* L.) cultivars by cryopreservation

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### Abstract

The shoot-tips of plants of 10 potato cultivars maintained by micropropagation were frozen (ultra rapid freezing) and cryopreserved in liquid nitrogen for three months. The survival rate of the thawed specimens exceeded 50%, and even reached 100% in the case of the cultivar Fénix. The average regeneration rate was between 2.5% and 22.0%, depending on the cultivar and the presence of extra plant hormones in the regeneration medium. Cytogenetic stability was confirmed by flow cytometry: no polyploid plants were found. The regenerated plants showed vegetative development characteristics of their respective cultivars.

**Key words:** meristems, germplasm, freezing, regeneration.

### Resumen

#### Conservación de cultivares de patata (*Solanum tuberosum* L.) mediante criopreservación

Se ha aplicado la criopreservación mediante el método de congelación ultrarrápida a 10 cultivares de patata mantenidos por micropropagación. Para ello se congelaron y conservaron en nitrógeno líquido meristemos apicales. La supervivencia tras la descongelación, transcurrido un periodo de tres meses, fue superior al 50% en todos los casos, alcanzando en algún cultivar, como Fenix, hasta el 100% de los meristemos congelados. Las tasas de regeneración se situaron entre el 2,5% y el 22,0%, dependiendo del cultivar empleado y el uso de fitohormonas en el medio MS sólido con agarosa. La estabilidad citogenética fue evaluada mediante citometría de flujo, no encontrando en ningún caso poliploides. Las plantas regeneradas mostraron un desarrollo vegetativo aparentemente idéntico a los cultivares originales.

**Palabras clave:** meristemos, germoplasma, congelación, regeneración.

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### Introduction

For several years, the Basque Institute of Agricultural Research and Development [BIARD]-NEIKER (*Instituto Vasco de Investigación y Desarrollo Agrario-NEIKER*) has maintained a partially characterised potato (*Solanum tuberosum* L.) germplasm bank (<http://www.neiker.net/patdb>). The collection contains material from 412 cultivars, 332 of which are commercial and the rest either local forms or cultivars developed at the Institute. The bank is a source of material for the genetic improvement of the species and the creation of new varieties (Ruiz de Galarreta *et al.*, 2000).

Potato germplasm is normally maintained using *in vitro* culture techniques employing media that retard

growth and controlled conditions of light and temperature. This works well for short and medium term preservation since material is always ready for use. However, the necessary periodic subculturing of plantlets in MS medium (Murashige and Skoog, 1962) has the disadvantage that infections may develop. There are also the risks of culture chamber breakdown and the appearance of somaclonal variation (Harding, 1991).

For the long term conservation of material, cryopreservation offers several advantages, such as reduced storage space, low maintenance (periodic refilling of the freezer tanks with liquid nitrogen is all that is needed), and the independence of survival on the duration of freezing (Bajaj, 1981).

Ultrarapid freezing (Bajaj, 1981; Schäfer-Menuhr *et al.*, 1996), slow controlled freezing (Towill, 1983; Harding *et al.*, 1991), vitrification (Schnabel-Preikstas *et al.*, 1992), and encapsulation and dehydration

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(Fabre and Dereuddre, 1990; Bouafia *et al.*, 1996) have all been investigated as methods of freezing the meristems or shoot apices of selected *Solanum* genotypes. Some of these methods are very laborious, complicated, time consuming, and only offer low regeneration rates (Benson *et al.*, 1989).

The aim of the present work was to improve and simplify the cryopreservation (ultrarapid freezing technique) of different potato cultivars, for the conservation and long term storage of their germplasm.

## Material and Methods

The plant material used included 10 potato cultivars: four obtained at NEIKER (Zorba, Gorbea, Draga, Fénix), two local cultivars (Cazona and Ganade) from Galicia (northwest Spain), and four commercial cultivars (Hydra, Isola, Kennebec and Red Pontiac).

Meristem samples 2-3 mm long and 0.5-1.0 mm thick (depending on the cultivar) were removed from 10-12 cm long, *in vitro* micropropagated plantlets. These were incubated at 23°C for 24 h in liquid MSTo medium (Towill, 1983) containing the salts and vitamins provided by MS medium plus 0.2 mg L<sup>-1</sup> gibberellic acid, 0.5 mg L<sup>-1</sup> indole acetic acid, and 0.5 mg L<sup>-1</sup> zeatin riboside.

After incubation, the meristems were placed in sterile Petri dishes containing cryoprotectant solution [DMSO (i.e., MSTo + dimethyl sulphoxide 10% w/v)] for 2 h at room temperature.

Aluminium laminae (0.7 cm × 2.0 cm and 0.03 mm thick) were cut and five drops of DMSO 10% (w/v) placed on top of each. One meristem was then placed in each drop. The laminae were introduced into cryotubes (two per tube), which were then only partially closed to allow the entry of liquid nitrogen from the freezer tank. Specimens were kept frozen for three months. A modified version of the ultrarapid freezing method of Schäfer-Menhur *et al.* (1997) was used. Table 2 shows the number of meristems frozen per cultivar.

To thaw the meristems, the aluminium laminae were removed from their cryotubes and placed in Petri dishes containing liquid MS medium at room temperature. The meristems thawed immediately and floated to the top of the medium.

Half the meristems of each cultivar were used in two different regeneration experiments. In experiment 1, five drops of MS medium containing 1% (w/v) low melting point agarose (Duchefa) were added to a Pe-

tri dish, and a meristem transferred to each drop before solidification. Each Petri dish was then filled with liquid MSTo medium (i.e., with hormones: 0.5 mg L<sup>-1</sup> zeatin riboside, 0.2 mg L<sup>-1</sup> gibberellic acid, and 0.5 mg L<sup>-1</sup> indole acetic acid); the solidified MS/agarose solid drops containing the meristems therefore appeared as 'islands' within the MSTo medium. The Petri dishes were then sealed and incubated for three months at 23°C. A 16 h photoperiod was established.

In experiment 2, exactly the same procedure was followed, except that the MS/agarose droplets also contained plant hormones (0.5 mg L<sup>-1</sup> zeatin riboside, 0.2 mg L<sup>-1</sup> gibberellic acid, and 0.5 mg L<sup>-1</sup> indole acetic acid).

After 14 days the meristems were examined using a microscope and callous structures could be seen. If no plantlets had regenerated from the meristem after 3 months, these calluses were transferred to modified callus medium (CM) (Keil *et al.*, 1989) (Table 1) to ensure plantlet regeneration. The plantlets obtained from both calluses and meristems were then transferred to solid MS medium.

Survival rate was determined by counting the number of meristems considered to be alive (by examining their colour and appearance) after thawing. The regeneration rate was calculated from the number of plants regenerated (at the end of the three month study period) from the initial number of frozen meristems. The ploidy level of the plants was estimated by flow cytometry using a PA analyser (Partec), quantifying the DNA in the leaf cells of the regenerated plants. Non-treated, original cultivar representatives of the same physiological age were used as controls.

**Table 1.** Composition of modified CM regeneration medium

Composition	Concentration (mg L <sup>-1</sup> )
Macro and Microelements MS*	4,410
Glucose	30,000
Glycine	2
Inositol	100
Vitamins:	
— Pyridoxine	0.5
— Thiamine	0.1
— Nicotinic acid	0.5
Hormones:	
— Gibberellic acid	0.02
— Zeatin riboside	2.00
— Naphthalene acetic acid	0.02

\* Murashige and Skoog (1962). CM: callus medium.

**Table 2.** Post/thaw survival rate and plants regenerated from 10 cryopreserved potato cultivars

Cultivar	Frozen meristems	Survival rate (%)	Meristems in regeneration medium	Plant hormones*	Regeneration rate (%)	Plants obtained
Cazona	240	53	63	+	17.5	21
			63	-	12.5	15
Draga	240	87	104	+	9.2	11
			104	-	2.5	3
Fénix	240	100	120	+	21.6	26
			120	-	8.3	10
Ganade	200	55	55	+	7.0	7
			55	-	1.0	1
Gorbea	200	50	50	+	17.0	17
			50	-	13.0	13
Hydra	240	90	108	+	10.8	13
			108	-	6.6	8
Isola	200	56	56	+	22.0	22
			56	-	16.0	16
Red Pontiac	300	90	135	+	10.0	15
			135	-	6.6	10
Kennebec	300	56	84	+	8.0	12
			84	-	5.3	8
Zorba	240	81	97	+	9.1	11
			97	-	6.6	8
Media		71.8		+	13.2	15.5
				-	7.8	9.2

\* 0.5 mg L<sup>-1</sup> zeatin riboside, 0.2 mg L<sup>-1</sup> gibberellic acid and 0.5 mg L<sup>-1</sup> indole acetic acid in MS/agarose medium.

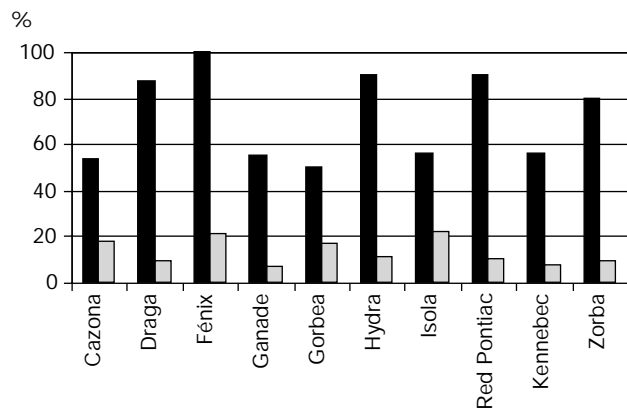
## Results

Table 2 shows the survival rate, the number of plants regenerated per cultivar and the number of meristems initially frozen.

The cultivar Fénix had the highest meristem survival rate (100%). Cazona, Ganade, Gorbea, Isola and Kennebec showed rates of around 50%. The remaining cultivars showed rates of around 80%. Significant differences were seen in regeneration rates depending on whether the regeneration medium contained extra plant hormones (i.e., hormones in the MS/agarose drop): 13.2% with extra hormones compared to 7.8% with hormones in the MSTo medium only ( $P=0.05$ ; least significant difference test). High survival rates clearly did not guarantee high regeneration rates (correlation rate  $r=0.08$ ) (Fig. 1).

Great differences were seen in the regeneration rates of the different cultivars. Isola and Fénix had the highest rates (over 20%) in the presence of extra plant

hormones, but less than half this value when hormones were only present in the MSTo medium. The Galician local cultivars Cazona and Ganade showed very different values (17.5% compared to 7% in the me-



**Figure 1.** Regeneration rate with extra plant hormones (grey), and survival rate (black), in 10 potato cultivars after cryopreservation.

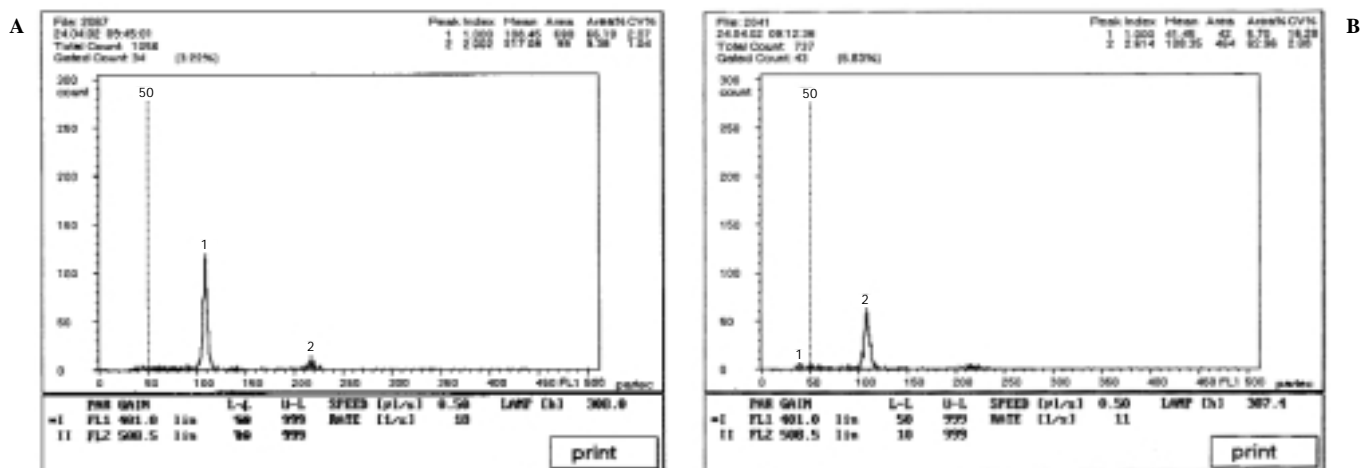


Figure 2. Ploidy determined by flow cytometry. A) Original Isola control. B) Regenerated Isola plant.

dium with extra hormones). Of the NEIKER cultivars, Gorbea showed a 17% regeneration rate in the presence of extra plant hormones, falling to 13% when these were present only in the MSTo medium.

Figure 2 shows the ploidy levels recorded by flow cytometry analysis of Isola plants. No differences were seen between regenerated plants and controls. In fact, no polyploid genotype was seen for any of the plants evaluated, and all the regenerated plants had phenotypes characteristic of their cultivar.

## Discussion

The post-thaw survival rate of the meristems was generally high: half the cultivars showed values of over 80%. These figures are in agreement with those obtained by Schäfer-Menuhr *et al.* (1996), in which all the cultivars used showed survival rates of > 50% (though notable differences were seen).

With respect to the regeneration rate, Mix-Wagner (1999), who used the cultivars Hydra and Isola, obtained values of 9%-16%, somewhat less than those obtained in the present study. However, Schäfer-Menuhr *et al.* (1996), achieved better survival rates with Kennebec than those reported here. Regeneration rates were greater in the presence of extra hormones, perhaps due to an increase in their local concentration. However, more work is necessary to confirm these preliminary findings, using growth regulators at different concentrations.

The difference between the survival and regeneration rates shows that cryopreservation involves two processes that need to be improved separately: freezing and regeneration. The survival rate was high, sug-

gesting that the process is already optimised. Better regeneration rates were achieved when plant hormones were added to the MS/agarose medium - nonetheless they need to be further improved. The regeneration rate was much more influenced by the genotype of the plant than the survival rate (Fig. 1). If improvements could be achieved, less material would need to be cryopreserved.

Schäfer-Menuhr *et al.* (1996) indicated that the longer it takes for a meristem to regenerate, the lower the vigour of the plant. This agrees with that observed in the present study; three months after the meristems were placed in the regeneration medium, their vigour gradually declined (results not shown).

In this type of culture procedure, there may be uncertainty about whether the regenerated plant has come from the meristem or the leaf that covered it. Plants that develop from callus, which is easily produced by the leaf, can show somaclonal variation. Those plants that regenerate first are more likely to have come from the meristem and are less likely to show such variation. In the present work, some cultivars, e.g., Zorba and Isola, were quicker to regenerate, but no molecular tests were performed to determine whether they had developed from the meristem or other tissue.

Mix-Wagner (1999) used RFLP and microsatellites as molecular markers to detect somaclonal variation, but found no anomalies in their regenerated plants. In the present study, no polyploids were detected by flow cytometry, even when regenerated from meristem callus cells. All the plants were phenotypically characteristic of their respective cultivars.

The present results, plus those of other authors, show that cryopreservation is a viable alternative in

the long term conservation of potato germplasm. Many genotypes can be preserved at a reduced cost, and the risks involved in manipulation and periodic subculturing eliminated. However, for experiments or genetic improvement programmes involving the periodic use of potato cultivars, *in vitro* maintenance is recommended alongside cryopreservation.

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