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Shoot-tip vitrification protocol for red chicory (*Cichorium intybus* L.) lines

C. Benelli*, A. Previati, A. De Carlo*, M. Lambardi***

* *Istituto per la Valorizzazione del Legno e delle Specie Arboree (IVALSA), Consiglio Nazionale delle Ricerche, Via Madonna del Piano, 10, 50019 Sesto Fiorentino (FI), Italy.*

** *Centro Sperimentale Orticolo "Po di Tramontana", Veneto Agricoltura, Via Moceniga, 7, 45010 Rosolina (RO), Italy.*

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Abstract: Shoot tips from *in vitro* stock plants of red chicory 'Rosso di Chioggia' line were cryopreserved by one-step vitrification. After two days of cold-hardening on hormone-free MS medium and loading for 30 min in a mixture of 2 M glycerol and 0.4 M sucrose at 25°C, shoot tips were dehydrated with PVS2 vitrification solution at 0°C for 60 min and plunged directly into liquid nitrogen. The post-thaw survival of shoot tips was achieved 79% when was cultured on recovery medium containing 0.5 mM BA. Observed regrowth, after six weeks of culture in the same medium composition, was 100%. Rooted cryopreserved microshoots showed good quality when transferred to the greenhouse. Preliminary results proved that the genetic fidelity of the cryopreserved line was maintained. The same vitrification protocol was then applied to three other red chicory lines, 'Rosso di Treviso precoce', 'Rosso di Treviso tardivo' and 'Castelfranco'. A simple and effective protocol for the cryopreservation of red chicory shoot tips has been successfully developed as a result of this study.

1. Introduction

In Italy, the major production area of red chicory (*Cichorium intybus* L. var. intybus), covering about 9 thousand hectares, is located in the Veneto region, one of the most economically important areas for vegetable production. An ancient typology, 'Rosso di Treviso Tardivo', that was progenitor of the present varieties of red chicory, was introduced into Italy in the 15th century. Over time, growers selected those plants having good production, while in recent years this leafy vegetable has undergone intense selection and breeding work, and several improved typologies have been produced (such as 'Rosso di Treviso precoce', 'Rosso di Verona', 'Rosso di Chioggia', 'Variegato di Castelfranco', and others) (Veneto Agricoltura, 2002), which are highly appreciated for their quality and productivity.

At the "Po di Tramontana" experimental farm in Rosolina (Veneto Agricoltura, Rovigo, Italy) a specific breeding program to select high-performance lines has been continuing for many years. Every year after in-field evaluation, the most valuable lines are introduced and maintained *in vitro* by subculturing every three weeks. The stock plants obtained from these lines are transferred to the greenhouse to produce high quality seeds to be used for the production of high quality red chicory. The costs of stock culture maintenance and the risks of contamination and decay of lines can be reduced by introducing cryopreservation as a tool for a long-term preservation. Cryopreservation involves the maintenance of plant propagules at ultra-low temperatures (-196°C, LN): under these conditions, biochemical and most physical processes are completely arrested and as such, plant material can be stored for unlimited periods.

Cryopreservation studies have been reported for Belgian endive (*Cichorium intybus* L. var. foliosum, cvs. Flash, Rumba and Carolus) by controlled-rate freezing (Demeulemeester *et al*., 1992; 1993) and encapsulation-dehydration techniques (Vandenbussche *et al*., 1993). Controlled-rate freezing is regarded as the traditional approach to plant cryopreservation. Although controlled-rate freezing has been effective for cryopreserving differentiated tissues (Reed and Uchendu, 2008), reports on cryostorage using this technique are limited. One of the practical limitations of the controlled-rate freezing approach is the need for an expensive programmable freezer (Engelmann, 2000). However, applying traditional (controlled-rate freezing) and new procedures (one-step freezing technique), the first examples of "cryogenic banks" are available today in several countries (Reed, 2001; Sakai and Engelmann, 2007). At the same time, the development

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of a vitrification system has made it possible to increase the number of plant species that can be cryopreserved (Towill and Bajaj, 2002; Lambardi and De Carlo, 2003, 2009; Sakai *et al*., 2008).

In the present study, a vitrification/one-step freezing procedure was developed using ‘Rosso di Chioggia’ shoot tips, with exposure of explants to the vitrification solution and rapid cooling by directly immersing in liquid nitrogen (LN). This procedure was then applied to three different red chicory typologies. In addition, validation of this method using molecular markers is presented with regard to maintenance of genetic stability.

2. Materials and Methods

Plant material

Microshoots of red chicory ‘Rosso di Chioggia (mp C7 4/212) were adventitiously induced from leaf portions through direct organogenesis (Fig. 1 a). The lines were obtained by isolating each adventitious shoot and transferring them onto a semi-solid MS (Murashige and Skoog, 1962) medium, supplemented with 30 g l^{-1} sucrose, 1.0 μM 6-benzyladenine (BA) and 7 g l^{-1} agar (proliferation medium). The pH of the medium was adjusted to 5.8 before autoclaving for 20 min at 121°C.

The shoot cultures were placed at 21±1°C under

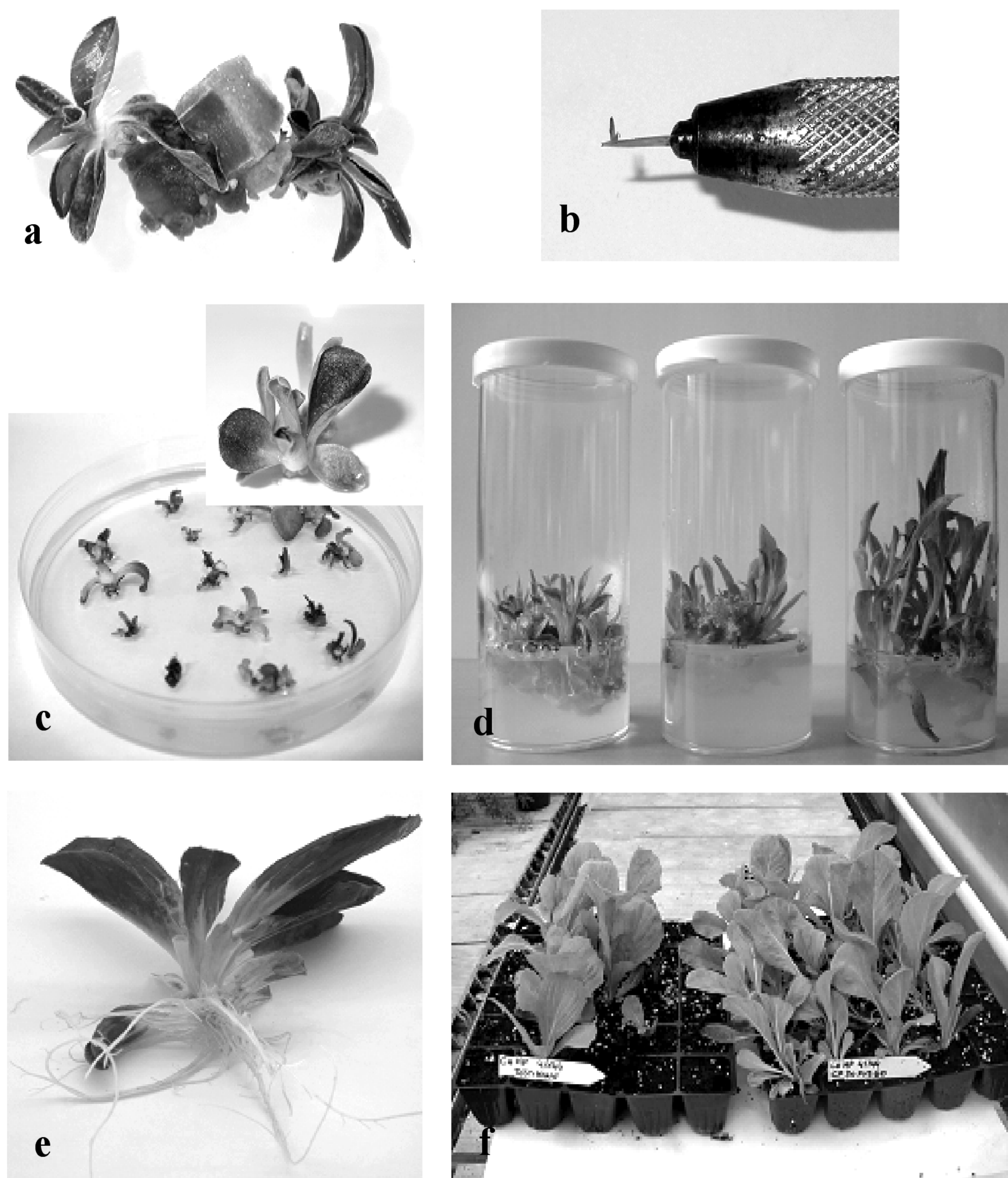

Fig. 1 - Cryopreservation of red chicory “Rosso di Chioggia” line (a-f). a - microshoots induced adventitiously from a leaf portion; b - a shoot tip just after excision from a shoot bud; c - shoot tip survival after three weeks and a well-formed shoot tip after cryopreservation with 60 min of PVS2 treatment (detail); d - regrowth in plastic cylinder on MS medium with 0.5 μM BA; e - cryopreserved rooted microshoot; f - plantlets from cryopreservation, after potting and acclimation in the greenhouse.

12-hr photoperiod conditions (60 μmol m^{-2} s^{-1} photosynthetically active radiation) and subcultured every three weeks (standard culture conditions). Short-day conditions avoided floral initiation.

Vitrification procedure

Before bud excision, stock plants were exposed to 4°C with an 8-hr photoperiod for a three-week cold-hardening period. Shoot tips (2-3 mm long), consisting of the apical meristem and 4-5 leaflets, were then excised from microshoots (Fig. 1b) under a stereo microscope in aseptic conditions, and were precultured on hormone-free MS medium supplemented with 0.09, 0.3 or 0.7 M sucrose for two days at 4°C under an 8-hr photoperiod to determine the effect of sucrose pretreatment on cryopreserved explant survival.

To induce dehydration, the shoot tips were loaded with a cryoprotectant (Loading Solution, LS; 2 M glycerol and 0.4 M sucrose) (Matsumoto *et al*., 1994) for 30 min at 25°C in 2-ml Nalgene® cryovials (10 shoot-tips per cryovial) and subsequently incubated at 0°C with the PVS2 (Plant Vitrification Solution 2; 30% w/v glycerol, 15% w/v ethylene glycol, 15% w/v DMSO in MS medium containing 0.4 M sucrose) (Sakai *et al*., 1990). In order to choose the longest possible exposure time of shoot tips to the vitrification solution while avoiding toxic effects, shoot tips were exposed to PVS2 for 30, 60, 90 or 120 min and evaluated for shoot tip survival. After dehydration, the samples were suspended in 0.6 ml of fresh PVS2 solution and rapidly frozen to –196°C by direct immersion in LN, where they were stored for at least 2 hr. For recovery, they were quickly rewarmed by plunging the cryovials into a 40°C waterbath (warming rate: about 150°C min^{-1}), unloaded from the PVS2 solution, washed for 20 min at 25°C in a liquid MS medium containing 1.2 M sucrose and finally plated onto the proliferation medium and maintained under standard culture conditions. Cryopreserved shoot tips were assessed for survival after three weeks.

In addition to proliferation medium, five different recovery media were tested to improve the regrowth of cryopreserved shoot tips: MS added with 0.5, 1.0 or 5.0 μM BA, MS with 1 μM Thidiazuron (TDZ) and MS with 0.5 g l^{-1} Activated Charcoal (AC). The role of AC has been discussed in many reports on plant tissue culture with different effects (Thomas, 2008).

Survival was defined as a percentage of green shoot tips after three weeks from thawing. Explants were transferred to plastic tubes (one shoot for each tube) and six weeks after LN treatment the percentage of plants demonstrating regrowth was assessed. Explants treated with PVS2 but not exposed to LN were used as controls.

In vitro *rooting of cryopreserved shoots*

Cryopreserved shoots were transferred to jars containing 100 ml of MS rooting medium with 20 g l^{-1} sucrose, 7 g l^{-1} agar and supplemented with 2.5 μM indole butyric acid (IBA). The jars were kept in a climatic chamber, at 21°C, under a light intensity of 40 μmol m^{-2} s^{-1} and a 12-hr photoperiod. After four weeks, the rooted microplantlets were transferred to *ex vitro* conditions. The plantlets were transferred to the greenhouse into pots with substrate containing both sterilised peat and sand in a ratio of 2:1. After five weeks, the plants were transferred to the field and their morphological stability was evaluated.

Cryopreserved chicory lines

The cryopreservation procedure, optimized for 'Rosso di Chioggia' shoot tips, was then tested on shoot tips from *in vitro* plants of three red chicory lines, 'Rosso di Treviso precoce' (TVP S5), 'Rosso di Treviso tardivo' (TVT) and 'Variegato di Castelfranco' (C90 S6'). All three lines are included in a program to safeguard and conserve red chicory in the Veneto region.

Encapsulation-vitrification procedure

'Rosso di Chioggia' shoot tips, cold-hardened at 4°C for two days, were encapsulated into 2% Na-alginate beads and treated with LS solution for 1 hr on a rotary shaker at 25°C. When this solution was removed, the beads were dehydrated by exposure to PVS2 for 2, 3 or 4 hr at 0°C, then placed in cryovials (five beads per cryovial) and plunged immediately into LN. Encapsulated shoot tips were thawed in a waterbath at 40°C for 3 min and placed on MS proliferation medium. Survival was assessed after three weeks.

Data analyses

A minimum of 30 samples were used for each treatment and each experiment was repeated twice. Statistical analysis of percentages was carried out by the χ^2 test or non-parametric statistical test, the Post Hoc Multiple Comparison Test (Marascuilo and McSweeney, 1977), both at $P \leq 0.05$.

Genetic stability assessments of cryopreserved plantlets

Random Amplified Polymorphic DNA (RAPD) analysis was applied on red chicory *in vitro* shoots to evaluate the genetic stability of cryopreserved and non-cryopreserved shoots (untreated control) collected from the same stock cultures. Genomic DNA was extracted from approximately 100 mg of leaves with a 'DNeasy Plant Mini Kit' (Quiagen). DNA concentration was determined using a spectrophotometer at 260 nm, and an aliquot of DNA was diluted to a working concentration of 20 ng μl^{-1}.

RAPD profiles were generated using 24 arbitrary 10-mers as primers, of which 10 primers were then selected for the reproducibility, the legibility and the

Table 1 - Nucleotide sequences of DNA primers used for RAPD analysis

Primer	Primer sequence
1253	GTT TCC GCC C
1247	AAG AGC CCG T
M2	ACA ACG CCT C
M3	GGG GGA TGA G
M10	TCT GGC GCA C
M13	GGT GGT CAA G
A1	CAG GCC CTT C
A5	AGG GGT CTT G
A9	GGG TAA CGC C
C7	GTC CCG ACG A

stability of the RAPD pattern: 1253, 1247, M2, M3, M10, M13, A1, A5, A9 and C7 (Table 1).

The amplification of DNA was performed according to Vettvori *et al*. (1996). Fragments were separated on 2% agarose gels by electrophoresis and visualized by ethidium bromide staining under UV light. For each primer, amplification reactions were repeated at least twice and only those having reproducible band partners were used. Minor fragments, which tend to be unstable in staining intensity, and therefore not reliable, were not considered.

3. Results

Influence of PVS2 times and preculture treatments

Prolonged exposure at 0°C to the vitrification solution appeared to be harmful for explants. Indeed, shoot-tip survival decreased after exposures of 90 min or more, while 30-min treatment was not enough to protect the explants during ultra rapid freezing (Table 2). When the incubation time was limited to 60 min, more than 72% of cryopreserved (+ LN) shoot tips survived after plating onto the proliferation medium (Fig. 1c). Incubation for 90 and 120 min in the PVS2 solution showed even a slight decrease in percentage survival of shoot tips without freezing (- LN). This result highlights the importance of incubation time with vitrification solution for shoot tip survival.

To enhance the shoot tips' osmotolerance to the vitrification solution, preculture treatments with different sucrose concentrations for two days at 4°C was applied before the LS and PVS2 treatments. However, frozen explant survival exhibited a significant decline when the shoot tips were pre-cultured on media containing high sucrose concentrations (Table 3), indicating that sucrose treatments are not appropriate to improve red chicory shoot tip survival.

All three red chicory lines can be successfully cryopreserved by loading shoot tips in PVS2 for 60 or 90 min, prior to freezing in LN. Among the lines, maximum explant survival ranged between 65% in 'Rosso di Treviso precoce' and 76% in 'Rossso di Treviso tardivo' and 'Variegato di Castelfranco', respectively with 60 and 90 min of PVS2 treatment (Table 4).

Table 2 - Survival of cryopreserved shoot tips of 'Rosso di Chioggia' after exposure to PVS2 for different time periods. Data were recorded three weeks after thawing (LN, liquid nitrogen)

PVS2 exposure time (min)	Shoot tip survival (%)[z]	
	- LN	+ LN
30	100 a	33.5 b
60	100 a	72.5 a
90	80 b	40.0 b
120	80 b	30.0 b

[z] In each column, percentages followed by different letters are significantly different at $P \leq 0.05$ by the post hoc Multiple Comparison test.

Table 3 - Effect of sucrose concentration in preculture medium (two days at 4°C) on 'Rosso di Chioggia' shoot tips cryopreserved following 60 min PVS2 treatment

Sucrose concentration (M)	Shoot tip survival (%)[z]
0.09	68.0 a
0.3	47.5 a
0.7	17.5 b

[z] Percentages followed by different letters are significantly different at $P \leq 0.05$ by the post hoc Multiple Comparison test.

Table 4 - Shoot tip survival percentage of three selected red chicory lines, following incubation and immersion in LN

Red Chicory Line	Loading time with PVS2 (min)	Shoot tip survival (%)[z]	
		- LN	+ LN
'Rosso di Treviso precoce'	60	80.0 a	65.0 a
	90	70.0 a	55.0 a
'Rosso di Treviso tardivo'	60	80.0 a	66.7 a
	90	90.0 a	76.7 a
'Variegato di Castelfranco'	60	90.0 a	50.0 a
	90	100 a	76.0 b

[z] For each select line and each column, percentages followed by different letters are significantly different at $P \leq 0.05$ by the χ^2 test.

Recovery media

The addition of BA to the recovery medium was found to be beneficial for post-thaw recovery of the shoot tips, even if with an increase of the concentration callus formation is stimulated. The highest post-thaw survival of 'Rosso di Chioggia' shoot tips was obtained with 0.5 µM BA (79%) (Table 5). When 1 µM TDZ was used, 33% of shoot tips survived, but after the first subculture (21 days), they stopped growing, after which no shoots developed. There was no survival of shoot tips on hormone-free medium supplemented with activated charcoal.

Table 5 - Effect of recovery media on 'Rosso di Chioggia' shoot tip survival (three weeks) and regrowth (six weeks) after thawing

Recovery medium	Shoot tip survival (%)	Shoot tip regrowth (%)[z]
0.5 µM BA	79.1 a	100 a
1.0 µM BA	47.6 b	95.0 a
5.0 µM BA	47.8 b	95.4 a
1.0 µM TDZ	33.3 c	0
0.5 g/l AC	0	--

[z] In each column, percentages followed by different letters are significantly different at $P \leq 0.05$ by the post hoc Multiple Comparison test.

A further increase of regrowth was easily achieved by transferring the explants in tubes containing proliferation medium with BA. The best concentration for the development of 'Rosso di Chioggia' chicory shoots was again 0.5 μM. Indeed, the plantlets obtained from this cytokinin treatment were generally taller and produced more shoot apices (Fig. 1d). After *in vitro* rooting (Fig. 1e), the microplantlets were transplanted into pots and successfully acclimated *in vivo* (Fig. 1f).

Field observations of the plantlets confirmed the morphological stability of acclimatized plants with the original mother plants (Fig. 2).

Fig. 2 - Experimental field, the arrow indicates the plot with cryopreserved 'Rosso di Chioggia' plants.

Vitrification vs encapsulation-vitrification

Cryopreservation by shoot-tip vitrification, using the PVS2 solution, was compared with the encapsulation-vitrification procedure in 'Rosso di Chioggia' line. The highest survival percentage of cryopreserved shoot tips was obtained by vitrification procedure (Table 6).

Table 6 - Shoot tip survival percentage of 'Rosso di Chioggia' shoot-tips after two different cryogenic procedures

Cryogenic procedure	Shoot tip survival (%)$^{(z)}$
Vitrification	76.0 a
Encapsulation - vitrification	35.2 b

$^{(z)}$ Percentages followed by different letters are significant at P ≤ 0.05 by the post hoc Multiple Comparison test.

The encapsulation-vitrification procedure proved to be less effective for the cryopreservation of the selected red chicory line. However, only a maximum of 35% explant survival was achieved when the longest treatment of the beads with PVS2 was applied; this percentage is markedly lower than that reported in the literature using an encapsulation-dehydration procedure in chicory (Vandenbussche *et al*., 1993).

Assessment of molecular stability by RAPD

To assess the genetic fidelity of plantlets regrown from cryopreserved shoot-tips, the RAPD patterns were compared with untreated samples of the same red chicory lines. Out of 24 primers screened, 10 selected primers produced clear and reproducible bands. The number of bands for each primer varied from five to twelve. Each primer generated a set of amplification products of a size ranging between 350 bp and 3000 bp.

Preliminary results proved that the genetic fidelity of the cryopreserved lines was maintained. For all 10 primers tested, RAPD fragment patterns of plantlets from cryopreserved shoot-tips did not show differences with respect to untreated shoots. Figure 3 represents amplified band patterns produced by two primers (1253 and 1247). Genetic fidelity was confirmed also by the other primers.

4. Discussion and Conclusions

'Rosso di Chioggia' shoot-tips cryopreserved using LS treatment for 30 min, PVS2 for 60 min at 0°C, and recovery on medium containing 0.5 μM BA resulted with a high survival rate (79%). This result is slightly inferior to that obtained in Belgian endive, cv. Flash (83%) where Demeulemeester *et al*. (1992) used a more complex procedure, consisting of cooling the explants to -40°C at a rate of 0.5°C min^{-1} prior to immersion in LN. In the present study, the vitrification protocol applied to different red chicory lines showed high survival percentages, even if tolerance to the PVS2 varied in different lines. Differences among lines can be considered to be genotype-dependent; these results are consistent with other experiences on cryopreservation in other plant species (De Boucaud *et al*., 2002; Kim *et al*., 2006; Benelli *et al*., 2009).

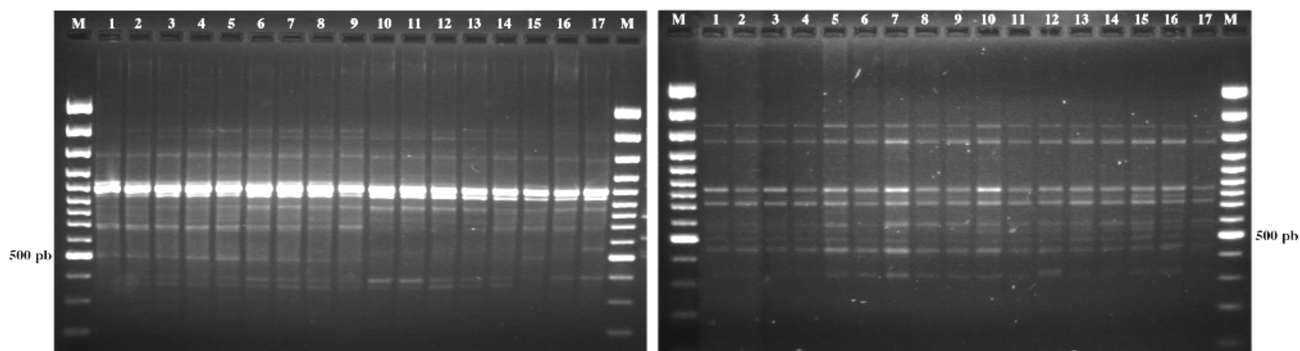

Fig. 3 - RAPD profiles generated with primers 1253 (left) and 1247 (right) from untreated and cryopreserved plantlets of 'Rosso di Chioggia' line. In both images, lanes M correspond to the 100-bp ladder; lanes 1-9 correspond to the amplification products from untreated samples, lanes 10-17 correspond to the amplification products from cryopreserved plantlets.

In many cases, preculture with sucrose can be very efficient to improve cryopreservation (Matsumoto *et al*., 1998), but such treatments applied on apical buds of several species did not induce increases in recovery after vitrification (Sakai, 2000). Sugar treatments can influence the membrane and protein composition (Ramon *et al*., 2002; Carpentier *et al*., 2005), influencing flexibility and permeability of the membrane. In red chicory shoot tips, preculture treatment on MS with 0.09 M sucrose for two days before PVS2 loading resulted in the best survival with respect to 0.3 and 0.7 M sucrose, but it did not improve the post-thaw survival percentage in the cryogenic procedure.

The type and concentration of growth regulators in the recovery medium is important for survival and regrowth of cryopreserved explants (Turner *et al*., 2001). Red chicory shoot tip survival (79%) was improved when the recovery medium contained 0.5 μM BA; callus formation was induced when the BA concentration was increased, in particular with 5 μM BA. Callus is not desired and represents a limitation in development of the cryopreserved explants. This phenomenon is observed in several species when a high concentration of BA was used (Wang *et al*., 2003). Demeulemeester *et al*. (1993) held the explants for one week on medium supplemented with plant growth regulators and then transferred them to hormone-free medium to avoid a callus phase in three varieties of chicory (Flash, Rumba and Carolus) after cryopreservation. This protocol was effective for restricting callus formation, but led to a decrease in survival percentage. In the present study, with the 'Rosso di Chioggia' line, we obtained the highest survival rate without callus formation by applying the lowest BA concentration and the best regrowth when the single cryopreserved shoot was transferred in tube.

Addition of activated charcoal in post-thaw recovery medium resulted deleterious for shoot tip survival and regrowth; whereas thidiazuron gave a low survival of explants after three weeks but no subsequent growth.

Rooted microshoots of 'Rosso di Chioggia', obtained from cryopreserved shoot-tips, exhibited high survival and vigour in the greenhouse.

Application of the encapsulation-vitrification procedure showed a limited survival of cryopreserved explants and further experimentation is needed to optimize the protocol for these red chicory lines.

A few genetic studies using molecular markers have been carried out on red chicory, mainly for the genetic characterization of commercial varieties (Barcaccia *et al*., 2003), and particularly RAPD markers were used to construct the genetic map of *C. intybus* (De Simone *et al*., 1997) to identify and to evaluate the phylogenetic relationships among cultivars of chicory (Koch and Jung, 1997; van Stallen *et al*., 2000, 2001; Barcaccia *et al*., 2003).

Assessment of the genetic stability of cryopreserved shoots can be performed with different techniques (Harding, 2004). In this study, RAPD markers were adopted because of their simplicity, rapidity and ability to screen a randomly large part of the genome.

Genetic stability was reported after RAPD analysis of cryopreserved plantlets and the untreated shoots. Comparison of the DNA patterns of control and frozen material did not reveal any variations caused by the cryoprotectant treatments or cryostorage.

The results obtained are confirmed by numerous studies that demonstrated that cryopreservation did not affect the genetic stability in various species, such as potato (Harding and Benson, 2000), grape and kiwi (Zhai *et al*., 2003), chrysanthemum (Martin and Gonzalez-Benito, 2005), *Citrus* spp. (Lambardi *et al*, 2007), and apple (Liu *et al*., 2008).

Moreover, the morphological analysis carried out in the field confirmed that there were no significant differences between plants derived from control and cryopreserved shoots.

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