Cryopreservation of Shoot Tips of Tetraploid Potato (Solanum tuberosum L.) Clones by Vitrification

DEBABRATA SARKAR* and PRAKASH S. NAIK

Division of Genetics and Plant Breeding, Central Potato Research Institute, Shimla-171 001, Himachal Pradesh, India

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In vitro-grown shoot tips of five tetraploid potato (Solanum tuberosum L.) clones were cryopreserved by vitrification. Excised shoot tips (0.5–0.7 mm) were pre-cultured on filter paper discs over half strength liquid Murashige and Skoog (MS) medium supplemented with 8.7 μM GA3 and different combinations of sucrose (0.3, 0.5 and 0.7 M) plus mannitol (0, 0.2 and 0.4 M) for 2 d under a 16 h photoperiod at 24 °C. The pre-cultured shoot tips were either successively loaded with 20 and 60% PVS 2 solutions or directly exposed to concentrated vitrification solution before physical vitrification during liquid nitrogen treatment. The vitrified shoot tips were warmed rapidly and treated with dilution mixture (MS+1.2 M sucrose) for 30 min before plating on regrowth medium. Addition of mannitol to the pre-culture medium improved survival of vitrified shoot tips. Direct dehydration of pre-cultured shoot tips with concentrated PVS 2 was detrimental to survival of vitrified shoot tips. Shoot tips pre-cultured on medium containing 0.3 M sucrose plus 0.2 M mannitol, and loaded with 20% PVS 2 for 30 min followed by 15 min incubation in 60% PVS 2 and 5 min incubation in 100% PVS 2 at 0 °C resulted in up to 54% survival after vitrification. About 50% of vitrified and warmed shoot tips formed shoots directly. Post-thaw culturing of vitrified shoot tips on medium containing an elevated level of sucrose (0.2 M) under diffuse light for the first week enhanced the survival rate. Continuous culturing of vitrified shoot tips on high-sucrose medium induced multiple shoot formation.

Key words: Solanum tuberosum L., potato, cryopreservation, germplasm conservation, in vitro conservation, meristems, shoot tips, tissue culture, vitrification.

INTRODUCTION

Minimal growth may be used for the conservation of potato (Solanum tuberosum L.) germplasm in vitro (Sarkar and Naik, 1998). This medium-term conservation approach is not only time-consuming and labour-intensive, but in addition may not even ensure good genetic stability of in vitro micropropagated plantlets (Harding, 1991). Cryogenic storage of shoot tips in liquid nitrogen has therefore been recognized as a major method for the long-term storage of potato germplasm. Storage in liquid nitrogen ensured a high degree of genetic (ploidy) stability in dihaploid potato clones and wild Solanum species (Ward et al., 1993). Work on the cryopreservation of potato shoot tips started as early as the late 1970s, and since then different methods have been developed using slow or ultrarapid cooling or other modified freezing approaches (Bajaj, 1977; Grout and Henshaw, 1978; Towill, 1981, 1984; Benson, Harding and Smith, 1989; Schäfer-Menuhr, Muller and Mix-Wagner, 1996). Some of these classical methods are time-consuming, require sophisticated freezing equipment and involve specific adaptation of cooling rates to optimize the results. Comparable results were not obtained in early potato cryopreservation work because of differential responses observed between and within groups and/or species. Except for the droplet freezing technique (Schäfer-Menuhr et al., 1996), no one method can be routinely applied to a wide range of species and accessions within species. In addition, many of these techniques involve regeneration of cryopreserved shoot tips through an intermediary callus phase. However, concerns have been raised regarding the genetic stability of plants regenerated in this way (Henshaw, O’Hara and Stamp, 1985).

In recent years, an alternate method has been developed to cryopreserve cells or small tissue segments through vitrification. Vitrification is the phase transition of water from a liquid directly into a non-crystalline amorphous phase, a glass, by an extreme elevation in viscosity during cooling (Fahy et al., 1984). In contrast to classical techniques based on freezing, cell dehydration in vitrification-based procedures is performed prior to freezing by exposure of samples to concentrated cryoprotective media and/or air desiccation followed by rapid cooling (Engelmann, 1997). In the encapsulation-vitrification technique, the explants are encapsulated in alginate beads and treated with vitrification solutions before freezing (Matsumoto and Sakai, 1995). Although the encapsulation-vitrification procedure was not employed in potato cryopreservation, efforts were made to cryopreserve potato shoot tips by vitrification based on the encapsulation-dehydration method (Fabre and Dereuddre, 1990; Bouafia et al., 1996). However, vitrification procedures based on encapsulation-vitrification and encapsulation-dehydration are lengthy, cumbersome and highly labour-
under a 16 h photoperiod (approx. 40 µmol m⁻² s⁻¹ light intensity) at 24 °C (A); sequential loading of pre-cultured shoot tips with 20% (30 min) and 60% (15 min) PVS 2 solutions followed by dehydration with concentrated PVS 2 for 5 min (B); direct dehydration of pre-cultured shoot tips with concentrated PVS 2 for 5 min (C); Data (%) are averaged over five genotypes. Bars with common letters are not significantly different at P ≤ 0.05, according to Student-Newman-Keul test.

with a highly concentrated vitrification solution before direct transfer to liquid nitrogen (Sakai, 1995). The vitrification procedure has been applied to a wide range of shoot tips (Yamada et al., 1991; Towill and Jarret, 1992; Matsumoto et al., 1997; Takagi et al., 1997).

To the best of our knowledge, no detailed work has been published on the cryopreservation of potato shoot tips by vitrification employing chemical additives. The present study was therefore undertaken to standardize an effective chemical-induced vitrification procedure for cryopreservation of potato shoot tips. Most vitrification studies reported so far suggest that the key to successful vitrification of cells or small tissue segments is the effective increase in the concentration of cellular solutes achieved by chemical pre-treatments. Therefore, one of the main objectives of the present study was to develop suitable pre-culturing conditions for potato shoot tips before vitrification in liquid nitrogen.

**MATERIALS AND METHODS**

**Plant material**

In vitro-grown plantlets of five potato (*Solanum tuberosum* L.) genotypes, viz. ‘Kufri Badshah’, ‘Kufri Chandramukhi’, ‘Kufri Lalima’, ‘Kufri Lauvkar’ and ‘Kufri Sindhuri’, were used in the present study. Disease-free stock cultures of these plants were multiplied and maintained over several years through shoot cuttings following the method described earlier (Sarkar, Chandra and Naik, 1997). Apical shoot tips about 0.5–0.7 mm long were dissected from 30-d-old plantlets. Shoot tips were pre-cultured on filter paper discs over half strength liquid MS (Murashige and Skoog, 1962) medium supplemented with 8.7 µM GA₃ and different (factorial) combinations of sucrose (0.3, 0.5 and 0.7 M) and mannitol (0.2 and 0.4 M) for 2 d under a 16 h photoperiod (approx. 40 µmol m⁻² s⁻¹ light intensity) at 24 °C.

**Vitrification procedure**

The vitrification procedure involved the following steps: (a) loading of pre-cultured shoot tips with cryoprotective solutions; (b) dehydration of the loaded shoot tips by concentrated PVS 2 vitrification solution (30% w/w glycerol, 15% w/w ethylene glycol, 15% w/w dimethyl sulfoxide in MS medium supplemented with 0.4 m sucrose; Sakai, Kobayashi and Oiyama, 1990); (c) plunging them into liquid nitrogen; (d) rapid warming (thawing) of vitrified shoot tips; (e) unloading of PVS 2 by transferring the shoot tips to dilution medium (MS + 1.2 m sucrose); and (f) post-thaw culturing of shoot tips.

Twenty pre-cultured shoot tips were placed in a 5 ml test tube and loaded with 3 ml 20% PVS 2 for 30 min at 24 °C. Subsequently, 20% PVS 2 solution was removed using a Pasteur pipette, and 3 ml of 60% PVS 2 was added. The tubes were placed in an ice bath and incubated for 15 min. After removal of the 60% PVS 2 solution, shoot tips were treated with 3 ml of ice cold concentrated PVS 2 for 5 min, and then transferred to 1 ml cryotubes (CRYOSTM, Sumitomo Bakelite Co., Japan). In the direct vitrification procedure, the pre-cultured shoot tips were not loaded with
Table 1. Analysis of variance for shoot tip survival and direct shoot formation during cryopreservation of potato shoot tips by vitrification following direct and sequential (20% PVS 2–60% PVS 2) exposure to concentrated PVS 2 solution

<table>
<thead>
<tr>
<th>Source</th>
<th>d.f.</th>
<th>Direct vitrification</th>
<th>Sequential vitrification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>4</td>
<td>578.25**</td>
<td>2067.22**</td>
</tr>
<tr>
<td>Sucrose</td>
<td>2</td>
<td>353.13**</td>
<td>3267.52**</td>
</tr>
<tr>
<td>Genotype x sucrose</td>
<td>8</td>
<td>119.8</td>
<td>6.78</td>
</tr>
<tr>
<td>Mannitol</td>
<td>2</td>
<td>4501.79**</td>
<td>10274.95**</td>
</tr>
<tr>
<td>Genotype x mannitol</td>
<td>8</td>
<td>363.1</td>
<td>6.25</td>
</tr>
<tr>
<td>Sucrose x mannitol</td>
<td>4</td>
<td>1083.92**</td>
<td>2075.86**</td>
</tr>
<tr>
<td>Genotype x sucrose x mannitol</td>
<td>16</td>
<td>1832</td>
<td>12.21</td>
</tr>
</tbody>
</table>

** P < 0.01.

In control experiments, the viability of shoot tips was assessed after: (a) pre-conditioning on different pre-culture media for 2 d at 24 °C; (b) sequential cryoprotection-dehydration by 20 and 60% PVS 2 solutions followed by concentrated PVS 2; and (c) direct cryoprotection-dehydration by concentrated PVS 2 without being loaded with diluted PVS 2 solutions.

Post-thaw culturing and viability

The vitrified (cryopreserved) shoot tips were plated on semi-solid MS medium containing 0.2 M sucrose, 5.8 μM GA₃, 1.0 μM BA and 6 gl⁻¹ Nobel agar (HiMedia, India), and cultured under 16 h diffuse light (approx. 6 μmol m⁻² s⁻¹) at 24 ± 1 °C. After 1 week, shoot tips were transferred onto standard shoot tip medium based on MS supplemented with 0.09 M sucrose, 2.9 μM GA₃, and 6 gl⁻¹ agar, and incubated under a 16 h photoperiod (approx. 40 μmol m⁻² s⁻¹) at 24 ± 1 °C. Shoot tip recovery was recorded at weekly intervals. Two types of growth were observed: regrowth of the shoot apex or callus formation. Survival and direct shoot formation were recorded as the percentage of the total number of shoot tips showing regrowth and direct shoot formation, respectively, 4 weeks after initial plating.
Sequential vitrification

dehydration. Variation due to genotypic differences was studied before and after cryoprotective loading and/or dehydrated. Variation due to genotypic differences was highly significant (\(P = 0.01\)) for shoot tip survival. There was a significant (\(P = 0.01\)) interaction between sucrose and mannitol for shoot tip survival in all the three control conditions studied (see Materials and Methods), suggesting that the effect of sucrose was not uniform over different levels of mannitol or vice versa (Fig. 1). After 2 d of preculturing on medium supplemented with 0.3–0.5 M sucrose or 0.3 M sucrose plus 0.2 M mannitol, more than 90% of the shoot tips survived (Fig. 1A). A significant reduction in viability was observed when shoot tips were pre-cultured on medium containing 0.7 M sucrose or 0.5 M sucrose plus 0.2 M mannitol. A higher concentration of mannitol (0.4 M) in the pre-culture medium was detrimental to shoot tip viability. After cryoprotective loading with diluted PVS 2 solution, about 70–80% of shoot tips survived when they were pre-cultured on medium containing 0.3–0.7 M sucrose or 0.3–0.5 M sucrose plus 0.2 M mannitol (Fig. 1B). Sucrose at the highest concentration (0.7 M) in combination with mannitol, or mannitol at the highest concentration (0.4 M) in combination with sucrose was detrimental to shoot tip viability following cryoprotective loading. In general, a reduction in viability occurred when the pre-cultured shoot tips were not cryo-loaded, but dehydrated directly with concentrated PVS 2 (Fig. 1C). Unloaded shoot tips recorded maximum survival when pre-cultured on medium supplemented with 0.3–0.5 M sucrose and 0.2 M mannitol.

RESULTS

In a preliminary experiment, excised apical shoot tips were either successively loaded with 20 and 60% PVS 2 solutions before vitrification or directly vitrified (without being loaded) with concentrated PVS 2. Little or no survival was observed in the vitrified and warmed shoot tips cooled to \(-196^\circ\)C in either of these cases. This suggested that preculturing (before vitrification) might be an essential step for successful cryopreservation of potato shoot tips by vitrification.

Control experiment

The effects of pre-culture media on shoot tip viability were studied before and after cryoprotective loading and/or dehydration. Variation due to genotypic differences was highly significant (\(P = 0.01\)) for shoot tip survival. There was a significant (\(P = 0.01\)) interaction between sucrose and mannitol for shoot tip survival in all the three control conditions studied (see Materials and Methods), suggesting that the effect of sucrose was not uniform over different levels of mannitol or vice versa (Fig. 1). After 2 d of preculturing on medium supplemented with 0.3–0.5 M sucrose or 0.3 M sucrose plus 0.2 M mannitol, more than 90% of the shoot tips survived (Fig. 1A). A significant reduction in viability was observed when shoot tips were pre-cultured on medium containing 0.7 M sucrose or 0.5 M sucrose plus 0.2 M mannitol. A higher concentration of mannitol (0.4 M) in the pre-culture medium was detrimental to shoot tip viability. After cryoprotective loading with diluted PVS 2 solution, about 70–80% of shoot tips survived when they were pre-cultured on medium containing 0.3–0.7 M sucrose or 0.3–0.5 M sucrose plus 0.2 M mannitol (Fig. 1B). Sucrose at the highest concentration (0.7 M) in combination with mannitol, or mannitol at the highest concentration (0.4 M) in combination with sucrose was detrimental to shoot tip viability following cryoprotective loading. In general, a reduction in viability occurred when the pre-cultured shoot tips were not cryo-loaded, but dehydrated directly with concentrated PVS 2 (Fig. 1C). Unloaded shoot tips recorded maximum survival when pre-cultured on medium supplemented with 0.3–0.5 M sucrose and 0.2 M mannitol.

Vitrification-cryopreservation experiment

The pre-cultured shoot tips were either directly vitrified or successively loaded with 20 and 60% PVS 2 solutions before vitrification with concentrated PVS 2 in liquid nitrogen. Analysis of variance for both vitrification conditions is presented in Table 1. The main effects of genotype and mannitol were highly significant (\(P = 0.01\)) for shoot tip survival and direct shoot formation in both vitrification conditions (i.e. direct and sequential) employed. Variation due to sucrose concentration in the pre-culture media was significant (\(P = 0.01\)) for both the survival characters, except for direct shoot formation in the direct vitrification condition. The genotype did not show any significant interaction with sucrose or mannitol, suggesting that the effect of sucrose or mannitol was uniform over the genotypes tested. Two-way interaction between sucrose and mannitol was highly significant (\(P = 0.01\)) for both characters. This

| Table 2. Survival of, and direct shoot formation from, cryopreserved shoot tips in five potato genotypes under two vitrification conditions |

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Direct vitrification</th>
<th>Sequential vitrification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Survival</td>
<td>% Direct shoot formation</td>
</tr>
<tr>
<td>Pre-culture: 0.3 M sucrose + 0.2 M mannitol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kufri Badshah</td>
<td>40 (39-21)*</td>
<td>25 (29-92)</td>
</tr>
<tr>
<td>Kufri Chandraamukhi</td>
<td>32 (34-15)</td>
<td>20 (26-26)</td>
</tr>
<tr>
<td>Kufri Lalima</td>
<td>35 (36-18)</td>
<td>18 (25-00)</td>
</tr>
<tr>
<td>Kufri Laukhar</td>
<td>25 (29-80)</td>
<td>10 (15-19)</td>
</tr>
<tr>
<td>Kufri Sindhuri</td>
<td>20 (26-45)</td>
<td>8 (16-60)</td>
</tr>
<tr>
<td>Pre-culture: 0.5 M sucrose + 0.2 M mannitol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kufri Badshah</td>
<td>35 (36-24)</td>
<td>18 (25-19)</td>
</tr>
<tr>
<td>Kufri Chandraamukhi</td>
<td>33 (35-11)</td>
<td>20 (26-26)</td>
</tr>
<tr>
<td>Kufri Lalima</td>
<td>37 (37-12)</td>
<td>23 (28-78)</td>
</tr>
<tr>
<td>Kufri Laukhar</td>
<td>25 (29-74)</td>
<td>10 (15-38)</td>
</tr>
<tr>
<td>Kufri Sindhuri</td>
<td>10 (24-81)</td>
<td>5 (10-64)</td>
</tr>
<tr>
<td>S</td>
<td>480</td>
<td>451</td>
</tr>
</tbody>
</table>

* % Data averaged over three replications and rounded; arc sine transformed means are within parenthesis.

Shoot tips were pre-cultured on media containing 0.3–0.5 M sucrose plus 0.2 M mannitol and cryopreserved following direct and sequential (20% PVS 2 → 60% PVS 2) exposure to concentrated PVS 2 solution before vitrification in liquid nitrogen.
showed that the effect of sucrose was not constant over different levels of mannitol or vice versa. The three-way genotype × sucrose × mannitol interaction was not significant, suggesting that sucrose and mannitol together do not have a major effect on the expression of genotypic variation over and above their individual effects.

In general, shoot tips pre-cultured on medium containing 0.3 M sucrose gave only 6% survival when they were vitrified directly with concentrated PVS 2 without being loaded sequentially with 20% (30 min) and 60% (15 min) PVS 2 solutions before vitrification. Data (%) were recorded after 4 weeks of post-thaw culturing of vitrified shoot tips and averaged over five genotypes. Bars with common letters are not significantly different at P ≤ 0.05, according to Student-Newman-Keul test.

A single shoot developed per vitrified shoot tip using the standard reculturing method (see Materials and Methods). However, if the vitrified shoot tips were cultured continuously on semi-solid MS medium containing 0.2 M sucrose, 5 µM GA₃ and 1.0 µM BA under 16 h diffuse light for the first week, and subsequently under a 16 h photoperiod of 40 µmol m⁻² s⁻¹ light intensity, multiple shoots developed. After 6 weeks of reculture on this high-

Fig. 3. Cryopreservation of potato shoot tips by sequential vitrification with PVS 2. Pre-freeze incubation effects of sucrose and mannitol on survival of, and direct shoot formation from, vitrified shoot tips following sequential cryoprotective loading with 20% (30 min) and 60% (15 min) PVS 2 solutions, and subsequent dehydration (5 min)-vitrification with concentrated PVS 2 in liquid nitrogen. Data (%) were recorded after 4 weeks of post-thaw culturing of vitrified shoot tips and averaged over five genotypes. Bars with common letters are not significantly different at P ≤ 0.05, according to Student-Newman-Keul test.

Within 2 weeks (Fig. 2). Following direct vitrification, maximum shoot tip survival (40%) and direct shoot formation (25%) were observed in ‘Kufri Badshah’ when the shoot tips were pre-cultured on medium containing 0.3 M sucrose plus 0.2 M mannitol, whereas ‘Kufri Sindhuri’ was the least responsive recording only 20 and 8% shoot tip survival and direct shoot formation, respectively (Table 2). Intermediate responses were observed in the other cultivars. The genotypic response to direct vitrification was more or less similar when shoot tips were pre-cultured on medium containing 0.5 M sucrose plus 0.2 M mannitol. However, in both pre-culture treatments, a reduction in direct shoot formation was recorded in all the genotypes following direct vitrification (Table 2).

The survival of vitrified shoot tips increased significantly when the pre-cultured shoot tips were loaded successively with 20 and 60% PVS 2 solutions before vitrification in liquid nitrogen (Fig. 3). Shoot tips pre-cultured on medium containing 0.3 M sucrose recorded about 27% survival following cryoprotective loading and vitrification. Increasing the sucrose concentration in the pre-culture medium improved the survival of vitrified shoot tips (38–42%). In general, a significant (P = 0.05) increase in survival of vitrified shoot tips occurred following pre-culturing on medium containing 0.3–0.5 M sucrose plus 0.2 M mannitol. A higher concentration (0.4 M) of mannitol in the pre-culture medium was detrimental to shoot tip viability following vitrification. Averaged over genotypes, the highest survival (54%) was observed when shoot tips were pre-cultured on medium supplemented with 0.3 M sucrose and 0.2 M mannitol, and loaded successively with 20 and 60% PVS 2 solutions before vitrification (Fig. 3). About 31% of vitrified shoot tips resumed normal shoot growth when pre-cultured on medium containing sucrose (0.5–0.7 M) alone. In comparison, about 50% of vitrified shoot tips formed shoots directly when they were pre-cultured on medium containing 0.3 M sucrose and 0.2 M mannitol (Fig. 3). Following sequential vitrification, maximum shoot tip survival (60%) and direct shoot formation (56%) were observed in ‘Kufri Badshah’ when the shoot tips were pre-cultured on medium containing 0.3 M sucrose and 0.2 M mannitol (Table 2). Minimum shoot tip survival (47%) and direct shoot formation (40%) were observed in ‘Kufri Sindhuri’ under the same pre-treatment and vitrification conditions. In all genotypes, a general reduction in survival and direct shoot formation occurred when shoot tips were pre-cultured on medium containing 0.5 M sucrose plus 0.2 M mannitol and cryopreserved by sequential loading with diluted PVS 2 solutions before vitrification. However, in contrast to direct vitrification, a higher percentage of vitrified shoot tips resumed direct shoot growth following sequential vitrification under both the pre-culture conditions (Table 2).

A single shoot developed per vitrified shoot tip using the standard reculturing method (see Materials and Methods). However, if the vitrified shoot tips were cultured continuously on semi-solid MS medium containing 0.2 M sucrose, 5 µM GA₃ and 1.0 µM BA under 16 h diffuse light for the first week, and subsequently under a 16 h photoperiod of 40 µmol m⁻² s⁻¹ light intensity, multiple shoots developed. After 6 weeks of reculture on this high-
sucrose (0.2 m) medium, about 30–50 shootlets developed from each vitrified shoot tip (Fig. 4A). The shootlets were multiplied, and they resumed growth to develop into plantlets under standard culture conditions (Sarkar et al., 1997) and were morphologically normal (Fig. 4B).

DISCUSSION

The present study showed that pre-conditioning of excised shoot tips was essential for successful cryopreservation of potato shoot tips by vitrification. It is well established that sucrose is an important pre-growth additive for the acquisition of desiccation tolerance during cryopreservation of shoot apices by vitrification. A high level of sucrose during pre-culture was reported to be essential in achieving high survival rates of cryopreserved shoot tips in a number of studies (Dumet et al., 1993; Gonzalez-Arnao, Moreira and Urra, 1996). However, in the current study, we observed that sucrose in combination with mannitol was more potent than sucrose alone for attaining effective pre-conditioning of potato shoot tips for vitrification. Mannitol was also reported to be most effective for reducing the water content and increasing the freeze resistance of sycamore and capsicum suspension cells during cryopreservation by conventional approaches (Withers and Street, 1977; Pritchard, Grout and Short, 1986a). The effects of this type of pre-growth have not been investigated in detail, but it seems probable that they include solute accumulation, reduction of isotonic water content, reduction in vacuolar volumes (Pritchard, Grout and Short, 1986b; Reed, 1989) and changes in membrane structure (Webb and Steponkus, 1990). In addition, we observed that sequential loading of pre-cultured shoot tips with 20 and 60% PVS 2 solutions before vitrification with concentrated PVS 2 greatly improved the survival of vitrified potato shoot tips. This beneficial effect may be due to the presence of DMSO, a highly penetrating cryoprotectant, in the loading solutions. Direct exposure of pre-cultured shoot tips to concentrated vitrification solution was detrimental to the viability of vitrified shoot tips. This harmful effect may be due to excessive osmotic stress or chemical toxicity exerted by concentrated PVS 2 (Matsumoto, Sakai and Yamada, 1994). It is particularly important that cryopreserved shoot tips are capable of producing shoots without intermediary callus formation. The optimum pre-culturing and loading conditions developed in the current study induced direct shoot formation from vitrified potato shoot tips. Post-thaw culturing of vitrified shoot tips under diffuse light and on medium containing an elevated level of sucrose (0.2 m) for the first week was found to be highly beneficial to shoot tip survival and direct shoot formation. It is well documented that cryopreserved shoot tips of potato require a low light level during the initial post-thaw culture phase (Henshaw et al., 1985). The beneficial effect of post-thaw culturing of vitrified shoot tips on a high-sucrose medium reported in this study might relate to some sort of favourable osmotic adjustment that allows the cryopreserved shoot tips to recover from the trauma of cryo-shock. Continuous culturing of vitrified shoot tips on this high-sucrose medium induced multiple shoots. This is highly advantageous for multiplying clones that show low survival after cryopreservation. However, there are potential risks of genetic or somaclonal variation in plants regenerated through adventitious shoot development (Benson et al., 1996). Thus, this mode of multiplication should only be used with caution until evidence is obtained of acceptable levels of trueness-to-type among the plantlets regenerated via multiple shoot formation.

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