e-ISSN:2320-0189
p-ISSN:2347-2308

Research & Reviews: Journal of Botanical Sciences

Cryopreservation of Petunia Shoot Tips Using Droplet-Vitrification Method

Jin-Mei Zhang[1], Bin Huang[1,2], Xiao-Ling Chen[1]*

[1]National Crop GeneBank, Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing 100081, China

[2]Fujian Agriculture and Forestry University, Fuzhou 350002, China

Short Communication

Received date: 12/05/2016

Accepted date: 13/07/2016

Published date: 15/07/2016

***For Correspondence**

Xiao-Ling Chen, National GeneBank, Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing 100081, China, Tel: +86 1062186691

E-mail: xlchencaas@126.com

Keywords: Cryopreservation, Droplet vitrification, Petunia, Shoot tips, TEM.

ABSTRACT

Recent advances in cryopreservation techniques have greatly enhanced our knowledge of the cryopreservation of clonal crops and recalcitrant-seeded species. Various species of Petunia (*Petunia × hybrida* Vilm.) are used both as popular horticultural plants and as model systems for plant molecular biology research. A previous study on conventional cryopreservation of petunia suspension cultures in liquid nitrogen may exhibit somaclonal variations in recovered cells, thereby necessitating the development of a cryopreservation technique using shoot tips as explant sources. A droplet-vitrification cryopreservation protocol of petunia shoot tips has been efficiently developed, and nowadays four petunia accessions have been successfully cryopreserved in National GeneBank of China using this method. Furthermore, histological observations of petunia meristem cells were performed at critical steps via transmission electron microscopy during droplet-vitrification. Cytological responses were observed after the preculture, loading and PVS2 steps. Further research is required to understand the cytological, biochemical, and molecular responses of cells during cryopreservation.

INTRODUCTION

Cryopreservation, the storage of biological materials at ultra-low temperatures in vapour or liquid nitrogen (LN, -130°C to -196°C) while maintaining cell viability and the ability to regrow after thawing, is a cost effective and space efficient technique [1]. It is presently the most promising long-term storage method for clonal crops and recalcitrant-seeded species [1]. With the rapid development of various cryopreservation techniques, more than 200 plant species have been successfully cryopreserved, including crops, trees, ornamentals, and wild plants [2,3]. Petunias (*Petunia × hybrida* Vilm.) are popular horticultural plants in Asia, America, and Europe [4]. Petunia germplasm can be conserved in plant gardens or *in vitro* GeneBanks, for example, four petunia cultivars were conserved in *in vitro* collection at the National Crop GeneBank of China (Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing, China); however, it consumes a lot of time and labour. Therefore, cryopreservation would be an effective method for conserving key collections of clonally propagated petunia cultivars.

Development of Cryopreservation Method of Petunia Shoot Tips

Cryopreservation of petunia suspension cell cultures has been reported by McLellan [5]. However, suspension cells recovered after exposure to LN may exhibit somaclonal variation. Therefore, there is a need for the development of cryopreservation protocols by using shoot tips as explant sources. On the basis of several vitrification and vitrification-based techniques available for plants [6,7], we developed a droplet-vitrification protocol for petunia shoot tips [8].

Several previous studies have reported that the orthogonal array tests contribute significantly to the development of cryopreservation methods for animals and plants [9-11]. In our study, we used orthogonal designs to efficiently determine the influence and optimal level of each factor with a reduced number of experiments. We have previously used an orthogonal design and one-factor experiments to optimise a carnation shoot tip cryopreservation procedure, and successfully determined the

significance of five variables using only 16 treatments [12]. In a full factorial experimental design for the development of droplet-vitrification protocol for petunia shoot tips, 216 treatment combinations would have to be performed. However, we successfully identified 3 factors, the age of *in vitro* plantlets (20 days), the concentration of sucrose in preculture solution (0.2 mol/L), and the recovery medium (MS basal medium [13], with half the concentrations of NH_4NO_3, KH_2PO_4, KNO_3, and sucrose), using 18 treatments based on the orthogonal experimental array [8] **(Figure 1).** Orthogonal experiments enable us to identify the preliminary levels of variables, and the follow-up one-factor experiments are necessary to further refine the levels of specific treatments, and improve the overall survival and regrowth levels. For example, for petunia shoot tips, follow-up one-factor experiments were performed for four variables, i.e., the preculture duration, the loading duration, the concentration, and the exposure duration of plant vitrification solution 2 (PVS2 [14]) [8].

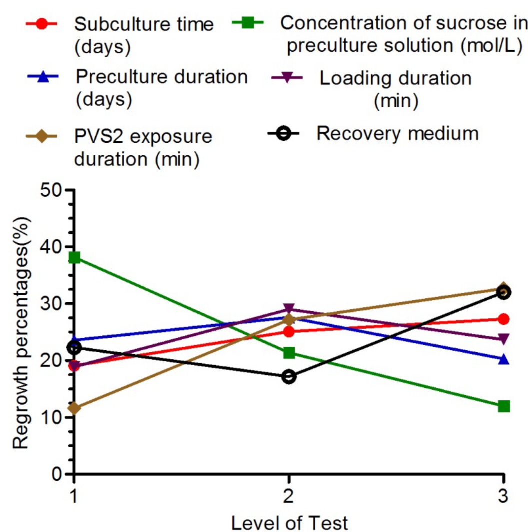

Figure 1. The effects of the subculture time, the concentration of sucrose in preculture solution, preculture duration, loading duration, PVS2 exposure duration, recovery medium on regrowth percentages after cryopreservation based on the orthogonal array. Level of treatments: sub-culture time (25, 20 or 15 days), the concentration of sucrose in preculture solution (0.2 M, 0.3 M or 0.5 M), preculture duration (0, 3 or 5 days), loading duration (20, 30 or 40 min), PVS2 exposure duration (10, 20 or 30 min), recovery medium (MS, MS supplemented with 0.5 mg/L BA, and MS supplemented with 825 mg/L NH4NO3, 85 mg/L KH2PO4, 950 mg/L KNO3 and 15 g/L sucrose).

Thus, in the optimized droplet-vitrification protocol, the petunia shoot tips were excised from 20-day-old *in vitro* plantlets and pre-cultured in MS liquid medium with 0.2 mol/L sucrose solution for 2 days. Subsequently, the pre-cultured shoot tips were incubated with loading solution for 30 min, and then dehydrated within PVS2 for 30 min at 0°C. Lastly, the treated shoot tips were frozen in the drops of PVS2 solution placed on aluminium foil strips, which were then rapidly immersed in LN. The cryopreserved shoot tips were then rewarmed in MS liquid medium with 1.2 mol/L sucrose and regrown in the recovery medium. Four petunia cultivars tested by using this optimised droplet-vitrification protocol showed average survival and regrowth percentages ranging from 67%-95% and 54%-80%, respectively. The evaluation of genetic stability, using simple sequence repeat markers, of these cultivars revealed no genetic changes in them during the cryopreservation process. These results indicate that the droplet-vitrification method would facilitate the development of programs for long-term conservation of petunia germplasm in Gene Banks. Currently, for each cultivar, 100-120 shoot tips have been cryopreserved in the National Crop GeneBank of China (Beijing, Chinese Academy of Agricultural Sciences, China).

Cytological Response of Meristem Cells to Cryopreservation Process under TEM

In vitrification, meristem cells of shoot tips must be sufficiently dehydrated to avoid lethal injury due to immersion in LN [2]. Many studies have revealed the importance of the pre-culture, osmoprotection, and PVS2 treatments in successful cryopreservation [15-17]. Understanding the ultrastructural changes in cells provides valuable information facilitating the explanation of their behaviour and responses during cryopreservation [18-20]. To investigate the tolerance of meristem cells of petunia during droplet-vitrification process, we observed the ultrastructural changes of cells in shoot tips from petunia cultivar “Niu 2” at critical steps using transmission electron microscopy. The samples were collected and fixed with 2.5% glutaraldehyde (m/v) for 12h at 25°C. The fixed samples were washed with 0.1 M phosphate buffer, followed by 1% osmium tetroxide, then washed with 0.1 M phosphate buffer, and subjected to serial dehydration with 30%, 50%, 70%, 80%, 90% and 100% acetone (v/v). The samples were embedded in Epon812 for polymerization, and then sliced into 1 μm semi-thin sections for the accurate positioning, and finally sliced 50-70 nm ultrathin sections with an ultramicrotome (LEICA UC6i, Germany).

Results showed that the tolerance of cells in shoot tips might be improved with the preculture, loading and PVS2 treatments. The meristems of the shoot tips after preculture, loading and PVS2 treatments, compared with fresh shoot tips, appeared to respond to osmotic stress **(Figure 2).** Many small vacuoles, rapid development of mitochondrion, and the increased heterochromatin were observed in the cells after preculture, loading and PVS2 treatments **(Figure 2).** Thus, this suggested that preculture and PVS2 treatment are necessary for successfully cryoprotection prior to LN exposure, which is also confirmed by orthogonal experiments and one-factor analysis [8].

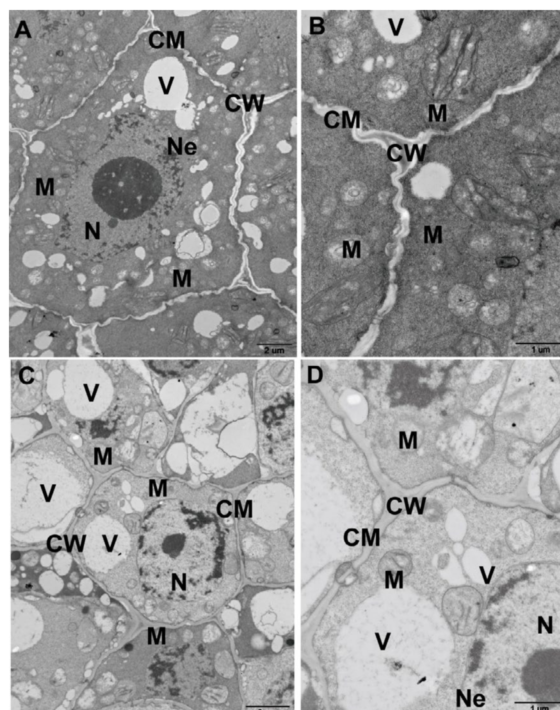

Figure 2. Ultrastructural observations of cells in fresh shoot tips (A and B) and shoot tips after preculture, loading and PVS2 treatments (C and D). Figure. 2A and 2B presented the feature of cells in fresh shoot tips (without any treatments) in the scales of 2 μm and 1 μm, respectively. Figure 2C and 2D presented the feature of cells in shoot tips after preculture, loading and PVS2 treatments in the scales of 2 μm and 1 μm, respectively. The letters represented: CW-cell wall, CM-cell membrane, N-nucleus, Ne-nucleus envelope, V-vacuole, M-mitochondria.

Meristem cells dehydrated in PVS2 solution showed that structurally preserved intact nuclei, cell walls, and rich organelles (mitochondria, plastid, etc.). All these features indicate a quite active cell metabolism. After LN exposure, some cells were able to repair the damage and survive during recovery culture, whereas some cells were extensively damaged and lethally injured.

DISCUSSION

The optimised droplet-vitrification method provided moderate to good regrowth for the four petunia cultivars tested. The ultrastructural investigations of the meristem cells during the cryopreservation procedures would help us to understand the stress responses of cells in shoot tips. Further studies are necessary to investigate the cytological, biochemical, and molecular responses of cells during cryopreservation.

ACKNOWLEDGMENTS

This study was supported by the Crop Germplasm Resources Protection, Cooperation Research on Collecting Techniques and Practice in Crop Genebank between China and United States of America (2014DFG31860), and Utilization Special Grant from the Ministry of Agriculture, and the Agricultural Science and Technology Innovation Program /Crop Germplasm Resources Preservation and Sharing Innovation Team.

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