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Full Length Research Paper

Evaluation of genetic stability in cryopreserved *Solanum tuberosum*

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Cryopreservation is a technology of high importance in the storage of plant germplasm for long periods; however, the practical application of this technology for the preservation of plant materials is useful only if it does not lead to the genetic changes in the plant of interest. In the present investigation, the genetic stability of potato (*Solanum tuberosum* L.) plantlets of the cultivars Agria and Marphona stored under cryopreservation and non-cryopreservation conditions was studied using amplified fragment length polymorphism (AFLP) technique. Also, flow cytometric studies were performed to detect if there were probably any changes in the level of polyploidy. Seven primer combinations were used in the AFLP studies. Agria plantlets kept under non-cryopreserved conditions were approximately of average 97% genetic similarity to those of the same cultivar stored under cryopreservation conditions. With the cultivar of Marphona, full (100%) homology was found between plantlets stored under cryopreservation and non-cryopreservation conditions. Comparative studies on the polyploidy levels of the plantlets of both cultivars conserved under two above-mentioned storage conditions indicated that cryopreservation technique did not cause any changes in polyploidy levels.

Key words: Cryopreservation, conservation, storage, genetic stability, potato.

INTRODUCTION

In Iran, potato is cultivated for more than two centuries, and more than 180000 hectares of the total cultivated lands of the country are under the cultivation with this crop. However, no serious attempt has been made toward the establishment of a gene bank for potato and its conservation until now.

The traditional methods of plant germplasm conservation and storage suffer from many difficulties. Plants are left exposed to destructive biological and non-biological factors under natural conditions. Conversions in the genetic structure of plants may occur because of the long-standing contacts and continuous interactions between plant and the environmental factors existing in the natural media. With certain plants, there are some difficulties in their sexual propagation. Also, the vegetative plant organs are weakly amenable for conservation. And lastly, vast space and many facilities are required for the propagation and conservation of plant germplasms.

To prevent the loss of important genetic information, cultivars are conserved in several collections of gene banks. In principal, there are two options for the medium to long-term storage of potato cultivars: storage as ***in vitro*** plantlets or microtubers (slow groth) and storage of meristems or shoot-tips in liquid nitrogen (cryo-conservation). There are well-established protocols for medium storage (Golmirzaie and Toledo, 1998) and cryopreservation of potato (Golmirzaie and Panta, 1997).

Slow-growth and microtuber regimes are used as a medium-term storage option. These techniques enable subculture intervals to be extended to between 12 months and 3 years. For extending the storage period, it is possible to use reduced temperature, reduced light conditions, and a modification of the media, particularly the addition of growth retardants (Wescott, 1981; Mix, 1983; Withers, 1987). The advantages of these forms of germplasm storage are that the collection is maintained under sterile conditions in controlled environment. Stored microplantlets of microtubers can be rapidly propagated

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from the ***in vitro*** cultures when required. The disadvantages are that the techniques require some special facilities and equipment, as well as well-trained personnel. The genetic stability of the cultures stored under these conditions needs further analysis.

Problems of ***in vitro*** conservation include high maintenance costs, risks of somaclonal variation and genetic instability, especially when growth retardants are used (Harding, 1991). Thus, cryopreservation appears to be a logical choice for long-term storage of potato germplasm with minimum space and maintenance requirements without genetic instability (Bajaj, 1978, 1985; Harding and Benson, 1994; Schoefer-Menuhr et al., 1996).

Cryopreservation is, so far, the only viable procedure for long-term germplasm conservation of vegetatively propagated species and, therefore, can be used for base collections. Cryopreservation implies the conservation of plant propagules at very low temperatures (below –150ºC), thus ensuring that all metabolic processes stop. In this way, subcultures are not required and the threat of somaclonal variation is reduced. Liquid nitrogen (–196ºC) is usually used as refrigerant, although freezers with working temperatures of –150ºC are now available. Cryopreserved material requires a limited space. However, power or liquid nitrogen supply must be guaranteed.

However, the conventional slow freezing method using a programmable freezer, which is complicated and time consuming, produced low rates of shoot formation and callus mediating recovery growth (Towill, 1984; Benson et al., 1989). For long term conservation, the availability and development of safe and cost effective techniques and subsequent high plant regeneration are the basic requirements.

The main obstacles to routine application of cryopreservation of plant germplasm were first the need to optimize the procedures for each plant species, and then the lack of information on the genetic stability of plants regenerated from cryopreserved material.

A major concern of curators is the genetic stability of conserved material. ***In vitro*** culture has been reported to induce genetic changes (somaclonal variation) in some cases (Scowcroft, 1984). Care should be taken to test that the ***in vitro*** culture phase does not produce genetic instability (Harding, 1999). Although not many studies have been performed yet, cryopreservation protocols seem to ensure genetic stability of the plant material. Sugarcane plants derived from cryopreserved embryonic clumps did not show field performance differences when compared with non-cryopreserved ones (González-Arnao et al**.**, 1999; Martínez-Montero et al**.**, 2002). Genetic stability of cryopreserved potato shoot tips has been studied through ploidy status or ribosomal genes stability (Harding, 1991; Benson et al**.**, 1996; Harding, 1997). Study of the DNA variation through amplified fragment length polymorphism assays have been carried out in strawberry, apple, grape, and kiwi (Hao et al**.**, 2001, 2002; Zhai et al**.**, 2003). To date, there is no substantial evidence to suggest that plants regenerated from cryopreserved shoot apices are genetically changed. In potato, DNA polymorphism observed was not induced by cryopreservation but by the whole process employed, including the tissue culture phase (Harding, 1997). In other works where variants were regenerated from cryopreserved apices, those were due to their chimeric structure (Fukai et al., 1994).

Genetic stability of cryopreserved material has been studied so far by the assessment of morphological (Towill, 1983), cytological (Lakhanpaul et al., 1996), biochemical (Chen and Beversdorf, 1992; Yoshimatsu et al., 1996) and non-polymerase chain reaction (PCR) generated molecular markers (Harding, 1991; Eksomtramage, 1993) without establishing accurately the effects of cryopreservation on the genome. The aim of this work was to assay the genetic stability of potato (***Solanum tuberosum***) ***in vitro*** plants regenerated from apices cryopreserved either by the slow freezing procedure or by the encapsulation-dehydration procedure. Evaluation of genome stability was performed by assessment of amplified fragment length polymorphism (AFLP) markers, which is a PCR-based technique. Ploidy and developmental competence of regenerated plants were also examined.

MATERIALS AND METHODS

Cryopreservation

Shoot tips (1-2 mm in length) of two potato cultivars (Agria and Marphona) were excised and then incubated for 24 h at 4ºC on the meistem tissue culture medium as a pre-culture step (Tovar et al., 1985). Such shoot tips were cryopreserved by the rapid freezing encapsulation and dehydration procedure. Shoot tips were encapsulated in 3% (w/v) Ca^{2+}/ alginate (Fabre and Derredre, 1990) and then pre-grown for 3 d on meristem medium containing increasing concentrations of sucrose (0.25 - 1 M). Following pre-growth step, the encapsulated shoot tips were desiccated for 2-4 h in an open empty sterile Petri dish exposed to a sterile air flow. The alginate beads containing the shoot tips were transferred into 2 ml sterile cryo-vials. Plunged into liquid nitrogen, they were maintained for 3 months. Alginate beads were thawed rapidly by plunging the cryo-vials in a water bath of the temperature of 37ºC and transferred to recovery medium. Recovers from the cryopreserved shoot tips were evaluated for their genetic stability over a period of 8 weeks.

Assessment of genomic stability

The AFLP analysis was conducted as described by Vos et al**.** (1995), but with minor modifications. For each genotype, young leaves of 30 plantlets were harvested and pooled. Total genomic DNA was extracted as described by Suhl and Korban (1996) and its quantity and quality were evaluated with a UV-Vis Photometer. 500 ng of DNA was digested with 2.5 units of ***Mse***I and ***Eco***RI restriction enzymes in a 40 µl final reaction volume using 4 µl of 10 × One-Phor-All buffer (10 mM Tris-HAc pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT, 50 ng/µl BSA; Boehringer Mannheim) and incubated at 37ºC for 1.5 h. The ***Eco***RI and ***Mse***I enzyme restriction sites of genomic DNA fragments were ligated to 50 mM ***Mse***I-adaptor and 5 mM ***Eco***RI-adaptor (MWG Biotech Ebersberg, Germany) using 2 units of T4 DNA ligase (Boehringer Mannheim) and 1 mM ATP per

Table 1. The seven selective AFLP primer combinations used in the evaluation of genetic stability in cryopreserved ***Solanum tuberosum***.

Primer combinations*	Sequences
E11- M17	5′-GATGAGTCCTGAGTAACAT-3′
	5′-GACTGCGTACCAATTCGTC-3′
E11- M35	5′-GATGAGTCCTGAGTAAGAG-3′
	5′-GACTGCGTACCAATTCGTC-3′
E46-MCAG	5′-GATGAGTCCTGAGTAACCC-3′
	5′-GACTGCGTACCAATTCGTC-3′
E46-M17	5′-GATGAGTCCTGAGTAACAA-3′
	5′-GACTGCGTACCAATTCAGG-3′
E8-M35	5′-GATGAGTCCTGAGTAACAT-3′
	5′-GACTGCGTACCAATTCAGG-3′
E8-M17	5′-GATGAGTCCTGAGTAAGAG-3′
	5′-GACTGCGTACCAATTCACT-3′
E8-MCAG	5′-GATGAGTCCTGAGTAACAA-3′
	5′-GACTGCGTACCAATTCGTC-3′

*_**Eco**_RI+NNN/_**Mse**_I +NNN primer combinations with three selective nucleotides on the 3´ end of either primer were used for selective amplification.

reaction. The volume of reactions mixtures were adjusted to 50 µl and incubated at 37ºC for 3 h. The resulting reaction product was diluted with 75 µl of autoclaved distilled water.

Preamplification was performed with the _**Eco**_RI/ _**Mse**_I primer combination with no selective nucleotide at the 3´ end (MWG Biotech, Ebersberg, Germany). The PCR preamplification was carried out in a 25 µl reaction volume with 0.3 µM _**Eco**_RI-primer, 0.3 µM _**Mse**_I-primer, 0.2 mM of each dNTP, 1.5 mM MgCl2, 1 × PCR buffer (10 mM Tris- Hcl pH 8.3 50 mM KCl, gelatin solution 1%), 0.5 units of DNA _**Taq**_ polymerase and 3.75 µl of diluted ligated DNA. For preamplification, the following cycle profile was used: 2 min at 72°C, 20 cycles: 30 sec at 94°C, 1 min at 60°C and 2 min at 72°C. The PCR product was diluted with 75 µl of sterile distilled water.

Seven _**Eco**_RI+NNN/_**Mse**_I +NNN primer combinations with three selective nucleotides in the 3´ end of either primer were used for selective amplification (Table 1). The AFLP primer names were abbreviated according to the standard nomenclature of AFLPs proposed by KeyGene (Mardi et al., 2005). 3.75 µl of diluted preselective PCR products were used as DNA templates for selective amplifications.The PCR selective amplifications were carried out in the same manner as PCR preamplifications. For selective amplification, the following cycle profile was used: 2 min at 94°C, 10 cycles: 30 s at 94°C, 30 s at 63°C (touchdown 1°C per cycle to 54°C), 2 min at 72°C and 23 cycles: 30 s at 94°C, 30 s at 54°C, 2 min at 72°C. All amplifications were performed in a BioRad thermocycler (BioRad Laboratories Inc., Hercules, CA, USA).

Amplification reaction products were separated on a 6% denaturing polyacrylamide gel in a 50 cm Sequi-Gen GT Sequencing Cell gel apparatus (BioRad Laboratories Inc., Hercules, CA, USA). The amplified fragments were detected by silver staining as described by Bassam et al. (1991). The resulting gels were scored manually.

Assessment of ploidy stability

Small piece of leaf was placed in a special small plate for flow cytometry (PAI, Partuc, Germany). Then, an aliquot of 400 µl of extraction buffer was added onto the sample, ground with a sharp blade, and the resulted liquid was purified through application of a filter. 1600 µl of DAPI (4,6-diamidino-2-phenyindole) reagent was added into a tube, and the tube was placed under the sensor of the instrument to get the peaks displayed on the monitor. With each experiment, the flow cytometry instrument was calibrated with its special plant standard. Rose (_**Rosa persica**_) is used as the plant standard for potato, because even with the octaploid level of potato, its peak does not locate on that of potato.

Phenotype evaluation

For the evaluation of the phenotype, regrown shoots were cultured onto hormone-free medium, propagated further and planted out in pots_**. In vitro**_ plants which had been treated with DMSO only but had not been put in liquid nitrogen of the respective cultivars were planted out as controls. The plants were observed during the growth period and the tubers were harvested for observing phenotypical changes (Schaefer-Menuhr et al., 1986).

RESULTS AND DISCUSSION

As indicated by the pattern of post-electrophoretic bands of selective PCR products obtained after determination of the sizes of all the bands and the corresponding scores, high homology was found between the samples of each cultivar studied from the stand point of the impacts of two conservation conditions. More detailed and exact results were obtained through analyses made using UPGMA algorithm approach and the calculation of similarity coefficient. According to the electrophoretic patterns obtained with six pairs of primers and Agria cultivar conserved under two cryopreservation and traditional conditions, the homology rates of 98, 94, 97, 100, 98 and 100% were obtained, with an average of 97.8%. With the cultivar, Marphona and with the comparison of two conservation approaches of cryopreservation and non-cryopreserva-

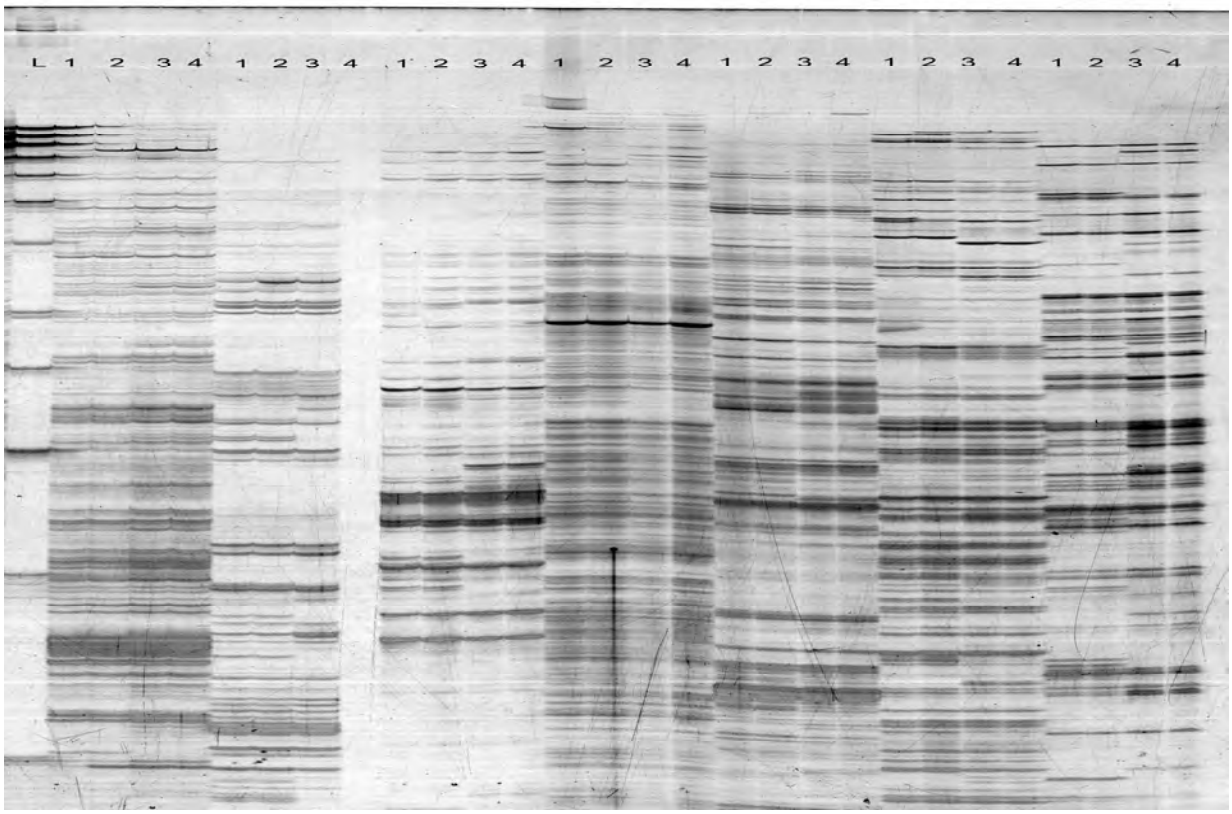

Figure 1. AFLP banding pattern of potato cultivars of Agria and Marphona preserved under usual traditional and long-term cryopreservation storage conditions.

tion, 100% homology was found with all seven primer pairs (Figure 1).

Helliot and co-workers (2002) studied the genetic stability of the terminal meristems of ***Prunus felenain*** plantlets stored for a long period of time and propagated, through a series of physiological, cytological, and molecular investigations, random amplified (RAPD) and AFLP, and concluded that there were not any significant discrepancies between the plants conserved under usual and cryoconservation conditions.

The genetic stability of plants recovered from frozen apices was evaluated by the use of AFLP markers. Such molecular markers are usually powerful tools for following potential changes in the DNA. Caetano-Annoles et al. (1991) suggested that AFLP polymorphism resulted from sequence variation within the primer complementary region. Moreover, small deletions or insertions into the restriction fragment could also generate AFLP markers. These rapid techniques needed very small amounts of plant material, which was compatible with the work on ***in vitro*** plants.

The studies with flow cytometry instrument and the plantlets from the selected cultivars, Marphona and Agria fresh and also conserved for a long period performed through the calculation of the proportion of the mode of the resulted peaks to the plant standard (rose) peak did not indicate any changes in the polyploidy levels (Table 2).

Table 2. The mode of the peaks obtained with potato (M_P) and rose (M_R) with Flow cytometry instrument.

Cultivar	M_P	M_R	M_P:M_R
Agria	129	69	0.53
Agria (cryopreserved)	124	67	0.54
Marphona	129	69	0.53
Marphona (cryopreserved)	130	74	0.56

Evaluation of the phenotype has been done by planting out re-grown plants of the two cultivars and comparing the phenotype with those of the respective control plants. Tubers of the two cultivars were planted out a second time. The number of plants failing to establish under the greenhouse conditions was very low. In general, the re-grown plants were very uniform in appearance and the tubers matched those of the unfrozen stock plants.

Schafer-Menuhr and co-workers (1996) did very comprehensive investigations on the preservation of old potato cultivars, and were successful with the conservation of an abundant number of these old cultivars through application of cryopreservation technology.

Schaefer-Menuhr et al. (1996) opined that the best method to investigate the genetic stability is to perform morphological studies. He and his co-workers studied 98 cultivars, of which only one plant was discerned as abnormal. They suggested the use of flow cytometry and DNA fingerprinting for genetic stability studies. However, they were of the opinion that although the Flow cytometric approach gives much detailed information on the changes of polyploidy levels, it is very difficult for a researcher to discuss the gained results. Their investigations also indicated that there were no genetic changes detectable through flow cytometry and fingerprinting in the plants preserved with the use of cryopreservation technique, and those conserved under usual conditions.

REFERENCES

Bajaj YPS (1978). Tuberization in potato plants regenerated from freeze-preserved meristems. Crop Improve. 5: 137-141.

Bajaj YPS (1985). Cryopreservation of germplasm of potato ***(Solanum tuberosum*** L.) and cassava ***(Manihot esculenta*** Crants): viability of excised meristems cryopreserved up to four years, Indian J. Exp. Biol. 23: 285-287.

Bassam BJ, Caetano-Anolles G, Gresshoff PM (1991). Fast and sensitive silver staining of DNA in acrylamide gels. Anal. Biochem. 196: 80-83.

Benson EE, Harding K, Smith H (1989). Variation in recovery of cryopreserved shoot-tips of ***Solanum tuberosunz*** exposed to different pre- and post-freeze light regimes. Cryo-Letters 10: 323-344.

Benson EE, Wilkinson M, Todd A, Ekuere U, Lyon J (1996). Developmental competence and ploidy stability in plants regenerated from cryopreserved potato shoot-tips. Cryo-Letters 17: 119-128.

Caetano-Annoles C, Bassam BJ, Gresshoff PM (1991). DNA amplification fingerprinting using very short arbitrary oligonucleotide primer. Bio/Technology 9: 553-557.

Chen JL, Beversdorf WD (1992). Cryopreservation of isolated microspores of spring rapeseed (Brassica napus L.) for in vitro embryo production. Plant Cell Tissue Organ Cult. 31: 141-149.

Eksomtramage T (1993). Cryoconservation de cals embryoge`nes pour la conservation des ressources ge´ne´tiques de canne a` sucre. The`se doctorale de l'universite´ de Rennes 1, France.

Fabre J, Derreude J (1990). Encapsulation-dehydration: a new approach to cryopreservation of Solanum shoot tips. Cryo-letters 11: 413-426.

Fukai S, Goi M, Tanaka M (1994). The chimeric structure of the apical dome of chrysanthemum (***Dendranthemagrandiflorum*** (Ramat.) Kitam.) is affected by cryopreservation. Sci Hort 57: 347-351.

Golmirzaie A, Toledo J (1998). Non-cryogenic, long-term germplasm storage. In: Hall,R. (ed). Methods in molecular biology, Vol.III: Plant cell culture protocols. Humana press, Netherlands, pp. 95-101.

Golmirzaie AM, Panta A (1997). Advances in potato cryopreservation by vitrification. CIP Program Report 1995-96. CIP, Lima, Peru. pp. 71-76.

Gonzalez-Arnao MT, Urra C, Engelmann F, Ortiz R, Delafe C (1999). Cryopreservation of encapsulated sugarcane apices: effect of storage temperature and storage duration. Cryo-Letters 20: 347-352.

Hao YJ, Liu QL, Deng XX (2001). Effect of cryopreservation on apple genetic resources at morphological, chromosomal, and molecular levels. Cryobiology 43: 46-53.

Hao YJ, Liu QL, Deng XX (2002). Analysis of ploidy and the patterns of amplified fragment length polymorphism and methylation sensitive amplified polymorphism in strawberry plants recovered from cryopreservation. Cryo-Letters 23: 37-46.

Harding K (1991). Molecular stability of the ribosomal RNA genes in ***Solamtm taberos~lm*** plants recovered from slow growth and cryopreservation. Euphytica 55: 141-146.

Harding K, Benson EE (1994). A study of growth, flowering and tuberization in plants derived from cryopreserved shoot-tips of ***Solanum tuberosum.*** Cryo-Letters 15: 59-66.

Harding K (1997). Stability of the ribosomal RNA genes in ***Solanum tuberosum*** L. plants recovered from cryopreservation. Cryo-Letters 18: 217-230.

Harding K (1999). Stability assessments of conserved plant germplasm. In: Plant conservation biotechnology (Benson EE ed.). Taylor and Francis Ltd, London, pp. 97-107.

Helliot B, Madur D, Drilewagner E, De Boucaud MT (2002). Evaluation of genetic stability in cryopreserved Prunus. In Vitro Cell. Dev. Biol. 38: 493-500.

Lakhanpaul S, Babrekar PP, Chandel KPS (1996). Monitoring studies in onion (***Allium cepa*** L.) seeds retrieved from storage at -20 ºC and -180ºC. Cryo-Letters 17: 219-232.

Mardi M, Buerstmayr H, Ghareyazie B, Lemmens M, Mohammadi SA, Nolz R, Ruckenbauer P (2005). QTL analysis of resistance to Fusarium head blight in wheat using a 'Wangshuibai' derived population. Plant Breed. 124: 329-333.

Mix G (1983). Langzeitlagerung von Kartoffelgenmaterial ***in-vitro***. Landbauforschung- Voelkenrode 33: 179-182.

Schaefer-Menuhr A, Mueller E, Wagner EM (1996). Cryopreservation: an alternative for long-term storage of old potato varieties. Potato Res. 39: 507-513.

Scowcroft WR (1984). Genetic variability in tissue culture: impact on germplasm conservation and utilization. International Board for Plant Genetic Resources Secretariat, Rome, p. 42.

Suhl IW, Korban SS (1996). A highly efficient method for isolating genomic DNA from plant tissues. Plant Tissue Cult. Biotechnol. 2: 113-116.

Tovar PR, Strada R, Schilde-Rentchler L, Dodds JH (1985). Induction and use of ***in vitro*** potato tubers. Cip Circular 13(4): 1-5.

Towill LE (1983). Improved survival after cryogenic exposure of shoot-tip derived from in-vitro plantlet cultures of potato. Cryobiology 20: 567-573.

Towill LE (1984). Survival of ultra-low temperatures of shoot-tips from ***Solanum tuberosum*** groups Andigena, Phureja, Stenotomum and other tuber-bearing ***Solanum*** species. Cryo-Letters 5: 319-326.

Vos P, Hoger R, Bleeker M, Reijans M, Van De Lee T, Hornes M, Frijteters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995). AFLP: a new technique for DNA fingerprinting. NucleicAcids Res. 23: 4407-4414.

Wescott RJ (1981). Tissue culture storage of potato germplasm. 1. Minimal growth storage. Potato Res. 24: 331-342.

Withers L (1987). Long-term preservation of plant cells, tissue and organs. Oxford Surv. Plant Mol. Cell Biol., 4: 221-272.

Yoshimatsu K, Yamaguchi H, Shimomura K (1996). Traits of Panax ginseng hairy roots after cold storage and cryopreservation. Plant Cell Rep. 15: 555-560.

Zhai Z, Wu Y, Egelmann F, Chen R, Zhao Y (2003). Genetic stability assessments of plantlets regenerated from cryopreserved ***in vitro*** cultured grape and kiwi shoot-tips using RAPD. Cryo-Letters 24: 315-322.