

Received 20 August 2003  
Accepted 31 October 2003

**CULTURE TREATMENTS FOR ENHANCING POST-THAW  
RECOVERY OF CRYOPRESERVED SUSPENSION CELLS OF  
POTATO CV. DESIREE**

BUSHRA SADIA<sup>1</sup>, PAUL ANTHONY<sup>1</sup>, KENNETH C. LOWE<sup>2</sup>,  
J. BRIAN POWER<sup>1</sup> and MICHAEL R. DAVEY<sup>1\*</sup>

<sup>1</sup>Plant Sciences Division, School of Biosciences, University of Nottingham,  
Sutton Bonington Campus, Loughborough LE12 5RD, UK, <sup>2</sup>School of Biology,  
University of Nottingham, University Park, Nottingham NG7 2RD, UK

**Abstract:** An efficient and reproducible protocol has been developed for the cryopreservation of cell suspension cultures of the potato (*Solanum tuberosum* L.) cv. Desiree. An evaluation was made of the effectiveness of different pre-culture and post-thaw treatments on cell growth, as measured by changes in biomass. Cell suspensions were cultured in UM medium supplemented with 0.25, 0.5, 0.625, 0.75 or 1.0 M sucrose prior to cryopreservation. Sucrose-treated cells were harvested from suspension and 0.75 ml packed cell volumes placed in 2 ml capacity polypropylene vials with 0.5 ml of chilled cryoprotectant (glycerol 46.0 g l<sup>-1</sup>, dimethylsulphoxide 39.0 g l<sup>-1</sup>, sucrose 342.0 g l<sup>-1</sup> proline 5.0 g l<sup>-1</sup>; pH 5.8). Cells were frozen at -0.5°C min<sup>-1</sup> from 0 to -35°C, held at -35°C for 35 min and stored, for 10 days, in liquid nitrogen (-196°C). The most effective pre-treatment, in terms of subsequent post-thaw cell viability as assessed by fluorescein diacetate uptake or triphenyltetrazolium chloride reduction, was culture with 0.75 M sucrose. For this treatment, the mean absorbance (490 nm) following triphenyltetrazolium chloride reduction was 88% greater (p < 0.05) than control and 59% greater (p < 0.05) for thawed cells also cultured on supporting filter paper discs.

**Key Words:** Potato, Cryopreservation, Cell Suspensions, Sucrose Pre-treatment

---

\* Corresponding author, e-mail: [mike.davey@nottingham.ac.uk](mailto:mike.davey@nottingham.ac.uk)

Abbreviations used: DMSO – dimethylsulphoxide; FDA – fluorescein diacetate; f. wt. – fresh weight; RAPD – Random Amplified Polymorphic DNA; s.e.m. – standard error of mean; TTC – triphenyltetrazolium chloride; UM – Uchimiyu and Murashige [12].

## INTRODUCTION

Potato (*Solanum tuberosum* L.) is normally propagated vegetatively due to high heterozygosity and low capacity for seed production. Genetic resource management is usually achieved by conserving plants and/or vegetative propagules, such as tubers [1]. Genetic manipulation approaches for potato improvement depend upon the routine maintenance of axenic shoots and suspensions as a source of totipotent cells from which protoplasts can be isolated. In turn, potato protoplasts provide material for increasing variability through genetic modification. For the latter, approaches have involved transformation by direct DNA uptake [2] and the introgression of foreign genes by somatic hybridization [3] and cybridization [4]. Whilst cell suspension cultures provide a readily accessible source of totipotent protoplasts, the routine sub-culture of such cell suspensions results in a gradual decrease in shoot regeneration capacity [5].

Previous reports have described cryopreservation protocols for potato shoot tips [6], root tips [7], pollen [8] and protoplast-derived cell colonies [9], by ultra-rapid or controlled, two-step freezing. However, there has been only one account of the cryopreservation of suspension cultured cells of potato, specifically of the cv. Red Pontiac [10]. One limitation of this previous study was that suspension cells were cryopreserved at  $-14^{\circ}\text{C}$  whereas, ideally, storage in liquid nitrogen ( $-196^{\circ}\text{C}$ ) is preferable. Therefore, the aim of the present study was to develop an efficient and reproducible cryopreservation protocol for cell suspension cultures of the commercially-important potato cv. Desiree, focusing on evaluating the effectiveness of pre-culture (especially sucrose) and post-thaw (filter paper support) treatments on cell growth. Additionally, this study has employed two complementary methods for measurement of cell viability following recovery from cryopreservation.

## MATERIALS AND METHODS

### **Plant materials, preparation of cell suspensions and culture conditions**

Cell suspension cultures were initiated from leaf-derived callus of the potato cv. Desiree, essentially as described by Scaramagli *et al.* [11] and maintained in UM medium [12]. Cells were used for cryopreservation 16 months after the initiation of suspensions. Three days after sub-culture, cells were allowed to settle, followed by replacement of 33 ml of the 50 ml aliquots of spent UM medium in the culture vessels with the same volume of new UM medium containing 0.38, 0.75, 0.94, 1.13 or 1.52 M sucrose. This resulted in final concentrations of *ca.* 0.25, 0.5, 0.625, 0.75 or 1.0 M sucrose in the UM medium in which the cells were incubated for 24 h prior to freezing. Suspensions were maintained alongside untreated (control) cells on a rotary shaker (60 rpm) at  $24 \pm 2^{\circ}\text{C}$  with a 12 h photoperiod ( $19.5 \mu\text{mol m}^{-2} \text{sec}^{-1}$ , Cool White fluorescent illumination).

**Cryoprotection and freezing of suspension cells**

Cells were cryopreserved as reported earlier [13]. Sucrose-treated cells were harvested from suspension using nylon sieves (100  $\mu\text{M}$  pore size) and 0.75 ml packed cell volume (0.5 g f.wt.) was placed (using a micro-spatula) into each 2 ml capacity polypropylene vial (Sarsted Ltd., Leicester, UK), followed by 0.5 ml of chilled cryoprotectant [13] (46.0 g  $\text{l}^{-1}$  glycerol, 39.0 g  $\text{l}^{-1}$  DMSO, 342.0 g  $\text{l}^{-1}$  sucrose, 5.0 g  $\text{l}^{-1}$  proline). The cryoprotection mixture was prepared in liquid UM medium; the pH was adjusted to 5.8 prior to filter sterilization (0.2  $\mu\text{m}$  pore size; Sartorius, Göttingen, Germany). All chemicals used to prepare the cryoprotectant mixture were of Analar grade, except for DMSO, which was spectroscopically pure.

Vials containing cells/cryoprotectant mixtures were held in aluminium cryo-canes, and chilled on ice for 1 h. Cells were frozen at a controlled rate ( $-0.5^{\circ}\text{C min}^{-1}$ ) from 0 to  $-35^{\circ}\text{C}$  and held at  $-35^{\circ}\text{C}$  for 35 min in a programmable freezer (Planer Cryo 10 Series, Planer Biomed, Sunbury-on-Thames, UK). Frozen cells were stored in liquid nitrogen ( $-196^{\circ}\text{C}$ ) for 10 days.

**Post-thaw recovery of cryopreserved cells**

Cells were thawed by plunging the vials into sterile water at  $45^{\circ}\text{C}$  for 3-4 min; excess cryoprotectant was removed from the vials under aseptic conditions using Pasteur pipettes. Thawed cells from individual vials were placed onto 20 ml aliquots of agar-solidified UM medium, in 9 cm diameter Petri dishes, with or without two superimposed 5.5 cm diameter Whatman No. 1 filter paper discs overlying the UM medium.

Cultures were maintained in the dark for 14 days at  $25 \pm 2^{\circ}\text{C}$  and transferred to light with a 16 h photoperiod (19.5  $\mu\text{mol m}^{-2} \text{sec}^{-1}$  of Cool White fluorescent illumination), following sub-culture onto new UM medium. All treatments were replicated 20 times (10 times each for the cells cultured with or without filter paper). Assessments of cell viability, using 2 standard protocols, were carried out 4 days and 10 days after the start of the post-thaw recovery phase. Control cells were maintained on semi-solid UM medium using the same incubation conditions as for the thawed samples. Cells were cultured for 28 d prior to biomass determination and re-establishment of cell suspensions.

**Measurement of post-thaw cell viability**

Post-thaw viability of cells was assessed by 2 established methods. The first involved the reduction of TTC and was based on a modification of the method of Steponkus and Lamphear [14], as described [15]. Absorbance was measured spectrophotometrically at 490 nm. TTC reduction was also determined for unfrozen (control) suspension cells, which were cultured in UM medium. TTC values were determined by assaying cell samples ( $n = 10$ ) from each Petri dish, following culture with or without filter paper supports.

The second method for assessing cell viability involved FDA staining. The protocol was based on the method of Widholm [16], as described by Ishikawa *et al.* [17]. Ten  $\mu\text{l}$  of a FDA stock solution ( $3 \text{ mg ml}^{-1}$  in acetone) were added to 10 ml of liquid UM medium. A 1.0 ml aliquot of this solution was mixed with 0.5 ml of cells from each treatment. Cells were observed under UV illumination after 5 min at room temperature. Cells were considered viable when they exhibited bright green fluorescence from the cytosol with unstained vacuoles. Cell viabilities were determined by counting at least 200 cells from each Petri dish.

#### **Assessment of the re-growth of thawed cells**

The post-thaw growth (recovery) of cell colonies was assessed by the measurement of f. wt. gain at 28 d after thawing and following removal of the cells from either medium or filter paper supports. For each experimental treatment, the f. wt. gain of cells was determined from 20 replicate Petri dishes (10 each for cells cultured with or without filter paper).

#### **RAPD analysis**

RAPD analysis was performed as reported [18]. Genomic DNA was isolated using a DNeasy Mini Kit (QIAGEN Ltd., Crawley, UK) from cryopreserved and unfrozen (control) suspension cells. Two primers each of 10 bases (OPA 2 and OPC 16) from Kits A and C (Operon Technology Inc., Alameda, USA) were used to screen for DNA modification by RAPD analysis.

#### **Re-establishment of suspension cultures and isolation of protoplasts**

Suspension cultures were re-established from thawed cells (28 d post-culture) by transferring 0.5 g f. wt. of cells from each Petri dish to 30 ml of liquid UM medium in 100 ml Erlenmeyer flasks under the same culture conditions as described previously. At 7 days intervals, spent culture medium was replaced by new UM medium, the volume of which was increased by 5 ml per flask at each sub-culture to a final volume of 50 ml per flask. Cultures were maintained for a further 35 days, prior to enzymatic isolation of protoplasts [19]. Protoplast yield was assessed by counting a minimum of 200 protoplasts per treatment using a haemocytometer. Protoplast viability, measured by FDA uptake [16], was assessed for cryopreserved and control cells.

#### **Statistical analyses**

Means and s.e.m. were used throughout; statistical significance between mean values was assessed using a conventional one-way ANOVA and identified using the Tukey-HSD test and Student's *t*-test [20]. A probability of  $p < 0.05$  was considered significant.

## RESULTS

### Assessment of cell viability by TTC reduction

The mean absorbance, as measured by TTC reduction, of unfrozen (control) and pre-treated, cryopreserved and thawed potato cells cultured with or without supporting filter paper discs is given in Tab. 1.

Tab. 1. Mean absorbance (490 nm) following TTC reduction, of thawed potato cells, after 4 and 10 days of culture with or without supporting filter paper discs.

Treatment	Absorbance (490 nm)			
	4 days		10 days	
	Cells cultured with filter paper	Cells cultured without filter paper	Cells cultured with filter paper	Cells cultured without filter paper
1	0.38 ± 0.04	0.20 ± 0.03	0.33 ± 0.06	0.34 ± 0.08
2	0.03 ± 0.03*	0.03 ± 0.01*	0.03 ± 0.01*	0.01 ± 0.01*
3	0.08 ± 0.02*	0.06 ± 0.01*	0.27 ± 0.08	0.17 ± 0.01*
4	0.08 ± 0.01*	0.07 ± 0.01*	0.37 ± 0.06	0.31 ± 0.07
5	0.17 ± 0.02*	0.11 ± 0.01*	0.62 ± 0.05*	0.54 ± 0.06*
6	0.05 ± 0.01*	0.06 ± 0.01*	0.24 ± 0.10*	0.10 ± 0.03*

Treatments: 1 = control (unfrozen), 2 - 6 = UM medium with 0.25, 0.5, 0.625, 0.75 or 1.0 M sucrose. Values are mean ± s.e.m. (n = 10). \*p < 0.05, compared to mean control value.

The mean absorbance of thawed cells after 4 days of culture was significantly ( $p < 0.05$ ) lower for all pre-culture treatments (treatments 2 – 6), compared to unfrozen (control) cells, when cultured with or without supporting filter paper discs overlaying semi-solid UM medium. The mean absorbance of cells initially pre-treated with 0.75 M sucrose (treatment 5) and cultured for 4 days post-thawing was 55% and 46% lower than that of control cells, with and without filter paper supports, respectively. After 10 days of culture, cells initially pre-treated with 0.75 M sucrose showed an increase in absorbance which was significantly ( $p < 0.05$ ) greater than the respective controls, with or without filter papers. There was no additional beneficial effect of the supporting filter paper discs. In contrast, cells subjected to initial pre-culture treatments involving 0.5 M sucrose (treatment 3) or 0.625 M sucrose (treatment 4) exhibited mean absorbances similar to control. For cells initially pre-treated with 0.25 M sucrose (treatment 2), the mean absorbance at 4 or 10 days was significantly ( $p < 0.05$ ) lower than the respective control (Tab. 1).

### Assessment of cell viability by FDA uptake

The mean viability of thawed cells, as assessed by FDA uptake, after 4 days of culture, was significantly ( $p < 0.05$ ) lower for all initial pre-culture treatments, compared to unfrozen controls (Tab. 2). This pattern was consistent with the

data obtained from TTC reduction assessments. Culture of cells with 0.75 M sucrose resulted in maximum cell viability compared to other pre-treatments. After 10 days of culture, cells pre-treated with 0.5 or 0.625 M sucrose showed increases in viability compared to the viability of cells at 4 days when the latter were cultured in the presence or absence of filter paper supports. Similarly, the viability after 10 days was significantly ( $p < 0.05$ ) greater for potato cells pre-treated with 0.75 M sucrose compared with that of controls, irrespective of filter paper supports. Cells pre-treated with 0.25 M sucrose failed to survive after 10 days of culture, which was consistent with data from the TTC reduction assessments.

Tab. 2. Mean viability, determined by FDA uptake, of thawed potato cells, after 4 and 10 days of culture with or without supporting filter paper discs.

Treatment	Survival (%)			
	4 days		10 days	
	Cells cultured with filter paper	Cells cultured without filter paper	Cells cultured with filter paper	Cells cultured without filter paper
1	76.2 ± 3.4	60.0 ± 5.4	65.2 ± 3.3	62.0 ± 5.6
2	0.4 ± 0.2*	0.20 ± 0.2*	0 ± 0*	0 ± 0*
3	37.2 ± 3.4*	22.6 ± 1.6*	47.0 ± 4.4*	41.0 ± 3.6*
4	35.0 ± 4.1*	30.0 ± 4.3*	50.4 ± 3.1*	42.2 ± 4.0*
5	50.0 ± 5.4*	37.0 ± 4.2*	80.0 ± 1.8	68.4 ± 2.3
6	13.4 ± 2.0*	8.0 ± 1.1*	11.0 ± 0.5*	7.2 ± 1.1*

Treatments: 1 = control (unfrozen), 2 - 6 = UM medium with 0.25, 0.5, 0.625, 0.75 or 1.0 M sucrose. Values are mean ± s.e.m. (n = 10). \* $p < 0.05$ , compared to mean control value.

Tab. 3. Effect of pre-culture treatments on mean f. wt. gain of cells following cryopreservation, thawing and culture for 28 d on semi-solid UM medium with or without supporting filter paper discs.

Treatment	F. wt. gain (g)	
	Cells cultured with filter paper	Cells cultured without filter paper
1	8.0 ± 0.4	9.5 ± 1.0
2	0.5 ± 0.1*	0.5 ± 0.1*
3	3.2 ± 0.8*	2.7 ± 1.0*
4	4.7 ± 0.5*	4.0 ± 0.3*
5	11.0 ± 0.7*	8.3 ± 1.0
6	0.5 ± 0.1*	0.4 ± 0.1*

Treatments: 1 = control (unfrozen), 2 - 6 = UM medium with 0.25, 0.5, 0.625, 0.75 or 1.0 M sucrose. Values are mean ± s.e.m. (n = 10). \* $p < 0.05$ , compared to mean control value.

### Post-thaw growth of cryopreserved potato cells

The mean f. wt. gain of cells not pre-treated or cryopreserved (control) and those cells cultured with or without supporting filter paper discs on semi-solid UM medium for 28 d after freezing and thawing, is summarized in Tab. 3.

The mean f. wt. gain was significantly ( $p < 0.05$ ) greater for cells pre-cultured with 0.75 M sucrose (treatment 5), followed by culture for 28 d after thawing on a filter paper support, as compared to control and all other treatments. However, when cells were cultured on semi-solidified UM medium without a filter paper support, the maximal increase in biomass occurred in the control treatment.

For cells initially pre-treated with 0.25 M sucrose (treatment 2) or 1.0 M sucrose (treatment 6), the mean f. wt. gain, irrespective of whether filter paper supports were used, was only a maximum of *ca.* 6% of the respective control mean value (Tab. 3), indicating a marked decline in growth and biomass production.

### RAPD analysis of thawed cell suspensions

RAPD analysis, using the DNA primers, OPA 2 and OPC 16, showed that there were no major differences in DNA fingerprints between control cells and thawed cells, irrespective of the pre-culture treatment (Fig. 1).

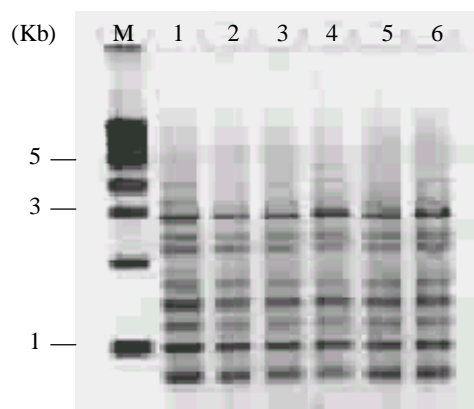


Fig. 1. RAPD profiles, generated by primer OPC 16, of potato suspension cells pre-cultured with 0.75 M sucrose, cryopreserved for 10 days, thawed and cultured with filter paper supports on semi-solidified UM medium for 28 d. Lane 1: control, Lanes 2-6: DNA from different cryopreserved suspension cells. M = Molecular markers in Kb.

### Protoplast isolation from re-established cell suspensions

Cell suspensions were initiated only from cells pre-cultured with 0.5, 0.625 or 0.75 M sucrose, and maintained after thawing on filter paper supports overlaying semi-solid UM medium. Such cells produced rapidly dividing friable suspensions. In contrast, for cells pre-cultured with 0.5, 0.625 or 0.75 M sucrose, and grown without filter paper supports, it was not possible to re-initiate suspensions because of the predominance of dead cells in these cases. Re-

established suspension cultures were used for protoplast isolation studies (Tab. 4). There were no significant differences, in terms of mean protoplast yields and viabilities, between control cells and cells pre-cultured before freezing with 0.5, 0.625 or 0.75 M sucrose.

Tab. 4. Yields and viabilities of protoplasts isolated from re-established cell suspensions.

Treatment	Protoplast yield (g f. wt. x 10 <sup>6</sup> )	Protoplast viability (%)
1	7.7 ± 0.4	78.6 ± 3.1
3	8.0 ± 0.3	76.0 ± 2.8
4	7.8 ± 0.4	78.0 ± 1.1
5	7.8 ± 0.3	81.5 ± 2.7

Treatments: 1 = control (unfrozen), 3 – 5 = UM medium with 0.5, 0.625 or 0.75 M sucrose. Values are mean ± s.e.m. (n = 5).

## DISCUSSION

This study demonstrates, for the first time, an efficient and reproducible protocol for the post-thaw recovery, from storage in liquid nitrogen, of cryopreserved suspension cells of the potato cv. Desiree. Pre-culture treatments involving sucrose significantly influenced the post-thaw cell viability. Pre-culture of suspension cells with 0.75 M sucrose was the most effective treatment to maximize post-thaw recovery of cells, compared with other treatments. Interestingly, sucrose has been used previously in cryopreservation protocols, as in the case of cell suspension cultures of *Helianthus tuberosus* [1], potato shoot tips [21] and cell suspensions of the potato cv. Red Pontiac [10]. For the studies involving cell suspensions of *Helianthus* and potato, the effective concentration range of sucrose was 0.58-0.88 M, consistent with that evaluated in the present investigation with cell suspensions of the potato cv. Desiree. However, one major difference between this earlier investigation and the present study was the duration of the sucrose pre-treatment. The present study involved treatment for 24 h that was significantly longer than the 1.5 h exposure used by Hellergren and Li [10]. This longer sucrose pre-treatment period may have facilitated the ability of potato cells to withstand freezing to a temperature of -196°C in liquid nitrogen.

A further finding from this investigation was that the inclusion of a filter paper support overlying the agar medium, on which the thawed cells were spread, maximized cell viability and biomass increase. This is consistent with previous studies using, for example, rice [22] in which it was speculated that the beneficial effects of using such supporting filter paper discs were due to enhanced diffusion of the cryoprotectants during the early post-thaw period.

TTC reduction, as utilized in the present study, is the most convenient method for multiple cell viability assessments, although Ishikawa *et al.* [17] reported an

over-estimation of freezing tolerance of suspension cultures of brome grass (*Bromus inermis*) with this assay. These authors suggested that viability assessments using the TTC reduction method should be confirmed by other viability assays. Consequently, in the present investigation, cell recovery following thawing was also assessed by FDA uptake and increases in biomass. In the present investigation, TTC reduction and FDA uptake gave comparable results for the viability of potato cells following thawing after 4 days and 10 days of culture.

The re-established potato cell suspensions were genetically stable, as based on DNA profiling using RAPD analysis. Hirai and Sakai [6] also reported the use of RAPD for assessing genetic stability of cryopreserved shoots of *S. tuberosum* cvs. Charly and Hertha and found no differences between cryopreserved and non-cryopreserved materials.

Cryopreservation is the preferred long-term option for conserving germplasms, used as experimental source material or generated through plant genetic manipulation programmes. In the case of potato, whilst improvements have been made in the cryopreservation and post-thaw recovery of shoot meristems, there are no reports of the freezing of cell suspensions of potato at temperatures below -14°C [10], and no protocols for the cv. Desiree. The procedure reported here might be applicable for the routine cryopreservation, post-thaw recovery and re-establishment of growth of cell suspensions of other potato cultivars and wild potato species.

**Acknowledgement.** BS was supported by The Commonwealth Scholarship Commission.

## REFERENCES

1. Stacey, G.N., Lynch, P.T. and Benson, E.E. Plant gene banking: Agriculture, biotechnology and conservation. **Agro-food Ind. Hi-Tech.** 10 (1999) 9-14.
2. Sawahel, W.A. Ultrasound-mediated stable transformation of potato tuber discs. **Biotechnol. Tech.** 10 (1996) 821-824.
3. Li, Y.G., Stoutjestijk, P.A. and Larkin, P.J. Somatic hybridization for plant improvement. in: **Morphogenesis in Plant Tissue Cultures**, (Soh, W.Y. and Bhojwani, S.S., Eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands, 2000, 363-418.
4. Galun, E., Aviv, D. and Perl, A. Cybridisation in Potato. in: **Biotechnology in Agriculture and Forestry**, Vol. 27, (Bajaj, Y.P.S., Ed.), Springer-Verlag, Heidelberg, 1994, 167-182.
5. Anthony, P., Davey, M.R., Azhakanandam, K., Power, J.B. and Lowe, K.C. Cryopreservation of Plant Germplasms: New Approaches for Enhanced Postthaw recovery. in: **Conservation of Plant Genetic Resources In Vitro**, Vol. 2, (Razdan, M.K. and Cocking, E.C., Eds.), Science Publishers, Inc., Enfield, NH, USA, 1999, 21-37.

6. Hirai, D. and Sakai, A. Cryopreservation of *in vitro*-grown meristems of potato (*Solanum tuberosum* L.) by encapsulation-vitrification. **Potato Res.** 42 (1999) 153-160.
7. Bajaj, Y.P.S. Tuberization in potato plants regenerated from freeze-preserved meristems. **Crop Improv.** 5 (1978) 137-141.
8. Towill, L.E. Liquid nitrogen preservation of pollen from tuber bearing *Solanum* species. **Hort. Sci.** 16 (1981) 177-179.
9. Bajaj, Y.P.S. Cryopreservation of potato somaclones. in: **Somaclonal Variations in Crop Improvement**, (Semal, J. Ed.), Martinus Nijhoff, Dordrecht, The Netherlands, 1986, 244-250.
10. Hellergren, J. and Li, P.H. Survival of *Solanum tuberosum* suspension cultures to  $-14^{\circ}\text{C}$ : The mode of action of proline. **Physiol. Plant.** 52 (1981) 449-453.
11. Scaramagli, S., Biondi, S., Leone, A., Grillo, S. and Torrigiani, P. Acclimation to low water potential in potato cell suspension cultures leads to changes in putrescine metabolism. **Plant Physiol. Biochem.** 38 (2000) 345-351.
12. Uchimiya, H. and Murashige, T. Evaluation of parameters in the isolation of viable protoplasts from cultured tobacco cells. **Plant Physiol.** 54 (1974) 936-944.
13. Swan, T.W., Ohare, D., Gill, R.A. and Lynch, P.T. Influence of preculture conditions on the post-thaw recovery of suspension cultures of Jerusalem Artichoke (*Helianthus tuberosus* L.). **Cryoletts.** 20 (1999) 325-336.
14. Steponkus, P.L. and Lamphear, F.O. Refinement of the triphenyl tetrazolium chloride method of determining cold injury. **Plant Physiol.** 42 (1967) 1423-1426.
15. Al-Forkan, M.A., Anthony, P., Power, J.B., Davey, M.R. and Lowe, K.C. Effects of *Erythro*<sup>TM</sup> on post-thaw recovery of cryopreserved cell suspensions of Indica rice (*Oryza sativa* L.). **Cryoletts.** 22 (2001) 367-374.
16. Widholm, J. The use of FDA and phenosafranin for determining viability of cultured plant cells. **Stain Technol.** 47 (1972) 186-194.
17. Ishikawa, M., Robertson, A.J. and Gusta, L.V. Comparison of viability tests for assessing cross-adaptation to freezing, heat and salt stress induced by abscisic acid in bromegrass (*Bromus inermis* Leyss) suspension cultured cells. **Plant Sci.** 107 (1995) 83-93.
18. Sriparaya, S., Blackhall, N.W., Marchant, R., Power, J.B., Lowe, K.C. and Davey, M.R. Relationships in pineapple (*Ananas comosus*) by random amplified polymorphic DNA (RAPD) analysis. **Plant Breed.** 120 (2001) 265-267.
19. Anjum, M.A. Effect of protoplast source and media on growth and regenerability of protoplast-derived calluses of *Solanum tuberosum* L. **Acta Physiol. Plant.** 20 (1998) 129-133.
20. Snedecor, G.W. and Cochran, W.G. **Statistical Methods**, 8th Edn, Iowa State University Press, Ames, 1989.

21. Grospietsch, M., Stodulková, E. and Zámecník, J. Effect of osmotic stress on the dehydration tolerance and cryopreservation of *Solanum tuberosum* shoot tips. **Cryolefts**. 20 (1999) 339-346.
22. Lynch, P.T., Benson, E.E., Jones, J., Cocking, E.C., Power, J.B. and Davey, M.R. Rice cell cryopreservation: The influence of culture methods and the embryogenic potential of cell suspensions on post-thaw recovery. **Plant Sci**. 98 (1994) 185-192.