A cryopreservation protocol for strawberry cell suspension cultures

Yongjie Wu[1,2], Florent Engelmann[3], Andrea Frattarelli[2] and Carmine Damiano[2]

[1] *Changli Institute of Pomology, Hebei Academy of Agricultural and Forestry Sciences, Changli Town 066600, Qin Huang Dao City, Hebei Province, P.R. China*

[2] *Istituto Sperimentale per la Frutticoltura, 00040 Ciampino Aeroporto, Rome, Italy*

[3] *IPGRI, 00145 Rome, Italy*

Introduction

Cryopreservation techniques have been developed for long-term conservation of more than 100 plant species cultured *in vitro* as protoplasts, cell suspensions, calluses, shoot apices, or somatic and zygotic embryos (Kartha and Engelmann 1994; Engelmann 1997). Cryopreservation is employed for conservation of plant genetic resources, but it is also applied to the conservation of biotechnological products, including metabolite-producing cultures and genetically engineered cell strains.

Although cell suspensions are a material of choice for genetic transformation experiments, so far leaf disks and calluses only have been transformed in the case of strawberry (James *et al.* 1990; Nehra *et al.* 1990; El Mansouri *et al.* 1996). Transformation from single cells is highly desirable, since this pathway overcomes the possibility of regenerating chimeric plants. Owing to the difficulty of obtaining transformed material, it is important to develop cryopreservation protocols that allow conservation of the transformed cell lines.

This paper summarizes the protocol developed for the cryopreservation of strawberry cell suspensions (Wu *et al.* 1997) and highlights the effect of several parameters within the freezing process on the viability of the cell suspension cultures.

Materials and methods

Cell suspensions were obtained from callus induced on leaf explants of strawberry (Blando *et al.* 1993) and cultured on a rotary shaker (90 rpm) at 25°C under a photoperiod of 16-h light/8-h dark, and a light intensity of 37 µmol m^{-2} s^{-1}. For cryopreservation, cells were sampled 5, 10 or 15 d after the last transfer to new culture medium and pretreated at 0°C for 60–180 min in PVS3 vitrification solution consisting of 50% (w/v) and 50% (w/v) glycerol (Nishizawa *et al.* 1993), with or without the addition of DMSO at 5%, then cooled at 0.5°C/min down to temperatures ranging between –35 and –45°C and immersed rapidly in liquid nitrogen. Thawing was performed either at room temperature or in a water-bath set at controlled temperatures between 20 and 40°C. Cells were then plated on standard solid medium and observed for regrowth.

Results

In the case of strawberry cell suspensions, pretreatment with various combinations of sucrose and DMSO did not ensure survival after cryopreservation (data not shown) and high survival rates (around 80% of

unfrozen control) were achieved when using the PVS3 vitrification solution, modified, or not, by the addition of 5% DMSO.

The influence on cell survival of two parameters, the effect of thawing regime and the effect of the duration of the last subculture was studied.

No viability was observed when rewarming was carried out in air at room temperature or in a water-bath at 40°C (Table 1). However, thawing at 20 or 30°C permitted high levels of viability with both cryoprotective solutions employed.

Table 1. Effect of thawing regime (in air at room temperature (RT) or in a water-bath thermostated at 20, 30 or 40°C) and of cryoprotective solution employed during pretreatment (MPVS3: PVS3 + 5% DMSO) on the viability (%) of cryopreserved strawberry cell suspensions. Cells were pretreated at 0°C for 1 h, then cooled at 0.5°C/min down to –40°C before rapid immersion in LN. (Adapted from Wu *et al.* 1997, with permission).

	Thawing regime			
Pretreatment	**RT**	**20°C**	**30°C**	**40°C**
PVS3	0	87	83	2
MPVS3	0	89	87	2

The viability of pretreated, prefrozen and cryopreserved cells decreased drastically with increasing duration of culture after the last transfer into new medium (Table 2).

Table 2. Effect of culture period (days) after last subculture on the viability (%) of pretreated, prefrozen (–LN) and cryopreserved (+LN) strawberry cell suspensions. Cells were pretreated with MPVS3, cooled at 0.5°C/min down to –40°C before immersion in LN, then thawed in a water-bath thermostated at 30°C. (Adapted from Wu *et al.* 1997, with permission).

	Days after subculture		
Pretreatment	**5**	**10**	**15**
pretreated	100	87	27
–LN	91	60	1.3
+LN	77	53	0.1

Discussion/conclusion

This study represents the first report of successful cryopreservation of cell suspension cultures using pretreatment with vitrification solutions followed by slow, controlled cooling.

The experiment on the effect of thawing procedure generated some interesting results. Total loss of viability after thawing samples in air at room temperature was probably due to recrystallization and ice crystal growth during the ensuing low rewarming rates. However, such dramatic differences in viability after thawing cell suspensions at temperatures in the range tested here have not been reported previously. A precise analysis of thawing rates and thermic events taking place during rewarming of the strawberry cell suspensions should be performed.

The importance of using cells during their exponential growth phase to achieve high viability, as notably demonstrated by Withers (1985), was re-emphasized during this study.

In conclusion, additional experiments will be necessary to determine optimal conditions for regrowth of cryopreserved cells and reinitiation of cell suspensions.

Acknowledgements

Yongjie Wu gratefully acknowledges the support provided by a Special Skills Training Award within the Italian Government funded training scheme of IPGRI. The authors thank the Istituto Sperimentale per la Frutticoltura for providing the research facilities.

References

Blando, F., A. Niglio, A. Frattarelli, S. Speranza and C. Damiano. 1993. Cell suspension cultures in strawberry: growth characterization and variability. *Acta Horticulturae* (*In Vitro* Culture) **336**: 257–262.

El Mansouri, I., J.A. Mercado, V. Valpuesta, J.M. Lopez–Aranda, F. Pliego–Alfaro and M.A. Quesada. 1996. Shoot regeneration and *Agrobacterium*-mediated transformation of *Fragaria vesca* L. *Plant Cell Reports* **15**: 642–646.

Engelmann, F. 1997. *In vitro* conservation methods. Pp. 119–162 in *Biotechnology and Plant Genetic Resources. Conservation and Use*. B.V. Ford–Lloyd, H.J. Newburry and J.A. Callow (eds.). CABI, Wallingford, UK.

James, D.J., A.J. Passey and D.J. Barbara. 1990. *Agrobacterium*-mediated transformation of the cultivated strawberry (*Fragaria x ananassa* Duch.) using disarmed binary vectors. *Plant Science* **69**,:79–94.

Kartha, K.K. and F. Engelmann. 1994. Cryopreservation and germplasm storage. Pp. 195–230 in Plant Cell and Tissue Culture, I.K. Vasil and T.A. Thorpe (eds.). Kluwer Academic Publishers, Dordrecht.

Nehra, N.S., R.N. Chibbar, K.K. Kartha, R.S.S. Datla, W.L. Crosby and C. Stushnoff. 1990. *Agrobacterium*-transformation of strawberry calli and recovery of transgenic plants. *Plant Cell Reports* **9**, 10–13.

Nishizawa, S., A. Sakai, Y., Amano and T. Matsuzawa. 1993. Cryopreservation of asparagus (*Asparagus officinalis* L.) embryogenic suspension cells and subsequent plant regeneration by vitrification. *Plant Science* **91**: 67–73.

Withers, L.A. 1985. Cryopreservation of cultured plant cells and protoplasts. Pp. 243–267 in *Cryopreservation of Plant Cells and Organs*. K.K. Kartha (ed.). CRC Press, Boca Raton, Florida.

Wu, Y., F. Engelmann, A. Frattarelli, C. Damiano and L.A. Withers. 1997. Cryopreservation of strawberry cell suspension cultures. *Cryo–Letters* **18**: 317–324.

Wu Y., Engelmann Florent, Frattarelli An., Damiano C. (2000).

A cryopreservation protocol for strawberry cell suspension cultures.

In : Takagi H. (ed.). Cryopreservation of tropical plant germplasm : current research progress and application.

Rome (ITA) ; Tsukuba : IPGRI ; JIRCAS, p. 357-359.

(JIRCAS International Agriculture Series ; 8). Cryopreservation of Tropical Plant Germplasm : Current Research Progress and Application : International Workshop, Tsukuba (JPN), 1998/10/20-23.

ISBN 92-9043-428-7.