

## CRYOPRESERVATION OF TROPICAL AND SUBTROPICAL FRUITS

S.K. Sharma, Maneesh Mishra and D.S. Yadav

Division of Horticulture (Biotechnology Lab.), ICAR Research Complex for NEH Region,  
Umroi Road, Umiam - 793 103, Meghalaya, India

Many of the tropical and subtropical fruit crops are reproduced vegetatively and there is an urgent need to preserve fruit crops through improved methods of vegetative propagation. Crop cultivars, parental lines, and experimental lines often require long-term preservation. Preservation is needed, also, for endangered wild species of all types.

Tissue culture technique is useful for the propagation and has several advantages like preservation of genotypes as well as decreased demand for land, manpower and decreased exposure, of axenic culture to crop-borne diseases and pests. But there are some problems with the maintenance and repeated transfers of cultures, the danger of contamination during each transferring which can cause elimination of line and genetic changes in the cultures during repeated subculturing. Loss of morphogenic potential, and other problems associated with tissue culturing, would be minimized by freeze tissues in living condition at very low temperatures such as in liquid nitrogen (-196°C). Successful cryogenic methods for doing this are now being developed. A brief review of such efforts and their use are described here.

Many tropical and sub-tropical fruit crops have proved recalcitrant to traditional cryopreservation methods. This can be overcome by vitrification of tissues that can be achieved by applying relatively simple dehydration and desiccation treatments (Benson et al., 1998). Wang et al. (1994) reported that excised embryogenic axes of mango were more tolerant to desiccation than whole seeds. After desiccation to 11% moisture content, 70% of the excised axes germinated in vitro. Embryonic axes of different moisture content lose their viability after 24 h in liquid nitrogen.

Villalobos et al. (1992) reported that dehydrated zygotic embryos of *Musa acuminata* and *M. balbisiana* were maintained in liquid nitrogen and successfully germinated after thawing at 40°C. Panis et al. (1992) reported that cryopreservation of cell suspension initiated from meristematic shoot tips of banana cv. Bluggoe (ABB) and wild BB diploid *M. balbisiana* was most efficient in presence of 7.5% DMSO. Regrowth was achieved after 100 days, when thawed cells, still surrounded by cryoprotectant.

Bhat et al. (1994) reported that air dried seeds of *M. balbisiana* with a moisture content of 13-18% were found to survive exposure to liquid nitrogen. After rapid thawing over 90% of the embryos germinated into seedlings. Panis et al. (1996) suggested that in banana embryogenic cell suspension cultures, extra cellular ice-initiation (-7.5°C) during slow freezing prevents excessive super cooling and enhances post thaw regrowth capacity. Panis et al. (1996) reported that preculturing of banana meristem for 2-4 weeks in MS medium, enriched with 0.3 to 0.5 M sucrose and then excised clumps were transferred to cryotubes and plunged directly into liquid nitrogen for storage. Panis (1996) described that embryogenic *Musa* cell suspension can be stored in liquid nitrogen, after slow freezing in the presence of DMSO. The process involves a precultivation period on media containing high concentration of sucrose followed by rapid freezing. Regeneration frequencies varied from 7.4 to 68.9%.

Pollen of 4 cultivars of lemon was stored in liquid nitrogen for 3.5 years. After 1 year, the germination rate of stored pollen was similar to that of fresh pollen (Ganeshan and Alexander, 1991). Nuclear cells of navel orange successfully cryopreserved for 40 days by vitrification. Nuclear embryos of sweet orange subjected to slow cooling at 0.5°C/min down to -42°C followed by immersion in liquid nitrogen survived. The highest survival rate (91%) was obtained with highly concentrated vitrification solution PVS2 (Sakai et al., 1991; Marin and Duran, 1988; 1994; Marin et al., 1993; Perez et al., 1997). Engelmann et al., (1994) observed for embryogenic callus of willow leaf and Chio mandarin, Cleopatra mandarin, Shamouti hamlin orange and Mexican lime were cryopreserved, increased DMSO concentration (10-15%) improved growth recovery after freezing.

Somatic embryos of korean native species (*C. maxima*, *C. grandis* x *C. junos*, *C. platymamma* x *C. junos*) were given pretreatment with MS medium containing 10% DMSO and 1.0 M sucrose. The most effective vitrification solution was 10% glycerol, 10% ethylene glycol and 5% DMSO in MS medium containing 1.0 M sucrose, and for preserving somatic embryo through gradual step freezing method (Oh et al., 1997). Normah et al. (1997) reported *C. aurantifolia* seeds can be successfully cryopreserved after desiccating them to moisture content of 12.93% while seeds *C. halimij* exhibited only 25% viability after cryopreservation at a moisture content of 9.5%.

Sterile tillers of *Vitis rupestris* were grown on a medium composed of 0.5-liter knop's solution, 0.5-ml Berthelot's micronutrients, thiamin, pyridoxine, nicotinic acid, Ca-pathothenate, inositol, biotin and 0.04-M sucrose. The survival after 24 days were 13% at 2°C, 46% at 7°C, 100% at 9°C and after 42 days 100% at 9°C. At the highest temperature the tiller could be stored upto 300 days without loss of viability and elongation of the tillers (Glazy, 1969). Ganeshan (1985) suggested that the technique of cryopreservation is useful for gene banks. He successfully cryopreserved pollen of 5 grape cultivars in liquid nitrogen. Moriguchi et al. (1988) reported that callus culture of *V. vinifera* x *V. labrusca* hybrid kyoho were stored successfully at 10°C for upto 360 days. *V. vinifera* cv. Koshusajaku

callus survived storage at both 10 and 13°C for 360 days when silicone was added to the medium.

Finkle *et al.* (1979) and Ulrich *et al.* (1979) have investigated the possibility of freeze conservation of a tropical palm tree, date palm (*Phoenix dactylifera* L.). Tisserat *et al.* (1985) reported that cryopreserved pollen of date palm or Deglet Noor dusted on freshly opened spathes of 10 years old Deglet Noor. Fruit yield and developments were similar in both frozen and non-frozen pollen. Bagniol *et al.* (1992) suggested that for cryopreservation of date palm, gradients may be exhibited both for outflow of water and the penetration of the cryoprotectants. MyCock *et al.* (1997) reported late globular/early tarpedo stage date palm (*Phoenix dactylifera*) embryos can continue normal growth and development after cryopreservation provided they are pretreated with a cryoprotectant mixture of glycerol and sucrose and then dried to water contents in the range of 0.4 - 0.7 g/g. Mater (1987) reported callus of date palm was treated with a cryoprotective mixture of PEG, glucose and DMSO and frozen to -25°C for 4 months. Freezing did not affect the potential of the callus for embryogenesis although growth during the first 2 months of culture was inhibited.

Yakuwa and Oka (1988) reported that either prefreezing of intact vegetative bud of mulberry at -10°C or 20°C followed by rapid thawing at 37°C or prefreezing at -20°C or 30°C followed by slow thawing at 0°C gave high percentage of survival. Embryonic axes of longer seeds with moisture content of 18% survived after 24 h in liquid nitrogen (Fu *et al.*, 1993). Fukai *et al.* (1994) reported shoot tips of Parsimmon (*Diospyros kaki*) preconditioned on medium containing 15 g sucrose/liter and stored at 10°C. Shoot explants survived for 30 weeks at 10°C.

## REFERENCES

- Bagniol, S., Engelmann, F. and Michaux, F.N. 1992. Histo-cytological study of apices from *in vitro* plantlets of date palm (*Phoenix dactylifera* L.) during a cryopreservation process. *Cryoletters*. 13 (6): 405-412.
- Benson, E.E., Lynch, P.T. and Stacey, G.N. 1998. Advances in plant cryopreservation technology: current application in crop plant biotechnology. *Ag-Biotechnology News and Information*. 10(5): 133-141.
- Bhat, S.R., Bhat, K.H. and Chandel, K.P.S. 1994. Studies on germination and cryopreservation of *Musa balbisiana* seed. *Seed Science and Technology*. 22(3): 637-640.
- Engelmann, F., Dambier, D. and Ollitrault, P. 1994. Cryopreservation of cell suspensions and embryogenic calluses of citrus using a simplified freezing process. *Cryoletters*. 15(1): 53-58.
- Finkle, B.J., Ulrich, J.M., Rains, D.W., Tisserat, B.B. and Schaefer, G.W. 1979. Survival of alfalfa, *Medicago sativa*, rice *Oryza sativa* and date palm *Phoenix dactylifera*, callus after liquid nitrogen freezing. *Cryobiology*. 16: 583.
- Fu, J.R., Xia, Q.H. and Tang, L.F. 1993. Effects of desiccation on excised embryonic axes of three recalcitrant seeds and studies on cryopreservation. *Seed Science and Technology*. 21(1): 85-95.
- Fukui, H., Ohba, H. and Nakamura, M. 1994. Low temperature storage of *in vitro* shoots of Japanese persimmon (*Diospyros kaki*). *Proc. International Plant Propagators Society*. 44 : 245-248.
- Ganeshan, S. 1985. Cryogenic preservation of Irape (*Vitis vinifera* L.) pollen. *Vitis* 24(3) : 169-173.
- Ganeshan, S. and Alexander, M.P. 1991. Cryogenic preservation of lemon (*Citrus limon* Burm.) pollen. *Gartenbauwissenschaft*. 56(5) : 228-230.
- Glazy, R. 1969. Recherches sur la croissance de *Vitis rupestris* scheele sain et court noue cultive *in vitro* a differentes temperatures. *Ann. Phytopathol.* 1 : 49-166.
- Marin, M.L. and Duran, V.N. 1988. Survival of somatic embryos and recovery of plants of sweet orange (*Citrus sinensis* (L.) Osb.) after immersion in liquid nitrogen. *Plant Cell, Tissue and Organ Culture*. 14(1) : 51-57.
- Marin, M.L. and Duran, Villa N. 1994. Cryopreservation of somatic embryos of Washington Navel Sweet Orange. 7th International Citrus Congress, Acireale, Italy. 313-317.
- Marin, M.L., Gogorcena, Y., Ortiz, J. and Duran, V.N. 1993. Recovery of whole plants of sweet orange from somatic embryos subjected to freezing thawing treatments. *Plant Cell Tissue and Organ Culture*. 34(1) : 27-33.
- Mater, A.A. 1987. Production and cryogenic freezing of date palm germplasm and regeneration of plantlets from frozen material. *Iraqi Journal of Agricultural Sciences 'ZANCO'* 5 (supplement) : 35-49.
- Moriguchi, T., Kozaki, I., Matsuta, N. and Yamaki, S. 1988. Plant regeneration from grape callus stored under a combination of low temperature and silicone treatment. *Plant Cell, Tissue and Organ Culture*. 15(1) : 67-71.
- MyCock, D.J., Berjak, P., Pammenter, N.W., Vertucci, C.W., Ellis, R.H., Black, M., Murdoch, A.J. and Hong, T.D. 1997. Cryopreservation of somatic embryos of *Phoenix dactylifera*. *Current Plant Science and Biotechnology in Agriculture* No. 30.
- Normah, M.N., Siti Dewi Serimala, M.N., Ellis, R.H., Black, M., Murdoch, A.J. and Hong, T.D. 1997. Cryopreservation of seeds and embryonic axes of several citrus species. *Current Science and Biotechnology in Agriculture* No.30.
- Oh, Sung Do, Oh, S.D., Altman, A. and Ziv, M. 1997. The effect of prefreezing treatment and cryoprotectants on the survival of cryopreserved somatic embryos and plant regeneration in Korean native citrus species. *Acta Horticulturae*. No. 447 : 499-505.
- Panis, B. 1996. Cryopreservation of banana (*Musa* spp.) germplasm. *Bull. Des Seances Academic Royale des*

Sciences d. outre Mer. 42(3) : 521-535.

- Panis, B., Dhed-a-D., Swennen, R., Adams, R.P. and Adams, J.E. 1992. Freeze-preservation of embryogenic *Musa* suspension cultures. Conservation of plant genes : DNA banking and *in vitro* biotechnology. 183-195.
- Panis, B., Totte, N. Nimmen, K. Van, Withers, L.A., Swennen, R. and Van, N.K. 1996. Cryopreservation of banana (*Musa* spp.) meristem cultures after preculture on sucrose. Plant Science Limerick. 121(1) : 95-106.
- Perez, R.M., Navarro, L. and Duran, V.N. 1997. Cryopreservation and storage of embryogenic callus cultures of several citrus species and cultivars. Plant Cell Reports. 17(1) : 44-49.
- Sakai, A., Kobayashi, S. and Oiyama, I. 1991. Survival by vitrification of nucellar cells of navel orange (*Citrus sinensis* var. *brasiliensis* Tanaka) cooled to -196°C. Journal of Plant Physiology. 137(4) : 465-470.
- Tisserat, B., Gabr, M.F. and Sabour, M.T. 1985. Viability of cryogenically treated date palm pollen. Date Palm Journal. 4(1) : 25-31.
- Ulrich, J.M., Finkle, B.J., Moore, P.H. and Ginoza, H. 1979. Effect of a mixture of cryoprotectants in attaining liquid nitrogen survival of cells. Fiziol. Rast. 15 : 749-756.
- Villalobos, V.M., Abbelnour, A., Adams, R.P. and Adams, J.E. 1992. Cryopreservation of *Musa* spp. and its potential for long-term storage of other tropical crops. Conservation of plant genes : DNA banking and *in vitro* biotechnology. 197-210.
- Wang, XiaoFeng; Fu, JiaRui; Wang, X.F. and Fu, J.R. 1994. Desiccation and cryopreservation of excised embryonic axes of mango seeds. Journal of South China Agricultural Univ. 15(3) : 88-92.
- Yakuwa, H. and Oka, S. 1988. Plant regeneration through meristem culture from vegetative buds of mulberry (*Morus bombycis* Koidz.) stored in liquid nitrogen. Annals of Botany. 62(1) : 79-82.

**Summary of completed/ongoing projects, funded by IERP, GBPIHED**