International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2017; 5(5): 560-564

Received: 27-07-2017
Accepted: 28-08-2017

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Cryopreservation of Plant Materials - A Review

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Abstract
Establishment of new plant varieties is getting important due to tremendous increase in population and changes in the human food behaviors. This leads towards the demand of new techniques and varieties in plant productions. Therefore some of the wild species and some other important plants species are in the extinction condition. Germplasm conservation of some of the important species is essential. Cryopreservation is a technique, which is used to preserve the reservoirs of genetic materials of plants by ultra low temperature. During the cryopreservation all biochemical activities significantly reduced and biological deterioration are stopped. The preserved materials can be used after many years. This review details the cryopreservation of plant genetic materials.

Keywords: Cryopreservation, plant genetic resources, low temperature, long term storage

Introduction
Biodiversity provides a source of compounds to the medical, food and crop protection industries (Panis and Lambardi, 2005) [21]. In agriculture germplasm are living genetic resources such as seeds or tissues that are maintained for the purpose of breeding. Plant Genetic Resources are highly important for food security and agro-biodiversity because they can be used to breed new or more productive crops that are resistant to biological and environmental stresses (Kaviani, 2011) [13]. Conservation of plant genetic resources ensures maintenance of agro biodiversity. Germplasm provides a raw material for plant breeders to develop various crops. For breeding program germplasm conservation is crucial one. Besides, many plant species are now in danger of becoming extinct (Panis and Lambardi, 2005) [21]. Their preservation is requisite for plant breeding programs.

Preservation only in field collections is risky, as valuable germplasm can be lost (genetic erosion) because of pests, diseases and adverse weather conditions. Moreover, the maintenance of clonal orchards is labour - intensive and expensive. Advances in biotechnology provide new methods for plant genetic resources and evaluation (Paunesca, 2009) [22]. Especially in the area of *in vitro* culture techniques and molecular biology provide some important tools for improved conservation and management of plant genetic resources (Ramanatha Rao and Riley, 1994; Withers, 1995) [25, 37].

Cryopreservation is a viable freezing of biological material and their subsequent storage at ultra low temperature at -196° C, therefore plant material can be stored for unlimited years. Cryopreservation or freeze-preservation at ultra-low temperature (-196 °C) is a sound alternative for the long-term conservation of plant genetic resources, since under these conditions, biochemical and most physical processes are completely arrested. As such, plant material can be stored for unlimited periods (Panis and Lambardi, 2005) [21]. Cryopreservation techniques are now used for plant germplasm storage at many institutes around the world (Niino, 2006) [18].

Principle of cryopreservation
Cryopreservation, developed during the last 25 years, is an important and the most valuable method for long-term conservation of biological materials. The main advantages in cryopreservation are simplicity and the applicability to a wide range of genotypes (Engelmann, 2004) [5]. Cryopreservation technique is based on the removal of all freezable water from tissues by physical or osmotic dehydration, followed by ultra-rapid freezing.

Cryopreservation includes classical and new techniques. Classical cryopreservation techniques have been developed in the 70-80s. They comprise a cryoprotective treatment followed by slow freezing (Kartha, 1985) [11]. The most common cryoprotective substances are

dimethylsulfoxide (DMSO), polyethylene glycol (PEG), sucrose, sorbitol and mannitol. These substances have the osmotic actions; however some of them such as DMSO can enter to cells and protect cellular integrity during cryopreservation (Rajasekharan, 2006) [24]. Classical cryopreservation methods are mainly used for freezing undifferentiated cultures such as cell suspensions and calluses (Kartha and Englemann, 1994) [12]. For freezing of differentiated tissues and organs such as seed, embryonic axes, shoot tips and zygotic and somatic embryos, new techniques include encapsulation-dehydration (ED), vitrification, encapsulation-vitrification, desiccation, pre-growth, pregrowth-desiccation and droplet freezing have been developed (Englemann, 1997) [4].

Cryopreservation implies the conservation of plant propagules at very low temperatures (below –150 ºC), thus ensuring that all the biological, biochemical and physiological activities are stopped due to lower temperature where therefore plant material can be stored for unlimited years. In this way, subcultures are not required and the threat of somaclonal variation is reduced. Liquid nitrogen is most widely used material for cryopreservation. Because liquid nitrogen is chemically inert, relatively low cost, non-toxic, non-flammable, readily available. Dry ice can also be used. Liquid nitrogen (–196 ºC) is usually used as refrigerant, although freezers with working temperatures of –150ºC are now available. At this temperature, cell division, metabolic, and biochemical activities remain suspended and the material can be stored without changes and deterioration for long time Walters *et al*. (2009) [36]. Cryopreserved material requires a limited space. However, power or liquid nitrogen supply must be guaranteed (Gonzalez-Benito *et al*., 2004) [8].

The biological effects of cooling are dominated by freezing of water that causes freezing injury. In cryopreservation, cryoprotectants help to replace the some of water to avoid ice crystals formation when frozen. That must be low toxicity and able to penetrate into cell. The most common cryoprotective substances are dimethylsulfoxide (DMSO), polyethylene glycol (PEG), sucrose, sorbitol and mannitol. These substances have the osmotic actions; however some of them such as DMSO can enter to cells and protect cellular integrity during cryopreservation (Rajasekharan, 2006) [24].

Most popular cryogenic procedures are vitrification (Sakai *et al*., 1990) [6, 7] and encapsulation/dehydration (Fabre and Dereuddre, 1990) [6, 7]. The difference between the two methods is the method of dehydration. In vitrification, cells are dehydrated by treatment in a highly concentrated solution such as PVS2 (Plant Vitrification Solution) solution (Sakai *et al*., 1990) [30]. In encapsulation/dehydration, cells are dehydrated by air-drying; then, dehydrated cells can be vitrified by rapid cooling (immersion into LN) and conserved safely for a long time.

For successful cryopreservation, it is essential to avoid the lethal intracellular freezing that occurs during rapid cooling in Liquid nitrogen (Sakai and Yoshida, 1967; Sakai, 1995) [29, 28]. The most damaging event during cryopreservation is the irreversible injury caused by the formation of intracellular ice crystals. (Dumet and Benson, 2000) [3]. The intracellular ice formations can avoided by Vitrification (production of a glossy state that is behaves like a solid due to increased viscosity without any crystallization). One of the best ways to prevent ice crystal formation at Liquid nitrogen without damage to membrane and an extreme reduction in cellular water is vitrification (Panis and Lambardi, 2005) [21].

In general, when a tissue is subjected to low temperatures, ice crystals will eventually form. These crystals may disrupt the cell membrane leading to the death of the cell. The goal of cryopreservation is to replace some of the water with other compounds that will not form large crystals when frozen. The most commonly used replacements are DMSO (dimethyl sulfoxide) and glycerol. These are mixed into a solution with media or serum in which cells are suspended and placed in a liquid nitrogen freezer. As the media begins to freeze, the salt concentration outside the cells will become greater than that in the cells and water will leave the cells to be replaced by the cryopreservative.

Cryopreservation media generally consists of a base medium, protein source, and a cryopreservative. The cryopreservative both protects the cells from mechanical and physical stress and reduces the water content within the cells, thus minimizing the formation of cell-lysing ice crystals. The protein source, often fetal bovine serum (FBS), also protects the cells from the stress associated with the freeze-thaw process.

Cryopreservation process

For successful cryopreservation, many factors such as source-plant status, starting materials, personnel, culture conditions, pretreatment conditions, cryopreservation methods, cryogenic facilities, regimes and post-thawing are involved (Reinhoud *et al*., 2000; Reed *et al*., 2004) [27, 26]. Cryopreservation involves storage of plant material (such as seed, shoot tip, zygotic and somatic embryos and pollen) at ultra-low temperatures in Liquid nitrogen (-196 °C) or its vapor phase (-150 °C). To avoid the genetic alterations that may occur in long tissue cultures storage, cryopreservation has been developed (Martin *et al*., 1998) [15].

For preservation plant tissue must be selected from the healthy plants. Small, young, rich in cytoplasm, meristematic cells survive better than the larger, highly vacuolated cells. Pre culture or pre growth involves pre culturing the germplasm on a medium supplemented with cryoprotectants such as sucrose or glucose before exposure to Liquid nitrogen. For pre culture Plant genetic resources were packed in cryotube or straw, and cryoprotectants were added. Packed specimens were gradually cooled from -20 ºC to -100 ºC using a programmable freezer or ethanol baths. Partial tissue dehydration can be achieved by the application of osmotically active compound to enhance plant stress tolerance. After making specimens freeze to a predetermined freezing-temperature, they are immersed in LN. The frozen cell/tissues are kept for storage at temperature ranging from – 70 to -196° C. Long term storage are best done at - 196° C (Daisuke Kami., 2012) [2].

For the recovery of germplasm after cryostorage, rapid rewarming is usually required to avoid recrystallisation (Towill, 1991) [34]. Cryopreserved tubes are warmed using hot water (40 ºC) for 1- 2 min. Frozen tips of the sample in tubes or ampoules are plunged into the warm water with a vigorous swirling action just to the point of ice disappearance. It is important for the survival of the tissue that the tubes should not be left in the warm water bath after ice melts. Just at point of thawing quickly transfer the tubes to a water bath maintained at room temperature and continue the swirling action for 15 sec, to cool, the warm walls of the tube. After that cryoprotectants are removed from a tube. After rewarming, samples are moved from the cryotube, and recultured. Determination of survival / viability done by using 2,3,5-Triphenyltetrazolium chloride (TTC) staining or Evans

blue staining or Fluoresciendiacetate [FDA] staining. Cryopreserved tissue should be genetically identical to non-treated phenotype and can directly produce normal plants (Dumet and Benson, 2000) [3]. A large number of reports showing no evidence of morphological, cytological, biochemical, or molecular alterations in plants from storage at -196 °C (Harding, 2004) [9].

Cryopreservation methods of plant genetic resources

Vitrification

The vitrification method has been the major cryopreservation method since Uragami *et al*. (1989) [35] developed it using asparagus culture cells. Plant tissues are added to the tube containing the loading solution (LS) for the osmoprotection. Beads in tubes are osmoprotected for about 30 min at room temperature (about 25 ºC). LS are the liquid culture medium in which sucrose (0.4 mol/L) and the glycerol (2.0 mol/L) were contained. After loading, LS is removed from a tube, and new vitrification solution is added for the dehydration of plant tissues. Many cryoprotectants are dissolved in the vitrification solution, and the optimal dehydration time using the solution changes greatly with treatment temperature. In many cases, the dehydration using the vitrification solution is performed at 0 ºC by the reason of the toxicity to plant cells. Plant Vitrification Solution 2 (Sakai *et al*., 1990) [30] is utilized most as the vitrification solution. They are immersed in LN after that. Cryopreserved tubes are warmed using hot water (40 ºC) for 1- 2 min and the vitrification solution is removed from a tube. After the removal of vitrification solution, unloading solution (the liquid medium supplemented with 1.2 mol/L sucrose) is added to a tube, and cryoprotectants are removed from plant tissues for 30 min at 25 ºC. In many cases, the above-mentioned liquid mediums (LS, PVS and unloading solution) were adjusted by pH 5.7-5.8, but without plant growth regulators. After unloading, samples are removed from the cryotube, and recultured.

Encapsulation-dehydration

The encapsulation-dehydration method was first reported by Fabre and Dereuddre (1990) [6, 7] using shoot apices of potato, and spread worldwide the same way as vitrification and encapsulation-vitrification. This method excels that of dehydration in that regrowth of plant germplasm after cryopreservation is markedly increased by encapsulating plant samples with alginate beads. In addition, encapsulated samples are difficult to be crushed with tweezers compared with the dehydration method.

Plant tissues are immersed in a calcium-free liquid medium supplemented with 0.4 mol/L sucrose and 30.0 g/L sodium alginate. The mixture (including a plant cell or tissue) was added drop by drop to the liquid medium containing 0.1 mol/L calcium chloride, forming beads about 5 mm in diameter. The above-mentioned liquid mediums (30.0 g/L sodium alginate and 0.1 mol/L calcium chloride) were adjusted by pH 5.7-5.8, but without plant growth regulators. Encapsulated germplasms are added to the culture bottle containing loading solution (LS) for the osmoprotection. Beads in the bottles are osmoprotected for 16 hrs at room temperature (25 ºC). LS is the liquid culture medium in which sucrose (0.75-0.8 mol/L) is contained. After loading, LS is removed from the bottle. Loaded samples are put on sterilized filter papers, and samples are dehydrated by silica gel for 3-7 hours before immersion in LN. After dehydration by silica gel, encapsulated samples are moved to a cryotube, and immersed in LN. Cryopreserved tubes are warmed using hot water (40 ºC) for 1 - 2 min. After rewarming, samples are moved from the cryotube, and recultured. In encapsulation dehydration, the addition of glycerol besides sucrose in LS reportedly enhances the regrowth percentage of cryopreserved samples. The optimal concentration of glycerol in LS is 0.5-2.0 mol/L for regrowth of cryopreserved specimens (Matsumoto & Sakai, 1995; Kami *et al*., 2008) [16, 17, 10, 16].

Encapsulation-vitrification

The encapsulation-vitrification method was reported first by Matsumoto *et al*. (1995) [16, 17] using shoot apices of *Wasabia japonica*, and then spread all over the world. The advantage of this method is that regrowth of plant germplasm after cryopreservation is markedly increased by encapsulating plant samples with alginate beads. The encapsulation of plant germplasm makes for less damage to samples during vitrification procedures (loading treatment and dehydration treatment).

Plant tissues are immersed in the calcium-free liquid medium supplemented with 0.4 mol/L sucrose, 30.0 g/L sodium alginate and glycerol (1.0-2.0 mol/L). The mixture (including a plant cell or tissue) was added drop by drop to the liquid medium containing 0.1 mol/L calcium chloride, forming beads about 5 mm in diameter. The above-mentioned liquid mediums (30.0 g/L sodium alginate and 0.1 mol/L calcium chloride) were adjusted by pH 5.7, but without plant growth regulators. Encapsulated specimens are added to the culture bottle containing LS for osmoprotection. Beads in the bottles are osmoprotected for 16 hours at room temperature (25 ºC). LS are the liquid culture medium in which sucrose (0.75-0.8 mol/L) and the glycerol (2.0 mol/L) were contained. After loading, LS is removed from a bottle, and PVS is added newly for the dehydration of plant tissues. The same as with vitrification, the dehydration using PVS is performed at 0 ºC in light of the toxicity to plant cells. After dehydration of PVS, encapsulated samples are moved to a cryotube containing fresh PVS, and immersed in LN. Cryopreserved tubes are warmed using hot water (40 ºC) for 1- 2 min and the vitrification solution is removed from the tube. After removal of the solution, unloading solution (supplemented with 1.2 mol/L sucrose; pH 5.7) is added to a tube, and cryoprotectants are removed from plant tissues for 30 min at 25 ºC. After unloading, samples are moved from the cryotube, and recultured.

Droplet method

The droplet method was first reported by Schafer-Menuhr *et al*. (1997) [31] using potato apices. The operating procedure is the same for vitrification. However, the LS immersion protocol differs compared with that in the vitrification method. After treatments by LS and PVS, plant samples are put on aluminum foil which is sterilized and cut small. One drop of PVS is dripped onto plant samples, and the whole aluminum foil is immersed in LN. The aluminum foil after cryopreservation is taken out from LN, and one drop of unloading solution supplemented with 1.0 mol/L sucrose is dipped onto to freezing samples. After rewarming, samples are moved from the cryotube, and recultured. In the droplet method, in order to make a plant sample cool quickly, In addition, the droplet method can reportedly obtain a high regrowth percentage after cryopreservation in tropical plants difficult to cryopreserved (Pennycooke and Towill, 2001; Leunufna and Keller, 2003; Panis *et al*., 2005) [23, 14, 20, 21].

Materials can be preserved

All parts of plants may be conserved by cryopreservation. Plant materials such as pollen, seeds, embryo, cells in plant tissue culture, buds, meristematic tissues and twigs can be preserved by using this method. Suspension or callus cultures, dormant buds, apical meristem, embryonic axes, seeds, somatic embryos and pollen also can stored in LN (Bell and Reed, 2002) [1].

Ex: numerous accessions of citrus, jack, almond, litchi, tea. Embryos of Conifer and P*inus*, shoot tips of *Malus*, *Pyrus*, *Prunus*, *Vitis vinifera.* Meristematic tissues of vegetatively propagated fruit trees, embryogenic callus, shoot tips, ovules, seeds and pollen of citrus **(**Gonzalez-Benito, M. E. *et al*., 2004) [8].

Different types of plant cell, tissues and organs can be cryopreserved, including cell suspensions, pollen, embryogenic cultures, somatic and zygotic embryos, shoot apices or meristems (Kaviani, 2011) [13]. For vegetatively propagated species, the most widely used organs are shoot apices excised from *in vitro* plant. In some woody species, cryopreservation of dormant buds has also been developed, and recovery was achieved by grafting (Towill and Forsline, 1999) [33]. Cryopreservation of *in vitro* cultured apices has certain advantages, among them that cryoprotective treatments can be applied to the shoots or apices while cultured *in vitro*. Besides, *in vitro* culture can be used for clonal propagation of the starting plant material and for virus-free plant production. Plant germplasm distribution in the form of *in vitro* cultures is usually less voluminous and improves health status. Furthermore, somaclonal variation is less probable to occur when recovery of plants is carried out directly from apices compared to other methods, for example by direct or indirect organogenesis (Scowcroft, 1984) [32]. In several cases, embryogenic cell lines have shown to be highly stable, although that stability may differ among species and could decrease with time in culture.

Merits and Demerits

Conventional methods of storage or preservation of germplasm has some limitations, they are expensive and time consuming. Preservation by only field collection may be the chance for loss of germplasm due to pest, disease and adverse weather condition. In conventional method a cell suspension culture is needed to be subculture every 7 to 10 days and callus cultures every 14 to 30 days. Furthermore, there is risk of possible loss through contamination or equipment failure. But in cryopreservation there is no need of any subculture and can maintain without any contamination.

It is pathogen free condition where large number of plants can store in little space, protection against natural disorders and preservation of rare genomes can attain and possible chance for development of cold acclimatization and frost resistance plants but Maintenance cost is high, required skilled person to perform, Plant may show genetic instability and lack of protocol for wider range of cells preservation and Cell/tissue may get damaged (Niino. T and Arizaga. M. V., 2015) [19].

It is important to have many choices of protocol for cryopreservation, because there are many types of plant propagules and plant species to be cryopreserved. The first thing that should be done is to determine how to make materials for cryopreservation, which are not only healthy and vigorous shoots, but also at a uniform stage and size. Secondly, it is necessary to determine whether the specimens are sensitive to some chemicals such as plant viteification solution or excess dehydration. Thirdly, the unloading step and regeneration medium should be reexamined. Current new protocols apply rapid cooling and warming by direct immersion in LN and unloading solution (Niino. T and Arizaga. M. V., 2015) [19].

Conclusion

Conservation and subsequent use of genetic resources are essential to meet the demand of future food security and also to conserve biodiversity. For this, Cryopreservation should be considered as a backup of plant germplasm. Cryopreservation technique is an effective approach for storage of plant cells, tissues, seeds and embryos. This can be a perfect and effective method for long term preservation of wide range of cells. It is a preservation method that enables plant genetic resources to be conserved safely, and cost-effectively. But, development of simple, reliable and cost effective method is essential.

Reference

1. Bell RL, Reed BM. *In vitro* tissue culture of pear: advances in techniques for micropropagation and germplasm preservation. Proceeding of the 8th IS on pear, Acta Hort., 2002, 596, ISHS.
2. Daisuke Kami. Cryopreservation of Plant Genetic Resources, Current Frontiers in Cryobiology, Prof. Igor Katkov (Ed.), 2012, ISBN: 978-953-51-0191-8.
3. Dumet D, Benson EE. The use of physical and biochemical studies to elucidate and reduced cryopreservation-induced damage in hydrated/desiccated plant germplasm. In: Engelmann F, Takagi H (eds) Cryopreservation of tropical plant germplasm, Italy, IPGRI, 2000.
4. Engelmann F. *In vitro* conservation methods. In: Ford-Lloyd BV, Newbury HJ, Callow JA (eds) Biotechnology and plant genetic resources: conservation and use. CABI, UK, 1997, 119-162.
5. Engelmann F. Plant cryopreservation: Progress and prospects. *In vitro* Cell Dev Biol. Plant. 2004; 40:427-433.
6. Fabre J, Dereuddre J. Encapsulation Dehydration – A new approach to cryopreservation of *Solanum* shoot-tips. Cryo-Letters. 1990; 11(5):413-426, ISSN 0143-2044.
7. Febre J, Dereuddre J. Encapsulation-dehydration: A new approach to cryopreservation of *solanum* shoot-tips, CryoLett. 1990; 11:413-426.
8. Gonzalez-Benito ME, Clavero-Ramírez I, López - Aranda JM. Review. The use of cryopreservation for germplasm conservation of vegetatively propagated crops. Spanish Journal of Agricultural Research. 2004; 2(3):341-351.
9. Harding K. Genetic integrity of cryopreserved plant cells: A review. CryoLetters. 2004; 25:3-22.
10. Kami D, Uenohata M, Suzuki T, Oosawa K. Cryopreservation of black chokeberry *in vitro* shoot apices. *Cryo-Letters.* 2008; 29(3):209-216. ISSN 0143-2044.
11. Kartha KK. Cryopreservation of plant cells and organs, CRC Press, Inc., Boca Raton, Florida, 1985.
12. Kartha KK, Engelmann F. Cryopreservation and germplasm storage. In: Vasil, I.K. and Thorpe, T.A. (eds) Plant Cell Tiss Cult Springer, 1994, 195-230.
13. Kaviani B. Conservation of plant genetic resources by cryopreservation. Australian J. Crop Sci. 2011; 5:778-800.

14. Leunufna S, Keller ERJ. Investigating a new cryopreservation protocol for yams (*Discorea* spp.). Plant Cell Reports. 2003; 21(12):1159-1166, ISSN 0721-7714.
15. Martin C, Iridono JM, Benito-Gonzales E, Perez C. The use of tissue culture techniques in the conservation of plant biodiversity. Agro-Food-Ind Hi-Tech. 1998; 9(1):37-40.
16. Daisuke Kami. Matsumoto T, Sakai A. An approach to enhance dehydration tolerance of alginate-coated dried meristems cooled to -196oC. *Cryo-Letters*, 1995; 16(3):299-306. ISSN 0143-2044.
17. Matsumoto T, Sakai A, Takahashi C, Yamada K. Cryopreservation of *in vitro*grown apical meristems of wasabi (*Wasabia japonica*) by encapsulation-vitrification method. Cryo-Letters, 1995; 16(2):189-196, ISSN 0143-2044.
18. Niino T. Developments in plant genetic resources cryopreservation technologies. In proceedings of the APEC workshop on effective gene bank management in APEC member economies. Suwon, Korea, 2006, 197-217.
19. Niino T, Arizaga MV. Review on Cryopreservation for preservation of potato genetic resources. Breeding Science. 2015; 65:41-52.
20. Panis B, Piette B, Swennen R. Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all Musaceae. Plant Science. 2005; 168(1):45-55, ISSN 0168-9452.
21. Panis B, Lambardi M. Status of cryopreservation technologies in plants (crops and forest trees). In: The role of biotechnology. Villa Gualino, 5-7 March, Turin, Italy 2005, 43-54.
22. Paunesca A. Biotechnology for endangered plant conservation: A critical overview. Romanian Biotech Letters. 2009; 14(1):4095-4104.
23. Pennycooke JC, Towill LE. Medium alternations improve regrowth of sweet potato (*Ipomoea batatas* {L.} Lam.) shoot tips cryopreserved by vitrification and encapsulation–dehydration. Cryo-Letters. 2001; 22(4):381-389, ISSN 0143-2044.
24. Rajasekharan PE. Seed cryoservation: problems and prospects. Paper presented at the ICAR Short Course on *In vitro* Conservation and Cryopreservation-New Options to Conserve Horticultural Genetic Resources, Banglore, India, 2006.
25. Ramanatha Rao V, Riley R. The use of biotechnology for conservation and utilization of plant genetic resources. Plant Genet Resour News. 1994; 97:3-20.
26. Reed BM, Kovalchuk I, Kushnarenko S, Meier-Dinkel A, Schoenweiss K, Pluta S *et al*. Evaluation of critical points in technology transfer of cryopreservation protocols to international plant conservation laboratories. Cryo Letters. 2004; 25:341-352.
27. Reinhoud PJ, Iren FV, Kijne JW. Cryopreservation of undifferentiated plant cells. In: Englemann F, Takagi H (eds) Cryopreservation of tropical plant germplasm, Italy, IPGRI, 2000.
28. Sakai A. Cryopreservation of germplasm of woody plants, in Biotechnology in Agriculture and Forestry 32, Cryopreservation of Plant Germplasm I, (Bajaj YPS, ed), Springer-Verlag, Berlin, Heidelberg, 1995, 53-69.
29. Sakai A, Yoshida S. Survival of plant tissue at super low temperature VI. Effects of cooling and rewarming rates on survival. Plant Physiol. 1967; 42:1695-1701.
30. Sakai A, Kobayashi S, Oiyama I. Cryopreservatioin of nucellar cells of navel orange (*Citrus sinensis Osb*. var *Brasiliensis* Tanaka) by vitrification. Plant Cell Reports, 1990; 9(1):30-33, ISSN 0721-7714.
31. Schafer-Menuhr A, Schumacher HM, Mix-Wagner G. Long-term storage of old potato varieties by cryopreservation of shoot-tips in liquid nitrogen. Plant Genetic Resources Newsletter, (October), 1997; 111(1):19-24. ISSN 1020- 3362.
32. Scowcroft WR. Genetic variability in tissue culture: impact on germplasm conservation and utilization. International Board for Plant Genetic Resources Secretariat, Rome, 1984, 42.
33. Towill LE, Forsline PL. Cryopreservation of sour cherry (*Prunus cerasus* L.) using a dormant vegetative bud method. Cryo Letters., 1999; 20:215-222.
34. Towill LE. Cryopreservation. In: Dodds JH (ed) *In vitro* methods for conservation of plant genetic resources, Chapman and Hall, London, 1991, 41-70.
35. Uragami A, Sakai A, Nagai M, Takahashi T. Survival of cultured cells and somatic embryos of *Asparagus officinalis* cryopreserved by vitrification. Plant Cell Reports, 1989; 8(5):418-421, ISSN 0721-7714.
36. Walters C, Volk GM, Towill LE, Ph L, Forsline. Survival of cryogenically-stored dormant apple buds: a 20 year assessment. Paper presented at the 1st International Symposium on Cryopreservation in Horticultural Species, Leuven, Belgium, 2009.
37. Withers LA. Collecting *in vitro* for genetic resources conservation. In: Guarino L, Ramanatha Rao V, Reid R (eds) Collecting plant germplasm diversity, Technical Guideline, CABI, Wallingford, UK, 1995, 51-526.