Plant Omics Journal POJ

POJ 3(6)177-182 (2010) ISSN:1836-3644

Cryopreservation by encapsulation-dehydration for long-term storage of some important germplasm: seed of lily [*Lilium ledebourii* (Baker) Bioss.], embryonic axe of persian lilac (*Melia azedarach* L.), and tea (*Camellia sinensis* L.)

Behzad Kaviani

Department of Horticultural Science, Faculty of Agricultural Science, Islamic Azad University, Rasht Branch, Rasht, Iran

Corresponding author: b.kaviani@yahoo.com

Abstract

Cryopreservation of germplasm in liquid nitrogen (LN) is a perfect method for the long-term conservation of plant genetic resources. Lily [*Lilium ledebourii* (Baker) Bioss.], Persian lilac (*Melia azedarach* L.), and tea (*Camellia sinensis* L.) were evaluated for long-term storage in liquid nitrogen (LN) at -196ºC. Encapsulation within alginate beads was shown to be beneficial in all studied species. Embryonic axes of *M. azedarach* L. and *C. sinensis* L. and seeds of *L. ledebourii* (Baker) Bioss. were encapsulated in MS medium supplemented with 3% Na-alginate, 100 mM $CaCl_2$ and 0.75 M sucrose and desiccated for 1 h under the laminar air flow. Embryonic axes and seeds were plunged into LN and held for at least 1 h. Following cryopreservation, embryonic axes and seeds were cultured on solid Murashige and Skoog (MS) medium. In *M. azedarach* L., survival rate after freezing was zero, for non-pretreated and non-encapsulated embryonic axes, also nil for pretreated and non-encapsulated embryonic axes, and 42% for pretreated and encapsulated embryonic axes. In *C. sinensis* L., non-encapsulated embryonic axes, even those pretreated with sucrose and dehydration, did not survive at all. However, pretreated and encapsulated embryonic axes may offer a better resistance after exposure to LN. In *L. ledebourii* (Baker) Bioss., control seeds did not survive after LN treatment. The rate of viability in non-encapsulated seeds pretreated with sucrose and dehydration was 75%. The rate of viability in pretreated, encapsulated seeds was 50%. In general, application of sucrose, dehydration and encapsulation can be suitable to increase tolerance of tissues to very low temperature (-196ºC) and be used as improved technique of cryopreservation in an extent number of species.

Keywords: Cryopreservation, encapsulation-dehydration, sucrose, *Melia azedarach* L., *Camellia sinensis* L., *Lilium ledebourii* (Baker) Bioss.
Abbreviations: MS_Murashige and Skoog; LN_Liquid nitrogen.

Introduction

More than one third of plant species are believed to be rare, endangered, and threatened by extinction. Their preservation is essential for classical and modern plant breeding and genetic engineering programs. Moreover, biodiversity of some plants provides a source of compounds to the pharmaceutical, food and crop protection industries. Preservation of plant material in field conditions (*in situ*) is difficult and risky sometimes, as valuable germplasm can be lost because of pests, diseases, human activities, climatic disaster and other biotic and abiotic hazards (genetic erosion). *In vitro* conservation of germplasm is one of the best methods for long-term storage of valuable genetic resources of many crops and forest species. Some reliable cryogenic procedures have been developed and the number of cryopreserved species has enormously been increased (Sakai, 1997; Panis and Lambardi, 2005). Cryopreservation is considered an ideal means for the long-term preservation of plant germplasm and is now successfully applied to a great number of plant species originating from tropical to temperate regions (Panis and Lambardi, 2005). Cryopreservation is considered as the only available method for safe long-term storage of plant genetic resources. These techniques have been used to conserve seeds of several species for long periods in germplasm banks. For the long-term conservation of plant germplasm without genetic alterations, cryopreservation at ultra-low temperature (-196ºC) is a perfect method and a valuable technique. It also provides the option for long-term backup of active collections that might be at risk. Recently, it was proven that cryotherapy can be successfully applied to eradicate viruses from some plants (Helliot et al., 2002; Wang et al., 2003; Uosukainen et al., 2007). Cryopreservation techniques include controlled freezing, vitrification, encapsulation-dehydration, dormant bud presser-vation, and combinations of these protocols are used in hundreds of species (Panis and Lambardi, 2005). In the encapsulation-dehydration method, explants are encapsulated in alginate beads (which can contain also mineral salts and organics), thus forming synthetic seeds or artificial seeds. Then, the artificial seeds are treated with a high sucrose concentration, dried down to a moisture content of 20-30% (under airflow or using silica gel) and subsequently rapidly frozen in LN. The

encapsulation-dehydration method has been successfully applied to a wide range of materials and the method has significantly improved the success of this strategy for cryopreservation (Grout et al., 2008). By this method, encapsulated pistachio embryos were shown to be able to undergo storage at low temperature (Onay et al., 1996). Encapsulated shoot tips of several tropical species have even been conserved at 20°C and 25°C (Maruyama et al., 1997) proving that encapsulation is a useful method for germplasm conservation. Since, cryopreservation induces a high stress to the plant tissues, the use of cryoprotectants such as penetrating and non-penetrating substances is necessary. Among these cryoprotectants, sucrose which increases the tolerance to dehydration and therefore contributes to maintaining the tissues' viability (Dumet et al., 1993) is greatly preferred. In the case of cryopreservation, and complementary to these cryoprotectants, encapsulation within alginate beads before dehydration was shown to be beneficial to the technique by increasing considerably the resistance of plant tissues to desiccation and freezing (Dereuddre et al., 1990; Bernard et al., 2002). Cryoprotectants increase the stability of the plasma membrane to withstand various stresses imposed by freezing and accelerate the recovery process after thawing (Uemura et al., 2009). Cryopreservation of biological tissues can be successful only if intra-cellular ice crystal formation is avoided. Crystal formation can be prevented through vitrification (Sakai, 2000). The cell cytosol can be concentrated through air drying, freeze dehydration, application of penetrating or non-penetrating substances (cryoprotectants), or hardening. For a solution to be vitrified at high cooling rates, a reduction in water content to at least 20-30% is required. Sugars play a very important role in the acquisition of resistance to desiccation and to freezing in LN (Suzuki et al., 2005 and 2006). Generally, tissues which have low water content, such as meristems, embryonic axes and seeds are more resistant to the stress of these techniques (Janeiro et al., 1996). All these tissues have greater genetic stability compared to the more disorganized callus and suspension systems. *Melia azedarach* L., *Camellia sinensis* L. and *Lilium ledebourii* (Baker) Bioss. are economically important. Wood of *M. azedarach* L. is desirable and usually used at industry, especially in making woody equipment. Different parts of this plant are used in medicinal and pharmaceutical. *C. sinensis* L. is an important species, especially its leaves are used in medicinal and pharmaceutical, also as a drink everywhere around the world. *L. ledebourii* (Baker) Bioss., the rarest lily, is extremely attractive and carefully under surveillance. It has excellent ornamental value, especially as a pot plant. In the present study, we examined a technique for cryopreservation of embryonic axes of *M. azedarach* L. and *C. sinensis* L. and seeds of *L. ledebourii* (Baker) Bioss. using a combination of encapsulation and dehydration. Tissues were encapsulated in alginate beads and treated with high levels of sucrose prior to dehydration and freezing.

Materials & methods

Plant materials

Embryonic axes of *M. azedarach* L. were isolated from the seeds of mature fruits collected from Mazandaran province in the northern part of Iran. Seeds of *C. sinensis* L. were obtained from ripe capsules and embryonic axes were isolated from the

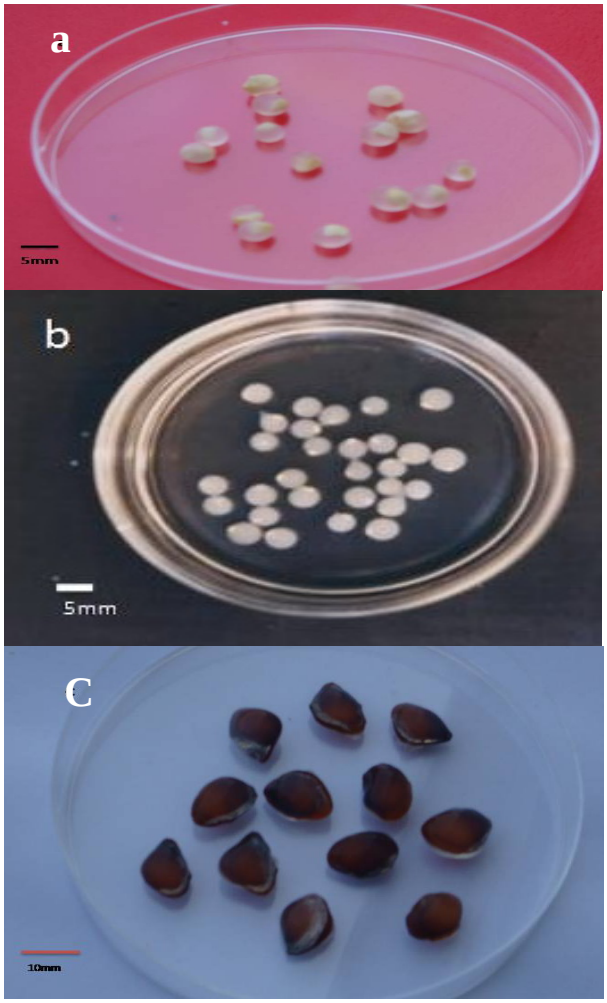

Fig 1. (a) Encapsulated embryonic axes (beads) of *Melia azedarach* L. (b) encapsulated embryonic axes (beads) of *Camellia sinensis* L. (c) encapsulated seeds (beads) of *Lilium ledebourii* (Baker) Bioss. Bead (capsule) is an artificial seed which is made by integration of Na-alginate and CaCl2. Encapsulation is a pretreatment that protect the germplasm against freezing.

seeds. These ripe fruits collected from Lahidjan, Guilan province in the north of Iran. Seeds of *L. ledebourii* (Baker) Bioss. were collected from Damash area of Guilan province in the northern part of Iran.

Disinfection procedures

Seeds of studied species were rinsed under tap water for 20 min. Seeds of *M. azedarach* L. were disinfected in 70% ethanol (v/v) for 1 min and 1% sodium hypochlorite (w/v) for 15 min and then rinsed three times in sterile water. Seeds of *C. sinensis* L. with no seed coat were surface sterilized in 96% ethanol (v/v) for 1 min, followed by 10% H_2O_2 for 10 min and 15% sodium hypochlorite (w/v) for 15 min and then rinsed three times with sterile water. Embyonic axes of *M. azedarach* L. and *C. sinensis* L. were excised from the cotyledons and placed in culture medium for experiments. Seeds of *L. ledebourii* (Baker)

Table 1. Effect of pretreatments of sucrose, dehydration and encapsulation on the viability of embryonic axes of *Melia azedarach* L. and *Camellia sinensis* L. and seeds of *Lilium ledebourii* (Baker) Bioss.

Treatment / Plant species	Viability %		
	Control	Pretreated with sucrose and dehydration	Pretreated with sucrose, dehydration and encapsulation
Melia azedarach L.	0	0	42
Camellia sinensis L.	0	0	100
Lilium ledebourii (Baker) Bioss.	0	75	50

Bioss. were disinfected in 70% ethanol (v/v) for 1 min followed by 0.5% sodium hypochlorite (v/v) for 10 min and rinsed three times with sterile water.

Culture media

Excised embryonic axes of *M. azedarach* L. and *C. sinensis* L. and seeds of *L. ledebourii* (Baker) Bioss. were placed in the culture medium for experiments. All experiments were done in MS (Murashige and Skoog, 1962) medium (basal salt mixture and vitamins) supplemented with 3% sucrose and 0.7% Agar-agar. Also, in *M. azedarach* L. medium, 2 mg l^{-1} glutamine and in *C. sinensis* L. medium, 200 mg l^{-1} caseine hydrolysate and 2 mg l^{-1} glycinin were added.

Encapsulation and osmoprotection procedure

Embryonic axes of *M. azedarach* L. and *C. sinensis* L. and seeds of *L. ledebourii* (Baker) Bioss. were individually suspended in liquid MS medium supplemented with 3% Na-alginate (w/v) and 0.75 M sucrose for 1 h with slow agitation. Then, embryonic axes and seeds was individually dispensed with a sterile forceps into liquid MS medium containing 100 mM CaCl2 and 0.75 M sucrose for 1 h with slow agitation (Fig. 1). In the case of controls with non-encapsulated embryonic axes and seeds, these were suspended in MS liquid medium and 0.75 M sucrose for 1 h.

Dehydration procedure

Encapsulated and non-encapsulated embryonic axes of *M. azedarach* L. and *C. sinensis* L. and seeds of *L. ledebourii* (Baker) Bioss. were dehydrated by transferring them to 9-cm empty open petri dishes and exposing them to the sterile airflow of a laminar flow cabinet at 25°C for 1 h.

Determination of the moisture content

To determine the moisture content, 20 encapsulated and non-encapsulated embryonic axes of *M. azedarach* L. and *C. sinensis* L. and 20 encapsulated and non-encapsulated seeds of *L. ledebourii* (Baker) Bioss. were maintained in MS liquid medium with 0.75 M sucrose for 1 h with slow agitation. Fresh weight was taken at each time period. After that they were dried under laminar air flow for 1 h. The dehydrated embryonic axes and seeds were weighted and dried in an oven at 110°C for 20 h. Moisture content was determined by subtraction of dry weight after 20 h at 110°C and expressed as a percentage of their initial fresh weight.

Cryopreservation and thawing procedure

For cryopreservation, dehydrated embryonic axes of *M. azedarach* L. and *C. sinensis* L. and dehydrated seeds of *L. ledebourii* (Baker) Bioss. were transferred to sterile 2 ml polypropylene cryovials (5 per cryovials) and immersed directly into LN and held for at least 1 h. For control a series of non-pretreated and non-encapsulated embryonic axes and seeds were plunged in LN for 1 h immediately after disinfection. Cryovials containing frozen embryonic axes and seeds were rewarmed rapidly in a water-bath at 37-38°C for 3 min. Embryonic axes and seeds were then removed from the cryovials and transferred to solid basal MS medium (0.8% Agar-agar) with 3% sucrose in 9-cm plastic petri dishes where they rehydrated and grew.

Recovery and viability assessment

Cryopreserved embryonic axes of *M. azedarach* L. and *C. sinensis* L. and cryopreserved seeds of *L. ledebourii* (Baker) Bioss. were cultured on solid basal MS medium (0.8% Agar-agar) with 3% sucrose in 9-cm plastic petri dishes. All treated and untreated (control) cultures were incubated in a growth chamber set for 16-h photoperiod, a light flux of 30 $\mu molm^{-2}s^{-1}$ and day/night temperatures of 20/25°C. After growth under standard conditions the percentage of survival in embryonic axes and seeds were recorded.

Statistical analysis

In every experiment, approximately 12 embryonic axes of *M. azedarach* L. and *C. sinensis* L. and 12 seeds of *L. ledebourii* (Baker) Bioss. were treated for each of three replicates. Data were subjected to analysis of variance (ANOVA) and significant differences between treatments were determined by Duncan's Multiple Range Test (DNMRT) using the statistical package for social science (SPSS ver. 14).

Results and discussion

Non-pretreated, non-encapsulated embryonic axes of *M. azedarach* L., *C. sinensis* L. and non-pretreated, non-encapsulated seeds of *L. ledebourii* (Baker) Bioss., control, did not survive after exposure to LN (Table 1). The same results were reported in many plants (Bernard et al., 2002; Reed et al., 2006). Contrary to this result, embryonic axes of *C. sinensis* withstood after freezing in LN without any pretreatment (Janeiro et al., 1996). The studies of Benelli et al. (2008) on *Pupulus* spp. germplasm demonstrated that all seeds of *P.trem-*

Table 2. Analysis of variance (ANOVA) for the effect of different pretreatments (sucrose, dehydration, and encapsulation) on viability percent of *M. azedarach* L., *C. sinensis*, and *L. ledebourii* (Baker) Bioss. after exposure to LN[a].

Source of variations		Df[b]	Sum of squares	Means of squares	F value	Prob.
M. azedarach L.	Between groups	2	3698.000	1849.000	75.986**	.000
	Within groups	6	146.000	24.333		
	Total	8	3844.000			
C. sinensis L.	Between groups	2	18688.889	9344.444	841.000**	.000
	Within groups	6	66.667	11.111		
	Total	8	18755.556			
L. ledebourii (Baker) Bioss.	Between groups	2	7505.556	3752.778	135.100**	.000
	Within groups	6	166.667	27.778		
	Total	8	7672.222			

a**: Significant at the 0.01 levels, b: Degrees of freedom

uloides maintained 100% viability after direct immersion in LN without any pretreatment. In this study, the percentage of germination in pretreated, non-encapsulated embryonic axes of *M. azedarach* L., and *C. sinensis* L. was nil. But the percentage of germination in seeds of *L. ledebourii* (Baker) Bioss., pretreated with sucrose and dehydration was 75% (Table 1). Blakesley et al. (1995) showed that the pretreatment of embryonic tissues of *Ipomoea batatas* with high levels of sucrose alone resulted in up to 28.6% survival. After dehydration the maximum survival demonstrated was 9.1%. The study of Suzuki et al. (2005 and 2006) on *Gentiana scabra* germplasm have revealed that pre-culturing with sucrose and desiccation induce high dehydration tolerance, the method has been found to be effective for cryopreservation. In most cases, cryopreservation of plant germplasm especially for tissues containing much water such as buds needs to remove excessive water content before the freezing (Bilavcik et al., 2008). Long-term pre-culture on medium containing high sucrose concentration showed a significant influence on the initial cultures response. Pre-culture with a high concentration of sucrose greatly increases the intracellular concentration which will act as the principal agent of tolerance to desiccation (Suzuki et al., 2006). Thus, concentration and time duration of sucrose and type and time duration of dehydration are varied in different species. Current study showed in *M. azedarach* L., the percentage of germination after exposure to LN was 42% for pretreated and encapsulated embryonic axes. In *C. sinensis* L., all pretreated and encapsulated embryonic axes withstood against LN. The percentage of germination in seeds of *L. ledebourii* (Baker) Bioss., pretreated with sucrose, dehydration and encapsulation was 50% (Table 1).

A 40% minimum regrowth standard was established as acceptable for most plant germplasms (Dussert et al., 2003). The studies of Caboni et al. (2008) on *Pyrus* spp. using encapsulation-dehydration showed the recovery of 60% of shoot tips after LN immersion and consisting in dehydration of Na-alginate beads for 2 days in 0.75 M sucrose and desiccation to 20% moisture content (fresh weight basis). The encapsulation-dehydration technique has been used for the cryopreservation of shoot tips from 9 different genera of hardwood species, among which *Malus*, *Pyrus* and *Prunus*. It is noteworthy that, for 50% of the species cryopreserved following an encapsulation-dehydration procedure, a shoot tip survival of 80% or more has been reported (Lambardi and de Carlo, 2003). Cryopreservation of coconut plumules by encapsulation-dehydration procedure allows successful (20-40%) regrowth level into leafy shoots, and the potential to regenerate a whole plant (approximately 90% success rate) free of diseases (Malaurie et al., 2009). As for seed cryopreservation, dehydration-freezing procedures have been successfully applied to intact seeds or embryonic axes of several woody species (Marzalina and Krishnapillary, 1999). The studies of Janeiro et al. (1996) on cryopreservation of embryonic axes and somatic embryos of *Camellia japonica* L. showed that none of the protective pretreatments applied to these experiments (desiccation, chemical protectors, hardening by culture at low temperatures and encapsulation in alginate beads) allowed cryopreservation of the somatic embryos (Janeiro et al., 1996). In contrary, Nukari et al. (2008) proposed that in cryopreservation of somatic embryos, encapsulation is especially useful. Totally, these studies showed being successful of the encapsulation-dehydration technique and pretreatment of sucrose for survival of germplasm in embryonic axes of *M. azedarach* L. and *C. sinensis* L. and seeds of *L. ledebourii* (Baker) Bioss. after exposure to LN. Data analysis for the effect of treatments on viability percent of *M. azedarach* L. ($F = 75.986$; $df = 2, 6$; $p<0.01$), *C. sinensis* L. ($F = 841.000$; $df = 2, 6$; $p<0.01$), and *L. ledebourii* (Baker) Bioss. ($F = 135.100$; $df = 2, 6$; $p<0.01$) showed that the differences between the survival rates of control and pretreated seeds were significant (Table 2). Similar results were obtained with a few plants such as *M. azedarach* L. (Bernard et al., 2002), *Cynodon* spp. (Reed et al., 2006) and *Camellia japonica* L. (Janeiro et al., 1996).

Current study demonstrated that the moisture content of *M. azedarach* L., and *C. sinensis* L. embryonic axes and *L. ledebourii* (Baker) Bioss. seeds after desiccation for 1 h and before exposure to LN was 15-20%. The study on cryopreservation of *M. azedarach* L. embryonic axes was revealed that the optimum moisture content was 16-18% (Bernard et al., 2002). It appears the optimum moisture content for germplasms of the more plants before exposure to LN is normally about 20% (Reed et al., 2006; Blakesley et al., 1995; Dumet et al., 2000). Reduction of water content to a critical level seems to be a necessary step for successful cryopreservation by encapsulation-dehydration because the cells must have no freezable water. Dehydration must be long enough to ensure sufficient cell dehydration, without cytotoxic effects. Studies show that alginate beads dried to 20% moisture vitrify on exposure to LN and form stable glasses that do not

form ice crystals on rewarming (Dumet et al., 2000). Thus, a prerequisite for successful application of encapsulation-dehydration technique is the avoidance of irreversible cell membrane damage caused by the formation of intracellular ice crystals. Ice crystallization can only be prevented through a reduction of the cellular water content, dehydration, to the strict minimum (Vertommeu et al., 2008). Membrane proteins likely play an important role in the acquisition of dehydration tolerance. In this study, embryonic axes of *M. azedarach* L. and *C. sinensis* L. and seeds of *L. ledebourii* (Baker) Bioss. were kept in MS liquid medium with 0.75 M sucrose for 2 h then dehydrated in sterile airflow of a laminar flow cabinet at 25°C for 1 h. Moisture contents of alginate-encapsulated embryonic axes of *M. azedarach* L. and *C. sinensis* L. and seeds of *L. ledebourii* (Baker) Bioss. were about 15-20%. Successful cryopreservation of embryonic axes of tea (*C. sinensis* L.) has been achieved using desiccation under sterile air flow as the only pretreatment (Chaudhury et al., 1991). Dumet et al. (1993) have pointed out that the meristem tissues can be dehydrated without major alterations and the better is its resistance to freezing in LN. Reed et al. (2006) showed that the encapsulation and dehydration cryopreservation protocol was most effective when combined with a 1 to 4 weeks cold acclimation period and dehydration to 19 to 23% moisture before exposure to LN. Many researchers have used a combination of encapsulation, high sucrose pretreatment and dehydration before exposure of gemplasm to LN (Blakesley et al., 1995; Dereuddre et al., 1990; Reed et al., 2006; Bernard et al., 2002).

Conclusion

For successful cryopreservation, it is necessary to induce high levels of dehydration tolerance in the plant cells, tissues and organs for preservation. It has been shown that in embryonic axes of *M. azedarach* L. and *C. sinensis* L. and seeds of *L. ledebourii* (Baker) Bioss., dehydration tolerance is efficiently increased by pre-culturing with sucrose. Also, encapsulation-dehydration improved the surviving of these plants after exposure to LN. Cryopreservation is now a viable long-term storage technique for the most plants germplasm. The encapsulation-dehydration technique has been successfully applied to a wide range of materials.

References

Benelli C, Capuana M, de Carlo A, Tsvetkov I (2008) Application of cryopreservation to the long-term storage of popular and aspen (*Populus* spp.) germplasm. 2nd meeting of working groups. Cryopreservation of crop science in Europe, Cryoplanet-cost action 871, Oulu, Finland, 20-23 February, pp. 47-48

Bernard F, Shaker-Bazarnov H, Kaviani B (2002) Effect of salicylic acid on cold preservation and cryopreservation of encapsulated embryonic axes of Persian lilac (*Melia azedarach* L.). Euphytica 123: 85-88

Bilavcik A, Faltus M, Zamecnik J, Casal RA, Jandurova OM (2008) Dehydration of grapevine dormant buds in relation to cryopreservation. 2nd meeting of working groups. Cryopreservation of crop science in Europe, Cryoplanet-cost action 871, Oulu, Finland, 20-23 February, pp. 32-33

Blakesley D, Al-Mazrooei S, Henshaw GG (1995) Cryopreservation of embryonic tissue of sweet potato (Ipomoea batatas): use of sucrose and dehydration for cryopreservation. Plant Cell Reports 15: 259-263

Caboni E, Condello E, Meneghini M, Palombi MA, Frattarelli A, Damiano C (2008) Progresses in cryopreservation of *Pyrus* spp. and evalution of genetic stability of the recovered shoots. 2nd meeting of working groups. Cryopreservation of crop science in Europe, Cryoplanet-cost action 871, Oulu, Finland, 20-23 February, pp. 36

Chaudhury R, Radhamani J, Chandel KPS (1991) Cryopreservation of embryonic axes of *Camellia sinensis* L. CryoLetters 12: 31-36

Dereuddre J, Scottez C, Arnaud Y, Duron M (1990) Resistance of alginate-coated axillary shoot tips of pear tree *(Pyrus communis* L., Beurre Hardy) *in vitro* plantlets to dehydration and subsequent freezing in liquid nitrogen: effect of previous cold hardening. CR Academic Sciences Paris III 310: 317-323

Dumet D, Block W, Worland R, Reed B, Benson E (2000) Profiling cryopreservation protocols for Ribes ciliatumm using differential scanning calorimetry. CryoLetters 14: 243-250

Dumet D, Engelmann F, Chabrillange N, Duval Y, Dereuddre J (1993) Importance of sucrose for the acquisition of tolerance to dessication and cryopreservation of oil palm somatic embryos. CryoLetters 14: 243-250

Dussert S, Engelmann F, Noirot M (2003) Development of probabilistic tools to assist in the establishment and management of cryopreserved plant germplasm collections. CryoLetters 24: 149-160

Grout BWW, Green JE, Benson EE, Harding K, Johnston JW (2008) Cryopreservation of encapsulated *Ribes* meristems and variation between accessions within a cultivar. 2nd meeting of working groups, Cryopreservation of crop science in Europe, Cryoplanet-cost action 871, Oulu, Finland, 20-23 February, pp 51

Helliot B, Panis B, Poumay Y, Swennen R, Lepoivre P, Frison E (2002) Cryopreservation for the elimination of cucumber mosaic and banana streak viruses from banana (Musa spp.). Plant Cell Reports 20: 1117-1122

Janeiro L, Vieitez AM, Ballester A (1996) Cryopreservation of somatic embryos and embryonic axes of Camellia japonica L. Plant Cell Reports 15: 699-703

Lambardi M, De Carlo A (2003) Application of tissue culture to the germplasm conservation of temperate broad-leaf trees. In: Jain S M, Ishii K, ed, Micropropagation of Woody Trees and Fruit. Kluwer AC Pub, Dordrecht, pp. 815-840

Malaurie B, Nan O, Bandupriya HDD, Borges M, Verdeil JL, Tregear J (2009) Cryopreservation as a tool for the anagement of coconut germplasm. 1st international symposium on cryopreservation in horticultural species, Leuven, Belgium, 6-8 April, pp. 103

Maruyama E, Kinoshita I, Ishii K, Ohba K (1997) Germplasm conservation of the tropical forest trees, Cedrela odorata L., Guazuma crinita Mart. and Jacaranda mimosaefolia D. Don., by shoot tip encapsulation in calcium-alginate and storage at 12-25°C. Plant Cell Reports 16: 393-396

Marzalina M, Krishnapillary B (1999) Recalcitrant seed biotechnology application to rain forest conservation. In: Benson E, ed, Plant Conservation Biotechnology. Taylor and Francis, London, pp. 265- 26

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15: 473-497

Nukari A, Wang Q, Uosukainen M, Laamaneu J, Rokka VM, Rantala S, Valkoneu J (2008) Evalution of encapsulation and droplet vitrification methods in gene preservation work. 2nd meeting of working groups. Cryopreservation of crop science in Europe, Cryoplanet-cost action 871, Oulu, Finland, 20-23 February, pp. 55-56

Onay A, Jeffree CE, Yeoman MM (1996) Plant regeneration from encapsulated embryoids and an embryogenic mass of pistachio, Pistacia vera L. Plant Cell Reports 15: 723-726

Panis B, Lambardi M (2005) Status of cryopreservation technologies in plants (crops and forest trees). In the Role of Biotechnology Villa Gualino, 5-7 March, 2005, Turin, Italy, pp. 43-54

Reed BM, Schumacher L, Wang N, Achino J, Barker RE (2006) Cryopreservation of bermudagrass germplasm by encapsulation dehydration. Crop Science 46: 6-11

Sakai A (1997) Potentially valuable cryogenic procedures for cryopreservation of cultured plant meristems. In: Razdan MK, ed, Conservation of Plant Genetic Resources In vitro. Cocking EC Science Publication, Inc, Enfield, pp. 53-66

Sakai A (2000) Development of cryopreservation techniques. In: Engelmann F, Takagi H, ed, Cryopreservation of Tropical Plant Germplasm. International of Plant Genetic Resources Institute, Rome, pp. 1-7

Suzuki M, Ishikawa M, Akihama T (2005) Cryopreservation of encapsulated gentian axillary buds following 2 step-preculture with sucrose and desiccation. Plant Cell, Tissue and Organ Culture 83: 115-121

Suzuki M, Ishikawa M, Okuda H, Noda K, Kishimoto T, Nakamura T, Ogiwara I, Shimura I, Akihama T (2006) Physiological changes in gentian axillary buds during two-step pre-culture with sucrose that conferred high levels of tolerance to desiccation and cryopreservation. Annals Botany 97: 1073-1081

Uemura M, Minami A, Kawamura Y (2009) Effect of low temperatures and cryoprotectants on plant plasma membranes. 1st international symposium on cryopreservation in horticultural species, Leuven, Belgium, 6-8 April, pp. 15

Uosukainen M, Laamanen J, Nukari A (2007) Cryopreservation in certified plant production. Advances in Horticultural Science 21: 258-260

Vertommeu A, Carpentier SC, Remmerie N, Witters E, Swennen R, Panis B (2008) Study of hydrophobic proteins and protein complexes involved in cryopreservation of banana (Musa spp.) meristems. 2nd meeting of working groups. Cryopreservation of crop science in Europe, Cryoplanet-cost action 871, Oulu, Finland, 20-23 February, pp. 32-33

Wang QC, Mawassi M, Li P, Gafny R, Sela I, Tanne E (2003) Elimination of grapevine virus A (GVA) by cryopreservation of in vitro-grown shoot tips of Vitis vinifera L. Plant Science 165: 321- 327