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Approaches for *in vitro* conservation of woody plants germplasm

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Abstract The conservation of plant genetic diversity aims at preserving as much as possible extant species by using innovative and complementary approaches to guarantee the effectiveness of the safeguarding strategies and to face present problems and future threats. The development and implementation of different *in vitro* conservation techniques have provided improvements for the international exchange of germplasm, for the storage of different *in vitro* culture forms and for products generated by biotechnology. These methods are also a valuable alternative to relieve the need of large lands extensions, where are traditionally kept reserve collections of trees belonging to many woody species. This chapter provides information of several study cases, describes some useful protocols, and aims at presenting a brief overview of currently available techniques for *in vitro* conservation to medium and long-term of woody plant germplasm.

Key words • Woody plant germplasm • Slow growth • Cryopreservation • *In vitro* culture • Biodiversity conservation • Liquid nitrogen

xx.x Introduction

Many woody species are seriously threatened in different parts of the world. They are highly affected by several natural and anthropogenic factors (Augusseau et al. 2006). Specifically, anthropogenic factors affect forests mostly through expansion of agricultural activity, settlement, deforestation, land fragmentation, and invasive species introduction (Mebrat and Gashaw 2013). The major causes identified of woody species loss are linked to rapid human population growth rates and poverty (FAO 2009). Through the years, many woody plants have supplied products (firewood, fruits, etc) that are very important for the subsistence of many localities and populations, who have traditionally relied on them to satisfy their needs (Shackleton et al. 2001; Walter 2001). According to FAO, the highest rates of woody species loss are in the tropics (FAO 2009). There are some records that suggest the reduction of the forested areas at half in the last 100 years (UNDP 2007), and studies which have found that 10% of tree species are under serious threat (Williams 1998). Therefore, the increasing global need for food and fiber, and the increasing trend of risks extinction for these species, highlight the urgent need for developing and employing reliable conservation strategies to guarantee the safeguarding of woody plant germplasm.

In general, an important criterion to preserve plant genetic diversity is the implementation of different complementary strategies of conservation. In that way is possible to take advantage of the benefits from each method and mitigate the uncontrollable specific deficiencies. Furthermore, it is also essential ensure the genetic stability of the stored material, and the effectiveness of the protocol in terms of reproducibility, regardless of the genotype response and of the type of biological material used.

Among the conservation approaches, there are two well recognized categories: *in situ* and *ex situ* conservation. *In situ* conservation represents the use of natural habitats to maintain the gene pool of plants in ecosystems under different environmental changes (Swaminathan, 1997). This method can be considered dynamic because may provoke evolutionary modifications in plants by the effect of biotic and abiotic factors. Under these conditions is possible to follow the adaptation of plants which are permanently subjected to a natural, and sometimes also artificial selection process. In this sense, the *in situ* conservation which

covers biosphere reserves, national parks and other protected areas increases the amount of diversity that can be conserved, but faces several problematic situations that imply the need of developing alternative strategies to support this important type of conservation (Karp et al. 1997). The selection of the appropriate strategy should be based on several criteria including the biological nature of the species, and the feasibility of applying the chosen methods (Engelmann 2012). The second conservation category which refers to *ex situ* approach is the preservation of plant germplasm outside its natural habitat (Heywood and Iriondo 2003). This involves different methods, some of which are also classified as dynamic, like botanic gardens and field gene banks, because both provide the opportunity of monitoring the evolutionary trajectory of samples during the storage. Other *ex situ* methods are classified as static, because safeguards the genes outside of the evolutionary context (Shands 1991). They are considered safest, not influenced by the climate conditions, and most cost-effective than dynamics methods. That is the case of *ex situ* alternatives such as seed banks and the storage of plant germplasm in liquid nitrogen (-196°C).

The development of plant biotechnology, especially the advances associated to *in vitro* culture and its adaptation to more than several thousand of plant species (George 1996; Gonzalez-Arno et al. 2014) has provided powerful tools to support and improve management and conservation of plant biodiversity (Withers 1995; Bunn et al. 2007).

The *in vitro* culture techniques have successfully supported the micropropagation of many Australian native plants, including numerous rare woody plant species (Taji et al. 1997). At the same time, they have provided new conservation approaches, which contribute to widen the availability of options for storing plant germplasm, allowing the establishment of an additional backup for different types of materials, either derived of *in vivo* plants and/or from various *in vitro* biological sources. Moreover, the *in vitro* cultures make easier the international exchange of plant germplasm from both practical and phytosanitary point of view. For instance, the entrance of fruit trees is forbidden during the growing season according to European and South American quarantine regulations, by contrast, the entrance of *in vitro* cultures is allowed at any time of the year (Hummer 1999). The application of *in vitro* techniques also provided a new valuable tool for fruit crops with the practice of micrografting. This technology consists of the placement in aseptic conditions of a maintained scion onto an *in vitro* grown rootstock. *In vitro* shoot tip grafting has often been applied for the improvement and rejuvenation of several fruit tree species, virus elimination,

and development of different physiological studies. Micrografting technique is used in quarantine as this method has a minimum risk for importing plants. Plant material derived from the *in vitro* micrografting can be further multiplied *in vitro* and acclimatized *ex vitro* (Rehman and Gill 2015). More recently, micrografting was also used as an efficient way for recovering cryopreserved adult shoot-tips of citrus (Volk et al. 2012).

The application of *in vitro* culture techniques has made possible the production of a new kind of germplasm, which includes cell suspension, embryogenic callus and somatic embryos. These biotechnological products constitute a novel source of material with important genetic information useful for breeding programs (Rao 2004), and provide expanded options for preserving plant biodiversity, but they require the use of *in vitro* methodologies for its own conservation (Gonzalez-Arno et al., 2014). The operational use of tissue culture techniques ensures regeneration, multiplication and recovery of the biological material before and after being subjected to either conservation methods. Therefore, this is a key prerequisite to start a research work when using an *in vitro* approach.

The aim of this chapter is to review and provide information about the available methods for the *in vitro* conservation of plant germplasm. Several study cases are presenting to illustrate the development and application of these biotechnological approaches in woody plants.

xx.x *In vitro* conservation approaches

xx.x Medium-term conservation

There are two identified approaches for *in vitro* conservation of plant germplasm: short to medium-term and long-term storage. In the first case, conservation is achieved with the reduction of *in vitro* growth of tissues by altering the usual formulation of culture medium either diminishing the nutrients concentrations, as well as including the addition of osmotically active compounds, or of growth retardants (Engelmann, 2011). These modifications may be combined or not with the reduction of culture temperature (Paulet and Glaszmann, 1994), light intensity, storage in darkness, and/or the regulation of available oxygen level (Lynch, 1999). The storage at low temperature (in the range of 0°C to 5°C) is

one of the major alternatives used for preserving cold tolerant species of temperate plants (Orlikowska 1992; Ashmore 1997; Shibli et al. 2006).

For medium-term storage, standard *in vitro* culture conditions can also be used when dealing with species that have a natural slow growing habit. Other possible options may be desiccation and/or encapsulation of the explants (Engelmann, 2012). Artificial seeds, which are produced by encapsulating plant propagules (shoot buds or somatic embryos) in a synthetic matrix, enable medium-term conservation of various plant species (Devi et al. 1998).

In general, the *in vitro* methods used to attain medium-term conservation allow the storage of biological material from quite a few months to 2–3 years (Cruz-Cruz et al. 2013), and as it was previously reviewed, this approach, which is based on the slow growth, can be induced under sub-optimal culture conditions by altering the micropropagation procedure.

xx.x Long-term conservation

The next challenge using *in vitro* techniques was to increase the storage duration to guarantee the long-term conservation of plant germplasm. However, this aim could only be possible with the total arrest of cellular division and of all metabolic processes. The effect produced by an ultra-low temperature, usually due to the storage of samples in liquid nitrogen (-196°C), allowed achieving this inanimate state under a new biotechnological approach, that is, using cryopreservation.

Currently, cryopreservation represents the safest alternative for long-term preservation of plant biodiversity without requiring continuous manipulations. However, the main stages involved in the cryopreservation procedure, such as cryoprotection, cooling/warming and recovery culture, play a crucial role in attaining success, and each successive step must be optimized whatever the cryogenic protocol selected and depending on the biological material used (Gonzalez-Arnao et al. 2014).

Plant cryopreservation techniques have significantly evolved over the past 26 years with the development of different mixtures of cryoprotectants named as Plant Vitrification Solutions (PVS) since 1990, the increasing of the cooling/warming rates during process since 2005, and with the partial combination of different cryogenic methods, which consequently

resulted in the establishment of new alternative techniques (e.g. Encapsulation-vitrification, Droplet-vitrification and V- or D-Cryoplate).

All these contributions have improved the effectiveness and the adaptability of the protocols to a larger number of species, and they are closely related on the one hand, to the encapsulation of tissues in calcium alginate gel, which has facilitated the manipulation and improved the tolerance of explants to drastic dehydration treatments that otherwise would have been lethal (Gonzalez-Arno and Engelmann 2006), and on the other, by the use of aluminium foil strips (Panis et al. 2005) and/or of cryoplates (Yamamoto et al. 2011; 2012) instead of cryovials, for carrying out the direct immersion of samples to liquid nitrogen during cooling, and the rapid plunge into unloading solution during warming at room temperature. These innovations have increased the probability of forming a glassy state at the low temperatures, which potentially would avoid the injuries due to the formation of ice crystals when the intracellular water could not be removed sufficiently of samples prior to immersion in liquid nitrogen. At the same time, they also help to avoid the destabilization of the non-crystalline solid produced, because the warming is also performed very rapidly.

Several exhaustive reviews and publications have described in details the most important characteristics of each cryopreservation techniques. They have always pointed out that the first cryogenic protocols developed, known as classical or conventional, are based on a freeze-dehydration process which induces the dehydration by the reduction of temperature usually at $0.5-1^{\circ}\text{C min}^{-1}$ down to around -40°C (slow freezing regime), followed by rapid immersion in liquid nitrogen (Gonzalez-Arno et al. 2014). The next important group of cryopreservation methods comprises the vitrification-based techniques, which are supported on the transition of the liquid viscous phase to an amorphous glassy solid at the glass transition (T_g) temperature (Fahy et al. 1984). This process is induced by a severe dehydration that takes place at a non-freezing temperature, due to the exposure of samples to highly concentrated cryoprotective solutions (PVS) and/or to physical drying conditions followed by a rapid or ultra-rapid cooling in liquid nitrogen. Among the vitrification-based techniques are found: i) Vitrification method, which involves treatment of samples with cryoprotective substances (loading), dehydration with a highly concentrated plant vitrification solution (PVS), rapid cooling and rewarming, removal of cryoprotectants and recovery. This procedure has been developed for shoot-tips, cell suspensions and somatic embryos of numerous different species (Sakai and Engelmann 2007; Sakai et al. 2008). ii)

Encapsulation-Dehydration, which basic protocol comprises encapsulation in calcium alginate beads, preculture of alginate coated samples in liquid medium with high sucrose concentration, evaporative air or silica gel desiccation to a water content in the bead around 20 % (fresh weight basis -fwb-), and rapid cooling in liquid nitrogen. Rewarming of the alginate encapsulated explants usually is performed at room temperature and for recovery, the beads are usually placed onto standard culture medium without having to extract the shoots or embryos from their alginate coating (Engelmann et al. 2008; Sherlock et al. 2005).

iii) Encapsulation-vitrification, which is a combination of encapsulation-dehydration and vitrification procedures, because samples are encapsulated in alginate beads, and then treated and cooled according to the vitrification technique described above (Sakai and Engelmann 2007).

iv) Droplet-vitrification, a protocol derived from the combination of the vitrification procedure with the droplet-freezing technique developed by Kartha et al. (1982). In this case, samples are treated with loading and vitrification solutions, and then placed on an aluminium foil strips in minute droplets of vitrification solution or just in one small drop, and the aluminium foil strip is directly immersed with the samples in liquid nitrogen (Sakai and Engelmann 2007).

v) V-Cryo-plate, which combines the encapsulation-dehydration and droplet-vitrification techniques. In this method, shoot tips are attached with a thin calcium alginate layer to an aluminium cryo-plate, loaded, treated with PVS, and then cooled by direct immersion of cryo-plates in liquid nitrogen (Yamamoto et al. 2011, 2012).

vi) D-Cryoplate, which the only difference with V-Cryoplate is that replaces the use of the PVS solution, by the desiccation under the laminar flow or employing silica gel, to dehydrate samples attached to the cryoplate and subjected to the loading treatment. Both cryoplate alternatives provide higher cooling and warming rates compared to other vitrification-based procedures (Engelmann 2014).

vii) Pregrowth technique, which is the culture of samples in the presence of cryoprotectants, followed by rapid immersion in liquid nitrogen.

viii) Desiccation, which consists of dehydrating explants or seeds usually by desiccation in the air current of a laminar airflow cabinet or with silica gel and then, direct immersion in liquid nitrogen. This method is mainly used for cryopreserving seeds. Finally, Pregrowth-Desiccation is the combination of the both previously mentioned methods and it is mainly used for cryopreserving meristematic cultures, small size seeds, polyembryonic cultures, zygotic embryos or embryonic axes extracted from seeds, respectively (Gonzalez-Arno et al. 2008). The cryopreservation of embryonic axis is a simple and useful strategy to preserve the germplasm of plants with recalcitrant seeds, because axes can be easily dried before their

rapid cooling in liquid nitrogen. In addition, according to Li and Pritchard (2009), recent evidences of less than expected longevity at conventional seed bank temperatures, and the innovations in the cryopreservation of recalcitrant-seeded species, both are important factors which are demonstrating that ultra-cold storage should be adopted for the long-term conservation of plants (Li and Pritchard 2009).

There is not a specific or unique protocol to guarantee the successful cryopreservation of plant germplasm. However, different important research groups around the world have identified empirically, the best cryogenic approaches which can potentially result more effective depending on the target material (i.e. cells, callus, shoot-tips, somatic embryos, etc). In Table X are summarized the most recommended methods according to the *in vitro* culture form that is desired to cryopreserve (González-Arno and Engelmann 2013). Moreover, for the large-scale application of cryopreservation, sometimes it is requires the complementary use of different cryogenic techniques (e.g. classical and/or some vitrification-based procedures), in order to improve the response of genotypes, cultivars or species, which are less tolerant to a specific protocol, or maybe are only required some additional modifications in the stages of cryoprotection, to achieve reproducible survival following the same technique (Gonzalez-Arno et al. 2008).

xx.x Importance of *in vitro* conservation of woody plant germplasm

For woody plants, the most conventional conservation methods are *in situ* and field genebanks as *ex situ* alternative. *In situ* conservation is considered the method of choice for conserving forest species and wild relatives (Brush, 1995). As regards fruit and timber trees, the field clonal collections (for vegetatively propagated species) and seed banks (for seed propagated species with orthodox seeds) are the *ex situ* traditional approaches for germplasm preservation. However, field collections require vast areas of lands, their management is very expensive, and the system is exposed to the risks arising from different kind of biotic and abiotic stresses. In addition, many woody species produce non-orthodox seeds, i.e. recalcitrant, which cannot be preserved under seed banks conditions (Lambardi et al. 2005). Therefore, the *in vitro* conservation methods play a significant complementary role.

At present, there are more reports about the development and adaptation of cryopreservation protocols for *in vitro* storing woody plant germplasm, than for medium-

term storage. This probably due to that cryopreservation (-196°C), currently provides more alternatives (controlled rate cooling, vitrification, encapsulation-dehydration, cryo-plate techniques, desiccation, etc) to preserve more different types of materials (e.g. seeds, embryonic axis, zygotic embryos, dormant buds) and *in vitro* forms (e.g. cells, callus, somatic embryos) in comparison with slow growth approach, which is usually performed using shoots and/or in *in vitro* plantlets. In addition, with the long-term storage in liquid nitrogen is eliminated the problem associated to the danger of maintaining big spaces required to store *in vitro* a backup of abundant collections, with the potential risks of contamination during the storage, and the somaclonal variation for some species (Blakesley et al. 1996).

Among the alternatives employed to induce the slow growth for different woody plant species, Shibli et al. (2006) reported *in vitro* medium-term storage by using high concentrations of sucrose, sorbitol or mannitol in the culture medium. This allowed reduction in growth at room temperature of bitter almond microshoots and extended the subculture interval to four months. On the other hand, it is already known the benefits of lowering the culture temperature as a convenient strategy for medium-term storage, especially when dealing with species of temperate origin. Shoot cultures of chestnut, oak and wild cherry were stored after 10 d of the last subculture at 2°C for up to 1 year without subculturing (Janeiro et al. 1995). Shoot cultures of *Castanea sativa* (cv. 'Montemarano') were stored for over 48 months at 8°C, and the combination with a low level of lighting resulted in a positive effect on survival for such conservation period (Capuana and Di Lonardo 2013). In Table Y are presented some additional successful examples of *in vitro* storage for medium-term.

As regards cryopreservation techniques, there are a lot of advances related to woody plants, since the first demonstrations that winter-hardy twigs survived after immersion in liquid nitrogen for one year (Sakai, 1960). Related to fruit trees, pear and apple, both are interesting cases to mention. For these species have been successfully adapted and large-scale applied several cryogenic techniques such as classical protocols with controlled rate cooling (Chang and Reed 2000; Chang et al. 1992), encapsulation-dehydration (Niino and Sakai 1992) and vitrification (Niino et al. 1992). A significant and important fact about the impact of cryopreservation of apple germplasm is that after a loss due to diseases, over 100

unique apple accessions were re-grafted from cryopreserved dormant buds, and the cultivars were successfully restored for propagation (USDA, ARS, 2013).

Cryopreservation of Japanese persimmon shoot tips has been also reported using different techniques as slow cooling (Matsumoto et al. 2004), vitrification (Matsumoto et al. 2001; Niu et al. 2012) and droplet-vitrification (Niu et al. 2012). However, the utilization of cryopreservation for persimmon germplasm is still limited. More recently, Matsumoto et al. (2015) reported the use of D-Cryoplate protocol with 10 persimmon cultivars, which resulted in high regrowth rates after cryopreservation, and may facilitate the long-term conservation in the genebank context.

For non-fruit forest trees, the same three cryopreservation approaches have been applied to meristematic tissues of some important timber and pulp species such as silver birch using controlled rate cooling with *in vitro* meristems (Ryynänen 1996), encapsulation-dehydration with *in vitro* axillary buds of *Eucalyptus* species (Páques et al., 2002), and vitrification with *in vitro* apical segments and buds of an aspen hybrid (Jokipii et al., 2004). Embryogenic conifer cultures (Park et al., 1998), seeds or isolated embryonic axes of *Azadirachta indica* (Berjak and Durnet, 1996) have been also successfully cryopreserved. Another significant advance is that several cryopreservation protocols have been already standardized for seeds of many native woody (over 30) species of Brazil, by using a rapid cooling regimen and a slow thawing at room temperature (Santos et al., 2012; 2013).

In Table Z are presented some additional successful examples of using cryopreservation techniques for the long-term storage of woody plant germplasm.

Study cases

xx.x *In vitro* medium-term conservation of forest species.

xx.x.x Family Meliaceae

Meliaceae is a large family of tropical and subtropical woody species, comprised of 50 genera and about 575 species of trees and (rarely) shrubs (Pennington and Styles 1975; Mabberley et al. 1995; Pérez-Flores et al. 2012) occurring in a variety of habitats, from rain forests and mangrove swamps to semi desert (Pérez-Flores et al. 2012). Various species are commercially important, used for vegetable oil, soap-making, insecticides, and highly prized

wood. Some of the best-known examples in the international timber-trade are American or true mahogany (*Swietenia* spp.), African mahogany (*Khaya* spp.), sapele (*Entandrophragma* spp.), Spanish cedar or cigar-box cedar (*Cedrela* spp.), toon or Australian red cedar (*Toona* spp.), besides several others from various parts of the world (Styles 1972).

Most of the timber-producing species are huge, dominant or emergent trees forming the major constituents of tropical rain-forest, secondary forest or other types of woodland. Timbers from members of the Meliaceae are in fact the backbone of the forest industry of many countries, but continuous exploitation of the natural forest has seriously depleted stocks of desirable specimens, notably in South America and parts of West Africa. Usually it is the best trees which are culled during exploitation so that potential sources of élite or superior genotypes are continually decreasing with consequent depletion of the gene pool (Styles 1972). With regard that, almost 50 years ago Lamb (1968) pointed out that *Cedrela* in Latin America and the Caribbean Islands had been so overcut that some species had been virtually eliminated. Supplies of mature trees of good form for seed production and tree breeding have remained since then only in remote and inaccessible places. Similarly, in *Swietenia* only trees of a bush-like form have survived in the Caribbean Islands, and are represented by the famous Cuban mahogany, *S. mahagoni* (Styles 1972).

Consequently, *ex situ* preservation of germplasm from members of the Meliaceae is necessary to safeguard the threatened diversity of this family, mainly due to the anthropogenic impact. In recent years, staff of the INIFAP (Jalisco, Mexico) and the IBONE (Corrientes, Argentina) has conducted researches aimed to evaluate the possibility of *in vitro* medium-term preservation of germplasm for Meliaceae species by using minimal growth techniques.

1.3.1.1 *Swietenia macrophylla* King. (mahogany)

The genus *Swietenia* Jacq. contains three species (*S. mahagoni* Jacq., *S. macrophylla* King, and *S. humilis* Zucc.) and two natural hybrids (Pennington 1981), occurring natively in the Neotropics (Figueroa 1994). They are medium-sized to large trees growing to 20-50 m tall, and up to 2 m trunk diameter. The genus is famed as the supplier of mahogany, one of the most beautiful and valuable tropical timber species. At first mahogany was yielded by *S. mahagoni*, a Caribbean species, which was so extensively used locally and exported that its

trade ended by the 1950s. These days almost all mahogany is yielded by the mainland species, *S. macrophylla* although no longer from its native locations due to the restrictions set by CITES (this species has been listed under Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora, CITES 2003).

S. macrophylla, known as “caoba” in Spanish and “mahogany” or “bigleaf mahogany” in English, grows in Central America from Yucatan southwards and into South America, extending as far as Peru, Bolivia and extreme western Brazil (Lamb 1966; Biswas et al. 2002; Mishra et al. 2014). Mahogany trees may reach 50 m in height and 0.75 to 1.2 m in diameter (Gueilfus 1994; Standley 1946). The economic value of *S. macrophylla* timber has resulted in their overexploitation for the last centuries. However, mahogany had always been obtained from natural forests (Snook 1993) because one of the main problems for the establishment of organized planting programmes is that within its natural area of distribution in the Americas, *S. macrophylla* trees are intensely attacked by the mahogany shoot-borer, *Hypsipyla grandella* Zeller (Lamb 1966; Lyhr 1992; Newton et al. 1993).

The overexploitation of natural populations and logging of the best individuals for commercial use have affected the genetic diversity of *S. macrophylla*. Consequently, it is necessary to design strategies to promote preservation and regeneration systems for this species, both *in situ* and *ex situ*. Mahogany seeds are classified as recalcitrant based on their limited desiccation tolerance and short storage life (Gómez et al. 2006). In this case, the *in vitro* tissue culture techniques are presented as an important tool for the establishment of *ex situ* germplasm conservation programs. In the last years, the INIFAP (Jalisco, México) staff has conducted studies to regenerating plants from seeds and adventitious buds (taken from field collected twigs), as well as to storing apical meristems using minimal growth *in vitro* culture for short- and medium-term preservation of mahogany germplasm.

Regarding to the preservation process, shoot apical meristems were dissected from *in vitro* plants of *S. macrophylla* belonging to a working collection initiated through seeds and axillary buds from adult trees collected in natural mahogany stands, as mentioned by Martinez et al. (2013a). After dissection, apical meristems were cultured on the multiplication medium composed of MS + 1.5 mg l⁻¹ BA + 0.5 mg l⁻¹ ANA (control treatment) or MS lacking plant growth regulators. Cultures were kept at 24, 18 or 16 °C. After 30 and 60 d of storage at different temperatures, the number and length of developed shoots was evaluated. Data from this work demonstrated that culturing on MS lacking plant

growth regulators and incubating at 16°C enables maintenance of *S. macrophylla* meristems for 60 d (Martinez et al. 2013a). In a subsequent study, the effect of sucrose/mannitol concentrations and mineral oil on meristems growth was evaluated. After dissection, shoot apical meristems were cultured on the multiplication medium (control treatment) or MS supplemented with different sucrose/mannitol concentrations (30/0, 20/10, 15/15, 10/20 and 0/30 g l⁻¹). A replica of each treatment was also carried out for adding 2 ml of mineral oil (J.T. Baker®) to the media. Cultures were kept at 28, 24 or 18 °C. Every 30 d of storage at different temperatures, the number and length of developed shoots as well as their vigor and oxidation were evaluated. Results from this experiment showed that adding MS with 15 g l⁻¹ sucrose and 15 g l⁻¹ mannitol and keeping cultures at 18°C was the best condition for preserving meristems during fifteen weeks without requiring subcultures of tissues. Following this procedure, the meristem growth was reduced while maintaining high survival (93%) and, after transferred to the multiplication medium, tissues showed good vigor, less oxidation and high number of shoots/explant compared with the control treatment. This methodology was also tested with meristems of *Tectona grandis* L. (teak, family Lamiaceae), which could be successfully preserved (100% survival) for fifteen weeks without requiring subcultures (Montiel-Castelán et al. 2016). Meanwhile, the addition of mineral oil to the media was not effective for *in vitro* preservation of mahogany or teak meristems. Researches on *in vitro* conservation of *Daucus carota*, *Vitis vinifera*, *Catharanthus*, *Valeriana wallichii* (Johnson 2002) and *Bacopa monnieri* L. (Sharma et al. 2012) match the efficiency of mineral oil as a preservation technique. However, in this study both survival and vigor significantly decreased, besides hyperhydric tissues were presented. Gaspar et al. (2002) indicate that hyperhydricity is an adaptive state induced by stress during *in vitro* culture.

More recently, studies have demonstrated that the previously developed storage procedure (culture of shoot apical meristems on MS + 15 g l⁻¹ sucrose + 15 g l⁻¹ mannitol and incubation at 18°C) was adequate to preserve *S. macrophylla* and *T. grandis* accessions for extended periods (18 and 11 months respectively) without requiring subcultures (unpublished data). *Ex situ* storage procedures are now available for the medium-term *in vitro* preservation of mahogany and teak. These approaches offer new opportunities for the conservation, sustainable management and utilization of these valuable timber trees.

1.3.1.2 *Cedrela odorata* L. (Spanish cedar)

The genus *Cedrela* contains seven species (Pennington 1981; Pennington and Muellner 2010) native to the tropical and subtropical New World from Mexico to northern Argentina (Verissimo et al. 1998; Cavers et al. 2003). *Cedrela* trees may reach 40 m in height and 1.2 m in diameter (Newton et al. 1995; 1999; Navarro et al. 2002). *C. odorata*, known as “Spanish cedar”, “red cedar” or “cedar”, is the most important and widely distributed species of the genus. Its wood is in high demand in the American tropics, as sawnwood, plywood, for molding and cabinet work. Having an agreeable, pleasant scent its wood is also used as packaging for Havana cigars. This species ranges from northern Mexico, through Central America and the Caribbean islands, to Brazil. It is found in tropical and sub-tropical and semi-humid climates, from sea level to close to 3,000 m above sea level, in Bolivia (Lamb 1968). It is usually found on well-drained soils. The species tolerates prolonged periods of drought (Lamb 1968; Salas 1993); it also grows in volcanic soils (Pennington and Sarukhán 1968).

As seen above, *C. odorata* is widely distributed throughout the neotropics and grows in mixed stands. However, the economic value of timber from this species has resulted in their overexploitation for the past two centuries and has prompted several studies concerning the sustainable production and use of *Cedrela* as a forest crop (Newton et al. 1995; 1999; Navarro et al. 2002; Cavers et al. 2003). As with *S. macrophylla*, an additional threat to the remaining trees and to organized planting programmes is that within its natural area of distribution in the Americas, *Cedrela* trees are intensely attacked by the mahogany shoot-borer (*Hypsipyla grandella*), to the extent that use of these trees as a forest crop has been halted until resistant cultivars can be selected for or bred (Keay 1996; O’Neil et al. 2001). Thus, it is hoped that breeding programmes can incorporate resistance to this plague to produce resistant cultivars and/or develop hybrids which could allow resistance in another species

In the last years, the INIFAP staff has conducted researches aiming to regenerate plants from seeds and axillary buds (taken from field collected twigs), as well as to storing apical meristems using minimal growth *in vitro* culture for short- and medium-term preservation of Spanish cedar germplasm. Regarding to the micropropagation systems, the initial step is to have a protocol for the establishment and multiplication *in vitro* that will generate duplicates

for preservation. Significant contributions were made lately in this sense by Sampayo et al. (2016), who suggest the establishment of apical and axillary buds on MS supplemented with 1.0 mg l⁻¹ BA, 0.01 mg l⁻¹ IAA, 30 g l⁻¹ sucrose, 2 g l⁻¹ activated charcoal (AC), 1 g l⁻¹ Polivinilpirrolidone (PVP) as antioxidant and 1 ml l⁻¹ Plant Preservative Mixture (PPM) as biocide. In terms to *in vitro* medium-term preservation of Spanish cedar, shoot apical meristems were dissected from *in vitro* plants of *C. odorata* belonging to a working collection initiated through seeds and axillary buds from adult trees collected in natural cedar stands, as mentioned by Martinez et al. (2013b). After dissection, apical meristems were cultured on the multiplication medium composed of MS + 1.0 mg l⁻¹ BA + 0.05 mg l⁻¹ ANA + 2 g l⁻¹ AC (control treatment) or MS + AC lacking plant growth regulators. Cultures were kept at 24, 18 or 16 °C. After 30 and 60 d of storage at different temperatures, the number and length of developed shoots was evaluated. Data from this work demonstrated that culturing on MS + AC lacking plant growth regulators and incubating at 16°C enables maintenance of *C. odorata* meristems for 60 d (Martinez et al. 2013b). Subsequent studies have demonstrated that as in *S. macrophylla* and *T. grandis*, adding MS with 15 g l⁻¹ sucrose and 15 g l⁻¹ mannitol and keeping cultures at 18°C allowed to preserve *C. odorata* accessions for extended periods (14 months) without requiring subcultures (unpublished data).

Nunes et al. (2003) have developed an *in vitro* preservation method for germplasm from *C. fissilis*, an economically important tree of the Brazilian Atlantic Forest. This method involves the medium-term storage of artificial seeds comprising alginate encapsulated propagules (shoot tips, cotyledonary and epicotyl nodal segments) at 25°C. Maximum post-storage (3 months) viabilities of 96-100% were achieved for encapsulated shoot tips and cotyledonary nodal segments stored on water-solidified agar (at 0.4-0.7% w/v). Encapsulated shoot tips stored on 0.4% (w/v) agar showed the highest longevity (44% survival rate after 6 months storage). Moreover, seeds of *C. fissilis* have been successfully cryopreserved (100% survival) by direct immersion in liquid nitrogen.

1.3.1.3 *Melia azedarach* L. (paradise tree)

The genus *Melia* L. contains about five species of flowering trees from the Old World tropics. *M. azedarach* is a native of west Asia, but naturalized throughout the warm countries. The tree is well known in India and its neighboring countries for more than 2000

years as one of the most versatile medicinal plants having a wide spectrum of biological activity. The timber tough and durable, which resembles mahogany, is used to manufacture agricultural implements, furniture, plywood, boxes, poles, tool handles; it is used in cabinet making and in construction because of its resistance to termites. Aqueous and alcoholic extracts of leaves and seed reportedly control many insect, mite and nematode pests. Its oil is used for preparing the candle wax, for pest control and lice killing. Fruit stones make ideal beads and are used in making necklaces and rosaries (Katende et al. 1995). The plant is known to contain several organic molecules i.e., terpenoids, flavonoids, steroids, acids and anthraquinones (Kumar et al. 2003).

The “paradise tree” (*M. azedarach* L. var. *gigantea*) has raised a great interest in Argentina due to the rapidity of its growth (50 cm in diameter at the height of 1.30 m and a 6 m long trunk which can be cut after 10 years) and because its wood displays characteristics similar to Spanish cedar (*C. odorata*), which makes of it an excellent forestry species (Kunkel 1978). It shows a very good adaptability to different types of soils and climatic conditions, it is highly resistant to insects and therefore much valued for reforestation programmes (Leonardis et al. 2000; Nardo et al. 1997). The wood is soft, easy to process manually or mechanically. It is used for making planks, furniture, coverings, frames, doors and windows (Mangieri and Tinto 1977). Paradise tree is conventionally reproduced through seeds, which results in highly heterozygous populations. Therefore, asexual propagation techniques were developed for multiplication of selected clones, including *in vitro* micropropagation using nodal explants (Ahmad et al. 1990; Domecq 1988; Thakur et al. 1990). However, very little work had been done to preserve *in vitro* germplasm of selected clones. Bernard et al. (2002) reported that embryonic axes were successfully recovered (83%) after 4 months of storage at 4°C (using 200µM salicylic acid). Although seed conservation at -20°C was also tried, only 43% of the seeds germinated at the end of the experiment (Engelmann and Takagi 2000).

Great progress has been made in the last fifteen years by the IBONE (Corrientes, Argentina) staff towards the optimization of paradise tree micropropagation and preservation. A first publication documented a successful *in vitro* regeneration procedure through shoot-tips of paradise tree (Vila et al. 2002), making possible the development of a preservation protocol that ensure the integrity of germplasm collections using minimal

growth *in vitro* culture for medium term preservation of selected genotypes (Scocchi et al. 2004).

Regarding to the preservation process, shoot apical meristems (0.5-0.6 mm in length, consisting of the dome and a pair of leaf primordia) were dissected from *in vitro* plants of *M. azedarach* L. var. *gigantea* (clone “El dorado”), belonging to a working collection initiated through axillary buds from an adult tree (more than 10 years old) as mentioned by Domecq (1988). After dissection, apical meristems were cultured on nutritive media composed either of MS, or half (1/2MS), or quarter strength (1/4MS), supplemented with different combinations and concentrations of BA and IBA, including the medium considered for optimum growth of paradise meristem (MS + 0.5 mg l⁻¹ BA + 0.1 mg l⁻¹ IBA) (Vila et al. 2002) as well as other 20 suboptimum media with reductions in the basal medium salts and/or reduction in the growth regulators concentrations (4 to 10 times less than that used in the optimum medium). In all cases, cultures were kept in a refrigerator at 4°C in continuous darkness. After 120, 240 and 360 d of storage at 4°C, apical meristems were transferred to shoot regeneration fresh medium (MS + 0.5 mg l⁻¹ BA + 0.1 mg l⁻¹ IBA) and incubated under standard culture conditions (27 ± 2°C and 14 h photoperiod, irradiance of 116 μmol m⁻² s⁻¹). After 60 d of culture, viability of recovered meristems was evaluated by recording the number of meristems capable of resuming growth and producing well developed shoots (with more than 25 mm long and with at least 6 expanded leaves). Root induction was obtained when the regenerated shoots were cultured on MS supplemented with 3.5 mg l⁻¹ IBA during four days and subsequent root formation in MS lacking growth regulators.

Apical meristems remained green or pale green during storage and basically they did not increase in length and never produced shoots at 4°C. However, when apical meristems stored for 120, 240 or 360 days were transferred to shoot regeneration fresh medium and incubated in light at 27°C, some of them remained alive and rapidly produced shoots. The percentage of meristems forming shoots was dependent on the storage duration and the medium composition. All meristems stored for 120 d were able to regenerate shoots with frequencies between 42 and 100%, independently of the storage medium. Meristems stored for 360 d only regenerated shoots when stored in certain culture media. Regarding to that, the reduction of the MS strength appears to be one of the most important factor to consider in order obtaining high survival rates of meristems after one year storage. The culture medium composed by 1/4MS + 0.5μM BA has allowed that 67% of the explants produce shoots (4-5

well developed shoots/explants) after 60 d on the regeneration medium. These results are in agreement with those reported for other tropical trees where the reduction of certain components of the culture medium in conjunction with the incubation at low temperatures has allowed germplasm storage for periods of 1-4 years (Engelmann 1997). Rooting of the regenerated shoots was induced in 60% of the cultures and the plants obtained were successfully transferred to the greenhouse. However, these results show that both shoot regeneration (67%) and rooting percentage (60%) are affected by the storage, because in the control cultures these values are around 100%. In conclusion, this investigation shows successful utilization of *in vitro* meristem culture for storage of *M. azedarach* during one year at 4°C without subculture or addition of fresh medium. It is a very simple and effective technique which appears as an alternative to the one reported by Bernard et al. (2002) which is based in the culture of embryonic axes.

1.3.2 *Gmelina arborea* Roxb. (melina)

Gmelina arborea (melina) is an important timber-yielding tree from the family Verbenaceae, naturally distributed in the moist deciduous forests of South East Asia. This is a species widely used in commercial plantations for timber production by the different uses of their wood. This medium-sized tree (30 m tall and 1.2-4.5 m trunk diameter) with clear bole of 9-15 m has received attention as a source of good quality pulp, medium density fiberboard (MDF) and plywood because of its beautiful white color and stronger fiber. *G. arborea* has potential as a material for higher-grade uses such as timber for buildings and for furniture (Kojima et al. 2009). It has also been valued for its medicinal properties. Almost all parts of this tree are used in folk medicine for treating various stomach disorders, blood diseases, fevers and skin problems (Sharma et al. 2001). Roots of *Gmelina* are used in commercial Ayurvedic preparations (Tewari 1995). The plant extracts are reported to exhibit anti-inflammatory and wound healing properties (Shirwaikar et al. 2003) and are also known to inhibit platelet aggregation (Faiza and Darakhshanda 1998). Chemical constituents of *Gmelina* include lignans (Anjaneyulu et al. 1977), flavonoids (Nair and Subramanian 1975), iridoid and phenylpropanoid glycosides (Hosny and Rosazza 1998) and an isoxazole alkaloid (Barik et al. 1992).

Melina seeds are classified as recalcitrant based on their short storage life (<1 year) (Prakash 1991). In this case, the *in vitro* tissue culture techniques are presented as an important tool for the establishment of *ex situ* germplasm conservation programs. In the last years, the INIFAP (Jalisco, México) staff has conducted studies aimed to *in vitro* regenerating plants as well as to storing melina germplasm using minimal growth techniques for short- and medium-term preservation. Regarding to the preservation process, shoot apical meristems were dissected from *in vitro* plants of *G. arborea* belonging to a working collection initiated through apical and axillary buds (taken from twigs collected from two years old plants growing in a greenhouse). In a preliminary study, apical meristems were cultured on MS lacking plant growth regulators and kept at 24, 16, 12 or 8 °C. Survival was assessed after 90 d of storage at different temperatures. Data from this work showed that incubating cultures at temperatures below 16°C generated tissues damage. In a subsequent assay, the effect of sucrose/mannitol concentrations added to MS on meristems growth was evaluated. After dissection, shoot apical meristems were cultured on MS supplemented with different sucrose/mannitol concentrations (30/0, 25/5, 20/10, 15/15, 10/20, 5/25 and 0/30 g l⁻¹) and incubated at 24 or 18 °C. Every 30 d of storage at different temperatures, the number and length of the developed shoots as well as their vigor and oxidation were evaluated. Results from this experiment demonstrated that adding MS with 15 g l⁻¹ sucrose and 15 g l⁻¹ mannitol and keeping cultures at 18°C was the best condition for preserving apical meristems during 10 months without requiring subcultures of tissues (unpublished data).

Meanwhile, Sukartiningsih et al. (2012) developed a method for synthetic seed formation and conservation by encapsulating axillary buds of *G. arborea* and storing under aseptic conditions. MS medium supplemented with 0.22 mg l⁻¹ BAP, 0.02 mg l⁻¹ ANA, 1.00 mg l⁻¹ IBA and different concentrations of sucrose (3, 6, 12, 24, and 36%) were used as the encapsulation media in combination with 4% (w/v) sodium alginate. The synthetic seeds were stored at 4, 10, 15, 20 or 25 °C for 1, 2, 3 and 4 weeks. After 4 weeks storage, synthetic seeds were effectively preserved (90% survival) at 15 and 20 °C and they successfully sprouted shoots in a succeeding culturing at 25°C. But those capsules stored at 4°C presented chilling injury and no recovered were recorded.

In Table 1 are presented some representative examples of medium-term conservation of woody plant germplasm.

<<Table 1>>

xx.x Cryopreservation of woody plant germplasm. Study cases

xx.x.x *Quercus* species

The genus *Quercus* (Fagaceae) comprises around 600 species and is distributed in the Northern Hemisphere, South of Malaysia and Colombian mountains (Mabberley 1987). Seeds of *Quercus* species are recalcitrant (Roberts and King 1980) and they cannot be stored in a dried state under conventional seed-banking conditions (Roberst 1973). Recalcitrant seeds of temperate origin species are generally less sensitive to desiccation than those of tropical (Berjak and Pammenter 2004). Recalcitrant seeds can be stored in a hydrated state, generally for short periods; however, the seeds will become more desiccation sensitive with time and, also, they will germinate, due to their ongoing metabolism (Pammenter and Berjak 2014). To widen storability, hydrated recalcitrant seeds of temperate species can be stored at low temperatures (but above zero) with aeration (Catalán Bachiller 1991). Antifungal treatments are usually also necessary. This hydrated storage can be used only for few months to a year. For long-term storage, cryopreservation could be a viable alternative (FAO 2014). Because of the large size of oak seeds, embryo axis cryopreservation is required, to allow a more uniform application of treatments and cooling/warming rates. Therefore, *in vitro* techniques are required for their recovery. The appropriate methodology for *in vitro* growth of embryonic axes should be established before attempting cryopreservation. The wounds produced by the excision of axes could enhance the production of reactive oxygen species and impair growth (Pammenter and Berjak 2014). In some instances, this problem can be overcome by the use of antioxidants or promoting antioxidant pathways (Bai et al. 2011, 2012; Naidoo et al. 2011).

In submitting the embryonic axis to low temperatures (to those of liquid nitrogen), intracellular ice crystal formation should be avoided and water should vitrify; therefore, free water should be reduced and cooling should be fast. Due to the potential damage of low water contents the water content window could be narrow; besides, the speed of drying is also critical. Embryo axis should be dried rapidly to avoid exposure to low water contents for long periods (Berjak and Pammenter 2008).

xx.x.x Quercus faginea (Gonzalez-Benito and Perez-Ruiz 1992)

Embryo axes

Embryo axes of *Q. faginea* Lam. had been successfully cryopreserved by previous desiccation (Gonzalez-Benito and Perez-Ruiz 1992). Green or just turning brown acorns were washed in soapy water; pericarp was removed and the embryo axis containing half of the acorn was surfaced sterilized (0.05% NaOH for 20 min) followed by three rinses in sterile, distilled water. Axes were excised in sterile conditions and subsequently blotted dried with sterile filter paper before placing them in the air flow of a laminar flow bench. After different times (1 to 8 h) axes were placed inside polyethylene cryovials and plunged in liquid nitrogen. Warming took place by immersion in sterile water at 40 °C. Axes were cultured on Woody Plant medium (Lloyd and McCown 1981) salts plus MS modified vitamins (1 mg L⁻¹ thiamine instead of 0.1 mg L⁻¹; Murashige and Skoog 1962) supplemented with 1.5 mg L⁻¹ BAP (6-benzyladenine). Incubation took place at 25°C in darkness for 1 week, and afterwards in a 16 h photoperiod with a photosynthetic photon flux density of 50 μmol m⁻² s⁻¹.

Control axes (non-desiccated, not immersed in LN) had 64% water content (fresh weight basis) and radicle started growing two weeks after culture. Maximum recovery (development of root or/and shoot) after 4 week culture was 60% for cryopreserved axes, when they had been desiccated for 3 h (21% water content).

xx.x.x Quercus ilex and Q. suber (Gonzalez-Benito et al. 2002).

Embryonic axis

Several factors were studied to cryopreserved embryonic axis of *Q. ilex* L. and *Q. suber* L. (Gonzalez-Benito et al. 2002). Acorns were sprayed with a fungicide mixture of 3 mL L⁻¹ Previcur (propamocarb 60.5% w/v) and 4 g L⁻¹ Benlate (methyl, 1-(butylcarbamoyl)-2-benzylimidazol carbamate 50% w/w) and kept in plastic bags (not tightly closed) at 5 °C until use. After extraction, desiccation or rewarming (depending on the treatment), axes were immersed for 15 min in an ascorbic acid solution (50 mg L⁻¹), and subsequently in 0.25 % NaClO for 5 min, followed by three rinses in sterile distilled water. Axes were cultured on WPM+ 0.1 mg L⁻¹ BA (N6-benzyladenine) for *Q. suber* or 1 mg L⁻¹ BA for *Q. ilex*. *In vitro*

incubation temperature played an important role in the appropriate development of *Q. ilex* axes, as 15°C was superior to 25°C: 50% shoot development vs 25% in control axes after twelve weeks culture in culture. On the other hand, *Q. suber* axes showed higher percentage of shoot development at 25°C (66%) than at 15°C (55%).

Axes were desiccated in the airflow of a laminar flow cabinet for different periods. *Q. suber* axes proved to be more sensitive to desiccation and cooling. Moderate desiccation to 34% (2 h desiccation) affected radicle elongation of *Q. suber* axes. Furthermore, desiccation to 18% (4h) was detrimental and survival decreased to 60%. Survival after cooling (immersion in LN inside cryovials) was low in *Q. suber* axes and was present as unorganised growth. In *Q. ilex* organised growth was decreased only after desiccation to 13 % (4 h), with radicle emergence being reduced from 80% (non-desiccated) to 30% and plumule elongation from 35% to 20%.

The effect of “ultra-rapid” cooling was studied in *Q. ilex* performed axes, by immersing them directly in sub-cooled liquid nitrogen. Sub-cooling was achieved by placing a container with liquid nitrogen in a chamber where the pressure was lowered to 30 mbar. At that pressure, nitrogen had solidified. When pressure was increased to atmospheric levels, liquid and solid nitrogen coexisted (-210°C). Axes were rapidly immersed in sub-cooled liquid nitrogen and kept for 1 minute; cooling rates of 2700 °C min⁻¹ were measured. Rewarming took place by rapidly immersing axes in WPM (11) liquid medium at room temperature for approximately 10 min. Subsequently, axes were immersed in an ascorbic acid solution (50 mg l⁻¹) for 15 min and afterwards surface sterilised. Although survival was high 94% for fast-cooled axes, the response as organised growth was not improved by this method compared with the standard cooling: 13% shoot elongation in embryo axes dried to 18% water content.

xx.x Other studies on cryopreservation of *Quercus* species

The strategies for forest biodiversity cryopreservation have been described (Häggman et al. 2008); among them cryopreservation is considered a viable strategy for the long-term conservation of plant cells (cell cultures, embryogenic cell cultures), seeds and embryos (somatic or zygotic).

xx.x.x Embryonic axes and plumules

Cryopreservation protocols of embryonic axes and plumules of *Quercus* species have been developed as an alternative strategy to seeds storage. As it has been mentioned before, *Quercus* seeds are recalcitrant and, therefore, they cannot be stored under conventional seed banking conditions.

There have been studies on the desiccation tolerance of embryo tissues of several *Quercus* species as a previous step for their cryopreservation. Black oak axes (*Q. kelloggii*, *Q. shumardii*, and *Q. velutina*) survived drying to water contents of 0.20–0.25 g g⁻¹ dry mass (ca. 17-20% water content fresh weight basis; Chmielarz and Walters 2007). Xia et al. (2014) studied the desiccation tolerance of axes of four *Quercus* different species, two from subtropical origin and two from temperate climate. They found that the species better adapted to drier environments did not produce the most desiccation-tolerant embryos, but those better adapted to freezing temperatures during winter, and that plumule tissues resulted in faster drying and were more sensitive to desiccation than radicles. There was a direct relationship between survival after desiccation and after cryopreservation. The higher sensitivity of plumules could be also related to a sudden increase of extracellular superoxide related to the damage associated to cotyledon excision (Pammenter and Berjak 2014).

Chmielarz et al. (2011) approached the cryopreservation of *Quercus robur* germplasm by using plumules extracted from embryos. The procedure implied the culture of plumules in 0.5 M sucrose solution (18 h), followed by sequential immersion in 0.75 M sucrose, 1.0 M sucrose and in 1.5 M glycerol (40 min each), and subsequent desiccation to 0.5-0.6 g H₂O g⁻¹ dry weight (33-38% fwb) with silica gel before direct immersion to liquid nitrogen. With this protocol survival rates of 51–76%, and 8–20% plumule regrowth were obtained.

xx.x.x Embryogenic cultures

Somatic embryogenesis is nowadays a useful strategy for the clonal propagation of selected genotypes of temperate hardwood trees (Pijut et al. 2014). The number of genotypes of *Quercus* species on which somatic embryogenesis has been induced has been increasing during the last years and, therefore, there has been an increasing interest in maintaining

those cultures. Many of the procedures developed are based on the use of vitrification solutions and fast cooling (direct immersion in liquid nitrogen).

Masses (4-6 mg) of globular-heart stage somatic embryos of *Q. robur* pre-treated resume embryogenesis after cryopreservation (Martinez et al. 2003), with a 70 % recovery rate. Masses were pre-culture on 0.3 M sucrose medium prior to immersion in the vitrification solution (PVS2; Sakai et al 1990) for 60–90 min prior to immersion in liquid nitrogen. A similar procedure was used with six embryogenic cell culture lines obtained from selected mature *Q. robur* trees (Sánchez et al. 2008), with recovery percentages of 57-92% after one year storage. After that period, genetic stability (study by RAPD markers) of cryopreserved somatic embryos and seedlings was demonstrated in five out of the six lines; in the remaining line, recovered seedlings were also stable.

A similar vitrification procedure to that used for *Q. robur* was used with *Q. suber* embryogenic cultures from mature trees, obtaining high recovery (88-93%) and germination and plant regeneration rates similar to those reached by non-cryopreserved cultures (Valladares et al. 2004).

Globular embryogenic clusters of several embryogenic lines obtained from *Q. ilex* mature trees have been successfully cryopreserved by vitrification (Barra-Jimenez et al. 2015). However, the differentiation capability was hindered by cryopreservation in one of the lines, which was related to genetic instability, detected by microsatellite markers.

Embryogenic calli has also being cryopreserved by air desiccation (Chmielarz et al. 2005). Before desiccation in the air flow of a laminar flow cabinet to 17.3 % water content (fwb), calli were cultured on medium with increasing sucrose concentration (0.25 M, 0.5 M, 0.75 M and 1 M).

All this previous works have contributed to the establishment of a cryopreserved collection of embryogenic cultures (globular or torpedo stages) of 51 *Q. suber* genotypes using a vitrification-based technique (Vidal et al. 2010). All lines withstood cryopreservation, and were able to produce new somatic embryos by secondary embryogenesis.

xx.x Cryopreservation of *Ilex paraguariensis* and wild relatives.

Ilex L., the only genus of the family Aquifoliaceae, is the largest genus of woody dioecious plants, with at least 600 species distributed in tropical, subtropical and temperate regions of both hemispheres (Galle 1997; Loizeau and Spichiger 2004). South America is considered one of the main areas of diversification of *Ilex*, together with East Asia (Loesener 1942; Lawrence 1951; Cuénoud et al. 2000). In southern South America the species are mostly found in northeastern Argentina, southeastern Brazil and eastern Paraguay (Gottlieb et al. 2005). Most species are deciduous or evergreen shrubs or small trees, but in the tropics the genus also includes some very large trees and a few climbers (Tsang and Corlett 2005).

The genus *Ilex* comprises several species with economic importance as crops and ornamentals. Some of them, commonly named “hollies,” such as “English holly” (*I. aquifolium*), “Japanese holly” (*I. crenata*) and “American holly” (*I. opaca*), have long been symbolic of Christmas, and have also been cultivated by nurserymen in Europe, Asia and the United States for landscaping. Various institutions and commercial breeders are developing hybrids with improved to winter and with more foliage (Hu 1989; Walden and Wright 1995). In South America, one of the most important species of economic and pharmacological interest is *I. paraguariensis*, popularly known as “yerba-mate” or “maté tree”. This is obtained in the native form and is widely cultivated in northeastern Argentina, eastern Paraguay and southern Brazil (Burris et al. 2012). The yerba-mate is characterized as an important product in the socioeconomic cultural context in their original regions. It is mainly consumed as “mate”, a hot beverage prepared by infusion that stimulate the central nervous system due to the presence of xanthic bases or alkaloids, such as caffeine and theobromine (Filip et al. 2001; Schinella et al. 2005). There are other products in the market made from the yerba-mate leaves, as blended teas, flavored tea, iced tea, and cosmetics that use *I. paraguariensis* extracts (Mosele 2002). Some investigations are expanding the use of this species in new products, exploring its probiotic properties (Preci et al. 2011; Ril et al. 2011).

Currently, the demand for food containing biologically active substances has increased, considering that consumers are seeking these products for a healthier life (Melo and Guerra 2002). The yerba-mate fits in this context, due to the numerous benefits to health it provides, such as hypocholesterolemic and hepatoprotective activity (Filip and Ferraro 2003; Açari et al. 2011), anti-inflammatory and anti-obesity effects (Bracesco et al. 2011), central nervous system stimulation, diuretic action (Castaldelli et al. 2011), inhibition of neoplastic cells

proliferation (Mejía et al. 2010), and antioxidant activity, preventing damages caused by free radicals (Bastos et al. 2007). Studies showed that among ten plant species used as teas, the yerba-mate was one that showed the highest antioxidant capacity (Asolini et al. 2006), which extracts polyphenol levels are higher than those of green tea and parallel to those of red wines (Gugliucci and Bastos 2009; Gugliucci et al. 2009).

On the natural distribution area of *I. paraguariensis* also occur a number of wild species of *Ilex* (like *I. brasiliensis*, *I. brevicuspis*, *I. dumosa*, *I. integerrima*, *I. pseudoboxus*, and *I. theezans*) which are sympatric with genuine maté (Giberti 1999). In the last years, *I. dumosa* ("yerba señorita") has received the most attention from plant breeders because of this species is resistant to the attacks of certain diseases which are common for *I. paraguariensis*. Moreover, with their leaves it is possible to make "maté" with less caffeine than with the ones from the genuine yerba-mate (Filip et al. 1999). Wild populations can be a valuable source of new genetic material for plant breeding. The natural diversity of plants growing in their natural habitats means that at least some individuals may carry genes of commercial importance, such as those which confer resistance to diseases and insects or are useful in stressful environments (Acquaah 2012). Therefore, it is crucial to secure as much biodiversity as is possible. However, in the maté-growing region (and their sympatric *Ilex* species) the risks of genetic erosion are high because the natural forest is gradually giving way to agroforestry and livestock production, a process accentuated by the low germinability of many species (especially that of *I. paraguariensis*). Moreover, market demands for uniformity concerning both quality and higher yields, clearly restricts genetic variation of a given crop species. Consequently, *ex situ* preservation of *Ilex* germplasm is necessary to safeguard the threatened diversity of this genus, mainly due to the anthropogenic impact. These species are usually preserved in field collections, at the risk of disease, pest, fire, drought and human damage, besides the genetic erosion (Giberti 1999; Zhang et al. 2014). Thus, research for alternative methods to field conservation for *Ilex* genetic resources became a priority.

So far, the only reports regarding the cryopreservation of *Ilex* germplasm were carried out in the IBONE (Corrientes, Argentina). Researches were aimed to evaluate the possibility of cryopreserving fruits, seeds, isolated embryos and shoot-tips of diverse South American *Ilex* species, as a complementary option to field collections.

xx.x.x Cryopreservation of *Ilex* fruits, seeds and zygotic embryos

Seed storage is the most effective and efficient method for the *ex situ* preservation of genetic resources of plants which produce orthodox seeds, by combining low storage costs (100 times cheaper than *in situ* preservation of individual trees) with ease of seed distribution and regeneration of whole plants from genetically diverse material as each seed is genetically different (Linington and Pritchard 2001; Li and Pritchard 2009). *Ilex* seeds are harvested at water contents above 30%, a feature that is often associated with recalcitrant behaviour (Berjak et al. 1989). However, the levels to which *Ilex* seeds will tolerate desiccation and low temperature storage are unclear. Moreover, seeds of *Ilex* species are individually enclosed by woody endocarp and have undeveloped embryos (mostly at heart stage) when fruits reach maturity (Martin 1946; Niklas 1987; Tsang and Corlett 2005; Dolce et al. 2007), resulting in a deep dormancy and low germination rate (Hu 1975; Hu et al. 1979). For example, *I. opaca* germinates in nature after one to three years and the germination rate is about one in ten million (Ives 1923). This extremely low germination rate constitutes a serious inconvenience for breeding and conservation programs, since it leads to a loss of potentially valuable genotypes. Besides, the small embryo size (160-350 µm in length) and the high level of dormancy of *Ilex* seeds, have hampered efforts to gain knowledge about their storage characteristics.

In recent years, the IBONE staff has conducted studies to evaluate the possibility to cryopreserving of fruits, seeds and zygotic embryos from seven South American *Ilex* species, using the vitrification, desiccation and encapsulation-dehydration techniques respectively. For all experiments, the source of plant material consisted in open pollinated ripened fruits (nuculanium) of *Ilex* spp. (Table 1) which were hand-harvested during summer (~3 months after anthesis) from trees growing in field.

xx.x.x Fruits

The first strategy for long-term preservation of *Ilex* germplasm involved the cryopreservation of fruits using the vitrification technique (Mroginski et al. 2006; 2011). Fruits were surface-sterilized by soaking them in 70% ethanol for 5 min, followed by immersion in an aqueous solution of 1.8% sodium hypochlorite and 0.1% Triton X-100[®].

Subsequently, fruits were rinsed three times with autoclaved distilled water. Superficially sterilized fruits were cold-pretreated (30 d at 4°C). Preconditioned fruits were placed in 5-ml cryovials and exposed to 2.5 ml of PVS3 [50% glycerol (w/v) + 50% sucrose (w/v)] (Nishizawa et al. 1993) vitrification solution for 60 min at 0°C prior to rapid immersion in LN (rapid cooling), or slowly cooled at 1°C min⁻¹ from +25 to -40°C (by using a Controller Rate freezing System, Gordiner Electronics, Inc. USA) and then immersed in LN (slow cooling). After storage in liquid nitrogen for 24 h, fruits were rewarmed by immersing the cryovials in a water-bath at 30°C for 1 min, and then washed three times for 15 min by replacing the PVS3 with an unloading solution composed of liquid MS medium supplemented with 1.2 M sucrose. For growth recovery, the rudimentary embryos at the heart stage were excised from seeds (Mroginski et al. 2011) and cultured *in vitro* on the germination medium consisting of quarter-strength MS supplemented with 0.1 mg l⁻¹ zeatin (Sansberro et al. 1998; 2001). The embryos were kept in a growth room at 27±2 °C, in the dark for 30 d and then transferred to light standard culture conditions (116 µmol m⁻² s⁻¹ PPF provided by cool white fluorescent lamps) for another 30 d. The embryo survival from fruits subjected to vitrification procedure was evaluated at 60 d after beginning the cultivation of the isolated embryos, through their germinability. Embryos from all the tested species did not tolerate cryostorage when vitrified fruits were rapidly cooled, but the ability to withstand immersion in LN increased when fruits were slowly cooled. Anyway, the embryo germinability from slow cooled fruits was scant.

xx.x.x Zygotic embryos

In another report, the IBONE staff described the successful crypreservation of *Ilex* zygotic embryos by using the encapsulation-dehydration technique (Mroginski et al. 2008; 2011). Fruits were surface-sterilized and rudimentary embryos were excised as above. After excision, embryos were preconditioned on semisolid germination medium supplemented with 0.3 M sucrose for 7 d, in dark conditions. Preconditioned embryos were encapsulated in 3 % sodium alginate (SIGMA-ALDRICH®), polymerized with calcium chloride (CaCl₂) at 0.1 M, to form calcium alginate capsules approximately 5 mm in diameter. Encapsulated embryos were then pretreated in liquid MS supplemented with 0.5, 0.75 and 1.0 M sucrose, by progressively increasing the concentration from 0.5 to 1.0 M for 24 h in each condition. The pretreatment was performed by placing the samples at 27°C on an orbital shaker at 100

rpm. After preculture, the beads were rapidly surface-dried on filter paper and dehydrated in 100 cm³ air-tight containers with 30 g silica gel (10 beads/container) for 5 h (equivalent to capsule moisture content -MC- of 25%, fw). Samples were then placed in 5-mL cryovials and rapidly immersed in LN (rapid cooling), or slowly cooled at 1°C min⁻¹ from +25 to -30°C and then immersed in LN (slow cooling). After 24 h storage in LN, samples were rewarmed by immersing the cryovials in a water-bath at 30°C for 1 min, and the beads were placed on semisolid germination medium for recovery. The recovered embryos were kept in a growth room at 27±2 °C, in the dark for 30 d and then transferred to light standard culture conditions for another 30 d. Survival was evaluated at 60 d after beginning the recultivation of the beads, through the germinability of the encapsulated embryos. Using the encapsulation-dehydration technique, it was increased the ability of *Ilex* embryos to withstand cryostorage (Table X). In most species, the slow cooling did not improve the survival of embryos. So, the use of rapid cooling is suggested, since no sophisticated facilities are necessary.

Table X. *In vitro* germination (%) of *Ilex* zygotic embryos from cryopreserved fruits (subjected to the vitrification technique) and encapsulated embryos (subjected to the encapsulation-dehydration technique). Data are the mean of three replicates ± SE.

Species	Fruits cryopreservation		Isolated embryos cryopreservation	
	-LN	+LN ⁽¹⁾	-LN	+LN ⁽²⁾
<i>I. brasiliensis</i>	73 ± 3	3 ± 3	56 ± 8	47 ± 13
<i>I. brevicuspis</i>	40 ± 6	0	8 ± 3	15 ± 8
<i>I. dumosa</i>	40 ± 6	13 ± 3	74 ± 14	67 ± 7
<i>I. integerrima</i>	43 ± 7	0	27 ± 12	50 ± 12
<i>I. paraguariensis</i>	40 ± 6	10 ± 6	50 ± 6	27 ± 3
<i>I. pseudoboxus</i>	67 ± 3	23 ± 3	23 ± 9	7 ± 3
<i>I. theezans</i>	67 ± 3	0	67 ± 3	65 ± 15

LN: Liquid Nitrogen

⁽¹⁾ Slow cooling.

⁽²⁾ Rapid cooling.

xx.x.x Seeds

More recently, it was evaluated the possibility of cryopreserving intact seeds from seven *Ilex* species using the desiccation technique (Dolce et al. 2016). Seeds were removed from fresh fruits, cleaned of the pulp and immediately used for assembly of experiment. Seeds were desiccated in 100 cm³ air-tight containers with 30 g silica gel (300 seeds/container) for 2 h intervals; up to 14 h. Desiccated seeds were placed in 2-ml cryovials and rapidly immersed in LN (rapid cooling). After 7 d storage in LN, samples were rewarmed by immersing the cryovials in a water-bath at 40°C for 2 min. Due to the deep dormancy and low germination rate of *Ilex* seeds when conventional methods are used, germinability was assessed through *in vitro* culture of intact/bisected seeds or isolated embryos, according to the previously optimised procedure for each species (Sansberro et al. 1998; Dolce et al. 2010; 2011; 2015). Cultures were kept in a growth room at 27±2 °C, under light standard conditions for 60 d (whole and cut seeds) or in the dark for 30 d and then transferred to light conditions for another 30 d (isolated embryos). Survival and plant development from seeds subjected to desiccation procedure was evaluated at 60 d after beginning the recultivation of the seeds or embryos.

The seeds MC decreased from an initial average of 40.9 ± 0.2% to 6.2 ± 0.1% after 14 h desiccation. Seeds of all species tested tolerated desiccation down to ~6% of MC with similar germination percentages to that in their respective control (non-desiccated and non-cryostored seeds). Moreover, intact seeds of the seven species could be successfully cryopreserved when they were desiccated to 6.4-8.4% (depending on the species) prior to immersion in LN with no reduction in the germinability compared with the control group. It is known that the most critical factor affecting cryopreservation of seeds is MC (Pritchard 2007), so it should be determined for each species the range of MC which allows seeds to tolerate cryogenic temperatures. Dehydration must be sufficient to avoid lethal intracellular freezing during cooling in LN, but not so intense to induce extended desiccation injury. In optimal cases, no significant difference is observed in the survival rates of desiccated control and cryopreserved material (Vertucci and Farrant 1995; Pammenter and Berjak 1999; Walters et al. 2002).

Data from this study demonstrated that *Ilex* seeds of the seven South American species tested did not show high sensitivity to dehydration and cryopreservation, as might be expected for tropical species which seeds are disseminated with high water content. Seeds

tolerated desiccation to ~6% MC without a loss in viability. Furthermore, these data allow to suggest a simply and cost-effective method for *Ilex* seed cryopreservation by using the desiccation technique. This is a very simply and cost-effective cryopreservation method because neither cryoprotectants nor any sophisticated facilities are necessary. Compared with cryopreservation of isolated embryos by encapsulation-dehydration technique, this protocol has advantages such as less consumption of time and labour and simplicity of the protocol.

xx.x.x Shoot-tips

Due to the allogamy of *Ilex* species, asexual reproduction would be of great value for the multiplication of select commercial lines of “mate”. Successful propagation is possible for juvenile material (Sansberro et al. 1999); however, like many other woody species, mature tissues show a low morphogenetic potential, which makes it difficult to clone mature trees by rooting cuttings or by *in vitro* techniques. In the same way, this makes it difficult the use of cryopreservation techniques for the safe long-term storage of selected genotypes. Anyway, despite not having optimized methodologies for *in vitro* propagation of *Ilex* species, studies have been initiated to evaluate the possibility of cryopreserving *I. dumosa* and *I. paraguariensis* shoot-tips (the two species currently cultivated for industrial purposes).

Shoot-tips (2-3 mm in length, consisting of the meristematic dome and two to three leaf primordia) were dissected from *in vitro* regenerated shoots obtained from cuttings of plants grown in a greenhouse after 45 d of *in vitro* establishment (Luna et al. 2003). After dissection, shoot-tips were preconditioned on semisolid multiplication medium consisting of quarter-strength MS + 0.1 mg L⁻¹ BAP, supplemented with 0.3 M sucrose for 48-72 h, using standard culture conditions. Apical shoot-tips were subjected to two cryopreservation techniques: encapsulation-dehydration and vitrification.

xx.x.x Encapsulation-dehydration (E-D).

Preconditioned apices were encapsulated in 3 % sodium alginate, polymerized with calcium chloride (CaCl₂) at 0.1 M, to form calcium alginate capsules approximately 5 mm in

diameter. Encapsulated apices were then pretreated in liquid MS supplemented with 0.5, 0.75 and 1.0 M sucrose, by progressively increasing the concentration from 0.5 to 1.0 M for 24 h in each condition. The pretreatment was performed by placing the samples at 27°C on an orbital shaker at 100 rpm. After preculture, the beads were rapidly surface-dried on filter paper and dehydrated in 100 cm³ air-tight containers with 30 g silica gel (10 beads/container) for 1 to 7 h. Samples were then placed in 5-mL cryovials and rapidly immersed in LN (rapid cooling), or slowly cooled at 1°C min⁻¹ from +20 to -30°C and then immersed in LN (slow cooling). After 24-48 h storage in LN, samples were rewarmed by immersing the cryovials in a water-bath at 40°C for 2 min, and the beads were placed on semisolid multiplication medium for recovery. The recovered apices were cultured for 1 wk in the dark before being transferred to standard culture conditions.

xx.x.x Vitrification (V).

Preconditioned apices were loaded in a 0.4 M sucrose + 2 M glycerol solution for 20-30 min at 27°C and exposed to PVS2 [30% glycerol (w/v) + 15% ethylene glycol (w/v) + 15% (w/v) DMSO + 0.4 M sucrose] (Sakai et al. 1990) or PVS3 [50% glycerol (w/v) + 50% sucrose (w/v)] (Nishizawa et al. 1993) vitrification solutions for 0, 30, 60, 90, 120 or 150 min at 27 or 0°C prior to rapid immersion in LN in cryovials with 3 mL of the respective PVS. After storage in liquid nitrogen for 24-48 h, apices were rewarmed by immersing the cryovials in a water-bath at 40°C for 2 min, and then washed three times for 15 min by replacing the PVS with an unloading solution composed of liquid multiplication medium supplemented with 1.2 M sucrose. After rewarming, apices were transferred to filter papers for 1-2 min to drain off excess liquid. For growth recovery, tissues were placed on semisolid multiplication medium, kept one week in the dark, and then transferred under standard culture conditions.

The survival from shoot-tips subjected to both cryogenic procedures (E-D and V) was evaluated at 30 d after beginning the recultivation of the explants. No survival was achieved from *I. dumosa* and *I. paraguariensis* shoot-tips subjected to cryostorage, whatever the cryogenic procedures and the freezing protocol (rapid or slow cooling) employed. With the E-D technique, survival of desiccated pretreated controls remained high (70-80%, depending of the species) until after 5 h dehydration (equivalent to capsule MC of ~25%), but it markedly decreased to 40% after 6 h dehydration and it fell to 0% after 7 h dehydration (equivalent to capsule MC of ~20%). Besides, PVS-treated controls shoot-tips showed high

survival rates (60-90%, depending of the PVS exposure duration) (data not published). These results suggest that the encapsulation and sucrose pretreatment or PVS exposure did not affect *per se* the viability of the shoot-tips, nor the dehydration up to MC of 25%. The negative results after cryopreservation of *Ilex* shoot-tips by E-D and V techniques would not be related to the high sensitivity of this tissue to sugars or desiccation (Figure X).

Consequently, further research is needed to explore other treatments before ultra-freezing for diverse protocols, that are conducive to better tissue protection during immersion in LN, in order to increase survival and regeneration efficiency. Assessment of cryopreservation protocols could be supported, for example, by differential scanning calorimetry studies, manipulation of preculture treatments to enhance tissue cryotolerance (e.g. cold hardening) (Paul et al. 2000), and post-thaw culture treatments to promote tissue recovery from cryoinjury (e.g., use of nursery cultures) (Hargreaves et al 2002). Moreover, biochemical and molecular biology studies on tissue culture recalcitrance and cryoinjury could potentially be more significant (e.g. to investigate the role of oxidative stress) (Benson 2000).

Cryopreserved collections of clonally propagated woody species have already been established in several countries, including USA (apple, pear, hazelnut), France (elm), Japan (mulberry) and India (almond, citrus fruits) (Reed 2002). For several other woody species, effective protocols of cryopreservation by vitrification approaches have been reported (Lambardi and De Carlo 2002; Lambardi 2002; Scocchi et al. 2004; Halmagyi et al. 2010; Volk et al. 2012; Silva et al. 2013; Soliman 2013) which would be used for cryo-stored germplasm collections. *Ilex* spp. cannot be ascribed to this list yet; however, efforts are continuing to overcoming the “recalcitrance” of these species to the conservation in LN.

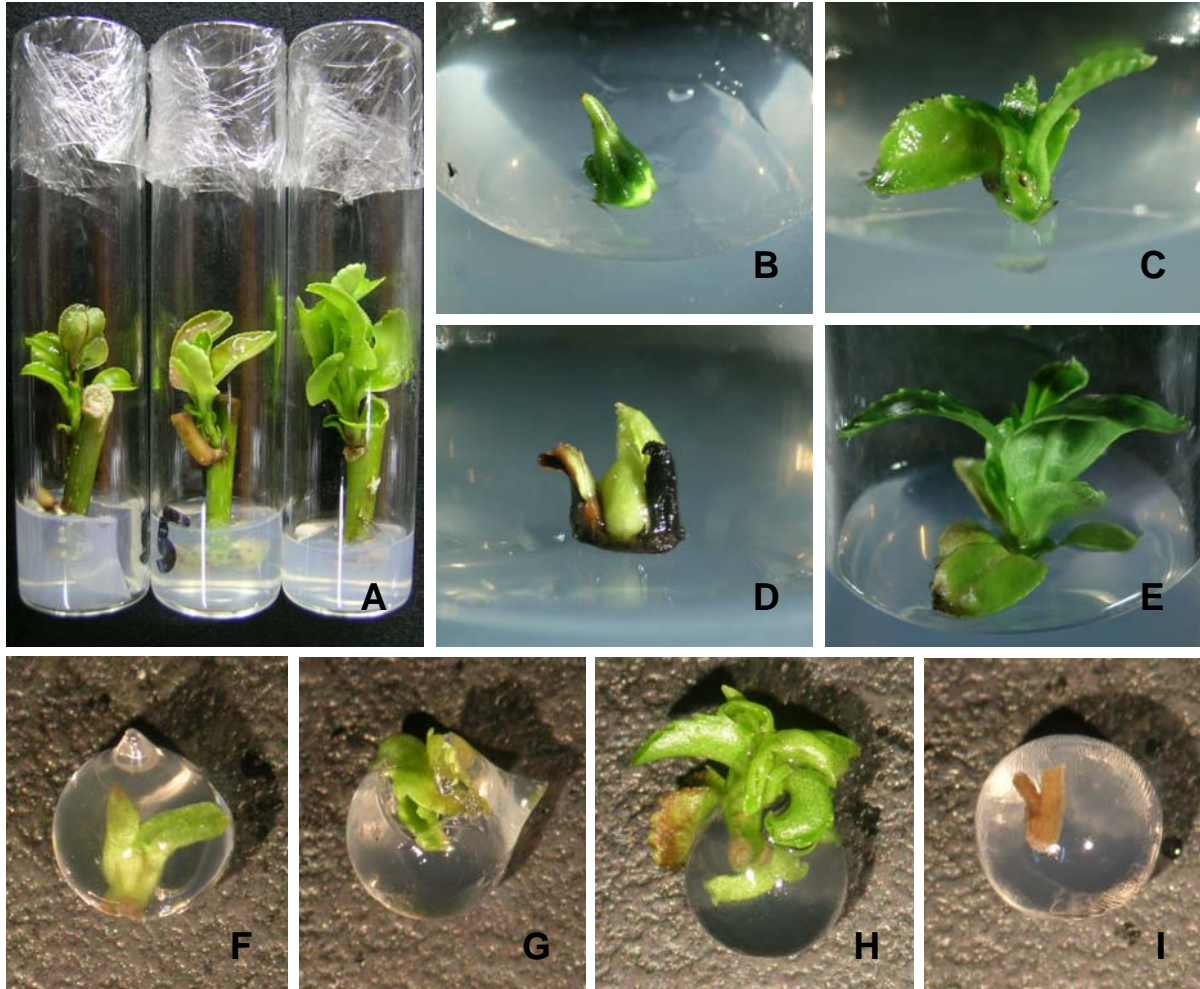


Fig. 1. Application of Vitrification and Encapsulation-Dehydration techniques for cryopreservation of *Ilex* spp. shoot tips. **A)** *In vitro* regenerated shoots after 45 d of cuttings establishment. **B,C,D,E)** *Ilex paraguariensis* shoot tips from the control treatments (-LN): untreated shoot tip after 30 (**B**) and 60 (**C**) days of culture; shoot tips treated with PVS3 for 120 min (**D**) or pretreated in liquid MS supplemented with progressively increasing sucrose concentration (**E**), after 60 days of culture. **F-H)** *I. dumosa* encapsulated shoot tips from the control treatment (-LN) with 4 h dehydration, after 15 (**F**), 30 (**G**) and 60 (**H**) days of culture. **I)** *I. dumosa* encapsulated shoot tips subjected to cryostorage, 10 days after recovery.

xx.x Family Meliaceae

xx.x.x *Melia azedarach*

As mentioned before, the “paradise tree” (*M. azedarach* L. var. *gigantea*) has raised a great interest in Argentina as an excellent forestry species. Over the last 15 years, great progress has been made towards the optimization of paradise tree micropropagation and cryopreservation; since the IBONE staff has conducted studies to regenerating plants from shoot apical meristems and zygotic embryos (Vila et al. 2002; 2003) and to cryopreserving such explants using diverse techniques (Scocchi et al. 2004; 2007). Moreover, since plants regenerated after cryogenic treatments should be genetically identical to the starting materials; the genetic fidelity of the plants obtained from successfully cryopreserved shoot apical meristems of paradise tree was investigated by using isozyme and RAPD markers.

xx.x.x.x Shoot-tips

The first strategy for long-term preservation of clonal germplasm from paradise tree involved the cryopreservation of shoot apical meristems using the encapsulation-dehydration technique (Scocchi et al. 2004). Shoot apical meristems (0.5-0.6 mm in length, consisting of the dome and a pair of leaf primordia) were dissected from *in vitro* plantlets of *M. azedarach* L. var. *gigantea* (clone “El dorado”). After dissection, apical meristems were preconditioned for 24 h on semisolid establishment medium consisting of MS + 2 μ M BA and 0.5 μ M IBA (Vila et al. 2002), using standard culture conditions. Preconditioned shoot-tips were encapsulated in 3 % sodium alginate (SIGMA-ALDRICH®), polymerized with calcium chloride (CaCl₂) at 0.1 M, to form calcium alginate capsules approximately 5 mm in diameter. Encapsulated embryos were then pretreated in liquid establishment medium supplemented with 0.5, 0.75 and 1.0 M sucrose, by progressively increasing the concentration from 0.5 to 1.0 M for 24 h in each condition. The pretreatment was performed by placing the samples at 27°C on an orbital shaker at 100 rpm. After preculture, the beads were rapidly surface-dried on filter paper and dehydrated in 100 cm³ air-tight containers with 30 g silica gel (10 beads/container) for 5 h (equivalent to capsule MC of 25%, fresh weight basis). Samples were then placed in 5-mL cryovials and rapidly immersed in LN (rapid cooling), or slowly cooled at 1°C min⁻¹ from +20 to -30°C and then immersed in LN (slow

cooling). After 1 h storage in LN, samples were rewarmed by immersing the cryovials in a water-bath at 30°C for 2 min, and the beads were transferred directly on semisolid establishment medium for recovery. The recovered shoot apical meristems were kept in a growth room at 27±2 °C, in light standard culture conditions. It was evaluated the survival percentage at 7 days, shoot proliferation percentage at 28 days and number of shoots/shoot apical meristem at 60 days after beginning the recultivation of the explants subjected to encapsulation-dehydration technique.

Using the encapsulation-dehydration technique, cryopreservation of shoot apical meristems of paradise tree can be achieved with high survival rates (slow cooling = 83 ± 2%; rapid cooling = 67 ± 2%) and shoot proliferation rates (slow cooling = 60 ± 1%; rapid cooling = 43 ± 1%). Plants regenerated after cryogenic treatments preserved genetic stability, when it was evaluated using the electrophoretic patterns of nine isozyme systems and RAPD profiles. The survival rates achieved following this encapsulation-dehydration protocol were comparable to those obtained by Bernard et al. (2002), who cryopreserved embryonic axes with a salicylic acid pretreatment. However, this cryopreservation procedure using an increasing sucrose concentration as the only cryoprotectant and controlled silica gel desiccation is preferable to others previously reported. It not only resulted in high survival rates, but it also represented the first protocol by which high shoot proliferation and clonal plants regeneration could be obtained, besides confirming their genetic stability. Regarding to that, it is known that *in vitro* propagation techniques based on shoot-tips culture are the most desirable for long-term germplasm conservation because this is an ideal explant with few differentiated cells that tend to remain genetically stable during the regeneration process (Kantha et al. 1980; Towill 1984; Mroginski et al. 1991).

xx.x.x.x Somatic embryos

Later, it was established a cryopreservation protocol for paradise tree somatic embryos by comparing three cryopreservation techniques: encapsulation-dehydration, pregrowth-dehydration and desiccation (Scocchi et al. 2007). Open pollinated immature fruits of *M. azedarach* L. var. *gigantea* were hand-harvested during late-spring (8-9 weeks after anthesis) from trees growing in field. *In vitro* culture of zygotic embryos and somatic embryogenesis

induction were conducted according to Vila et al. (2003). Somatic embryos were subjected to three cryopreservation techniques:

Encapsulation-dehydration (E-D).

It was adapted from that developed for shoot-tips cryopreservation (Scocchi et al. 2004). The beads were dehydrated in containers with silica gel for up to 4 h, thereby reaching 21-26% of MC (fwb). Same beads were dehydrated without sucrose pretreatment. Desiccated beads were then placed in 5-mL cryovials and rapidly immersed in LN (rapid cooling), or slowly cooled at $1^{\circ}\text{C min}^{-1}$ from +20 to -30°C and then immersed in LN (slow cooling). After 1 h storage in LN, samples were rewarmed by immersing the cryovials in a water-bath at 30°C for 2 min. Somatic embryos were then transferred to recovery medium (quarter-strength MS, 3% sucrose, no growth regulators) under standard culture conditions.

Pregrowth-dehydration (P-D).

Non-encapsulated mature somatic embryos were submitted to the same pretreatment as for the encapsulation-dehydration technique, dehydrated with silica gel from 90.5% MC (0 h) to 13.5% MC (4 h), and then cooled rapidly (direct immersion of the cryotubes in LN) or slowly ($1^{\circ}\text{C min}^{-1}$ from $+20^{\circ}\text{C}$ to -30°C followed by immersion of the cryotubes in LN). Rewarming and growth recovery took place as described previously.

Desiccation (D).

Non-pretreated and non-encapsulated mature somatic embryos were desiccated with silica gel from 83.5% MC (0 h) to 9.3% MC (4 h) and then cooled rapidly (direct immersion of the cryotubes in LN) or slowly ($1^{\circ}\text{C min}^{-1}$ from $+20^{\circ}\text{C}$ to -30°C , followed by immersion of the cryotubes in LN). Rewarming and growth recovery took place as described previously.

Recovery (%) was assessed after 3 weeks by counting the number of cryostored somatic embryos which developed into plantlets and/or produced adventitious embryos, and gave rise to fully developed plantlets. Among the three cryopreservation techniques tested, only E-D and P-D led to successful results after slow cooling. No survival was achieved after rapid cooling, whatever the technique used. The highest recovery rates were achieved after 3 h

dehydration, reaching 36% survival with E-D technique (21% of MC) and 30% survival with P-D technique (19% of MC). This work reported for the first time the cryopreservation of paradise tree somatic embryos. The recovery percentages achieved were intermediate, so it might be possible to further improve the results by refining the pretreatment conditions, e.g. by increasing the duration of treatment with the successive sucrose media employed, or by using higher final sucrose concentrations, or by testing other techniques such as vitrification, encapsulation-vitrification or droplet-vitrification (Sakai and Engelmann 2007). Finally, the protocol established would have to be validated using additional paradise tree accessions.

xx.x Cryopreservation of Citrus germplasm. Study cases.

Citrus fruits are recognized as an important food widely consumed throughout the world, which are characterized by the distinct aroma and delicious taste, becoming an inseparable part of our diet. Citrus fruits are grown in more than 140 countries, most of the crop grows on either side of a belt around the equator covering tropical and subtropical areas of the world 35°N and 35°S latitudes with cultivation and production concentrated in major regions in the Northern Hemisphere (Ramana et al. 1981; UNCTAD 2004).

The origin of citrus fruit is identified full of interesting legends. There exists the belief that citrus is native to the subtropical and tropical areas of Asia, originating in certain parts of Southeast Asia including China, India, and the Malay Archipelago (Ramana et al. 1981; Gmitter and Hu 1990). According to ancient Chinese documents, in the earliest reference of citrus fruits are mentioned mandarins and pummelos as highly prized tributes only available for the imperial court. Lemon was originally grown in India and sweet oranges and mandarins are indigenous to China. Later, some research has suggested that the true origins of citrus fruit are Australia, New Caledonia (off eastern Australia), and New Guinea (Anitei 2007). The spread of citrus also advanced to northern Africa and southern Europe, and the first introduction of citrus to America was achieved by Spanish and Portuguese explorers. Currently, the commercial production, processing, and global trade of citrus are huge, and have significantly increased since then. Since several years ago, citrus have been considered as the most important fruit in the world (Ramana et al. 1981; UNCTAD 2004).

Citrus genetic resources are traditionally maintained as living collections in field genebanks, because most species produce seeds with recalcitrant or intermediate storage

behaviour and numerous cultivars are seedless. However, as it has been already reviewed, plant material in field conditions remains exposed to pests, diseases and other natural hazards (Gonzalez-Arno et al. 1998; 2013; Nagy and Attaway 1980). Greenhouses are also an advisable alternative for conserving selected materials under controlled conditions, protected from pest and insect borne disease; however, these houses demand special technical facilities, in addition to considerable expenses to maintain a limited number of specimens (Duran-Vila 1995). Therefore, cryopreservation is currently the safest and most appropriated method for long-term storage of these problem species.

So far, many studies have been conducted on cryopreservation of citrus germplasm from the first reports since the 80s. As result, effective cryopreservation protocols have been developed for different type of plant tissues and organs (Marin and Duran-Vila 1988; Sakai et al. 1990; Olivares-Fuster et al. 2000; Cho et al. 2001, 2002a, 2002b, 2002c; Gonzalez-Arno et al. 1998; 2003; Lambardi et al. 2004; Kaya et al. 2013).

In general, cryopreservation of citrus germplasm has been performed using seeds (Mumford, and Grout 1979), ovules (Gonzalez-Arno et al. 2003), embryogenic axes (Radhamani and Chandel 1992), somatic embryos (Marin and Duran-Vila 1988; Gonzalez-Arno et al. 2003), embryogenic calluses and cell suspensions (Engelmann et al. 1994; Gonzalez-Arno et al. 1997; Sakai et al. 1990, 1991), pollen (Ganeshan and Alexander 1991; Rajasekharan et al. 1995), and shoot-tips from juvenile (Gonzalez-Arno et al. 2003) and adult plants (Volk et al. 2013).

From the biotechnology point of view, cryopreservation of embryogenic callus and ovules has an especial interest for breeding citrus programs based on protoplast fusion (Olivares-Fuster et al., 2000), as well as, cryopreservation of shoot-tips from adult plants is the most desirable approach for preservation of citrus genetic resources. Plants regenerated from adult shoot-tips are true to type and do not have juvenility characteristics. Therefore, they will not require several years to flower and produce fruits (Gonzalez-Arno et al. 2003; Volk et al. 2013).

xx.x.x Callus and Cell cultures

Cryopreservation of embryogenic callus can be used for the long-term storage of totipotent lines. Embryogenic cultures have shown to be suitable as a source for protoplast

isolation, somatic hybridization, and plant regeneration (Olivares-Fuster et al., 2000). Therefore, cryopreserved embryogenic callus has the advantage that can be readily used for further biotechnology applications anytime, without needing to start a process of callus formation each time, which in addition is an erratic, time consuming, season and genotype dependent process. The availability of frozen material contributes to solve this limitation.

xx.x.x Embryogenic callus (Gonzalez-Arno et al., 1997)

Ovules were excised under sterile conditions from immature fruits collected 2 to 8 weeks after anthesis. The fruits were surface-disinfected by soaking for 20 min in a 2% (v/v) sodium hypochlorite solution containing 0.1% (v/v) wetting agent Tween 20, and the excised ovules were cultured on semisolid MS (Murashige and Skoog, 1962) medium containing 100 mg.L⁻¹ *i*-inositol, 0.2 mg.L⁻¹ thiamine HCl, 1 mg.L⁻¹ pyridoxine HCl, 1 mg.L⁻¹ nicotinic acid, 50 g.L⁻¹ sucrose, 500 mg.L⁻¹ malt extract and supplemented with 10 g.L⁻¹ agar. Ovules were cultured in dark for 2-3 months.

When an embryogenic mass of friable callus (white to yellow) started appearing, calluses were removed from the ovules, and then subcultured every 4-6 weeks on the same medium and culture conditions until obtaining enough material. For developing cryopreservation experiments, calluses were used 3-4 weeks after the last subculture.

About one-third of sterile 2 ml cryotubes were filled with fragments of calluses which were pretreated in liquid medium with sucrose concentrations (0.15, 0.3, 0.6 or 0.9 M) or glycerol (0.1, 0.3, 0.5, or 0.7 M) combined with Dimethylsulfoxide (DMSO) for 1 h at 0°C. DMSO was added progressively to the liquid medium over a period of 30 min until the final concentration (5 or 10%, v/v) was reached.

Freezing was performed using a programmable freezer (Bio-Cool, FTS Systems, USA) at different slow freezing rates (0.2, 0.5 or 1 °C.min⁻¹) from 0 °C to -20°, -40°, -60° or -80 °C before the rapid immersion in liquid nitrogen. Seeding was induced in samples at -10°C. After cryopreservation, samples were kept for at least 1 h at -196 °C. Rapid thawing was carried out by plunging the cryotubes in a water-bath at +40 °C, and calluses were then transferred directly (without washing) to recovery medium. Calluses, placed onto pieces of filter papers were successively transferred to fresh medium after 1 h, 24 h and 7 days of

culture. The survival rate was evaluated 60 d after freezing, and corresponded to the percentage of calluses which had increased in size during the recovery period.

As result, the best cryopreservation conditions for citrus embryogenic calluses comprised the use of mixture DMSO 5 % + sucrose 0.9 M or DMSO 10 % + sucrose 0.6 M for 1h at 0 °C, and freezing at 0.5 °C.min⁻¹ down to -40 °C before immersion into liquid nitrogen. This protocol allowed obtaining up to 50 % of viability, and recovery was evident with the formation of a new mass of calluses and then, of several somatic embryos.

xx.x.x Cell suspensions

The first cryopreservation report with nucellar cells of citrus (Sakai et al., 1990, 1991) and using the vitrification solution PVS2, which had been recently developed at that time, was performed with cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka). Cells were sufficiently dehydrated with the highly concentrated solution PVS2 [30% glycerol (w/v) + 15% ethylene glycol (w/v) + 15% (w/v) DMSO + 0.4 M sucrose] (Sakai et al. 1990) at 25 °C for 3 min, then transferred to plastic cryotubes and finally, directly plunged into liquid nitrogen. Citrus cells were successfully cryopreserved by vitrification and the average rate of survival was about 90% (Sakai et al. 1990).

By using a classical protocol with slow freezing regime was performed cryopreservation of cell suspensions of *Citrus deliciosa* Tan. After a one-hour cryoprotective treatment with 0.15M sucrose and 5 % DMSO, cells were prefrozen to -40°C at 0.5 °C.min⁻¹ and immersed in liquid nitrogen. After rapid thawing, survival ranged between 50 and 60% of unfrozen controls. Proliferation in liquid medium was optimal after a 5-day recovery period on solid medium. Production of somatic embryos was higher with cryopreserved cultures than with unfrozen control cultures (Aguilar et al., 1993)

A simplified cryopreservation process (using Mr. Frosty device) was experimented with a cell suspension of willow leaf mandarin and embryogenic calluses of six varieties of Citrus. Its efficiency was comparable to that of the standard freezing protocol developed-previously for these materials, which required the use of a programmable freezing apparatus. The cell suspension could be frozen without modifying the original pretreatment conditions (0.15 M sucrose + 5 % DMSO). Embryogenic calluses of five varieties out of six experimented

withstood cryopreservation with the simplified freezing process. Optimal results were obtained by increasing the DMSO concentration to 10 or 15 % (F. Engelmann et al. 1994),

In general, embryogenic callus and suspension cell cultures have been cryopreserved with high level of success using different protocols based either on dehydrative slow cooling or vitrification procedures. Very high survival rates have been obtained with callus and suspension cultures originating from a wide range of genera, species and cultivars. In all instances cryopreserved callus produced embryos and whole plants. Despite some limitations, the conservation of embryogenic callus and cell suspensions allows preservation of material in a form ready for biotechnological applications.

xx.x.x Cryopreservation of Ovules and Somatic embryos (Gonzalez-Arno et al., 2003)

Ovules are immature seeds which can be recovered in large numbers from ovaries and small fruits even from seedless cultivars. In polyembryonic species ovules cultured *in vitro* produce true-to-type plants through the development of somatic embryos from nucellar tissues.

Ovules of ‘Pineapple’, ‘Navel Foyos’ and ‘Parent Navel’ sweet orange (*Citrus Sinensis* Obs.), ‘Duncan’ grapefruit (*C. paradise* Macf.), ‘Fino’ lemon (*C. limon* (L.) Burm. f.), common mandarin (*C. deliciosa* Ten.) and sour orange (*C. aurantium* L.) were excised from immature fruits collected 2 to 8 weeks after anthesis collected from field grown trees of the germplasm bank maintained at Instituto Valenciano de Investigaciones Agrarias (IVIA), Valencia, Spain. The fruits were surface-disinfected by soaking for 20 min in a 2% (v/v) sodium hypochlorite solution containing 0.1% (v/v) wetting agent Tween 20. Ovules were excised from cut open fruits under sterile conditions and cultured overnight on semisolid MS (Murashige and Skoog, 1962) medium containing 100 mg.L⁻¹ *i*-inositol, 0.2 mg.L⁻¹ thiamine HCl, 1 mg.L⁻¹ pyridoxine HCl, 1 mg.L⁻¹ nicotinic acid, 50 g.L⁻¹ sucrose, 500 mg.L⁻¹ malt extract and supplemented with 10 g.L⁻¹ agar.

Cryopreservation of ovules was performed following the basic protocol of encapsulation-dehydration technique. Ovules were encapsulated in calcium alginate (3 %) beads and pregrown in liquid medium with various sucrose concentrations (0.75, 1.0 or 1.25 M) for various pregrowth durations (1 to 7 d.). In some cases, the sucrose concentration in the pregrowth medium was increased progressively (0.3 M + 0.5M+ 0.75M; 0.75 M + 1 M or

0.75 M + 1 M + 1.25 M) by transferring samples after 1, 3 or 4 days in culture to media with the higher sucrose concentrations. Encapsulated ovules were then dehydrated at room temperature down to 20-25 % of moisture content (MC, fresh weight basis) under the sterile air current of the laminar flow cabinet, and placed in sterile 2 mL polypropylene cryotubes for freezing performed either rapidly by direct immersion of the cryotubes in liquid nitrogen or slowly, by cooling at $0.5\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$ from $20\text{ }^{\circ}\text{C}$ to $-40\text{ }^{\circ}\text{C}$ before immersion in liquid. Survival of ovules was evaluated after 6-9 months of culture by counting the number of ovules which formed embryos.

The results obtained before cryopreservation, demonstrated that ovules were able to withstand high sucrose concentrations (0.75M, 1M and 1.25M) even under prolonged treatments. However, survival was always very low and erratic after the immersion in liquid nitrogen (ranged from 1 to 16% depending on citrus specie). Other cryopreservation approaches as vitrification and encapsulation-vitrification procedures were also assayed in parallel, but both resulted completely unsuccessful (González-Arno, unpublished results). It would be interesting and advisable to try the most recent cryogenic approaches concerning to V and/or D-Cryoplate techniques (Yamamoto et al. 2011; Niino et al. 2013), which would allow the manipulation of abundant number of ovules attached to cryo-plates, and increasing to ultra-rapid the cooling rate.

xx.x.x Somatic embryos (Gonzalez-Arno et al. 2003)

The first report of cryopreservation of somatic embryos of citrus, referred to embryos derived from *in vitro* cultured ovules of 'Washington navel' sweet orange and included a conventional cryogenic protocol (Marin and Duran-Vila 1988; Marin et al. 1993). Careful selection of embryos at early developmental stages was required to achieve survival, which represented an initial drawback in the freezing procedure. The availability of sufficient material at the right physiological stage under tissue culture conditions was limited, since this is an uncontrolled biological process. Moreover, the results after cryopreservation could not be improved either by selecting uniform embryo populations or by modifying the cooling rates during freezing. Survival was always low and erratic, ranging from 3.7% to a maximum of 30.5%. Recovery of cryopreserved cultures was in the form of secondary embryogenesis originating from the surviving zones of the embryos. Therefore, this first

protocol set up was unsuitable for long-term conservation of Citrus germplasm (Duran-Vila 1995).

Later, further cryopreservation studies focused on using the encapsulation-dehydration approach. Somatic embryos were induced *in vitro* from two biological sources: ovules isolated from immature fruits and from thin cut layer explants of stigma, style and ovaries (Carimi et al. 1999).

Ovules of the following eleven genotypes were assayed: ‘Washington Navel’, ‘Navalete’, ‘Sucreña’, ‘Washington Foyos’ and ‘Bonanza’ sweet orange, common mandarin and ‘Page’ ((*C. paradise* x *C. tangerina*) x *C. clementina*), ‘Calabacita’ and ‘Cajel’ sour orange, volkamer lemon (*C. volkameriana* Ten. & Pasq.) and alemow (*C. macrophylla* Wester). Ovules were cultured on the medium described above and under a light regime of 16 hrs light/8 hrs dark with a photosynthetic photon flux (PPF) of 43 $\mu\text{mol.m}^{-2}\text{s}^{-1}$.

Thin cut layer explants from stigma, style and ovaries of the following thirteen genotypes were assayed: ‘Pineapple’ sweet orange, ‘Sevillano’ sour orange, ‘Fino’ lemon, ‘clemenules’ clementine (*C. clementina*), ‘Avana Apireno’ mandarin, ‘Fortune’ (*C. clementina* x *C. tangerina*), ‘Kinow’ (*C. nobilis* x *C. deliciosa*), satsuma (*C. unshui* (Mak.) Marc.), ‘Star Ruby’ grapefruit, Rangpur lime (*C. limonia* Obs.), Rough lemon (*C. jambhiri* Lush), volkamer lemon and alemow. Stigma, style and ovaries were obtained from flowers collected before opening and surface-disinfected by immersion for 10 min in a 2% (v/v) sodium hypochlorite solution containing 0.1% (v/v) wetting agent Tween 20, followed by three 3-min rinses in sterile distilled water. After disinfecting, the flowers were opened under sterile conditions and whole pistils dissected with a scalpel. Stigmas, style and ovaries were cut perpendicularly to the longitudinal axis at a thickness of 0.4-0.5 mm. The thin cut layers were placed with the abaxial surface towards the semisolid culture medium supplemented with 7 g.L⁻¹ agar. Lighting culture conditions were the same as described above. For the cryopreservation experiments, embryos were used when they became available either at globular, torpedo, and heart shape stages, without any specific selection of a physiological stage. Long term maintenance of the embryos in the original culture media was avoided to minimize the occurrence of a secondary embryogenesis. Survival of somatic embryos was evaluated after 1 month of culture by counting those which germinated.

Thin cut layer explants from stigma, style and ovaries cultured *in vitro* regenerated somatic embryos only from ‘Fino’ lemon, ‘Avana apireno’ mandarin and ‘Star Ruby’

grapefruit out of 13 genotypes assayed. Nevertheless, they were a useful additional source for producing citrus somatic embryos of different species. An illustration of somatic embryos obtained by *in vitro* culture of thin cut layer explants is presented in Figure 2.

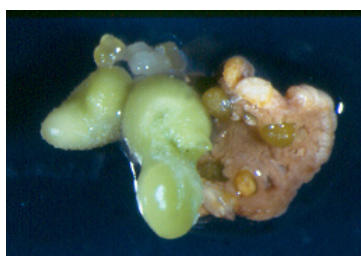


Fig. 2 Citrus somatic embryos derived from thin cut layer explants cultured *in vitro*.

The best cryopreservation conditions irrespective of the source used for obtaining the somatic embryos, comprised the encapsulation of tissues in calcium alginate beads (3%), preculture in liquid medium containing 0.75 M sucrose (24 h), desiccation of beads with silica gel down to around 20% (fresh weight basis), rapid immersion in liquid nitrogen followed by slow rewarming. In general, survival was high and ranged between 75 and 100% after cryopreservation. Results obtained with explants derived from stigma, style and ovaries, respectively, are shown in Table 3.

Table 3. Survival of cryopreserved somatic embryos obtained by *in vitro* culture of thin cut layer explants. Alginated coated embryos were 1d-pregrown in 0.75M sucrose and desiccated down to 20-25% moisture content prior to rapid cooling.

Species	Survival (%)*		
	Pregrowth	Desiccation	Freezing
Lemon (Fino lemon)	100 (4/4)	67 (6/9)	76 (29/38)
Mandarin (Avana Apireno)	73 (8/11)	90 (9/10)	95 (19/20)
Grapefruit (Star Ruby)	100 (3/3)	-	100 (10/10)

-: not tested, * In brackets, number of surviving ovules out of treated ones.

Reprinted from Gonzalez-Arnao et al., 2003

In all instance, after cryopreservation the whole embryo remained green and germinated producing normal plantlets (Figure 3).



Fig. 3. Adventitious embryos produced from a cryopreserved encapsulated somatic embryo originated from ovules.

Following *in vitro* culture of ovules or thin cut layer explants, the embryos that become available are usually in different development stages. Therefore, the selection required for the application of the classical cryogenic protocol earlier reported (Marin and Duran-Vila 1988) is cumbersome and very inconvenient. However, this difficulty was overcome using the encapsulation-dehydration method, which always allowed consistent high survival rates without any previous selection.

Somatic embryos are easily obtained from all polyembryonic species and they regenerate true to type plants. Therefore, the encapsulation-dehydration approach for somatic embryos can be efficiently used for the conservation of citrus germplasm.

Cryopreservation of shoot-tips

Juvenile shoot-tips (Gonzalez-Arno et al., 1998, 2000)

Seeds of *Poncirus trifoliata* (L) Raf. were devoid of testa and surface sterilized for 10 minutes in 0.7 % sodium hypochlorite solution with few drops of tween 20. After 3-4 rinses with sterile distilled water, seeds were aseptically transferred in test tubes on semi-solid MS medium (Murashige and Skoog, 1962) supplemented with 50 g.L⁻¹ sucrose, 0.5 mg.L⁻¹

benzylaminopurine (BAP), 0.25 mg.L⁻¹ indole butyric acid (IBA), 40 mg.L⁻¹ adenine, 750 mg.L⁻¹ malt extract and 10 g.L⁻¹ agar. They were maintained at 26 °C under 8 h light/ 16 h dark photoperiod under a light intensity of 40 μmol.m⁻².s⁻¹. Shoot-tips (size: 0.5-1 mm) were excised from *in vitro* plants 20 d after the last subculture and left overnight on standard medium for recovery.

Cryopreservation experiments were carried out following the basic protocol of encapsulation-dehydration technique. Shoot-tips were encapsulated in alginate (3 %) beads and pregrown in liquid medium with various sucrose concentrations (0.3 to 1 M) for various pregrowth durations (1 to 10 d.). In some cases, the sucrose concentration in the pregrowth medium was increased progressively by daily transfer of apices in media with higher sucrose concentration from 0.3 up to 1 M. Encapsulated shoot-tips were then dehydrated at room temperature down to 20-25 % of moisture content (MC, fresh weight basis) under the sterile air current of the laminar flow cabinet and transferred in sterile 2 mL polypropylene cryotubes. Samples submitted to pregrowth with progressive increase of sucrose concentrations were desiccated under the same conditions down to several MCs (36, 28, 23, 17 and 15 %). After desiccation, freezing was performed either rapidly by direct immersion of the cryotubes in liquid nitrogen or slowly, by cooling at 0.5 °C.min⁻¹ from 20 °C to -40 °C before immersion in liquid nitrogen, using a programmable freezer (Bio-Cool, FTS Systems, USA). Samples were kept for at least 1 h at -196 °C. For thawing, the cryotubes were placed under the air current of the laminar flow cabinet for 2-3 min. Beads were then transferred in Petri dishes on standard semi-solid medium. Shoot-tips were re-cultured for the first week in the dark, and then transferred under standard lighting conditions.

The best cryopreservation conditions determined for shoot-tips of *Poncirus trifoliata* rootstock comprised pregrowth in liquid medium with 0.5 M sucrose (3-4 d) or in media with increasing sucrose concentrations (0.3M/24 h + 0.5M/24 h + 0.75M/24 h), desiccation down to around 20 % of water content in the beads, and freezing preferably slow. These optimal conditions allowed achieving survival with shoot-tips of two additional citrus rootstocks: Troyer citrange and Carrizo citrange. The protocol involved preculture with the progressive increase (from 0.3 M up to 0.75 M) in sucrose concentration, desiccation to 20–25% MC and slow freezing. Survival (%) of shoot-tips from the three citrus rootstocks species after preculture, dehydration and slow freezing is shown in Table 4. Growth recovery

of juvenile citrus shoot-tips after cryopreservation occurred directly without callus formation (Fig. 4).

Table 4. Effect of encapsulation-dehydration protocol on the survival (%) of apices from three different citrus species (reprinted from Gonzalez-Arnao et al., 2000).

Species	Survival (%)		
	Pregrowth	Dehydration	Slow freezing
<i>Poncirus trifoliata</i>	100	90±5	44±4
Troyer citrange	100	83±4	36±3
Carrizo citrange	100	95±5	55±5

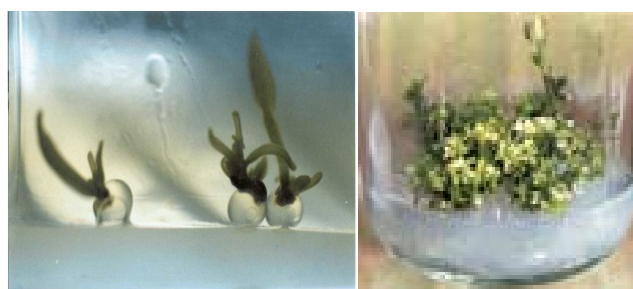


Fig. 4. Growth-recovery and regeneration of citrus juvenile shoot-tips cryopreserved by encapsulation-dehydration.

xx.x.x Adult shoot-tips

Later, the next significant achievement was the cryopreservation of adult citrus shoot-tips performed by Volk et al., 2012. Shoot tips were excised from actively growing vegetative flushes of protected trees, surface-disinfected, and precultured overnight on semi solid medium with 0.3 M sucrose, before the exposure to loading (2 M glycerol + 0.4 M sucrose) for 20 min and vitrification (PVS2) solutions for 30 or 60 min at 0 °C, prior to direct immersion in liquid nitrogen. Rewarmed shoot tips post-cultured overnight on survival

medium were then micrografted on 'Carrizo' seedling rootstocks to produce whole plants. Micrografted shoot tips recovered quickly and rooted plants could be transferred to the greenhouse within months. Regrowth of whole plants after micrografting averaged 53 percent for cryopreserved shoot tips of cultivars representing eight Citrus and Fortunella species (Volk et al., 2012). It was also found that freezing on foils resulted in higher levels of survival than freezing in vials. For the seven cultivars included in the experiment, the average viability of shoot tips cryopreserved using cryovials was 53%, and the viability of shoot tips cryopreserved on foil strips was 83% (Bonnaert et al, 2014)

xx.x.x Seeds and Embryonic axis

King et al. (1981) determined that the longevity of various citrus seeds was improved when the storage temperature and the moisture contents of seeds were decreased.

Seeds of species tolerant to desiccation can be successfully cryopreserved by direct immersion and storage in liquid nitrogen after partial desiccation (air drying or silica gel drying), without any cryoprotective treatment. For species, whose seeds are sensitive to desiccation, partially dehydrated embryonic axes excised from seeds can also be cryopreserved by direct immersion in liquid nitrogen (Duran-Vila et al. 1997). Marzalina and Krishnapillay (1999) reported that MC of citrus seeds should be reduced to 20% or less to achieve high germination after storage in liquid nitrogen.

Normah and Serimala (1995) reported that *Citrus aurantifolia* seeds can be successfully cryopreserved at -196 °C after desiccating them to a moisture content of 12.93 % (50% viability) while seeds of *C. halimii* presented only 25 % viability after cryopreservation at moisture contents of 9.5 %. The Seeds of *C. hystrix* were highly sensitive to desiccation as they failed to germinate when the moisture contents were reduced to 27 % and thus did not survive cryopreservation. On the other hand, the embryonic axes of the three Citrus species gave higher percentage of survival after cryopreservation. Survival rate was 100 % in *C. aurantifolia* and *C. halimii* embryonic axes with moisture contents of 9-11 % and 16.6 % respectively. With *C. hystrix* axes, the highest survival rate obtained was 60 % at a moisture content of 11.04 %.

Mumford and Grout (1979) and Lambardi et al. (2007) have reported high tolerance of *C. limon*, *C. halimii* and *C. volkameriana* seeds to dehydration below 10% MC.

Seeds of trifoliolate orange (*Poncirus trifoliata* (L.) Raf.) are sensitive to desiccation, and could not withstand reduction in moisture level below 20%, whereas the excised embryonic axes could be easily desiccated to moisture levels as low as 14% without much loss in viability, and could be successfully cryopreserved in liquid nitrogen (Radhamani, J. & Chandel, K.P.S. Plant Cell Reports (1992) 11: 372)

Intact seeds of two citrus species were desiccated in a sterile air flow to 16% (*C. sinensis*) or 10% (*C. aurantium*) moisture content, and immersed in liquid nitrogen. After cryopreservation, both citrus species showed a high (93% germination) survival (Lambardi et al. 2004).

Embryonic axes of *C. sinensis* [L.] Osb. ('Pineapple' sweet orange) of *C. reticulata* Blanco (mandarin) and *C. limon* L. (lemon) were successfully cryopreserved using the encapsulation-dehydration technique and the best recovery rate was attained by axes with water content in the range of 0.15–0.17 g H₂O/g dry mass. Sufficiently dehydrated embryonic axes that were cooled to –196°C developed into seedlings without any intermediary callus formation, after transfer to recovery medium (Santos and Stushnoff, 2014).

xx.x.x Pollen

Application of cryogenic techniques to preserve pollen viability for prolonged durations has been found suitable for conservation of Citrus germplasm (Kobayashi et al. 1978) For instance, pollen of four citrus cultivars (*C. limon*, *C. aurantifolia*, *C. sinensis* and *Poncirus trifoliata*) were collected and cryopreserved by direct immersion in liquid nitrogen (-196 °C) for 3.5 years (Ganeshan and Alexander, 1991). This indicates that rapid cooling to an ultra-low temperature had no a harmful effect on the biological integrity of pollen under storing cryogenic conditions.

In Table 5 are presented some examples of cryopreservation of woody plant germplasm.

«Table 5»

Conclusion

This chapter reviewed the progresses of different *in vitro* approaches for the conservation and safeguarding of woody plant genetic biodiversity. Several study cases were presented and described to exemplify the protocols developed for storing the germplasm to medium and long-term using different *in vitro* procedures.

The advances of *in vitro* conservation techniques have enhanced the availability of reliable strategies and provided some useful approaches, not only for woody plants, but also for improving the *ex situ* preservation of plant biodiversity in general. Slow growth could be more widely used to provide safer alternatives to the field gene bank for species that are readily propagated *in vitro* (Withers and Engelmann, 1997), while cryopreservation is the safest option to support the storage of cell lines and of embryogenic cultures, to support the genetic transformation and the biotechnological breeding programs, in addition, it is a valuable complementary alternative for orthodox seeds in the context of seed bank. Moreover, cryopreservation is very important for the long-term conservation of biodiversity of tropical and subtropical forest species, and for many other plants with recalcitrant seeds and/or vegetatively propagated (Gonzalez-Arno et al., 2014).

There is not a unique recipe for conservation, but certainly, there is a common concern about using the science to protect plant biodiversity around the world. Now, it is well recognized that the most appropriate strategy is combining approaches of *ex situ* and *in situ* methods to complement the conservation of plant germplasm.

Currently, the slow growth and cryopreservation techniques have been significantly integrated to many conservation programs, to the benefit of both germplasm management and research. Various research institutes, private companies and genebanks around the world have implemented these approaches to maintain important collections and/or back-ups of diverse woody species with different purposes. Such is the case of Instituto Valenciano de Investigaciones Agrarias (IVIA), Valencia, Spain, where is kept a large cryopreserved collection of calluses to support the breeding projects of citrus species (Olivares-Fuster et al., 2000). The private company Sylvagen in Vancouver, Canada, which keeps conifer embryogenic cell lines, and NCGR (Corvallis) and NCGRP (Fort Collins) both in USA, which maintain collections of several temperate fruit trees (Hummer & Reed 2000).

The basic and practical knowledges and the comprehension of the physiological response of different plant species are continuously improved allowing their better adaptation and increasing the effectiveness of these in vitro methods. In much cases, the storage protocols developed are shared with other researchers, genebanks, and the expertise is used worldwide to extend much more the benefits of these biotechnological approaches for the safe preservation of woody plant germplasm.

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