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Cryopreservation of germplasm of tamarillo (*Solanum bataceum* Cav.)

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biodiversidade e Biotecnologia Vegetal, realizada sob a orientação científica da Doutora Sandra Correia (Centro de Ecologia Funcional) e do Professor Doutor Jorge Canhoto (Universidade de Coimbra)

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Part of these results, regarding the cryopreservation of embryogenic masses of tamarillo, will be submitted to the journal *Trees*.

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2.4-D – 2.4-Dichlorophenoxyacetic acid

CE – Embryogenic callus

Ctr -80 ºC – -80 ºC control

Ctr CH – Cold hardening and preculturing control

Ctr DMSO – DMSO control

Ctr LN – Liquid nitrogen control

Ctr N – Normal control

Ctr PVS2 – PVS2 control

DMSO - Dimethyl sulphoxide

LN – Liquid nitrogen

MS – Murashige and Skoog culture medium

NAA – 1-Naphthalene acetic acid

PVS2 – Plant vitrification solution 2

RT – Room temperature

SE – Somatic embryogenesis

SUMMARY

Germplasm preservation has a very important role in current breeding and conservation programs. Due to either the genetic alterations that plant breeding may impose and to the extinction rate numerous plants are facing, it is crucial to store genetic resources to minimize biodiversity losses. The success of *in vitro* conservation depends on the storage duration. For long-term storage, cryopreservation is the only method available. Slow cooling and vitrification procedures were tested on tamarillo's (*Solanum betaceum* Cav.) embryogenic tissues and vitrification was found to be an adequate method to apply to this species.

Tamarillo is a solanaceous tree from the Andean region and economically important due to its fruits high nutrient content. One of the most important biotechnological tools that can be applied to this species is somatic embryogenesis (SE), which protocol was elaborated at the Laboratory of Plant Biotechnology of the University of Coimbra. Induction of somatic embryos on this species can be achieved through a "two-step" process. In this procedure zygotic embryos and leaf segments are first exposed to MS (Murashige and Skoog) medium with an auxin and high concentrations of sucrose forming embryogenic and non-embryogenic masses (that can be subcultured). Embryogenic masses are then transferred to an auxin-free medium to allow somatic embryos development. However, maintenance of the *calli* requires frequent subcultures making the process labour-intensive and space-consuming. Furthermore, in long-term cultures, karyotype aberrations and other events causing somaclonal variation occur resulting in atypical embryos.

Four different lines of embryogenic masses were submitted to a 5-day cold hardening stage (4 ºC in the dark) and a 3-day preculture on a hormone-free MS medium with an increasing sucrose concentration of 0.25 M, 0.5 M and 1 M, 24 h each (4 °C in the dark). For slow cooling the tissues were treated with cryoprotectant DMSO solution (60 and 90 min) and slowly cooled to an intermediate temperature of -40 °C, promoting the cytoplasm

vitrification, upon a subsequent immersion in liquid nitrogen (LN). In vitrification, a loading solution was added (30 min) at room temperature (RT) for osmo-protection. The loading solution was then removed and changed by plant vitrification solution 2 (PVS2) for 60 min. at 0 ºC, time after which the PVS2 solution was renewed and the embryogenic masses immersed in liquid nitrogen. Following rapid thawing in a water bath at 40 °C the *calli* were then transferred to the same induction medium (MS supplemented with 5 mg L^{-1} picloram or 2 mg L^{-1} 2.4-dichlorophenoxyacetic acid, 90 g L^{-1} sucrose and 2.5 g L^{-1} Phytagel). For vitrification, before placing the tissues in recovery conditions, the PVS2 solution was replaced with a washing solution (30 min at RT).

The quantification was carried out by monitoring the samples weight during four months at regular intervals of one month. The quantification of the tissues was performed during the exponential growth stage, therefore, the *calli's* growth rate was calculated using an exponential regression of mass over time. The average growth rate of cryopreserved *calli* reached about 0.5 g/month for three lines (50 % less than normal control). As for germinated plants, a 50 % decrease was observed for cryopreserved *calli* when compared to the normal control.

The same procedures were applied to tamarillo's somatic embryos and meristematic cells. However, the time to get trustable results goes over the time-lapse for the presentation of this thesis. More time will be needed to obtain firm conclusions.

The results so far obtained have shown that cryopreservation by PVS2 vitrification is a viable method to maintain embryogenic masses of tamarillo for it was effective for three of the four lines tested.

Key words: Embryogenic masses, Shoots, Somatic embryos, Vitrification, Woody species

RESUMO

A preservação de germoplasma é extremamente importante nos atuais programas de melhoramento genético e de conservação. Devido às alterações genéticas que o melhoramento de plantas pode impor e às taxas de extinção de numerosas plantas, é crucial armazenar recursos genéticos a fim de minimizar a perda de biodiversidade. Os métodos de conservação *in vitro* dependem da duração de armazenamento pretendida e para armazenamento a longo prazo, a criopreservação é o único método atualmente disponível. Os métodos de criopreservação, *slow cooling* e vitirificação, foram testados em tecidos embriogénicos de tamarilho (*Solanum betaceum* Cav.) e a vitrificação mostrou ser o método mais eficaz a aplicar nesta espécie.

O tamarilho é uma árvore da família das solanáceas proveniente da região andina e economicamente importante devido ao alto teor nutritivo dos seus frutos. Uma das ferramentas biotecnológicas mais importantes que podem ser aplicadas a esta espécie é a embriogénese somática (SE), cujo protocolo foi elaborado no Laboratório de Biotecnologia Vegetal da Universidade de Coimbra. A indução de embriões somáticos nesta espécie pode ser alcançada através de um processo “two-step". Neste procedimento embriões zigóticos e segmentos foliares são primeiramente expostos a um meio MS com auxina e concentrações elevadas de sacarose formando massas embriogénicas e não-embriogénicas (que podem ser subcultivadas). As massas embriogénicas são depois transferidas para um meio isento de auxina para permitir o desenvolvimento de embriões somáticos. No entanto, a manutenção dos calos requer subculturas frequentes o que consome muito espaço e torna o processo trabalhoso. Além disso, quando em cultura a longo prazo ocorrem alterações do cariótipo e outros eventos que causam variação somaclonal, resultando em embriões atípicos.

Neste trabalho, quatro linhas diferentes de massas embriogénicas foram submetidas a uma etapa de 5 dias de habituação (4 °C no escuro) e uma pré-cultura de 3 dias em meio MS (Murashige e Skoog) sem hormonas e com um aumento progressivo na concentração de sacarose: 0,25 M, 0,5 M a 1 M (24 h cada, 4 °C, no escuro). No método de arrefecimento lento (*slow cooling*), os tecidos foram tratados com a solução crioprotetora de DMSO (60 e 90 min) e lentamente congelados até a uma temperatura intermédia de -40 °C, de forma a promover a vitrificação do citoplasma, após imersão em azoto líquido (LN). Na vitrificação para osmo-protecção, à temperatura ambiente (RT), foi adicionada uma solução de carregamento (loading solution; 30 min). Posteriormente essa solução foi substituída pela solução Plant Vitrification Solution 2 (PVS2) durante 60 min. a 0 ºC, tempo após o qual a solução PVS2 foi renovada e as massas embriogénicas foram imersas em azoto líquido. Depois da descongelação rápida num banho a 40 °C, os calos foram então transferidos para o mesmo meio de indução (MS suplementado com 5 mg L^{-1} picloram ou 2 mg L^{-1} ácido diclorofenoxiacético, 90 g L^{-1} sacarose e 2,5 g L^{-1} de Phytagel). Para a vitrificação, antes de colocar os tecidos em condições de recuperação, a solução PVS2 foi substituída com uma solução de lavagem (Washing solution; 30 min RT).

A quantificação foi executada através da monitorização do peso das amostras durante quatro meses, em intervalos regulares de um mês. A quantificação dos tecidos foi realizada durante a sua fase de crescimento exponencial, consequentemente, a taxa de crescimento dos calos foi calculada utilizando uma regressão exponencial de massa/tempo. A taxa de crescimento média dos calos criopreservados atingiu cerca de 0,5 g/mês para três linhas (50% menos do que o controlo normal). Também foi observada uma diminuição de 50% de plantas germinadas para calos criopreservados quando em comparação com o controlo normal.

Os mesmos procedimentos foram aplicados a embriões somáticos e meristemas de tamarilho. No entanto, resultados conclusivos só poderão ser obtidos posteriormente ao período de defesa desta tese.

Os resultados obtidos até agora mostraram que a criopreservação por vitrificação com PVS2 é um método viável para manter massas embriogénicas de tamarilho uma vez que foi eficaz para três das quatro linhas testadas.

Palavras-chave: Embriões somáticos, Espécies lenhosas, Massas embriogénicas, Meristemas Vitrificação

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Germplasm preservation has a very important role in current breeding and conservation programs because it is crucial to store genetic resources to minimize biodiversity losses. From the several methods of conservation that can be used cryopreservation is the only available method that ensures long-term storage (Ozudogru and Lambardi, 2016).

Previous works have shown that embryogenic *calli* of tamarillo are instable in culture, and after one year of subculture, changes in the karyotype have been observed (Currais *et al.*, 2013). Thus, it is important to develop a strategy to maintain these tissues without modifications in the chromosome number to assure the true-to-type cloning of the obtained plants.

Since no work regarding tamarillo's conservation has ever been done, cryopreservation using slow cooling and vitrification procedures were tested on tamarillo's (*Solanum betaceum* Cav.) embryogenic tissues, somatic embryos and shoots in order to define which could be the most efficient approach and it is here described for the first time.

1.2 CRYOPRESERVATION

1.2.1 Biotechnology in conservation of plant biodiversity

Plant preservation is important to ensure plant biodiversity and conservation due to the rate of extinction numerous plants are facing (approx. one third of all worldwide existing plants) (IUCN, 2016). Also, maintaining biodiversity, is crucial for classical and modern plant breeding programmes (Panis and Lambardi, 2006; Popova *et al.*, 2015). Advances in the field of biotechnology were crucial for the entitled modern plant breeding programmes which lead to the production of a new, high valued, germplasm including clones obtained from elite genotypes, cell lines with singular features and genetically

transformed material (Engelmann, 2011). Although conservation of wild, rare and endangered species is imperative, most conservation activities have mainly concerned crop species (Engelmann, 2011; Ozudogru and Lambardi, 2016).

Conservation of these species is mainly in *ex situ* field collections. However, this way of conservation has some disadvantages since external conditions, as diseases and adverse weather conditions, can affect the plants. Moreover, the maintenance of clonal orchards is labour-intensive, expensive and requires huge facilities. It is in this context that biotechnological resources, specifically *in vitro* cultures, are increasingly being seen as an ideal alternative for plant conservation (Panis and Lambardi, 2006; Engelmann, 2011).

In vitro culture is, by definition, the establishment and maintenance of cells, tissues, organs or *calli* under aseptic laboratory conditions (Canhoto, 2010), and it is the most specialized form of genetic resources *ex situ* preservation. *In vitro* propagation protocols have been described for numerous plants and the advantages that tissue culture provides are mainly: 1) the possibility of propagating a given plant genotype at high multiplication rates, 2) the achievement of disease free plants by meristem culture and, 3) the overall decrease of space requirement and costs due to the size of the explants (Engelmann, 2011).

The *in vitro* conservation methods depend on the period of storage duration that is intended. For short and medium-term storage the purpose is to decrease the growth rate. To achieve this, it is necessary to adjust environment conditions, like decreasing the temperature or light, and/or, especially if dealing with more cold-sensitive species, adjust culture medium, as reducing the sugar content or attenuating the minerals elements. However, other parameters, as type of explants, their physiological state, genetic stability, type of culture vessel and the necessity of continuous inputs of material, influence this practice. Also this is not a solution for long-term storage duo to genetic erosion. For long-

term storage, cryopreservation is the only current method available and will be more thoroughly discussed ahead (Engelmann, 2011; Ozudogru and Lambardi, 2016) (Fig. 1).

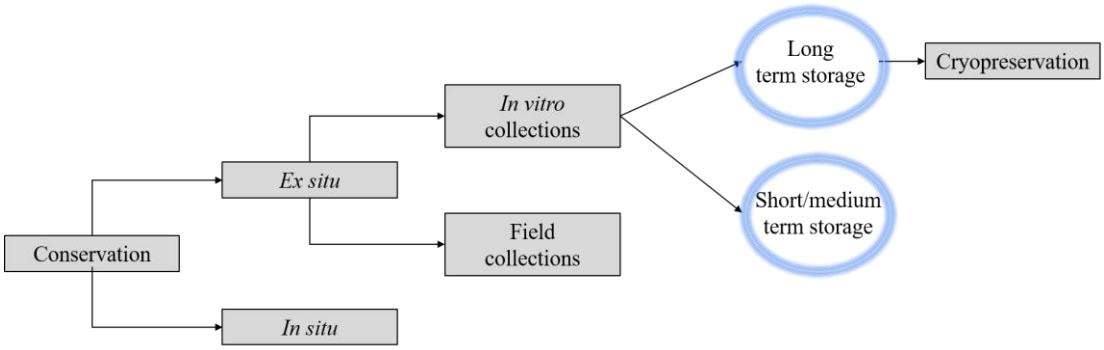


Figure 1 - Diagram situating the role of cryopreservation in conservation of biodiversity.

1.2.2 General aspects of cryopreservation

Cryopreservation is an *in vitro* conservation technique for long-term storage that uses ultra-low temperatures to preserve micro-organisms, small multicellular organisms, complex cellular structures, organs, tissues, primary cells, established cell lines, proteins and nucleic acids (Barun, 2015). The first mammalian cells to be successfully cryopreserved were spermatozoa due to the discovery of the cryoprotective effect of glycerol (Polge *et al*., 1949, cited in Barun, 2015) and since this finding as well as an increase understanding of the causes of cryo-injury, numerous methods have been developed, improved and applied for different types of cells, tissues and organs (Barun, 2015). At cryogenic temperatures metabolic activity is ceased which implies the blocking of biological growth and development of the cryopreserved material. In theory, if the material of interest survives the cryopreservative method, it can be stored for unlimited time (Lambardi *et al.*, 2005; Ozudogru and Lambardi, 2016).

Cryopreservation of biological tissues can only be successful if intracellular ice crystal formation is avoided since these crystals may disrupt the cell membrane causing

irreversible damage. A similar type of avoidance can be found in nature where some plants synthetize specific substance such as sugars, proline and proteins in order to lower their cells freezing-point when at sub-zero temperatures (Panis and Lambardi, 2006). Although this is the theoretical basis of cryopreservation it cannot be applied when dealing with the ultra-low temperatures of cryopreservation (-196 ºC). At this temperature, crystal formation, without an extreme reduction of cellular water, can only be prevented through "vitrification" (the physical process of transition of an aqueous solution into an amorphous and glassy state) which can be achieved by controlled or fast freezing rates and concentrated cellular solutions (Panis and Lambardi, 2006). The goal is to replace some of the water in the cell and its surrounding medium with other compounds (cryoprotectants) so to minimize the damage to the biological material during water physical modifications. When exposed to liquid nitrogen (-196 ºC), the extracellular medium freezes which leads to an increase of its salt concentration becoming a hypertonic solution. This concentration gradient promotes water to leave the cell and its replacement by cryoprotectants (Barun, 2015) (Fig. 2).

Despite the important economic and scientific advantages that this method can provide, basic aspects of cryopreservation are still to be explained to a level that can ensure the efficient application of this technology to any case, as cell lines of closely related species require different parameters and even same cell lines have reproducibility problems when tested in different laboratories (Heine-Dobbennack *et al.*, 2009).

1.2.3 Cryopreservation techniques

Cryoprotective agents

As previously referred, for the success of cryopreservation it is vital to replace some of the intra and extracellular water with other compounds, the cryoprotective agents. Although the mechanism of action of most of these substances are still far from being understood (Panis and Lambardi, 2006), their purpose is to increase the extracellular osmolality, promoting dehydration during cooling, and to cohere residual water, therefore decreasing the damage to the biological material during ice formation. Two main categories of cryoprotectants can be distinguished: 1) penetrating and, 2) non-penetrating (Barun, 2015) (Fig. 2).

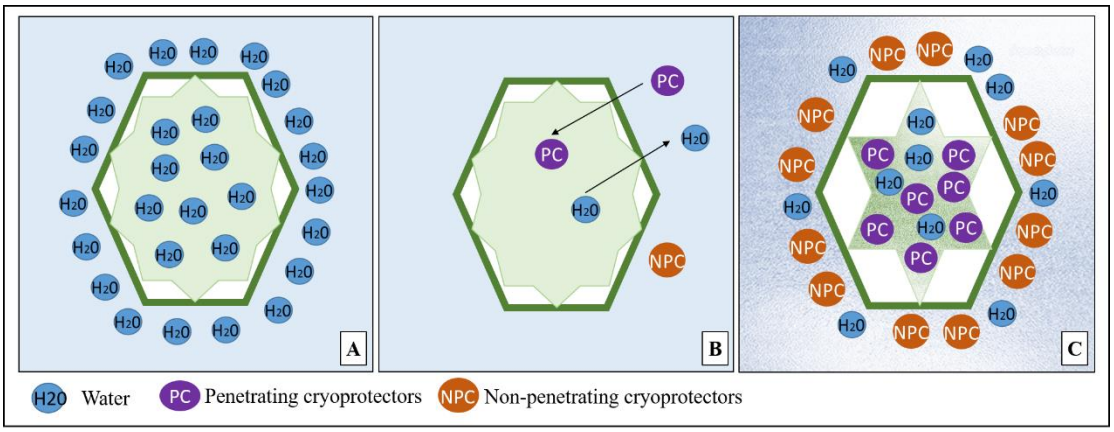


Figure 2 - Schematic illustration of the dehydration process promoted by cryoprotectants. (A) Cells normal environment with high water content; (B) Application of penetrating and non-penetrating cryoprotectants which replace some of the water in the cells and its surroundings preparing the tissues to the low temperature of LN; (C) In LN, crystal formation can only be prevented through "vitrification" (the physical process of transition of an aqueous solution into an amorphous and glassy state) which can be achieved by controlled or fast freezing rates and concentrated cellular solutions.

The penetrating agents are substances for which the cell membrane is permeable and, as so, can enter the cell. Those include compounds like dimethyl sulphoxide (DMSO), methanol, propylene glycol, ethylene glycol and glycerol (Barun, 2015). For several

applications DMSO is preferred for it is extremely fast penetrating the cells, however when its toxicity is problematic, glycerol or amino acids, like proline, are commonly the chosen substitutes (Panis and Lambardi, 2006).

The non-penetrating agents are compounds for which the cell membrane is not permeable to and therefore incapable of entering the cell promoting an osmotic dehydration. This category includes substances like sugars (sucrose and glucose), sugar alcohols (glycerol and mannitol), yolk serum, skim milk and high molecular weight compounds as polyethylene glycol (PEG) (Panis and Lambardi, 2006; Barun, 2015).

Several substances have been tested as cryoprotectants, however glycerol and DMSO are the most commonly used for they have demonstrated more effectiveness for preserving a larger rage of material from cells to small multicellular organisms (Barun, 2015). Nevertheless, the choice on the type of cryoprotectants to use will ultimately depend on the cryopreservation method to apply.

Cryopreservation methods

For the use of cryogenic storage we can assume two main approaches: 1) slow-cooling (Fig. 3A) and, 2) vitrification (Fig. 3B) (Reed *et al.*, 2004).

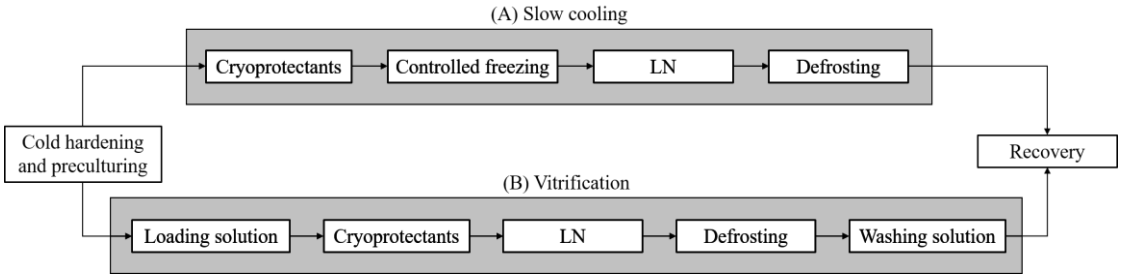


Figure 3 – Cryopreservation methods: (A) slow cooling and; (B) vitrification.

The first method, slow-cooling or slow-freezing, involves a programmed decrease of temperature. Here the tissues are submitted to a treatment with cryoprotectant solutions (sucrose and DMSO) and slowly cooled (-1 ºC / min) in a freezing container (Nalgene® Mr. Frosty) to an intermediate temperature of -40 °C, promoting the cytoplasm vitrification, upon a subsequent immersion in liquid nitrogen (LN). The goal of this technique is to dehydrate the cells down to a minimum safe level where ice formation is non-lethal (Panis and Lambardi, 2006; Ozudogru and Lambardi, 2016). It was the first technique developed, although it requires specific equipment, it uses relatively nontoxic cryoprotectants, takes little technician time, is excellent for storing large plant collections do to the amount of samples that can be processed at one time and has proven itself effective for temperate plants (Barun, 2015).

The second method, vitrification, implies the transition of liquids into solids without ice crystal formation. It involves an exposure to highly concentrated cryoprotective solutions (glycerol, ethylene glycol, DMSO and sucrose) that desiccate and penetrate cells in order to form a highly viscous intracellular solution that forms a metastable glass avoiding ice formation at low temperatures (Lambardi *et al.*, 2005; Ozudogru and Lambardi, 2016). Although cryoprotectants are more toxic than in the slow-cooling method, it is a fast procedure and enables fast recovery, despite the need for careful timing to change solutions, it is commonly used and is effective for both temperate and tropical plants (Reed *et al.*, 2004).

Regardless of the technique applied there are some important key steps for both procedures: 1) culture condition, 2) cold hardening, 3) preculture, 4) cryopreservation methods and, 5) recovery after LN storage (Panis and Lambardi, 2006).

Culture condition refers to the state of the cells before being submitted to cryopreservation. Here aspects like the cells age have an important role in their ability to

survive and develop functional embryos after submitted to LN. Studies reveal that the cells capacity is inversely proportional to their age, this meaning that their viability will decrease throughout the time they are maintained in culture (Ozudogru and Lambardi, 2016).

The second and third steps refer to an adaptive metabolism, a natural defence mechanism that increases the plants ability to survive unfavourable environmental conditions, in this case low temperatures. To do so, the plant rises the osmotic value of the cell solutes by increasing substantially compounds like protein, sugars and amino acids (proline) (Panis and Lambardi, 2006). To induce the expression of the genes responsible for these compounds variations, the tissues are placed at 4 ºC. However, this is not enough to achieve the moisture necessary for vitrification. Thus it is imperative to promote dehydration tolerance induced by preculturing cells in a medium with an increasing concentration of chemical substances like sugars and amino acids (Panis and Lambardi, 2006).

Dehydration tolerance is a progressive measure that is continued in step four by exposure to solutions with penetrating and non-penetrating cryoprotectors to avoid and protect cells from ice crystal formation, as referred previously. Subsequent to this adaptive metabolism, cells are prepared to be submitted to LN.

Finally, cells recovery after LN storage is a vital step to ensure the success of the technique once in this reverse process the dangers of the physical modifications of the water are also present. To overcome this, it is imperative to thaw the tissues rapidly by plunging them directly in a 40 ºC bath and culturing them in an appropriate media (Panis and Lambardi, 2006).

1.2.4 Applications and future perspectives

Cryopreservation has already been successfully applied to a number of different plant material with several advantages:

a) For asexual propagated species, cryopreservation has a wide applicability such in species coverage as in the number of genotypes in a specie. This technique has been productive for root and tubers, fruit trees, ornamental and plantation crops, from both temperate and tropical plants (Engelmann, 2011). Lists of species successfully cryopreserved can be consulted in several publications (Engelmann, 2004; Ozudogru and Lambardi, 2016).

b) Applicability for recalcitrant seed species, in contrast with the previous ones, is still in a very initial stage due to the limited research in the area preformed only by a few teams worldwide. Although there are extensive lists of plant species, whose embryos and/or embryonic axes have already been cryopreserved, when thoroughly analysed only a limited number of those species are in fact recalcitrant seed species, which indicates that there is still space for improvement in this field (Engelmann, 2011).

c) Similarly to recalcitrant seed species, cryopreservation of rare and endangered species is still limited. However, there have been developed protocols for orchids (Hirano *et al.*, 2009), bryophytes and fens (Pence, 2008), wild *Citrus* (Malik and Chaudhury, 2006; Lambardi *et al.*, 2007; Hamilton *et al.*, 2009), several endangered Australian and Japanese species (Turner *et al.*, 2001; Tanaka *et al.*, 2008), etc.

d) When considering this method for large-scale germplasm conservation, there is an increasing number of gene banks and botanic gardens that have adopted this method for different types of material, including seeds, plant tissues, apices, *calli*,

spores or pollen of some species (Engelmann, 2011; Ozudogru and Lambardi, 2016).

e) Cryopreserved cultures enable constant availability of plant material for environmental, toxicological and biotechnological studies.

Although this technique is being used in situations as described above, there are in need new technical methodologies to increase the efficiency and applicability (Popova *et al.*, 2015). Research in this area is mostly performed in universities, institutes, botanical gardens and gene banks, and it is expected that new findings, especially about physiological mechanisms, will contribute expressively in the development of the techniques. It is important, however, to refer that an appropriate conservation strategy must consider both *in situ* and *ex situ* approaches (Engelmann, 2011).

1.3 TAMARILLO

1.3.1 Characterization and distribution

Tamarillo, *Solanum betaceum* Cav. syn *Cyphomandra betacea* (Cav.) Sendt, is a small solanaceous tree native to the South America sub-tropical Andean region from Colombia to Chile (Correia and Canhoto, 2012) (Fig. 4A). From the Andean region it spread to Central America and West Indies, and later to islands of Azores and Madeira and South Europe. It was first introduced in Australia and New Zealand in the late 1800's (TamarilloGrowersAssociation, 2008). The trade name tamarillo appeared in 1967 in New Zealand, where there was an increase of its production and consumption, as a manner to distinguish the plant from its relative tomato (*Solanum lycopersicum* L.) (Morton, 1987). The origin of the name is believed to be a combination of the Maori word "Tama", which implies leadership, and "rillo" from the Spanish word "amarillo"

(yellow), once the yellow tamarillo was the first variety produced, and only in the 1920's the red variety was developed (TamarilloGrowersAssociation, 2008).

Tamarillo is a 2 to 4-meter-high perennial tree, with 10-30 cm deciduous leaves (Fig. 4B). It is on the top of its branches that small groups of flowers develop and although they may appear in different periods throughout the year, they normally blossom in the transition of summer to fall (Fig. 4C). They mainly self-pollinate, characteristic that may be responsible for the low variability observed in natural populations (Barghchi, 1986, cited in Correia, 2011). Tamarillo produces edible fruits, 5 to 10 cm long and 3 to 5 cm wide, egg-shaped and sharp at both ends, isolated or in groups of 3 to 12 units, reaching maturity between October and April. The epicarp's colour, can be dark-red, orange, yellow or a blend of these colours, it is thick and has an unpleasant taste. As for the pulp, it may present the same range of colours as the epicarp, it is consistent, juicy and has a sweet-sour taste (Fig. 4D).

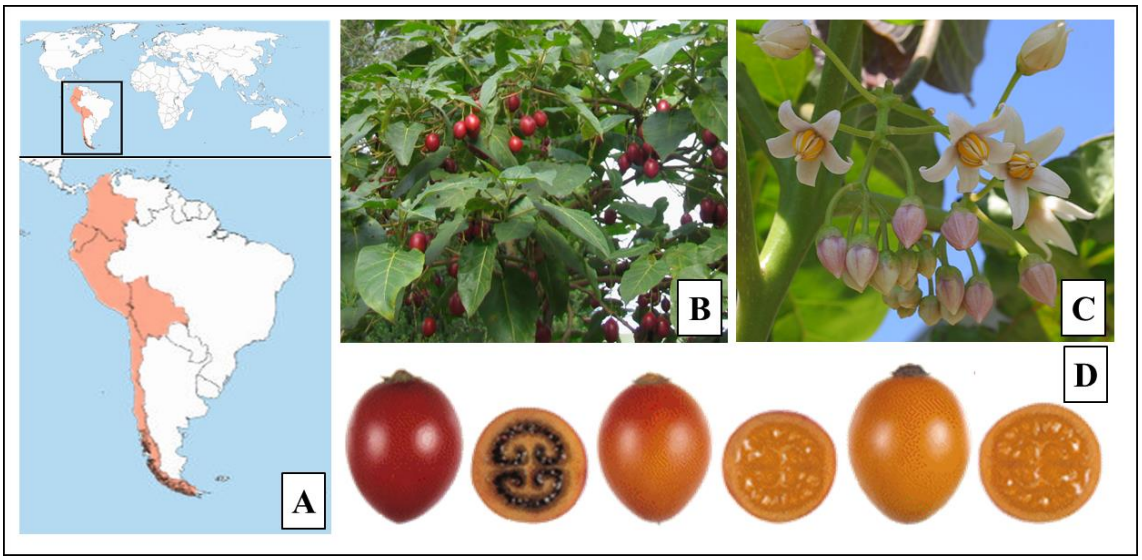


Figure 4 – Tamarillo origin and general aspects (A) Andean region from where tamarillo is native; (B) Tamarillo tree with red fruits; (C) Tamarillo's flowers; (D) Tamarillo's fruits range of colours. (http://funnelandspade.blogspot.pt/2010/06/tamarillos-also-known-as-tree-tomatoes.html; http://www.fancyplants.de/en/exotichome/nwhspec/tamarillo/).

1.3.2 Economic relevance and production

The Solanaceae family, also known as the nightshade family, includes 3000 to 4000 species of which about half belong to the genus *Solanum* (SOL, 2014) (Fig. 5 Above).

The family is highly diversified in habitat, morphology and ecology raging from trees or shrubs to vines, epiphytes and annual plants. Economically, the Solanaceae are the third most important taxon for it includes ornamental and medicinal plants (SOL, 2014) and has the most variable of crops species for agricultural utility including *Solanum lycopersicum* (tomato), *Capsicum* spp. (peppers), *Solanum melongena* (eggplant), *Solanum tuberosum* (potato) and *Nicotiana tabacum* (tobacco) (Mueller *et al.*, 2005). Beyond these well-known plants, this family has less popular species, like tomatillo (*Physalis philadelphica*), naranjilla (*Solanum quitoense*) and tamarillo (*Solanum betaceum* Cav.), that are now increasing their popularity and interest and have, therefore, agricultural and economic potential (Bohs, 1989; FAO, 2016).

Several solanaceous are nowadays models to study: 1) the evolutionary interface between plants and people, for the intensive human selection on these crops coupled with their highly conserved genome organization, allow to explore the basis of phenotypic diversity and adaptation to natural and agricultural environments and, 2) different biological systems as fruit ripening (tomato), plant defence (tomato and tobacco), anthocyanin pigments (petunia) (SOL, 2014) and acquisition of embryogenic ability (tamarillo) (Correia and Canhoto, 2012).

Commercially, tamarillo's fruits have special interest for food industry (Fig. 5 Bellow), to fresh consumption and to produce juices and jams, mostly because of: 1) its high nutrient content, namely vitamins C (30-45 mg/100 g) and E (1.86 mg/100 g), provitamin A, proteins (1.5-2 g/100 g), anthocyanins (antioxidant) and some minerals like potassium

and iron and, 2) its low carbohydrate content (7.7 g/100 g) and low caloric value (approx. 28 cal/100 g) (McCane and Widdowson, 1992, cited in Correia, 2011). Compounds like anthocyanins and carotenoids have been subject of several studies (de Rosso and Mercadante, 2007; Kou *et al*., 2008; Hurtado *et al*., 2009, cited in Correia, 2011) for they may have biological, therapeutic and preventive antioxidant properties thus making this plant a potential valuable resource whose fruits and compounds can eventually improve human health. In fact the commercial production of tamarillos began during World War II precisely as a supply of vitamin C once the availability of more common fruits was restricted. Today the demand for the fruit remains strong due to an increasing awareness of its unique flavour and nutritional qualities (TamarilloGrowersAssociation, 2008) and intensive plant breeding programmes to obtain new cultivars more attractive to consumers were implemented in countries such as New Zealand and USA (Meadows, 2002, cited in Correia, 2011). It is currently grown in California, Argentina, Colombia, Ecuador, Venezuela and New Zealand (Correia and Canhoto, 2012).

Figure 5 – Top: Several species belonging to the genus Solanum; Bellow: Different utilities for tamarillo's fruits. (https://solgenomics.net/documents/img/solanaceae-image-small.jpg; http://www.bite.co.nz/recipe/2159/Tamarillo-bacon-and-feta-salad/; http://www.peplers.co.nz/; http://www.yummly.com/recipes/tamarillo-fruit; https://www.naturespride.eu/products/tamarillo).

Tamarillo cultivars propagation can be accomplished through seeds and cuttings (Prohens and Nuez, 2001). While cuttings develop in small plants with hanging branches which

need an artificial support, seeds produce upright trees with higher branches but do not assure genetic uniformity, vital when propagating selected genotypes (Prohens and Nuez, 2001). When achieving uniformity is the objective of a cultivar, one has to use asexual methods of propagation. Traditional techniques have revealed themselves inadequate for improving cultivars because of the low success of cross-pollination, the high incidence of incompatibility and phytosanitary issues (Mossop, 1977, cited in Correia, 2011). A valid alternative for this plant's breeding are biotechnological methods as *in vitro* cloning and genetic transformation (Barghchi, 1998, cited in Correia and Canhoto, 2012).

1.3.3 *In vitro* regeneration systems

Tamarillo micropropagation methods have been described in various assays (Correia and Canhoto, 2012). *In vitro* cloning can be achieved through: 1) axillary shoot proliferation, which was the first method to be applied (Cohen and Elliot, 1979, cited in Correia, 2011); 2) organogenesis, obtained on leaf explants (Guimarães *et al.*, 1996; Obando and Jordan, 2000) and, 3) somatic embryogenesis, first obtained from mature zygotic embryos and hypocotyls cultures, and later in follow-up studies, from other explants (Guimarães *et al.*, 1996; Lopes *et al.*, 2000; Canhoto *et al.*, 2005).

Micropropagation through axillary shoot proliferation (Fig. 6A), has the advantage of not requiring induction of new meristems for they already exist on the explant, which reduces significantly the regeneration time. Moreover, once *callus* is not formed in this process, the risk of obtaining genetic variations does not occur and all the regenerated plants are genetic and phenotypically identical. In other micropropagation methods *callus* formation is frequent, increasing the probability of occurring chromosomic and genetic anomalies and consequently originating plants with some variations (Canhoto *et al.*, 2005). Also this

technique enables plant exponential multiplication which does not occur for organogenesis and somatic embryogenesis (Canhoto, 2010).

Organogenesis (Fig. 6B) is a process where a unipolar structure (root or shoot) is formed on the explants tissue (leaves, stems, roots, hypocotyls, petals or sepals) and whose vascular system is connected directly to the tissue. In this process there has to be an induction of a new meristem which is possible due to a ratio of auxins/cytokinins or the presence of oligosaccharides in the culture media. There are two processes in organogenesis: 1) direct organogenesis, where meristems are formed on the explants in culture, develop in shoots and after rooting, form new plants and, 2) indirect organogenesis, in this case, gems develop on a callus that was previously formed on the explant and then proceed to rooting (this is the most common process) (Canhoto, 2010). Although this is a potential successful method once theoretically it could develop as many plants as the number of cells of the explant, other limitations may occur, such as contaminations and phenols oxidations and the recalcitrance to regeneration that some species have, especially species from true grasses and woody plants (Chawla, 2003).

Finally, for somatic embryogenesis (SE) (Fig. 6C), the advantage of obtaining embryos is that, because they are a bipolar structure, they do not need rooting so overall the regeneration time is considerably shorter. Also, once the embryos have a unicellular origin this is an ideal method to regenerate genetically modified plants; the new plants are free of diseases; and because embryos are not protected by the seed, this technique enables studying the embryos physiology during its development.

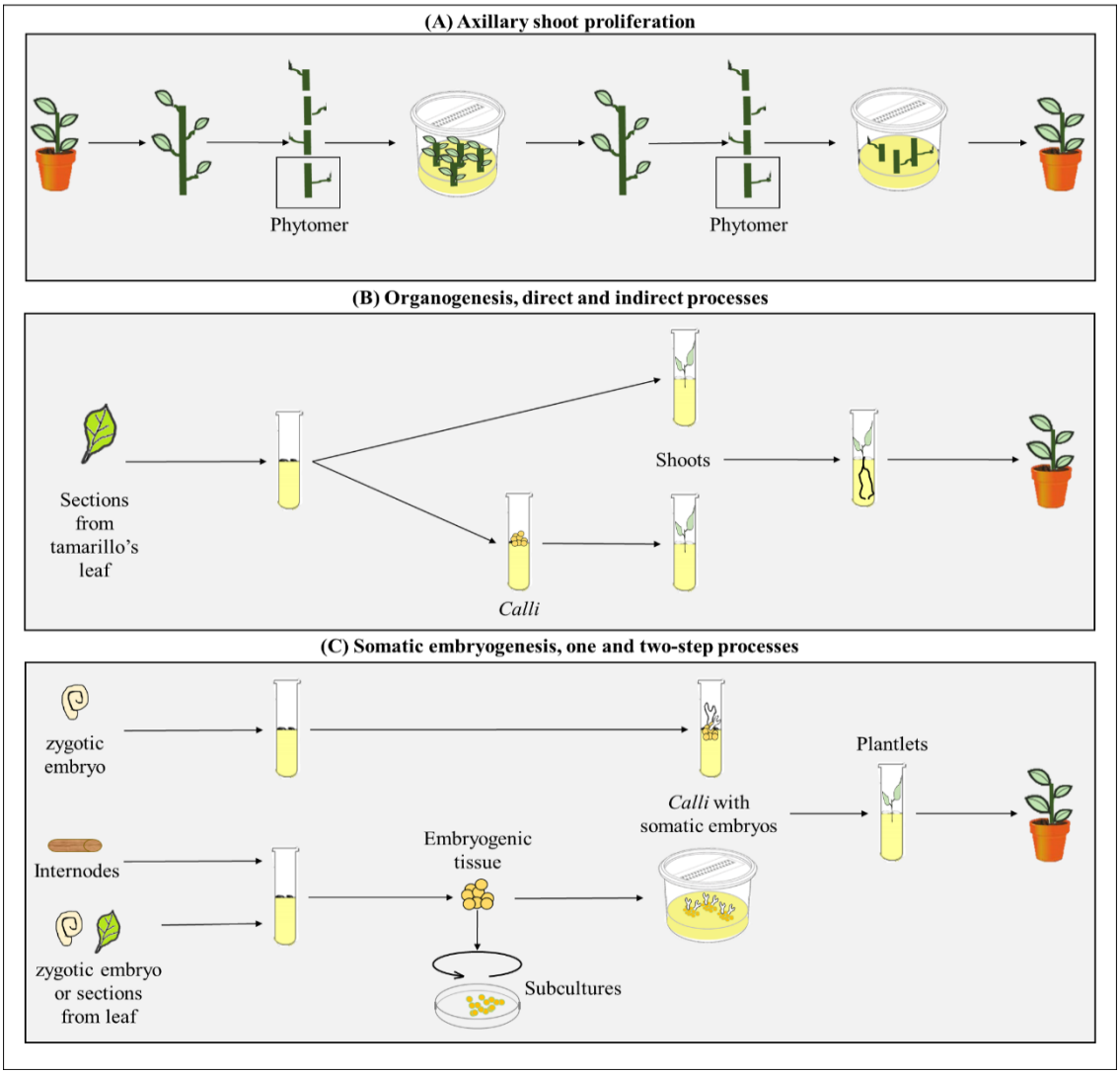


Figure 6 - Tamarillo micropropagation methods: (A) Axillary shoot proliferation; (B) Organogenesis; (C) Somatic embryogenesis.

Somatic embryogenesis in tamarillo was successfully described for the first time in 1988 by our laboratory through its induction on mature zygotic embryos and hypocotyls (Guimarães *et al.*, 1988). Subsequently, the same research group (Laboratory of Plant Biotechnology from the University of Coimbra) elaborated an effective protocol of SE from different kinds of explants (Guimarães *et al.*, 1996; Lopes *et al.*, 2000; Correia and Canhoto, 2012), making this process a model, for studying and understanding the cytological and molecular mechanisms involving embryo's formation and development,

and a process with important applications for plant cloning, genetic transformation and cryopreservative methods (Correia and Canhoto, 2012).

Different explants of tamarillo, as young leaves, mature zygotic embryos, cotyledons and hypocotyls, have the ability to initiate embryogenic cultures despite many selected species, in particular trees, have been reported to have problems to be cloned due to the recalcitrance of adult material for SE induction (Thorpe and Stasolla, 2001; Bonga, 2012). For tamarillo a way to circumvent this issue is to induce SE in leaf segments from *in vitro* plants established by axillary shoot proliferation (Correia and Canhoto, 2012). SE induction is achieved by submitting the explants to an auxin-rich culture medium supplied with high sucrose levels (9%) which raises SE induction efficiency up to 85% (Canhoto *et al.*, 2005). The process has two different paths: "one-step" embryogenesis and "two-step" embryogenesis.

In the first process, explants such as zygotic embryos are exposed to a basic Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with 1-Naphthaleneacetic acid (NAA) and high sucrose content. From the hypocotyl region of the zygotic embryos, *calli* with somatic embryos are formed after a 4 to 6 weeks of culture (Guimarães *et al.*, 1996). Once the somatic embryos are induced and matured in the same NAA medium this process is called "one-step" SE and has been effective in other species such as *Feijoa sellowiana* (Canhoto and Cruz, 1996); and *Myrtus communis* (Canhoto *et al.*, 1999).

The second process is a "two-step" procedure where, first the explants from young leaves or zygotic embryos are exposed to MS media with picloram or 2.4-dichlorophenoxyacetic acid (2.4-D), respectively (Fig. 7A-B), with high concentrations of sucrose. After 4 to 6 weeks of culture slow growing embryogenic and non-embryogenic *calli* are induced (Fig. 7C-E) and subsequently (8 to 10 weeks of culture) clusters of embryogenic tissue, with continuous proliferation, are formed. This embryogenic tissue can be maintained in

subculture, and in a second step, the sucrose content is lowered, the hormones are removed from the embryogenic tissue's media and somatic embryos are developed (Guimarães *et al.*, 1996; Lopes *et al.*, 2000; Canhoto *et al.*, 2005) (Fig. 7F-G). For both processes in order to enable the embryos development it is required to transfer them to a MS 3% sucrose (light conditions).

Somatic embryos formation is not a synchronised event, but during the process the morphological evolution of these structures is similar to the one that occurs during zygotic embryogenesis in which the embryos pass through globular, heart-shaped, torpedo and cotyledonal phases and often present a suspensor-like structure (Correia and Canhoto, 2012). However, if working to evaluate plant regeneration through SE, it is difficult to establish an efficient quantification method duo to the continuous *calli* proliferation, the asynchronous embryo development and the presence of anomalous embryos (Correia and Canhoto, 2012).

For plantlets conversion (Fig. 7H-J), before field conditions, the plantlets are transferred to the greenhouse to be submitted to an acclimatization stage, phase in which there is a high level of plant mortality that seem to be associated to abnormal plant development and unrelated to the embryos abnormalities at the initial stages, once studies have revealed that these embryos during growth recover to a normal phenotype (Canhoto *et al.*, 2005).

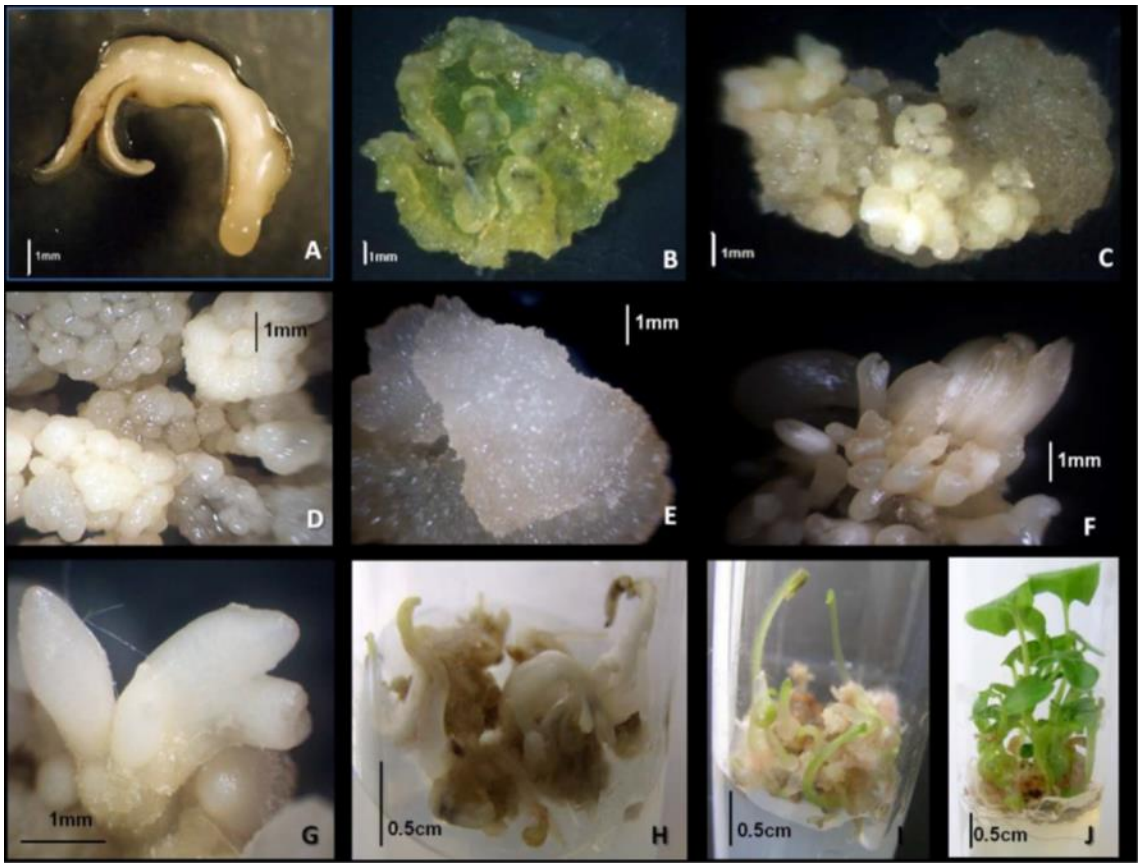


Figure 7 - Somatic embryogenesis in tamarillo. (A) Zygotic embryo on a TD medium; (B) Leaf explant on a TP medium; (C) Embryogenic and non-embryogenic (darker) zones formed on a leaf explant; (D) Embryogenic tissue; (E) Non-embryogenic callus; (F) and (G) Somatic embryos at different developmental stages induced in an auxin free MS medium; (H-J) Somatic embryo conversion and plantlet development on MS medium (adapted from Correia and Canhoto, 2012).

As these conditions are well characterized in tamarillo, this plant stands out as a model to understand this morphogenic process, being its advantages: 1) the possibility to induce SE in different organs derived from adult trees, 2) the storage of the embryogenic tissue in culture for medium-term without losing its embryogenic potential, 3) the embryos conversion into plantlets is simple and effective once it is not required a big amount of initial callus and, 4) the obtainment of embryogenic and non-embryogenic callus from the same explant which is particularly interesting once it may lead to the identification of genes that promote or inhibit the embryogenic process (Correia and Canhoto, 2012).

The fact that this system in tamarillo enables one to acquire embryogenic tissue with high proliferation rate makes SE a tool with great biotechnological potential. However, some lines in long-term culture become unstable and there have been detected variations in the embryogenic tissue as polymorphisms and chromosome abnormalities (Currais *et al.*, 2013). Studies related to this issue revealed that plantlets obtained from five-year old cultures displayed more deviations than the ones obtained from one-year cultures (Currais *et al.*, 2013).

1.4 OBJECTIVES

Given the importance of cryopreservation for genetic preservation and general necessity to understand the mechanisms involved in the process in order to improve and extend the applicability, cryopreservation methods were tested on different tamarillo's tissues/organs.

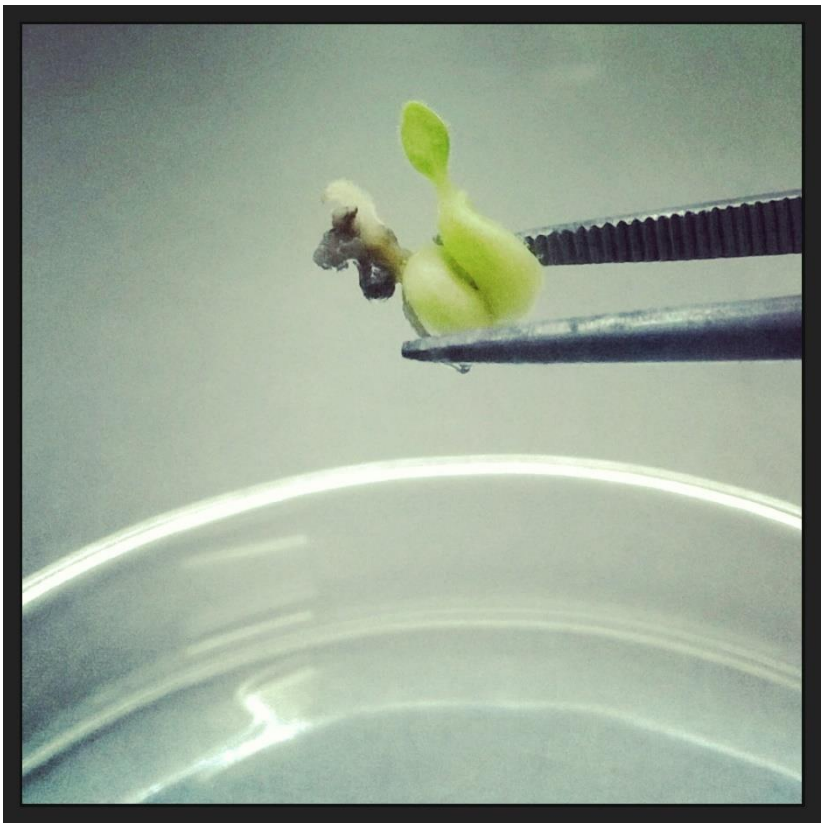
As previously discussed in this chapter, tamarillo has particular interest for: 1) food industry, owing it to its fruits attributes and, 2) academic knowledge and research once biotechnological, morphological and physiological studies may be performed due to the effectiveness of protocols already established for this species. Regarding the food industry, biotechnology may provide uniform and more profitable cultures. This may be achieved with classical and modern plant breeding programmes where characteristics, like the size of the fruits, are manipulated. It is certainly understood that with such modifications in the tamarillo's genetics the long-term storage of its germplasm is imperative for safeguarding biodiversity. Regarding academic knowledge biotechnology research may improve the production of *in vitro* collections. Because of the genetic instability of the cultures, it is vital to preserve the germplasm of all the different varieties

of tamarillo and cryopreservation is possibly the only safe solution available. In this setting, it would be important the cryopreservation of tamarillo's embryogenic tissues, somatic embryos and shoots.

Due to the fact that no work in this area has yet been articulated, the main objective of this study was to establish an efficient cryopreservative method to apply to tamarillo's germplasm preservation. To do so, our experimental design was set to: vary physiological factors, by submitting *calli*, embryos and shoots, from different genotypes of tamarillo, to LN; and test different cryopreservation methods, namely slow cooling and vitrification, in order to select the most adequate for this species.

CHAPTER 2

MATERIALS AND METHODS



2.1 PLANT MATERIAL, CULTURE MEDIA AND CONDITIONS

2.1.1 Embryogenic masses

The embryogenic *calli* used (Fig. 8A) were previously induced from different explants in different years at the Laboratory of Plant Biotechnology from the University of Coimbra (Table 1). This enabled the study of physiological variation (genotype and *calli* age) response to cryopreservation.

Table 1 - *Calli* lines used in the cryopreservation study, with reference to their origin, time in culture and the type of medium where their induction was achieved.

Calli lines	Origin	Time in culture	Induction medium
CE1	Zygotic embryo	5 years	TD
CE2	Leaf explant	5 years	TP
CE3	Leaf explant	3 years	TP
CE4	Leaf explant	1 years	TP

Somatic embryogenesis induction medium TP, used for leaf explants, consisted in MS (Murashige and Skoog, 1962) with 5 mg/L of Picloram; while TD medium, applied to zygotic embryo explants, contained MS with 2 mg/L of 2.4-D. Both media were supplemented with 9% of sucrose and their pH adjusted at 5.7 before autoclaving. When using solid media, 2.5 g/L of Phytagel (Sigma) was added, and after autoclaving, the media was distributed in Petri dishes plates (100 x 20 mm, with 20 mL each). The explants were maintained in the dark at 25 ºC. Embryogenic masses were subcultured in the same media and conditions in which were induced.

2.1.2 Somatic embryos

Somatic embryos (Fig. 8B) were obtained by maturation of masses of the CE4 callus in MS basal medium with 4% sucrose, pH at 5.7 and 2.5 g/L of Phytagel (maturation medium). Polypropylene Combiness © microboxes containers (210 ml of capacity and L

filter lids) were used with 50 ml of maturation medium and they were placed in the dark at 25 ºC for 4 weeks.

2.1.3 Apical and axillary shoots

The apical (Fig. 8C) and axillary shoots (Fig. 8D) used were sectioned from a denominated TLC line, micropropagated in our laboratory through axillary shoot proliferation. Explants were excised from 2 months old plants (time since last proliferation) and were submitted to LN. The use of these structures intended to allow a comparative analysis of the different structures capacity to survive LN and the efficiency of the type of cryopreservative method applied.

The plantlets were maintained in polypropylene Combiness © microboxes containers (565 ml of capacity, XXL filter lids), with 80 ml of a MSBA medium, which consists in MS base medium supplemented with 0.2 mg/L 6-benzylaminopurine (BAP), in a controlled growth chamber (25 ºC, 16h/8h photoperiod).

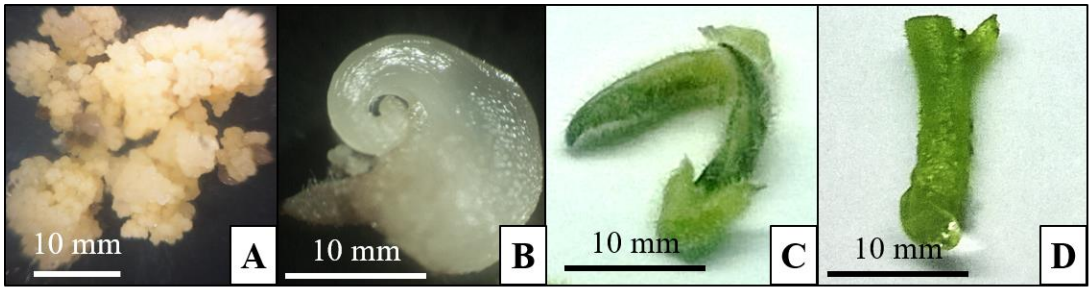


Figure 8 - Tamarillo's structures used in this work: (A) Embryogenic masses; (B) Somatic embryos; (C) Apical shoot and; (D) Axillary shoot.

2.2 COLD HARDENING AND PRECULTURING

The plant material was placed 5 days at 4 ºC in the dark, in the same culture media in which they were subcultured: embryogenic tissue in TP or TD media, somatic embryos in maturation medium and shoots in MSBA medium (Fig. 9).

After this time and also at 4 ºC / dark, cell dehydration was promoted by placing the plant material in Petri dishes with solid MS media supplemented with an increasing sucrose concentration: first day in 0.25 M sucrose, second day in 0.5 M and third day in 1 M.

This was applied for both slow cooling and vitrification methods (Fig. 9).

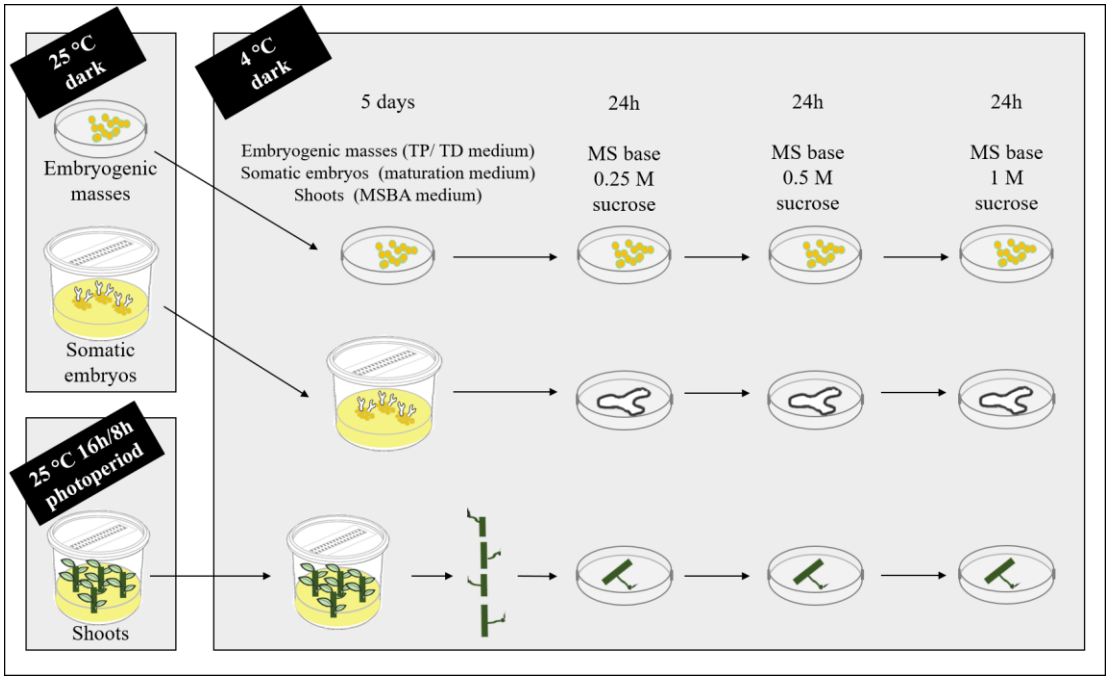


Figure 9 - Cold hardening and preculturing steps used to prepare the plant materials for cryopreservation. Embryogenic masses, somatic embryos and shoots were placed in the dark at 4°C during 5 days, time after which they started a dehydration process achieved by placing them in MS media with an increasing sucrose concentration.

2.3 CRYOPRESERVATION TECHNIQUES

As stated previously, different cryopreservation techniques were tested in order to analyse which was/were the most effective for tamarillo and which was/were more adequate for each plant material used (*calli*, somatic embryos or shoots).

2.3.1 Slow cooling

After sucrose preculture, embryogenic masses (200 mg of CE1, CE2, CE3 and CE4), somatic embryos and shoots were transferred to cryovials. At 0 ºC (in ice) the material

was submitted to 3 applications of cryoprotectant solutions: in the first application, 500 µl of MS with 180 g/L sucrose (solution A) and 100 µl of MS with 180 g/L sucrose and 15% DMSO (solution B), were added to the cryovials for a period of 22.5 min.; after this time 200 µl of solution B were added for another 22.5 min; and finally in the third application, 200 µl of solution B were applied for 45 min. At the end of this treatment the final solution in each cryovial had 1 ml MS + 180 g/L sucrose + 7.5% DMSO. After this 90 min. treatment, the cryovials were placed in freezing container, Nalgene® Mr. Frosty (86 mm × 117 mm) at -80 ºC for 1 h. This specific equipment allows the temperature inside the cryovials to decrease 1 ºC / min. reaching -40 ºC approximately after 1 h (temperature at which ice nucleation stabilizes). At this time cryovials were passed immediately to LN in Nalgene® CryoBox™ Boxes 5x5 (Fig. 10). Another time of exposure to the cryoprotective solutions was also tested, being the total time 60 min. (15'+15'+30').

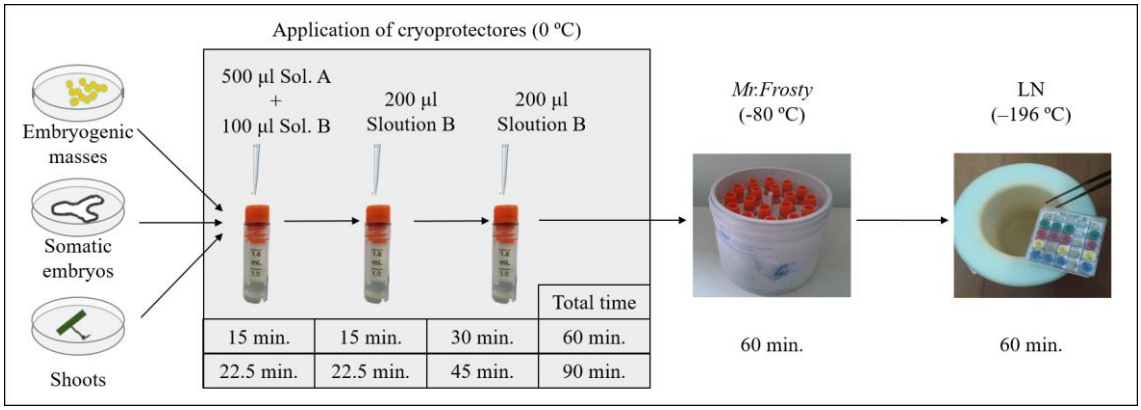


Figure 10 – Schematics of the steps of the slow cooling method. Plant tissues, after cold hardening and preculturing, were submitted to cryoprotective solutions (60 or 90 min. total application time), then they were transferred to Mr. Frosty and placed for 60 min. at -80°C. Finally the tissues were submerged in LN.

2.3.2 Vitrification

Embryogenic masses (200 mg), somatic embryos and shoots were placed in cryovials. At room temperature (RT) 0.5 ml of loading solution [2 M glycerol (w/v) + 0.4 M sucrose

(w/v) autoclaved] were added to each cryovial, being the cells exposed to this solution for 30 min. in order to protect them from the effect of ice crystals. After this period the loading solution was removed and changed with 0.5 ml of PVS2 [30 % glycerol (w/v) + 15% ethylene glycol (w/v) + 15% DMSO (w/v) + 0.4 M sucrose in MS, pH at 5.7, autoclaved] at 0 ºC (in ice) for 1 h. After this hour, the PVS2 solution in the cryovials was renewed, the vials were placed in Nalgene® CryoBox™ Boxes 5x5 and submerged in LN (Fig. 11).

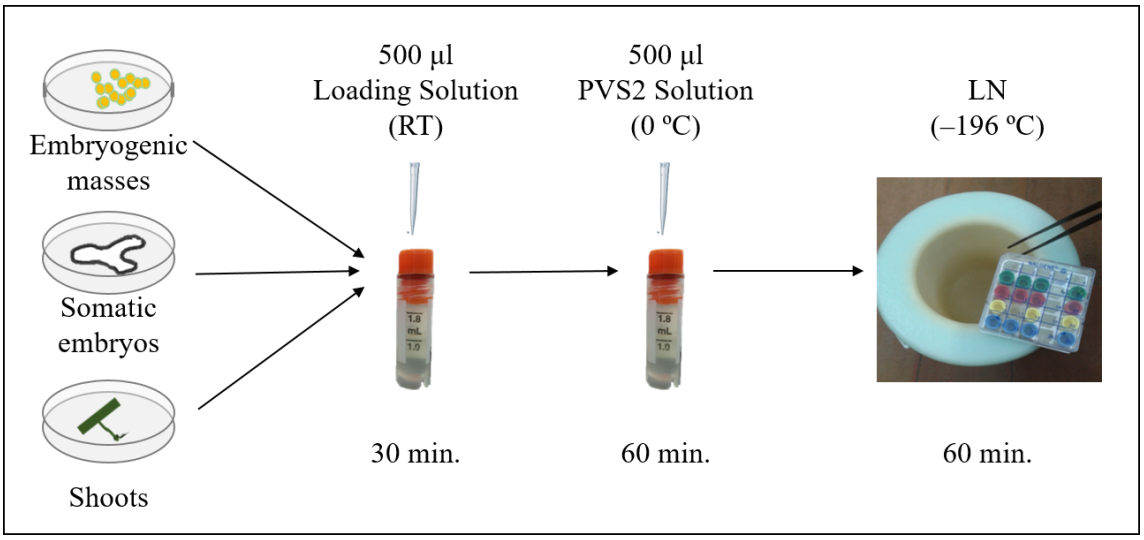


Figure 11 - Schematics of the steps of the vitrification method. Plant tissues, after cold hardening and preculturing, were submitted to a loading solution for 30 min. which prepared them for the cryoprotective treatment with PVS2, that lasted for 60 min. After this, the tissues were ready to be submerged in LN.

2.3.3 Droplet/vitrification

The different plant materials were placed in 60 mm x 15 mm Petri dishes with loading solution at RT for 30 min. and then retained in 5 µl drops of PVS2 for 1 h at 0 ºC. The PVS2 drops were placed on aluminium foil strips (5 mm x 15 mm, previously washed in acetone and autoclaved) and submerged directly in LN (Fig. 12).

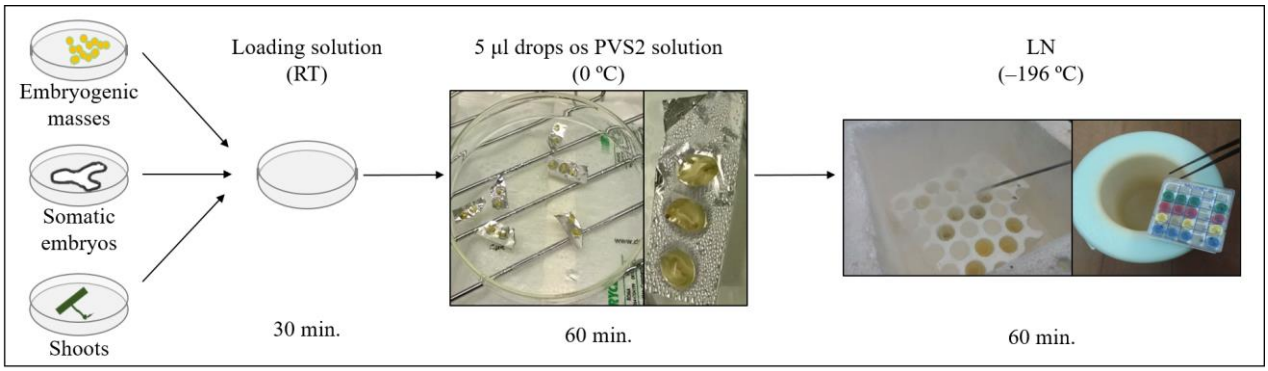


Figure 12 - Schematics of the steps of the Droplet/vitrification method. Plant tissues, after cold hardening and preculturing, were submitted to a Loading solution for 30 min. which prepared them for the cryoprotective treatment with PVS2. The tissues were retained in the drops of PVS2 solution and after 60 min. the aluminium foil strips containing the PVS2 drops and the tissues were submerged directly in LN.

2.3.4 Encapsulation/vitrification

For this procedure before starting dehydration, the tissues must be involved in alginate capsules. This can be achieved by placing the material (previously washed with a sterile MS solution without calcium) in a Petri dish (60 mm x 15 mm) with a MS (calcium free) 3% alginate solution (autoclaved) for 20 min. and then pipetting this mixture one drop at a time into a sterile flask with 25 mL of a 100 mM calcium chloride ($CaCl_2$) solution. The alginate in contact with $CaCl_2$ forms beads closuring the tissues inside, only then, the cells are ready to be submitted to cold hardening and preculture and vitrification procedures as explained above (Fig. 13).

An alternative to this method was tested in embryos and shoots. The encapsulation procedure was the same but instead of engaging the vitrification it was applied the slow cooling process.

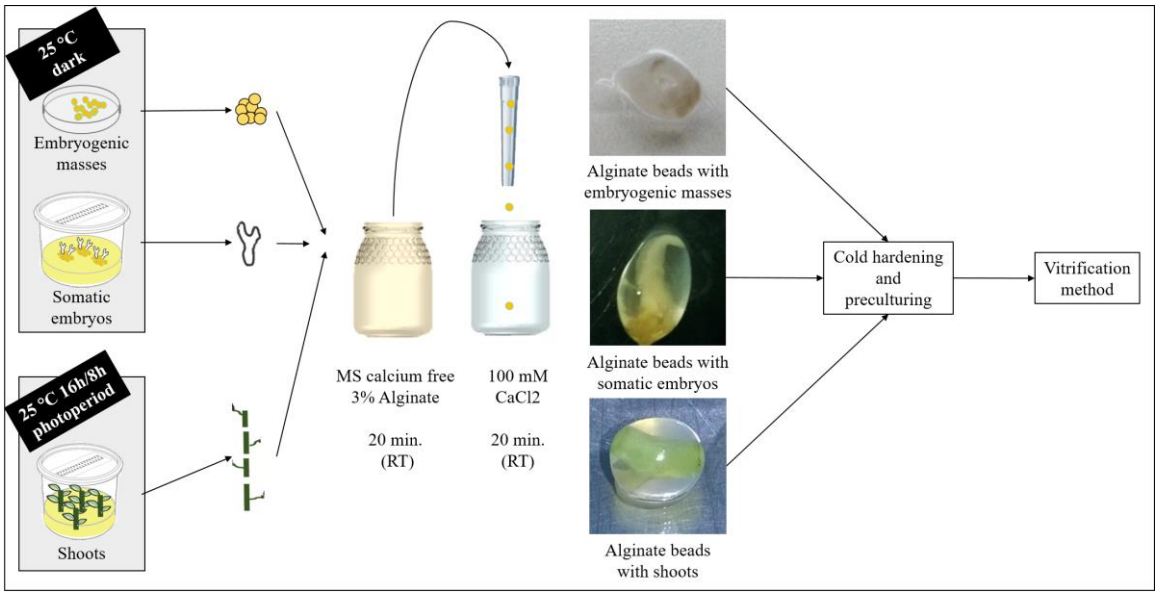


Figure 13 - Schematics of the Encapsulation procedure. Plant tissues were placed in an alginate solution and subsequently in a calcium solution. The alginate in contact with de calcium formed beads retaining the tissues inside. Only then the tissues proceeded to cold hardening and preculture and the vitrification method (previously described).

2.4 CRYOPRESERVATION EXPERIMENTAL DESIGNS

2.4.1 Embryogenic masses

Cryopreservation

The tissues used were removed from subcultures, obtained by SE "two-step" process, (Fig. 14A). The experimental design was planned so that we would be in conditions to compare the *calli's* average growth rate between them throughout the cryopreservative methods, by removing some plant material (200 mg) in control stages (Fig. 14B and C). The first control (normal *calli*) is a common control stage for both methods and it refers to the *calli* that were maintained in subculture and were not submitted to any step of the cryopreservation techniques. For vitrification method there were three control stages (Fig. 14B): cold hardening and preculturing control was taken after the cells were exposed to low temperatures and to the sucrose dehydration process; PVS2 control, to monitor the possible cell damage caused by the toxicity of the PVS2 solution; And finally the LN

control, taken after the plant tissues were submerged into LN. For slow cooling four controls were executed (Fig. 14C): cold hardening and preculturing control, taken in the same conditions as for vitrification; DMSO control, removed after the cells had been submitted to the cryoprotective treatment; -80 ºC control, as a regulator of the effectiveness of Mr. Frosty and; LN control, taken after the cells were exposed to the ultra-low temperatures of LN.

TTC viability test

For each tissue in LN, a viability test was performed by submitting cells to a 1% Tetrazolium/PBS solution for 30 min. in the dark.

Recovery

Regardless of the method used to prepare the tissues, their exposure to LN was for 1h, time after which they were thawed rapidly in a 40 ºC bath (approximately 1 min.). The recovery conditions were determined by the *calli's* line: for CE1 the recovery was managed on TD media, for CE2, CE3 and CE4 on TP. All lines were recovered in the dark at 25 ºC.

However, when performing vitrification treatment, before placing the tissues in recovery conditions, the PVS2 solution in the cryovials was changed for 0.5 mL of washing solution (MS medium with 1.2 M sucrose, pH at 5.7, autoclaved) for 30 min. at RT (the droplet stripes were submerged in washing solution in Petri dishes). Also for vitrification, two recovery conditions were tested: 1) in solid media (TP or TD with 2.5 g/L Phytagel) in the dark at 25 ºC, and; 2) in liquid media (TP or TD) in the dark at 25 ºC with agitation.

Somatic embryos conversion and plant acclimatization

Calli maturation into somatic embryos (or the somatic embryogenesis second step) enables the validation of the cryopreservation methods for embryogenic tissues when obtaining normal and functional embryos on the *calli* after LN exposure. To do so, the embryogenic tissues were set to continue the SE process from the stage where it was removed, embryogenic tissue in subculture (Fig.14A), and were placed in polypropylene Combiness © microboxes containers (210 mL of capacity, L filter lids) with 50 mL of MS medium with 4% sucrose, 2.5 g/L of Phytagel, pH 5.7 and without growth regulators, in the dark at 25 ºC. After maturation of the embryos these were transferred to glass test tubes with 12 mL of MS medium with 3% sucrose, 2.5 g/L of Phytagel, pH 5.7 and placed in a controlled growth chamber (25 ºC, 16h/8h photoperiod). After embryos germination (with well-developed roots), they were carefully removed from the glass test tubes, properly washed to eliminate all residual medium and placed in plastic vases, with a ratio of 3:2 of universal substrate and perlite, in a controlled climatic chamber (25 ºC, 70% humidity and a 16h/8h photoperiod).

Quantification

After the cryopreservation procedures, the *calli* were monitored and quantified. The quantification was made by monitoring the samples weight during four months in regular intervals of one month. This enabled us to draw a four month growth curve for each *calli* in each step of the procedure. The quantification of the tissues was performed during the exponential growth stage, therefore, we calculated the *calli's* growth rate using an exponential regression of mass/time.

Statistical analysis

Analysis of variance (ANOVA) was applied to determine significant differences between the *calli's* average growth rates for each step of the vitrification method and, where applicable, the different means were compared by Tukey's t-test ($p<0.05$). For each line we worked with an n=3 for Normal and PVS2 controls, an n=4 for cold hardening and preculturing control and an n=9 for LN controls. The analysis was carried out using GraphPad Prism 6 statistical software.

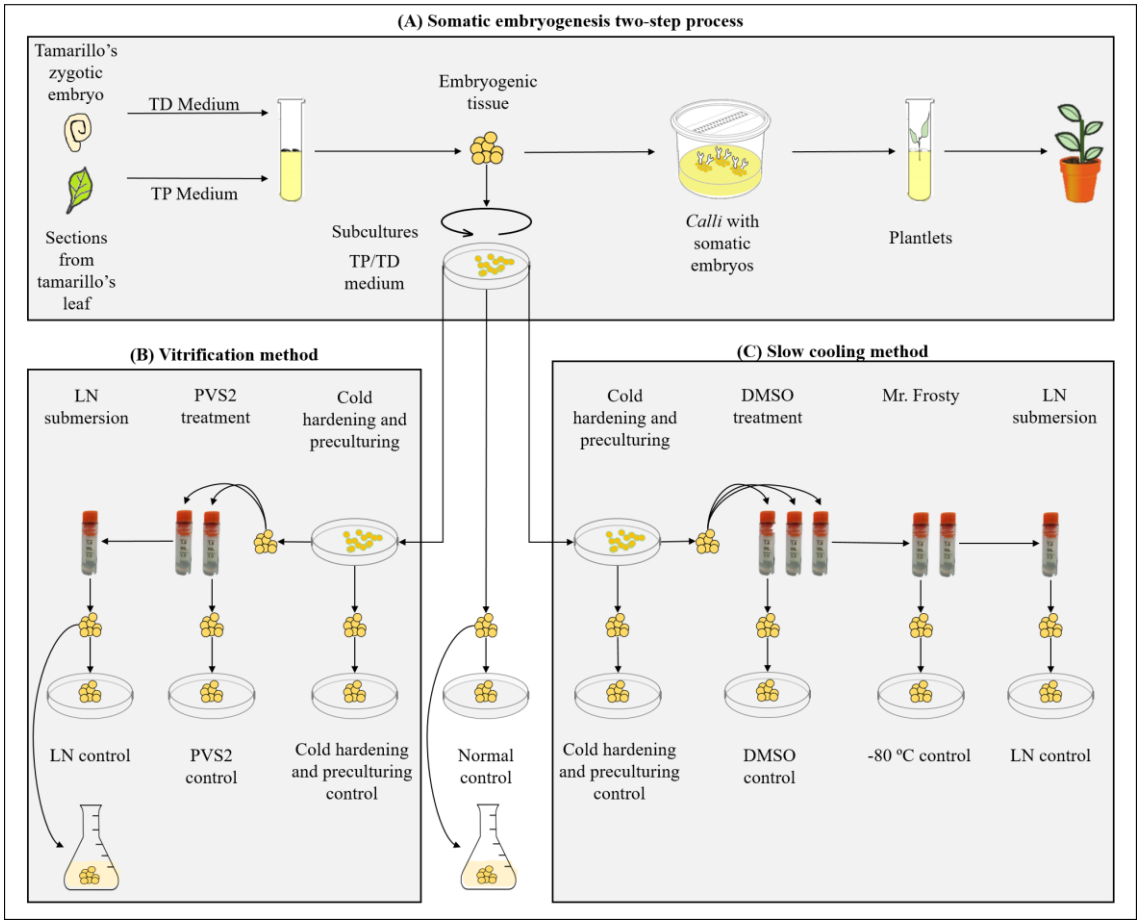


Figure 14 - Schematic drawing of the experiment design concerning the tamarillo's embryogenic masses. The *calli* used were removed from subcultures achieved by two-step SE (A) and submitted to vitrification (B) and slow cooling (C) methods. Throughout the steps of both cryopreservation techniques, several controls were taken and cultured in solid and liquid media for 4 months. After this time the *calli* were set to continue the SE process from the step from where they were removed and finish the process.

2.4.2 Somatic embryos

Cryopreservation

The somatic embryos used were achieved by maturation of CE4 callus maintained in subcultures and obtained by SE two-step process, (Fig. 15A). The experimental design was elaborated so that we would be able to compare the number of germinated embryos throughout the cryopreservative methods, by removing some embryos in control stages (Fig. 15B and C). The control stages were the same as the ones performed for embryogenic tissues. Three controls for the vitrification method (Fig. 15B): cold hardening and preculturing, PVS2 control and LN control. For slow cooling four controls were executed (Fig. 15C): cold hardening and preculturing control, DMSO control, -80 ºC control and LN control.

TTC viability test

For each trial in LN, a viability test was performed by submitting somatic embryos to a 1% Tetrazolium/PBS solution for 30 min. in the dark.

Recovery

The recovery procedure was equal as for the embryogenic tissues, however in this case the embryos recovery was managed by placing them in polypropylene Combiness © microboxes containers (210 ml of capacity, L filter lids) with 50 ml of MS medium with 3% sucrose, 2.5 g/L of Phytagel, pH 5.7 and placed in a controlled chamber (25 ºC, 16h/8h photoperiod).

Quantification

After the cryopreservation procedures, the somatic embryos were monitored and quantified. The quantification was made by registering the number of germinated

embryos in each control stage. This enabled to analyse the differences between the cryopreservative methods and between the control steps.

An average percentage of germination was calculated for each control:

$$\frac{(\text{Number of germinated somatic embryos} * 100)}{\text{Number of initial somatic embryos}}$$

The number of replicas used were: n=3 for normal control; n=5 for cold hardening and preculturing, DMSO, -80 ºC, and PVS2 controls and; n=5 for both slow cooling and vitrification LN controls.

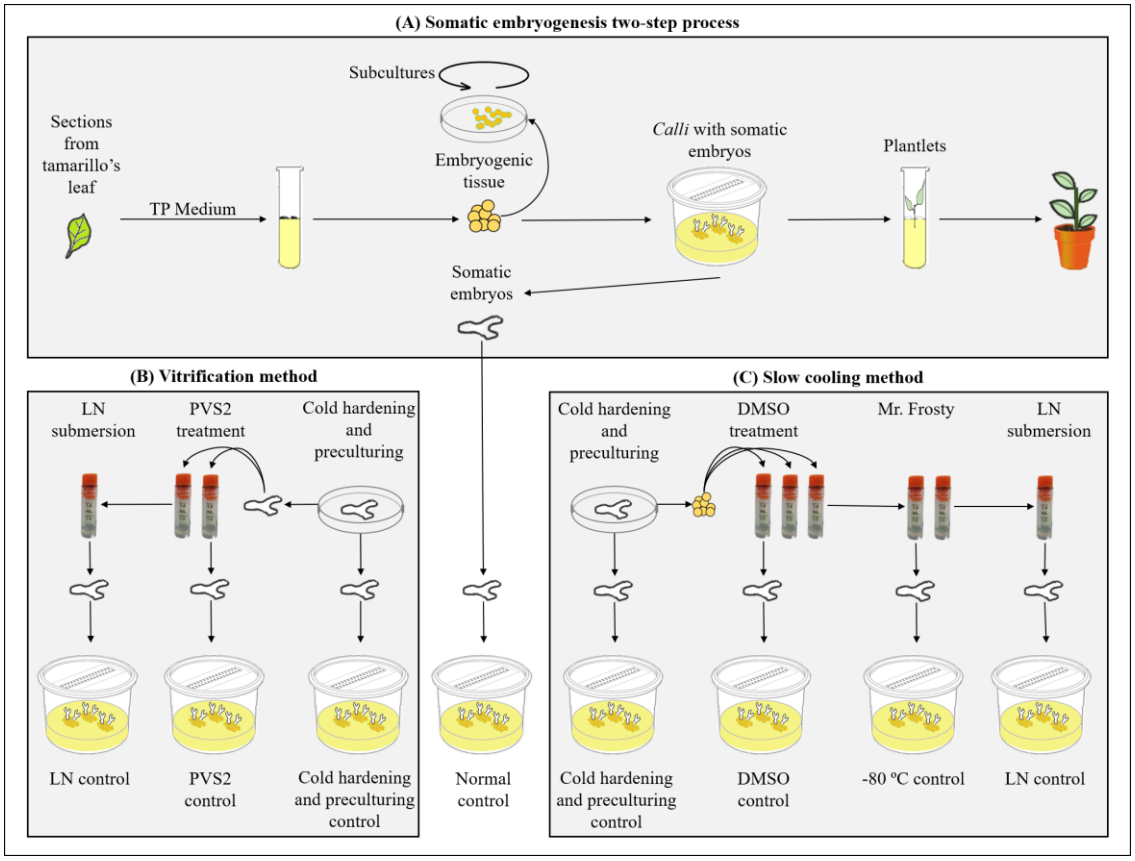


Figure 15 - Schematic drawing of the experiment design concerning the tamarillo's somatic embryos. The embryos used were achieved by maturation of callus in subculture which was obtain by two-step SE (A) and submitted to vitrification (B) and slow cooling (C) methods. Throughout the steps of both cryopreservation techniques, several controls were taken and placed in germination medium, so that we would be in conditions to compare the number of germinated embryos between the controls.

2.4.3 Apical and axillary shoots

Cryopreservation

The plantlets used were removed from the micropropagated plants (TLC line) in culture at our laboratory. The tamarillos were propagated by sectioning the plant stems in phytomers and placing them in the same MSBA medium, eventually each phytomer originates a new plant (Fig. 16A). The experimental design was elaborated so that we would be able to compare throughout the cryopreservative methods, the number of shoots that developed into plants by removing them in control stages (Fig. 16B and C). The stages from where the controls were removed were the same as for the previous two structures: first two controls normal and cold hardening and preculturing, common for both cryoprotective methods. Two for the vitrification method (Fig. 16B): PVS2 control and the LN control. And three for slow cooling (Fig. 16C): DMSO control, -80 ºC control and LN control.

Recovery

The recovery procedure was equal as for embryogenic tissues and somatic embryos, however in this case the shoots recovery was managed in polypropylene Combiness © microboxes containers (565 ml of capacity, XXL filter lids), with 80 ml of a MSBA medium, which consists in MS base medium supplemented with 0.2 g/L 6-benzylaminopurine or benzyl adenine (BAP), in a controlled chamber (25 ºC, 16h/8h photoperiod).

Quantification

After the cryopreservation procedures, the shoots were monitored and quantified. The quantification was made by registering the number of developed shoots in each control

stage. This enabled to analyse the differences between the cryopreservative methods and between the control steps.

The average percentage of recovery was calculated in the same conditions as it was for somatic embryos. Here we worked with an n=3 for normal and cold hardening controls and n=5 for all the other stages.

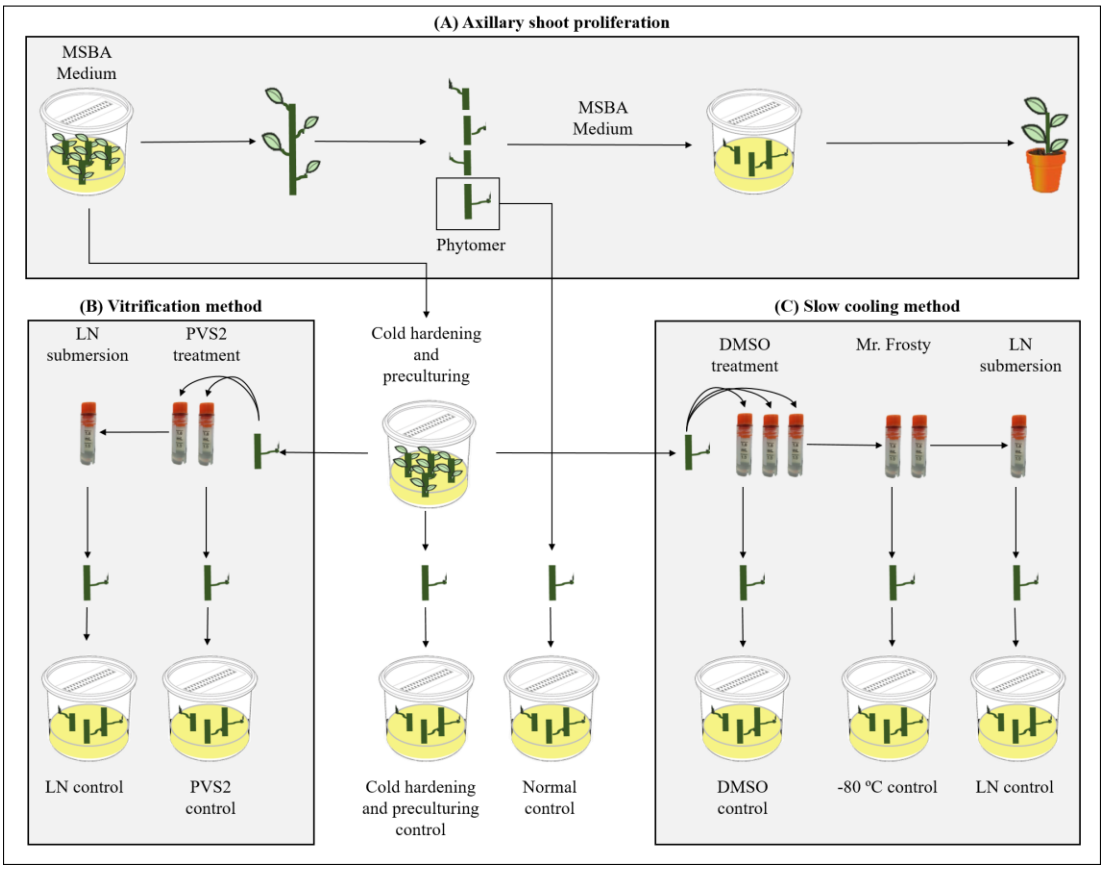


Figure 16 - Schematic drawing of the experiment design concerning the tamarillo's apical and axillary shoots. The phytomers used were achieved by sectioning plant stems from a micropropagated culture from our laboratory (A). They were submitted to vitrification (B) and slow cooling (C) methods, and throughout the steps of both cryopreservation techniques, several controls were taken and placed in MSBA medium, so that we would be in conditions to compare the number of developed shoots between the controls.

CHAPTER 3

RESULTS

3.1 CRYOPRESERVATION OF EMBRYOGENIC TISSUES OF TAMARILLO

Slow cooling and vitrification methods were applied to CE1, CE2, CE3 and CE4 lines aiming to select the most adequate cryoprotective method for tamarillo's embryogenic tissues. In a first stage a qualitative analysis was performed (Fig. 17). All lines survived slow cooling (positive TTC viability test; Fig. 17B1, 3), however the CE1, CE2 and CE3 lines were unable to recover from the cryotemperatures as perceptible by the tissues browning and consequent lack of growth four months later (Fig. 17B2). The CE4 line had no growth, although the tissue remained viable (Fig. 17B4). The vitrification resulted in survival for all *calli* lines (TTC viability test; Fig. 17C1, 3). However, in this case, four months later the tissues survived (Fig. 17C2) with exception for the CE4 which was unable to recover (Fig. 17C4). The multiplication of the embryogenic tissues submitted to vitrification was possible to observe by analysing the new proliferating whitish tissue developing over some browned tissues (Fig. 17D1) and by their increment in mass (except CE4 callus; Fig. 17D2).

After vitrification had been selected as a reliable technique, the *calli's* response to some vitrification method variations was tested, namely droplet/vitrification and encapsulation/vitrification, however these methods were unviable for the cells once they were not able to survive the cryoprotective treatment and consequently the LN temperatures.

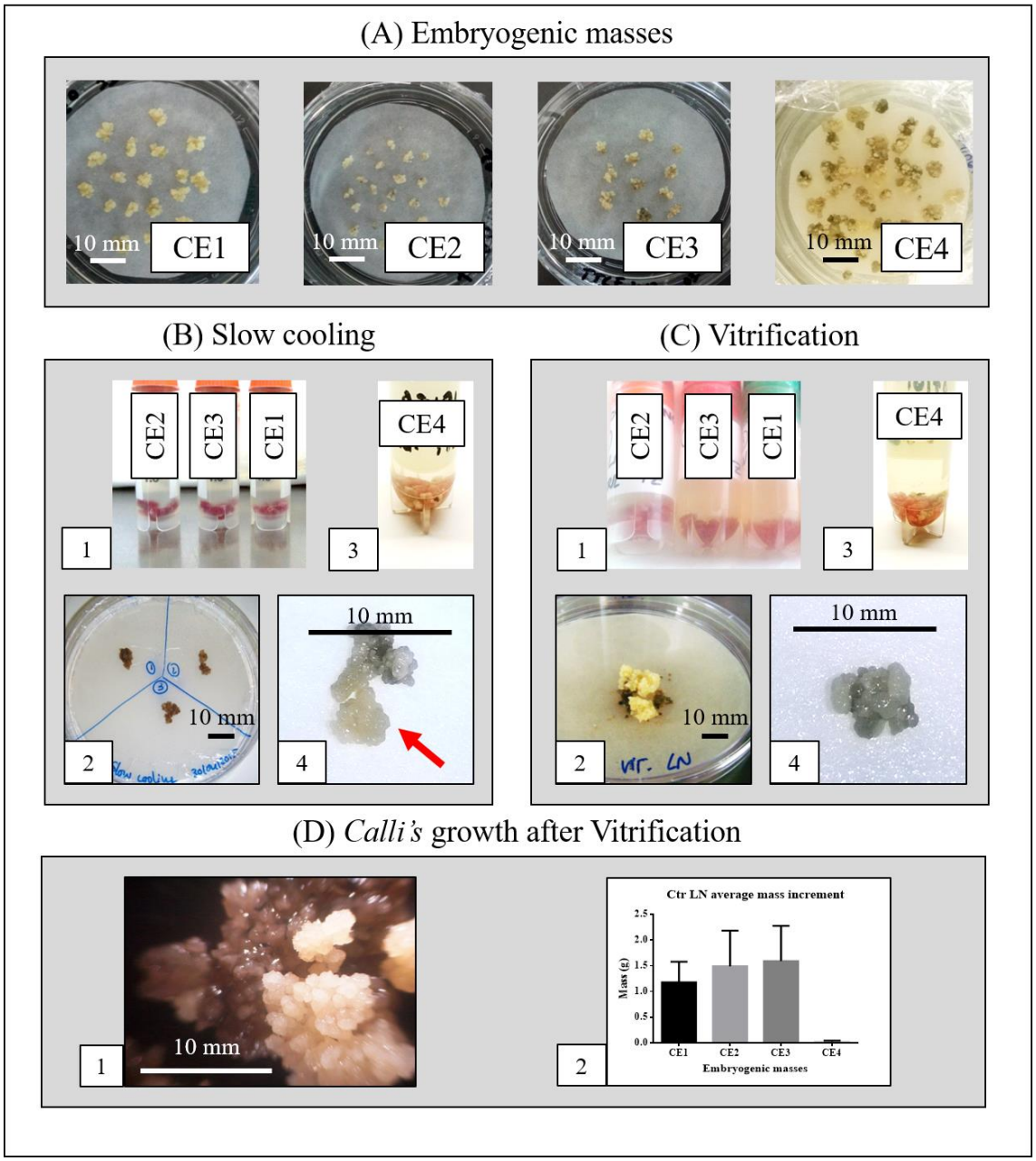


Figure 17 –Embryogenic *calli's* response after slow cooling and vitrification methods. (A) Control subcultures of the embryogenic tissues tested CE1, CE2, CE3 and CE4; (B) Analysis of the *calli's* response to the slow cooling method by TTC viability test (B1,3) and its status after four months of recovery (B2,4); (C) Analysis of the *calli's* reaction to the vitrification method where a TTC viability test was evaluated (C1,3) and its conditions after four months of recovery (C2,4); (D) *Calli's* response to vitrification after four months of recovery were new cells can be observed (D1) and the overall increment in mass for the tested lines, with exception for CE4 (D2).

Vitrification was selected over slow cooling after this initial qualitative analysis. The next question to discuss was to compare the vitrified *calli's* growth/recovery. To do so, the *calli's* average growth rates were compared between them in each control step of the

vitrification process (Fig. 18): after control growth, after cold hardening and preculture conditions, after PVS2 and after the LN step. We can observe that: for the normal control (ctr N), in two months, the CE1 line grew faster than CE2 and CE4 (both lines with no significant differences) and finally the CE3 line with the lowest value (ANOVA; $p < 0.05$).

After submitting the tissues to cold hardening and preculturing (ctr CH), during the four months of monitoring, the *calli's* growth rates decreased when compared to the normal control and became more homogenous with no differences among them (ANOVA; $p < 0.05$).

For the PVS2 control (ctr PVS2), it wasn't observed a decrease in the growth rates from the previous step to this one, as observed from the normal to the cold hardening control. The highest growth occurred in CE1 and the lowest in CE4 line (ANOVA; $p < 0.05$).

Finally, the controls from liquid nitrogen submersion (ctr LN) did not differ in growth, with exception for the CE4 which was unable to recover from the cryotemperatures. It was interesting to notice that also from the previous step (ctr PVS2) to this one there was no change in the *calli's* average growth rates.

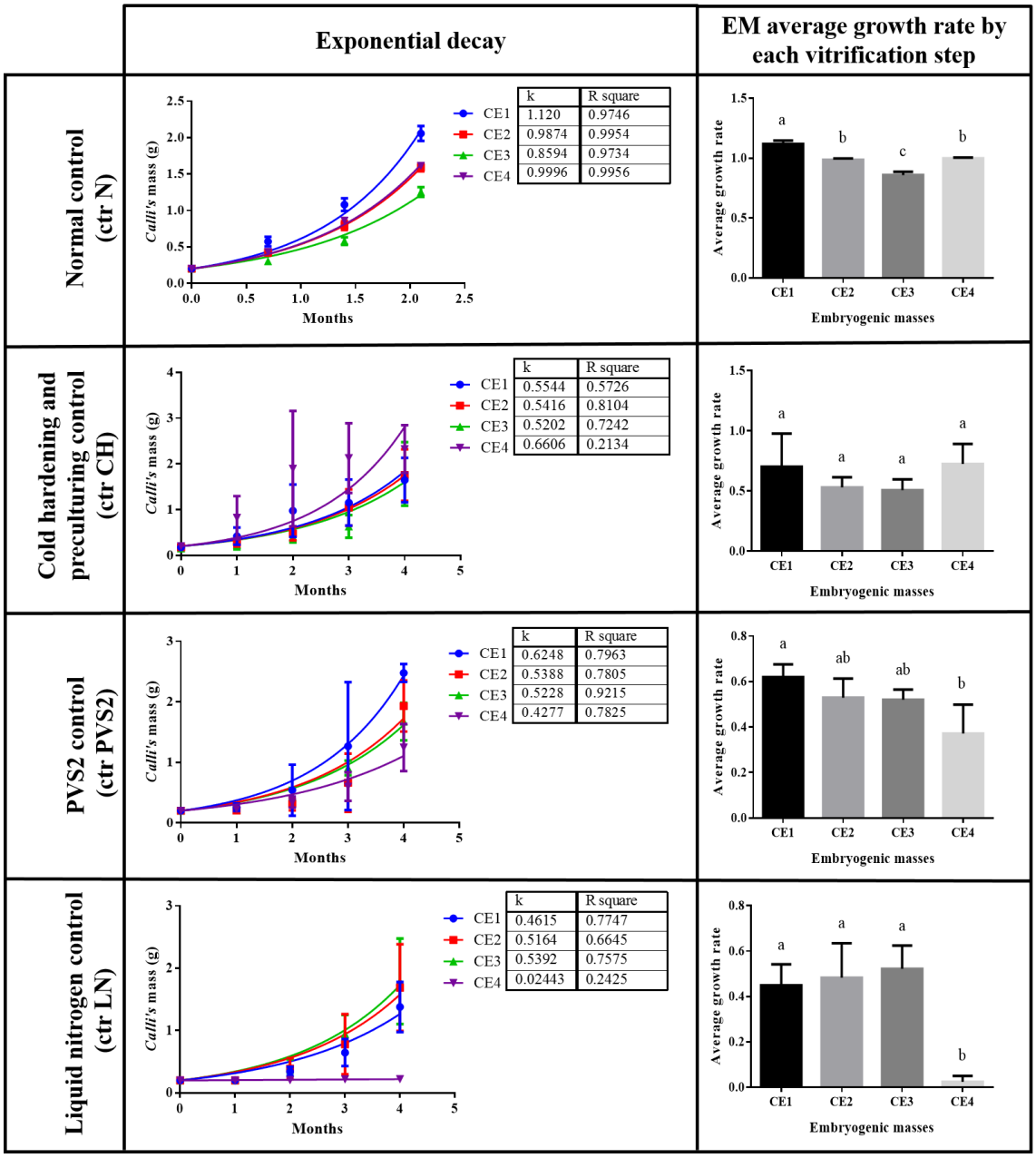


Figure 18 – Embryogenic *calli's* average growth rates (k; as the slope of an exponential regression over time) for each step for the vitrification method: normal control (ctr N), cold hardening and preculture control (ctr CH), cryoprotectant PVS2 control (ctr PVS2) and liquid nitrogen control (ctr LN). (Mean ± SD). ANOVA on k values; columns with the same letter were not statistically different at p=0.05.

The next question to address was whether growth was affected by the media state: solid / liquid. Before vitrification, the *calli* cultured in liquid media grew faster than the ones cultured in solid media (t-test; Table 2). However, after vitrification, recovery was successful only in solid, except for CE4 (Table 2). Although the tissues in liquid medium did not proliferate in the four months after cryopreservation, when comparing the *calli's*

physic characteristics, the cells recovering in liquid medium maintained the yellowish features in comparison with the brownish colour the ones placed in solid medium showed (Fig. 19).

Table 2 - Comparison of the *calli's* average growth rates before (ctr N) and after (ctr LN) cryopreservation. (Mean ± SEM; t-test; $p < 0.05$; "*" indicate statistical differences).

Calli lines	Normal *calli* (ctr N) Solid medium	Normal *calli* (ctr N) Liquid medium	Cryopreserved *calli* (ctr LN) Solid medium	Cryopreserved *calli* (ctr LN) Liquid medium
CE1	1.120 ± 0.016	1.168 ± 0.025	0.449 ± 0.031 *	0.072 ± 0.018
CE2	0.987 ± 0.006	1.161 ± 0.011 *	0.483 ± 0.051 *	-0.099 ± 0.016
CE3	0.859 ± 0.016	1.332 ± 0.028 *	0.522 ± 0.034 *	0.008 ± 0.024
CE4	0.999 ± 0.004	1.146 ± 0.004 *	0.023 ± 0.009 *	0.039 ± 0.034

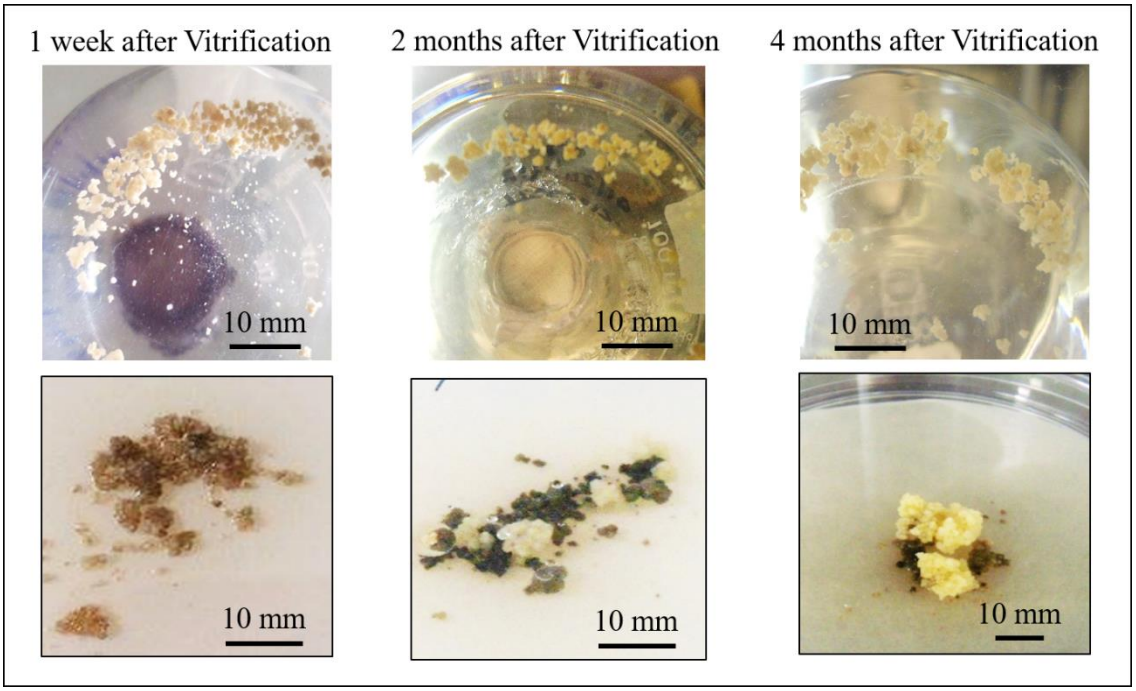


Figure 19 – *Calli's* morphology four months after vitrification, when recovered in liquid and solid media.

The validation of vitrification as an effective cryoprotective method for tamarillo was achieved by obtaining viable somatic embryos and therefore plantlets from the cryopreserved embryogenic tissues. This was accomplished by setting the tissues to

complete the “two-step” SE process from the stage from where they were removed (Fig. 20).

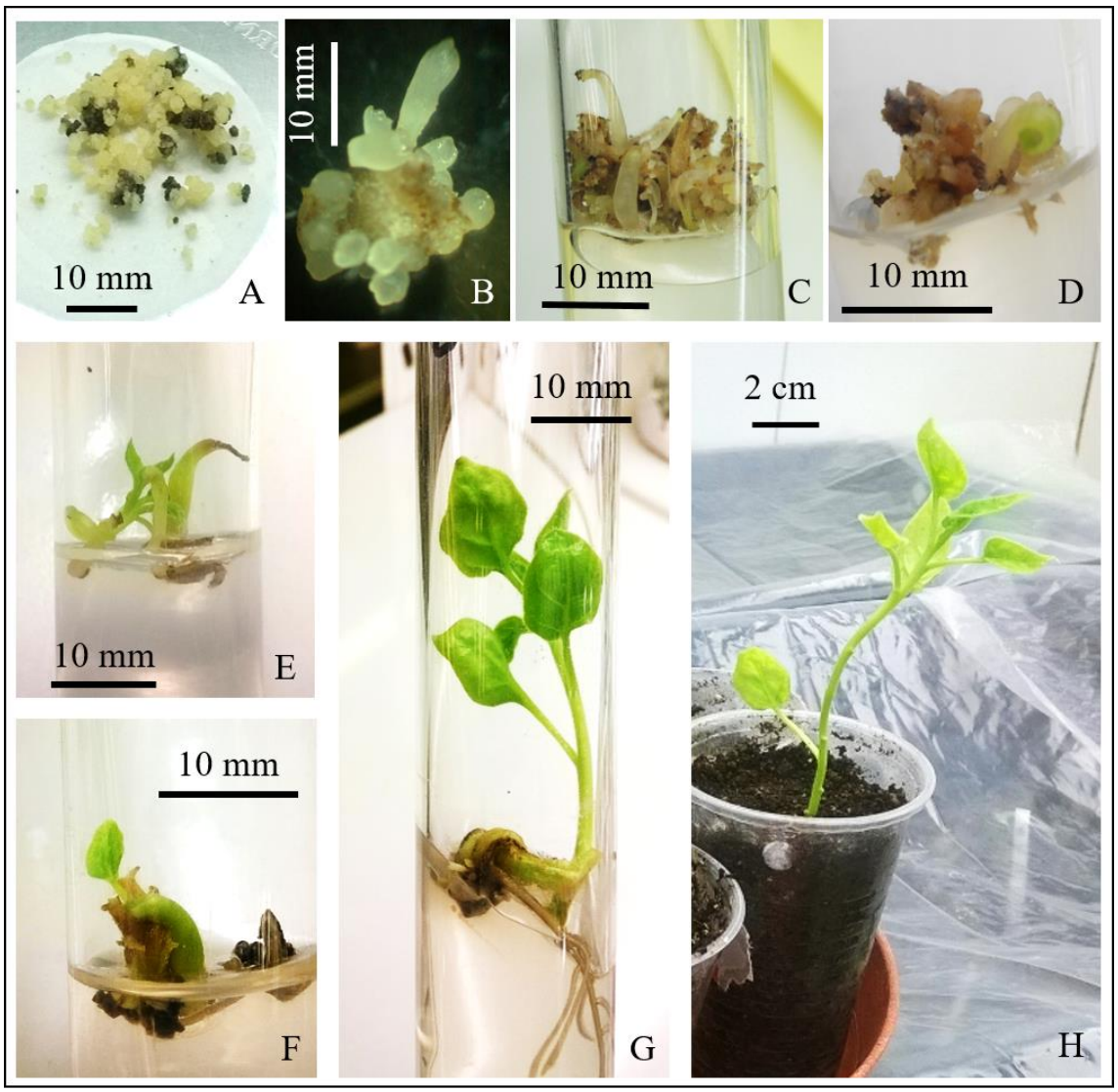


Figure 20 – Final stages of the “two-step” SE for the cryopreserved embryogenic tissues: (A) *Calli* proliferation; (B) Somatic embryos developing on the callus; (C) Somatic embryos in germination medium; (D) Embryo initializing germination observable by the green tone and by the small root; (E-G) Plantlet development; (H) Plant acclimatization.

For the normal *calli* (ctr N), CE1 formed less embryos than CE2, CE3 and CE4 (Table 3) and there was a gradual increase in the number of germinated plants from CE1 to CE4. Relatively to the cryopreserved *calli* (ctr LN) there was a gradual increase in the number of embryos from CE1 to CE3 likewise in number of germinated plants.

When analysing the tissues individually CE1 ctr N developed somatic embryos, but they were unable to germinate. As for CE2, 0.2 g of ctr N developed the same number of somatic embryos as the ctr LN, however the number of germinated plants originated from the ctr LN embryos was lower (1:2). For the CE3 line, 0.2 g of ctr N originated less embryos than the ctr LN (1:2), however, similarly to the CE2 line, the number of germinated plants originated form ctr LN was inferior to ctr N and in the same proportions (1:2).

Finally, the only data available for the CE4 line is the one from the normal tissue (ctr N). It had higher embryogenic capacity than the older lines, with higher number of somatic embryos and plantlets produced (Table 3).

Table 3 - Number of somatic embryos of CE1 – CE4 lines achieved form normal and cryopreserved *calli*, the number of somatic embryos per 0.2 g of embryogenic tissue and the number of plantlets they originated. "-"= no data.

Calli		Initial mass (g)	Somatic embryos	Somatic embryos/0.2 g	Plantlets	Germination (%)
CE1	ctr N	3.24	46	3	0	0.0
	ctr LN	3.78	0	0	0	0.0
CE2	ctr N	3.67	220	12	90	40.9
	ctr LN	3.7	197	11	47	23.9
CE3	ctr N	5.35	338	13	224	66.3
	ctr LN	5.22	627	24	245	39.1
CE4	ctr N	0.50	301	120	242	80.6
	ctr LN	-	-	-	-	-

3.2 CRYOPRESERVATION OF SOMATIC EMBRYOS OF TAMARILLO

Somatic embryos achieved through "two-step" SE applied to the CE4 callus were tested for cryopreservative method. All the cryopreservative techniques described before were executed (Fig. 21).

The embryos did not survive slow cooling (TTC viability test), although a shade of red on the embryos indicate that some of the embryogenic cells attached to the embryos, as

seen before, were capable to endure the process (Fig 21D). For vitrification, the results were the same with some red hue in some regions of the embryos (Fig. 21E).

By the time this thesis was written, although some embryos were clearly dead, others were still apparently alive (Fig. F-H). With the data available, in one month, in average, 80% of the normal control embryos (ctr N) and 6% of the DMSO control (ctr DMSO) embryos germinated. All other stages had no positive results.

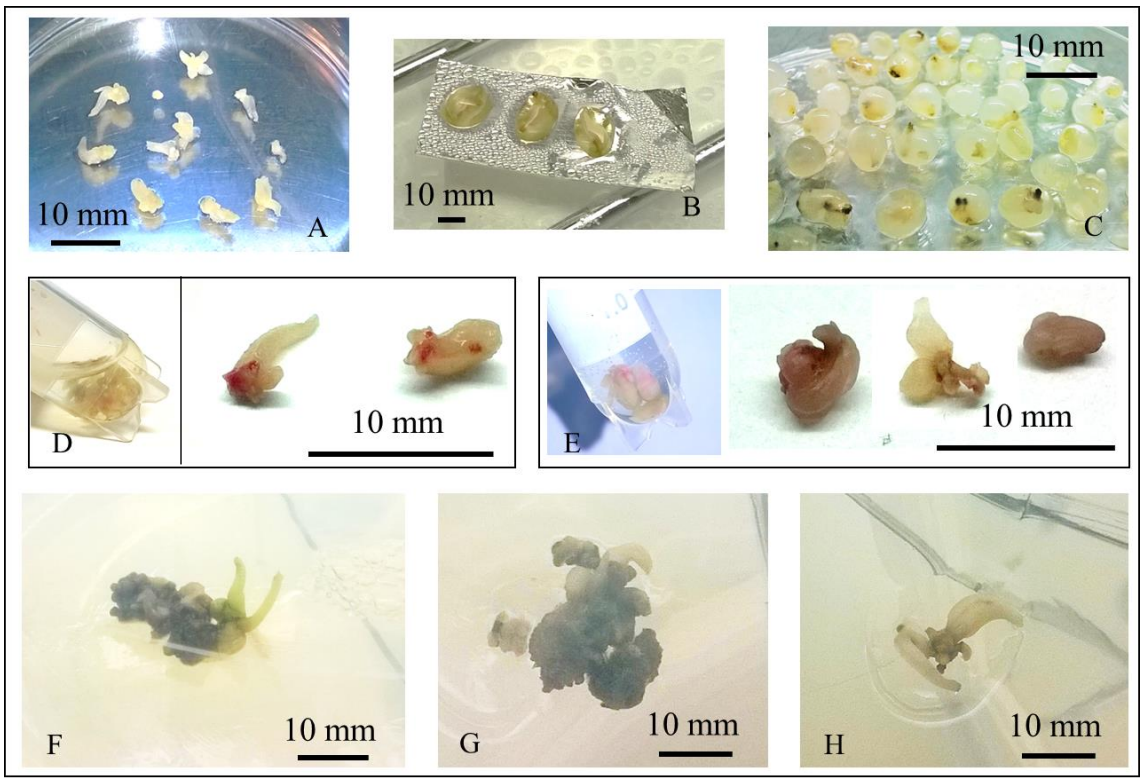


Figure 21 – Cryopreservation assays with tamarillo's somatic embryos: (A) CE4 somatic embryos; (B) PVS2 treatment for droplet/vitrification; (C) Somatic embryos retained in alginate capsules for encapsulation/vitrification; (D) TTC viability test for slow cooling and a detail of two embryos submitted to this test; (E) TTC viability test for vitrification and some embryos in which the test was applied; (F) Germinated embryos from the DMSO control; (G) Embryos from the slow cooling LN control, with apparently both dead and alive embryos; (H) Embryos from the vitrification LN control.

3.3 CRYOPRESERVATION OF APICAL AND AXILLARY SHOOTS

Tamarillo's apical and axillary shoots were used to test the cryopreservative methods (Fig. 22A-D). Three months after the shoots cryopreservation, some shoots were still apparently viable (Fig. 22E-J) with development of the shoots for normal, cold hardening, DMSO, DMSO/Encapsulation, PVS2 and PVS2/Encapsulation controls but no positive results for the others (Table 4), however, some of the cryopreserved shoots are still apparently alive. Still, similarly as for what occurred with the somatic embryos experiments, time showed itself insufficient to conclude the trial.

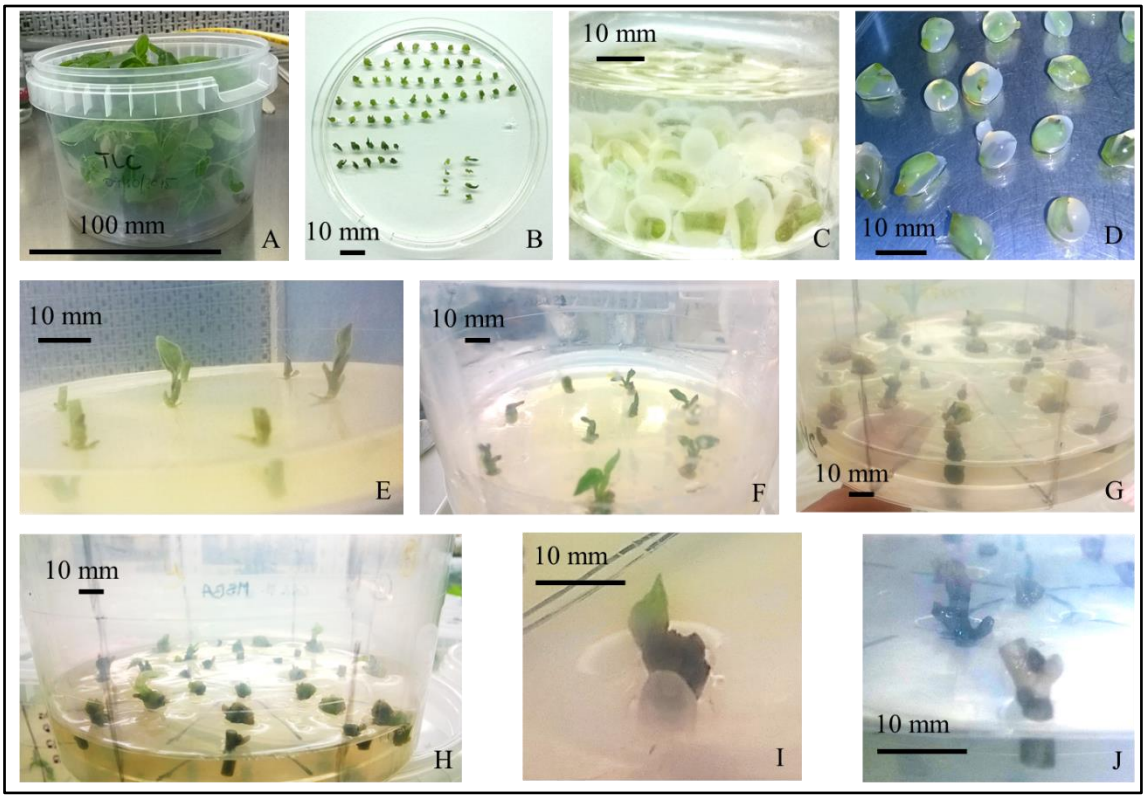


Figure 22 – Cryopreservative testing in tamarillo's shoots: (A) TLC line in culture from where the phytomers were removed; (B) Shoots in cold hardening and preculture; (C; D) Encapsulation of the shoots; (E) Two week normal control shoots in development; (F) Three months cold hardening and preculture control; (G) Three months DMSO control; (H) Three months PVS2 control; (I) Three months DMSO/Encapsulation control; (J) Three months LN controls.

Table 4 – Average percentage of developed shoots in each stage of the cryopreservative methods

	Control stages	**Shoot development (%)**
Common controls for all methods	Ctr N	93
	Ctr CH	50
Slow cooling	Ctr DMSO	73
	Ctr -80 ºC	0
	Ctr LN	0
Slow cooling/encapsulation	Ctr DMSO/Encap.	40
	Ctr -80 ºC/Encap.	0
	Ctr LN/Encap.	0
Vitrification	Ctr PVS2	47
	Ctr LN	0
Encapsulation/vitrification	Ctr PVS2/Encap.	13
	Ctr LN/Encap.	0
Droplet/vitrification	Ctr PVS2	0
	Ctr LN	0

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1 CRYOPRESERVATION OF EMBRYOGENIC TISSUES

The trials to test the cryoprotective methods, slow cooling and vitrification, on embryogenic tissues showed that, although all *calli* initially survived both cryopreservative procedures, after four months only the tissues that endured the vitrification method recovered. This finding suggest that vitrification is better than slow cooling for embryonic tissue preservation.

Immediately after being removed from the LN *calli* survived both procedures. However, once only vitrified cells recovered, it may suggest that, the PVS2 from vitrification is more effective than DMSO protecting the cells from cryoinjuries and therefore an effective cell protecting solution, at least in three of the four tested lines. These results are sustained by other studies in which cryopreservation in woody species improved with PVS2 solution (Panis and Lambardi, 2006).

The four tamarillo's lines of embryogenic masses tested had different origin and time of subculture. It was previously shown that physiological factors may influence the effectiveness of the technique (Ozudogru and Lambardi, 2016). However, consistently with the reported for other species such as *Quercus ilex* (Barra-Jiménez *et al.*, 2015) or *Picea mariana* (Touchell *et al.*, 2002), here the physiological response to the vitrification was similar, despite the *calli's* genotype tested, once CE1 had different origin than the other tissues. Hence it seems that the method is effective despite the age of the tissues, as CE2 and CE3 had the same origin but differ in time of subculture.

However, for the CE4 line the situation is substantially different. It was expected to survive vitrification for it has the same origin as C2 and C3 but, for this more recent callus, the method was ineffective. This suggests that there may be an optimal time in

which to apply the vitrification related to the stability of the tissues in culture. This subject hasn't been investigated before and needs some future attention.

The use of distinct lines was also important so that the *calli's* ability to grow and develop viable somatic embryos could be evaluated. When in subculture the *calli's* growth rates decrease from CE1 to CE3. This may be explained with their progressive loss of embryogenic ability over time and convergence to a non-embryogenic profile, which is a type of tissue that proliferates in a considerable higher rate than the embryogenic one (Correia and Canhoto, 2012). This loss of embryogenic competence is sustained with the increase in the number of plantlets obtained from CE1 to CE3 normal control. Although CE1 and CE2 are in culture for the same time, they were achieved from different structures which may indicate that the embryogenic tissues obtained from leaves maintain embryogenic ability longer than the obtained from zygotic embryos. The prevalence of embryogenic capacity associated with the *calli's* origin has been reported, for example, in wheat (*Triticum aestivum* L.) where embryogenic tissues obtained from young zygotic embryos maintain embryogenic competence longer than the ones induced in older zygotic embryos (Hess and Carman, 1998).

The differences in the normal *calli's* growth rates disappeared once they started the vitrification treatments. As observed in the controls taken throughout the procedure, from cold hardening and preculture the embryogenic masses did not differ in growth. This could be explained by some level of synchronization of molecular mechanisms induced by the stress conditions the cells are submitted when prepared for cryotemperatures. This is an important step for cryopreservation for it induces high frost resistance and accumulation of substances that influence cryopreservation success (Bilavcik *et al.*, 2012; Barra-Jiménez *et al.*, 2015).

An important question in the optimization of methods for cryopreservation is whether solid or liquid media should be used in the *calli's* recovery. Liquid medium may improve the cells recovery immediately after removal form LN, probably due to the readiness in access to the nutrients in the medium and in a more homogenised way. However under these conditions the cells were unable to proliferate and produce embryos. This can probably be explained by the fact that, in embryos development, there is a need for polarization of the cells contents (Dodeman *et al.*, 1997) and, once the use of liquid medium requires agitation, this polarization may not be well set. Although somatic embryos can be achieved in liquid medium for some species (Kreuger *et al.*, 1995; Kosky *et al.*, 2002), it is not possible for tamarillo and no research has been done regarding cells recovery from LN and maturation in somatic embryos in liquid conditions. It would be interesting to analyse this issue in detail in the future for it may be positive to recover the cells in liquid medium in a first stage but then transfer them to a solid one.

As for the maturation of the *calli* in somatic embryos, it was expected an increase in number of embryos from CE1 to CE3 for both normal and cryopreserved tissues. This was true, except for normal CE3 line which developed the same number of embryos than the normal CE2 line. It is possible that an error occurred when counting the embryos and that the real number would be more similar to the one obtained for the cryopreserved CE3.

Nevertheless, when comparing the percentage of germinated plants obtained from the embryos, the increase of plantlets from CE1 to CE3 was observed. CE1 did not originate plantlets and CE2 germinated half the plants than CE3. This proportion was maintained when comparing the cryopreserved tissues. However it is interesting to notice that the percentage of germination from both CE2 and CE3 normal tissues was twofold higher to the cryopreserved ones, which may indicate that cryopreservation has some impact on the

viability of the embryos developed from cryopreserved tissues. This impact is common to occur once the percentage of recovery can be between 30 to 100% in other species (Sakai *et al.*, 2008).

CE4 line differs from the others. The normal control developed a large number of embryos which were more viable that the ones from the older lines corroborating with the postulated effectiveness of more recent lines to produce viable embryos (Currais *et al.*, 2013). Although very effective this tissue could still be in an adaptation stage and this could be the reason why the cryopreservation techniques were unfruitful. Possibly due to the higher instability of this tissue, the cold adaptive molecular mechanisms are not successfully activated and the callus is not well prepared for cryotemperatures, and in fact, although the CE4 survived the cryoprotective treatment (ctr PVS2), it was not able to recover from the LN immersion (ctr LN). Again, there are no comparable studies relating the time of callus induction with the success of cryopreservation. This is therefore another subject needing further research.

In summary, the experiment showed that vitrification was an efficient method for ¾ of the tested lines and is therefore the actual best option to apply for tamarillo's embryogenic tissue, although some optimizations to the procedure are required.

4.2 CRYOPRESERVATION OF SOMATIC EMBRYOS AND SHOOTS

Tamarillo´s embryos did not survive the cryoprotective methods, for there were no positive results from the TTC viability tests, but taking into account the embryos morphological features after submitted to cryotemperatures, this could be untrue. The shoots survived the cryoprotectant treatments for slow cooling as for vitrification, but it is still unknown if they will be able to recover from the cryotemperatures, for there are

some meristems that are presumably dead and others that appear to be alive. Studies so far indicate that vitrification has been the preferred method to apply to shoots from hardwood species, as *Quercus suber, Olea europaea* or *Castanea sativa* (Panis and Lambardi, 2006), therefore it is expected that tamarillo's shoots will respond better to this technique also.

One month of recovery for somatic embryos and four for shoots are still very premature times to withdraw any conclusions, however, once physiological characteristics may influence cryopreservation (Ozudogru and Lambardi, 2016) there should have been tested different genotypes, so that the recovery time could in fact be the limiting variable and not the tissue. Also, the embryos tested resulted from the CE4 line that, as seen before, was the only *calli* line that didn't respond to the cryopreservative methods and therefore again, a different genotype should have been tested.

However, considering the lack of results at this time, it cannot be said that there is a viable cryopreservative method to apply to tamarillo's somatic embryos and shoots, hopefully in some time it will be possible to achieve some conclusion in this matter

Nevertheless, results seem to indicate that it is preferable to apply cryopreservation to embryogenic tissues once they were the only structure with clear recovery.

4.3 CONCLUSIONS

Although there are still lacking some results from somatic embryos and shoots, it seems cryopreservation is more efficiently applied to embryogenic tissues. For this tissue, the only viable cryopreservative technique was the vitrification since it: (1) does not interfere with the cells capacity to proliferate, despite a 50% reduction in the *calli's* average growth

rates in the four months subsequent to cryopreservation and, (2) has an impact of 50 % decrease on the embryos viability and ability to germinate.

By the time the experiments were conducted, the CE2 had been subcultured for five years, the CE3 for three and CE4 for one. The results suggest a possible optimal age for applying the vitrification method to the tissues, sometime between two years (time necessary for *calli's* physiological constancy) and four years of subculture (time after which the *calli* become unstable and start losing their embryogenic potential). However there should be additional studies related to this issue in the future for it may be an important factor in the success of cryopreservation.

Also, there is still space for optimization of the cryopreservation technique for: (1) different times of exposure to the solutions used in the vitrification method, especially the PVS2 solution, could be tested and, (2) adjustments in the time the tissues recover, first in a liquid medium, with higher access to nutrients, and subsequently in solid medium, where they are placed in more stable conditions, could be performed.

Overall, with the results so far obtained it has been shown that cryopreservation by PVS2 vitrification is a viable method to maintain embryogenic masses of tamarillo.

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