Application of Cryoconservation for Genotype Independent Methodology on Potatoes

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Application of Cryoconservation for Genotype Independent Methodology on Potatoes

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LIST OF CONTENTS

List of Acronyms

Institutes and conventions

CAES HRO: Central Agricultural Experiment Station, Hokkaido Research Organization

CIAT: International Center for Tropical Agriculture

CIP: International Potato Center of Peru

FAO: Food and Agriculture Organization of the United Nations

GLKS: The Groß Lüsewitz Potato Collection

INIBAP: International Network for the Improvement of Banana and Plantain

INIFAP: Instituto Nacional de Investigación Forestal Agrícola y Pecuaria de México

IRD: Recherche pour le Dévelopment of France

IPK: Leibniz Institute of Plant Genetics and Crop Plant Research of Germany

KAES HRO: Kitami Agricultural Experiment Station, Hokkaido Research Organization

NCGRP: National Center for Genetic Resources Preservation

NBPGR: National Bureau of Plant Genetic Resources

NAC: National Agrobiodiversity Center of Republic of Korea

RDA: Rural Development Administration of Republic of Korea

NARO/NIAS: National Agriculture and Food Research Organization

NICS: National Institute of Crop Science

RDA: Rural Development Administration of Republic of Korea

SARC: Shimane Agriculture Research Center of Japan

Technical terms

ABA: abscisic acid

CBF: C-repeat-binding factor

COR15A: cold regulated 15A

COR15B: cold regulated 15B

COR47: cold regulated 47

CH: cold hardening

DRE/CTR*: cis* elements

DREB: dehydration response element binding protein

DREB1A: dehydration response element binding protein1A

DREB1B: dehydration response element binding protein1B

DREB1C: dehydration response element binding protein 1C

GRs: genetic resources

HOS1: ubiquitin-protein ligase

ICE1: inducer of C-repeat-binding factor expression 1

KIN1: stress-responsive protein / stress-induced protein 1

KIN2: stress-responsive protein / stress-induced protein 2

GA: gibberellin acid

LN: liquid Nitrogen

TC: treatment control

PoGRs: potato genetic resources

PGRs: plant genetic resources

PVS2: plant vitrification solution 2

PVP: polyvinyl peroxidone

RAPD: restriction fragment length polymorphism

Summary

Environmental stresses affect plant growth and can decrease crop productivity. Temperature, which influences the metabolism of organisms, is a key abiotic factor that determines the growing season and geographical distribution of plants. Adaptation to environmental stresses is accompanied by physiological and developmental changes (Gehan *et al.* 2015). Potato (*Solanum tuberosum* L.) is a crop consumed worldwide that is affected by climate change. It is necessary to seek conservation strategies for diverse potato cultivars.

The Mexico genebank holds potato strains with specific resistance to diseases, such as late blight, as well as breeding lines that bear the resistance traits. An objective of potato conservation is the development of practical and successful cryopreservation protocols for *in vitro* grown potato shoot tips, which can be used and are being implemented by cryo-banking institutions around the world (Machida-Hirano *et al.* 2015). Potato cryopreservation can efficiently complement field and *in vitro* conservation of potato, providing a means of preserving genotypes difficult to preserve by other methods.

Cryopreservation is an important tool for the long-term storage of plant genetic resources and efficient cryopreservation protocols have been developed for many plant species. Practical procedures, developed using *in vitro* tissue culture, can be a simple and reliable preservation option for potato genetic resources, which are allogamous, rather than maintaining them by vegetative propagation in genebanks.

Cryopreserved materials provide a long-term backup of field collections. Occurrence of genetic variation in tissue culture cells during prolonged subculturing, can be avoided with suitable cryopreservation protocols that provide high regrowth. This can facilitate systematic strategic cryo-banking of potato genetic resources. To ensure high regrowth rates after being exposed to the cryoprotectants and liquid nitrogen (LN), pre-treatment steps with low temperature exposure prior to the freezing process must be customize for each protocol.

To enhance understanding of the effect of cold hardening and dehydration methods on cryopreserved potato genotypes, several osmoprotective concentrations, exposure times, media culture and pre and post-LN treatments where evaluated. Potato (*Solanum tuberosum* L.) cultivars exposed to low temperature treatment showed differential regrowth rate responses among genotypes, due to cold-hardening exposure (5º C) prior to the cryopreservation procedure (Valle-Arizaga *et al*. 2017a). The D cryo-plate method has been shown to be successful for long-term cryopreservation of potato cultivars and can be easily implemented in conservation institutions with

a user-friendly and low-budget methodology that can reduce the cost of conservation of potato germplasm *in vitro*.

Keywords: cryopreservation, D Cryo-plate, DMSO droplet, droplet vitrification, encapsulation vitrification, potato genetic resource, V Cryo-plate*, Solanum tuberosum* L.

Chapter 1

General introduction

1.1 Introduction

Currently we are encountering global warming and climate change as a reality, challenging the conditions for food production under more severe stress patterns. Environmental stresses affect plant growth and decrease crop productivity extremely. One conditional abiotic factor is the temperature, which influences the metabolism of organisms, thus is a key factor determining the growing season and geographical distribution of plants, through response/adaptation to environmental stresses with physiological and developmental change (Chinnusamy *et al*. 2003; Awasthi *et al.* 2015).

It is important to look over the mechanisms and conservation strategies impacting over the cultivars affected by the current climate change. Potato (*Solanum tuberosum* L.) is an essential crop consumed worldwide. Mexico is one of original countries of potato and holds potato strains with specific resistance to diseases such as late blight, as well as cardinal breeding lines that bear the resistance trait. Conservation of cardinal species and cultivars have become a priority to ensure current and future breeding programs for potato. It is imperative to assess the development of practical and successful preservation protocols of potato genetic resources (PoGRs) and indicate the direction of safe and efficient long-term storage of plant genetic resources (PGRs), providing for safeguarding of genotypes difficult to preserve, wild types and other species decided as priority collections (Machida-Hirano 2015; Watanabe 2015).

1.2 *Ex situ* preservation

1.2.1 Preservation of plant genetic resources

Ex situ preservation of PoGRs is the storage of seeds or plant materials under artificial conditions to maintain their long-term viability and availability for use. Globally, genebanks are employing seed storage, field collections, *in vitro* storage (tissue culture or cryopreservation) for *ex situ* preservation of PGRs (Hammer 2006; Deke 2008).

However, the germplasm of many plantation crops and fruits trees cannot be preserved as their seeds are recalcitrant and embryo degenerates early; moreover, germplasm of vegetatively propagated crops cannot be stored on a long-term basis, it must be grown and multiplied periodically in nurseries or fields (Bajaj 1995). In addition, vegetatively propagated crops are genetically heterozygotes because of their allogamous nature. They can be maintained only by vegetative propagation such as cutting, grafting or layering. Preservation of PGRs in genebanks is

mostly by vegetative propagation due their allogamous nature and many genebanks are maintaining germplasm as field collections (Niino and Valle-Arizaga 2015).

1.2.2 Seed Genebank

Storage of PGRs that produce orthodox seeds, which are tolerant to low moisture content and low temperatures, at appropriate temperature and humidity, is the most convenient *ex situ* preservation method. Many major seed crops are included this category. However, recalcitrant seeds, which are sensitive to low moisture content and low temperatures, do not survive if they are stored under the standard storage conditions used for orthodox seeds. This category of seeds includes several important tropical and sub-tropical tree species. There is one more category recognized as intermediates between orthodox and recalcitrant seeds and known as intermediate seeds, which can tolerate combinations of desiccation and low temperature (Breese 1989; FAO 2014).

1.2.3 Field genebank

Field genebanks maintain living plants. Field genebanks are used for the plants which to produce non-orthodox seeds or no seeds and are vegetatively propagated. The vegetatively propagated plants comprise of two types, the perennial and annual/biennial. The former can be maintained in the field for long period without replanting but for the latter re-planting is necessary annually or biennially. The preservation of these PGRs requires an adequate area of land and continuous maintenance. Vegetatively maintained PGRs are vulnerable to loss from natural disasters and damage caused by pests and diseases (Reed 2010; FAO 2014).

1.2.4 *In vitro* genebank

In vitro genebanks are the means to overcome the disadvantage of the field genebanks and reflect progress in plant tissue culture techniques. Preserved *in vitro* germplasm can be propagated and regenerated into plantlets in a sterile and pathogen free environment. *In vitro* genebanks are used for species with an established tissue culture system. To maintain *in vitro* germplasm, it should be subcultured after specific periods of time under standard culture conditions to avoid deterioration and/or contamination of materials. Several slow growth (minimal growth) methods have been established for short (3 months) to middle (3 years) term storage using low temperature, minimal nutrition, growth retardant and so on, singularly or in combination (Oka and Niino 1997). A drawback of tissue culture storage is the induction of genetic variation or mutation during

prolonged subculturing. For this reason, minimal growth method is desirable for preservation of *in vitro* materials reduce the subculture interval. Selection of explant is also important *in vitro* storage as somaclonal variation from cultured cells and callus may occur more easily compared to *in vitro* shoot cultures (Kaczmarezyk *et al.* 2011). Hence there is a preference for using shoots for *in vitro* storage by minimal growth method. Details regarding preservation methods can be found at the website link: http://cropgenebank.sgrp.cgiar.org/index.php/procedures-mainmenu-43/conservation-mainmenu-198.

1.2.5 Cryobanking

Besides field banks, orthodox seed collections and *in vitro* collections, cryopreservation has demonstrated a high stability and recovery of the species conserved by this methodology during the last decades. Cryopreservation is becoming an increasingly used method for the long-term storage of PGRs (Bajaj 1995). Cryopreservation requires only a minimum of space and low level of maintenance. Methods for cryopreservation have been developed for many plant species and further research is being conducted to enable adoption of this approach even more broadly (Li and Pritchard 2009; Pritchard *et al.* 2013). A timely book 'Plant Cryopreservation: A Practical Guide' was published to aid in the use of cryopreservation techniques globally, for the preservation of all forms of plant biodiversity (Reed 2008). Also, an updated 'Genebank Standards for Plant Genetic Resources for Food and Agriculture' was issued from Commission on Genetic Resources for Food and Agriculture (FAO 2014 http://fao.org/docrep/meeting/027/mf804e.pdf). In this book, one chapter discusses genebank requirements for *in vitro* culture and cryopreservation. Thus, cryopreservation techniques using *in vitro* shoot tips are recognized as a long-term storage tool for PGRs.

Recent research on cryopreservation has focused on practical procedures for genebank storage, thereby enabling cells and meristems to be cryopreserved by direct transfer into liquid nitrogen (LN). The development of simple and reliable methods for cryopreservation facilitates cryo-banking. Optimal cryopreservation conditions produced high levels of regrowth after LN storage (González-Arnano *et al.* 2008; Keller *et al.* 2008; Wang *et al.* 2008; Kaczmarezyk *et al.* 2011). The status of the main cryo-stored germplasm, apart from potato, is shown (Table 1.1). Seed preservation at super low temperature (by vapor of or liquid phase of LN) has been successfully achieved for a wide range of crop species by the standard seed bank protocol. There are several large cryopreserved collections of orthodox seeds. In National Center for Genetic Resources Preservation (NCGRP) USA preserves approximately 10% of the seed accessions preserved (over 37,000 accessions) have been cryopreserved. Whereas, more than 1,200 seed accessions of 50

species have been cryopreserved at the National Bureau of Plant Genetic Resources (NBPGR), India and 400 *Panax ginseng* seed accessions have been cryopreserved in National Agrobiodiversity Center, Rural Development Administration (NAC, RDA), Rep. Korea. At the Institute de Recherche pour le Dévelopment (IRD), France, a cryopreserved collection of coffee germplasm (7 species, over 500 accessions) have been also stored safely in LN even though it is a non-orthodox seed (Niino and Valle-Arizaga 2015). Some temperate woody plants can be cryopreserved by using dormant buds (Towill and Ellis 2008) and this cryopreservation method is called 'Cryopreservation of dormant buds'. This method is now applied to *Malus* spp. (Forsline *et al.* 1998), *Morus* spp. (Niino 2000; Rao 2009) and *Ulmus* spp. (Harvengt *et al*. 2004) at four different Institutes having a large scale cryo-storage infrastructure (Table 1.1).

The large scale cryo-storage of *in vitro* shoot tips has been accomplished at several Institutes by optimizing cryopreservation protocols (Table 1.1). The International Network for the Improvement of Banana and Plantain (INIBAP) has been maintaining the *Musa* spp. cryobank collection of over 700 accessions by the droplet vitrification method (Panis *et al.* 2005; Panis 2008). The other crops which have been cryopreserved in cryo-banks, are *Allium sativum* (Kim *et al*. 2004a, 2004b, Keller 2005), *Juncus effuses* (Niino *et al*. 2013), *Manihot esculenta* (Escobar *et al.* 1997), *Menta* L. (Senula *et al.* 2007), *Pyrus* spp. (Reed 1990), *Rubus* spp. (Reed 1988), *Wasabia japonica* (Matsumoto *et al.* 1994, 1998).

1.3 Potato genetic resources

1.3.1 Field collection

Potato is one of the most important food crops for food security. There are more than 4,500 varieties of *Solanum tuberosum* L. (Hils and Pieterse 2009). Preservation of potato genetic resources in genebanks is mostly by vegetative propagation due their allogamous nature and many genebanks are maintaining PGRs as field collections. But it is impossible to have a hundred percent of certitude with this methodology due natural disasters, pest and high cost maintenance.

1.3.2 *In vitro* collection

To maintain *in vitro* potato in optimal conditions, the accession requirements should be catered and at the same time establish a standardized methodology to be applied by the Genebanks. At the International Potato Center (CIP), Peru, the *in vitro* collection consists of 4,062 accessions that are maintained under slow growth conditions (Table 1.2). The medium used contains MS salts, 40 g/L sorbitol, 20 g/L sucrose, 2 mg/L glycine, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine, 0.4 mg/L thiamine and 8 g/L agar. Cultures are maintained at 6 - 8° C under 22 μmol/m²s illumination

and 16 hrs of light. This allows *in vitro* plantlets to be stored for approximately 2 years without sub-culturing (Panta 2014). The Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Germany maintains 2,855 potato accessions *in vitro* at 4° C as microtubers (Table 1.2). The cycle of slow growth maintenance consists of a warm phase with long-day at 20° C for 2 to 3 months, a microtuber induction phase with short-day at 9° C for 2 to 4 months and a cold storage period, in which microtubers are stored at 4° C for 12 to 15 months (Keller *et al*. 2006; Niino and Valle-Arizaga 2015). Besides these Institutes, *in vitro* storage of PGRs is conducted at many other Institutes, such as National Institute of Crop Science (NICS), Rural Development Administration (RDA), Rep. Korea, National Forestry, Central Potato Research Institute, Shimla, India, Crops and Livestock Research Institute, Mexico and National Institute for Agricultural Research, Chile. Almost all Institutes mentioned above have started research on cryopreservation of PGRs as an alternate of *in vitro* storage.

1.3.3 Cryostorage

During the last 25 years, several cryopreservation techniques have been established based on the conventional slow freezing method. These techniques such as the vitrification method, encapsulation/dehydration method and encapsulation/vitrification method, involve the steps of extraction of freezable water from the tissue cells before cooling (Reed 2008). As a result, vitrification of internal solutes takes place during cooling. Modified techniques have been developed which further reduce the chance for lethal ice-crystal formation through the application of ultra-fast cooling and rewarming rates. These techniques are called the droplet vitrification method, V cryo-plate method and D cryo-plate method (Panis *et al.* 2005; Yamamoto *et al.* 2011b; Niino *et al.* 2013). Detailed descriptions of these cryopreservation protocols can be found in Reed (2008). The status of potato cryo-banks of *in vitro* grown shoot tips globally is shown (Table 1.2). IPK, Germany, and CIP, Peru, are two of the largest potato genebanks. Both institutes have been applying cryo-storage to potato and achieved large cryo-bank collections with over 1,456 and 869 accessions, respectively. The cryo-storage of the potato shoot tips have been also established at NCGRP, USA, NAC, RDA, Rep. Korea, and NARO, GRC, Japan.

1.4 Base knowledge of cryopreservation

4.1.1 The concept of Vitrification

Cryopreservation is based on the reduction and subsequent interruption of metabolic functions of biological materials by decreasing the temperature with LN (-196° C), while maintaining viability. At -196° C, almost all the cellular metabolic activities are quiescent, and the

cells can be preserved in such state for a long-term. It is essential to avoid lethal intracellular freezing that occurs during rapid cooling in LN and warming to maintain the viability of hydrated cells and tissues (Sakai and Yoshida 1967). Cells and tissues that are to be cryopreserved in LN, need to be sufficiently dehydrated before being immersed in LN.

There are two types of liquid-solid phase transitions in aqueous solutions. (a) Ice formation is the phase transition from liquid to ice crystals, and (b) vitrification is a phase transition from a liquid to amorphous glass that avoids crystallization (Sakai *et al*. 2008). Water is very difficult to vitrify because the growth rate of crystals is very fast, even just below freezing point. However, highly concentrated cryo-protective solutions such as glycerol are very viscous and are easily supercooled below -70° C. This allows them to be vitrified on rapid cooling (Sakai 1997; Sakai *et al*. 2008). Vitrification refers to the physical process by which a highly concentrated cryoprotective solution supercools to very low temperatures and finally solidifies into a metastable glass without crystallization (Fahy *et al*. 1984). Vitrification had been proposed as a method for the cryopreservation of biological materials because of the potentially detrimental effects of extracellular and intracellular freezing might be avoided (Luyet 1937). Thus, vitrification is an effective freeze-avoidance mechanism for hydrated cells and tissues. As glass fills space in a tissue, it may contribute to the prevention of additional tissue collapse, solute concentration, and pH alteration during dehydration. Operationally, glass is expected to exhibit a lower water vapor pressure than the corresponding crystalline solid, thereby preventing further dehydration. Because glass is exceedingly viscous and stops all chemical reactions that require molecular diffusion, its formation leads to dormancy and stability overtime (Burke 1986).

In any cryopreservation method, whole specimens or partial parts of specimens, which are in sufficient concentration of cytosol, can vitrify by rapid cooling into LN. In the plant vitrification method, plant vitrification solution (PVS) is used which is an extremely concentrated solution (7 - 8 M) of cryoprotectants. The most applied PVS is PVS2 solution which contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulfoxide (DMSO) and 0.4 M sucrose in basal MS medium (Sakai *et al.* 1990; Matsumoto and Niino 2014). This solution is supercooled below -70° C and vitrified at about -115° C without any detectable freezing events (Sakai *et al*. 1990, 1991).

1.4.2 Cryopreservation

Preservation of *in vitro* shoot tips and somatic embryos at cryogenic temperatures is considered a suitable alternative that can ensure the long-term security of vegetatively maintained germplasm. Once stored in LN, germplasm can be kept for apparently almost unlimited periods, and as a result cryopreservation is the most appropriate for long-term storage of base collections.

Cryopreservation is often combined with tissue culture preservation for *in vitro* storage, due cryopreservation protocols are usually preceded by tissue culture, except when preserving seeds, pollen and dormant buds (Engelmann and Takagi 2000; Reed 2010; Niino and Valle-Arizaga 2015). In the vitrification method, cells and tissues must be sufficiently dehydrated with plant vitrification solution without causing injury to be capable of vitrifying upon rapid cooling into LN. High survival of *in vitro* grown materials is determined not only by the cryogenic protocol itself, but also by the physiological conditions of the materials to be cryopreserved. This means that some steps for acquisition dehydration tolerance or low temperature tolerance are crucial in the cryopreservation procedure. Following procedures such as preconditioning, preculture and osmoprotection (loading treatment) are vital for successful cryopreservation (Sakai *et al.* 2008).

1.4.3 Longevity and genetic stability of post LN storage

Germplasm will survive for a long time in cryogenic storage, it is not known for exactly how long. Estimates of the actual shelf life of cryogenically stored material are critical for efficient gene banking, but are difficult to obtain because of instrument limitations or the extended times required for measurements. The seeds of *Brassica oleracea* cryo-stored in LN for 20 years maintained their viability up to 97% after storage, but the seeds stored at -18° C for 25 years had lower viability (11%) (Walters *et al.* 2004). Cryogenic storage clearly prolonged shelf life of lettuce seeds with half-lives projected as ~450 and ~2600 years for fresh lettuce seeds stored in the vapor and liquid phases of liquid nitrogen, respectively (Walters *et al.* 2004).

Maintaining viability and genetic stability during storage is also important for cryopreserved *in vitro* shoot tips. After the development of vitrification methods, a few research publications appeared to suggest the exact viability and genetic stability of materials after long-term cryo-storage. Recently, Caswell and Kartha (2009) demonstrated that it was possible to cryopreserve *in vitro* strawberry and pea meristems in LN for 28 years. In the case of *in vitro* grown strawberry meristems, there was no decrease in the percentage of viable meristems stored for 8 weeks or 28 years. This result significantly extends the reported duration of successful cryopreservation of plant meristems and provides corroborating evidence to the theory that plant meristems may be cryopreserved indefinitely (Caswell and Kartha 2009). Also, for *in vitro* grown wasabi shoots, there was no significant differences of regrowth and morphological characteristics among 10 year cryo-storage, 2 hrs cryo-storage, treated control and control by vitrification method (Matsumoto *et al.* 2013). In biochemical analyses of sinigrin, which is a chemical precursor of the mustard oil, there was no significant difference of concentration level among them. All restriction fragment length polymorphism (RAPD) fragment patterns of 10 year cryo-storage tested were identical to those of 2

hrs in cryo-storage. From these results, Matsumoto *et al.* (2013) concluded that wasabi plants derived from shoot tips cryopreserved for 10 years by vitrification method are genetically stable. Charoensub *et al.* (2004) suggested that callus formation might increase the frequency of genetic variants. However, optimized cryogenic techniques with suitable conditions can provide high survival after rewarming. High survival is attributed to a lower degree of injury incurred by explants during cooling and rewarming. This indicates that a high level of survival after cryopreservation is necessary to reducing genetic changes.

1.4.4 Development of cryo-plate methods

Several protocols have been developed for cryopreservation of potato including the DMSO droplet method (Schäfer-Menuhr *et al*. 1997), vitrification method (Golmirzaie and Panta 2000), encapsulation vitrification method (Hirai and Sakai 1999), droplet vitrification method (Kim *et al*. 2006; Panta *et al.* 2014) and gelled droplet vitrification (Hirai 2011). These methods have been used for cryo-storage of potato genetic resources in genebanks around the world. However, the implementation of cryo-storage as a routine preservation method is still limited because it requires skillful manipulation and involves cumbersome steps such as osmoprotective and dehydration treatments and transfer of samples (Yamamoto *et al.* 2011b). Consequently, standard cryopreservation protocols are labor intensive and fraught with problems associated with mistiming solution exposure or over manipulating propagules in solution. These disadvantages may impede the wider utilization of cryostorage for important plant genetic resources.

The cryopreservation method using aluminum cryo-plates has been reported recently for genetic resources of several crops including strawberry, Dalmatian chrysanthemum, mint, mulberry, carnation and mat rush shoot tips/buds (Sekizawa *et al.* 2011; Yamamoto *et al*. 2011a, b, 2012a, b; Niino *et al.* 2013). Aluminum cryo-plates could be used for dehydration of shoot tips either by the vitrification protocol using various vitrification solutions (termed as the V cryo-plate method) or simply air-dehydration (termed as the D cryo-plate method). Both methods are “user-friendly” procedures that can ensure very high cooling and warming rates over 4000° C min^{-1} of treated explants (Yamamoto *et al.* 2011b; Niino *et al.* 2013). As a result, very high regrowth with various plant species has been obtained after cryopreservation (Niino *et al.* 2013).

The V cryo-plate and D cryo-plate protocols were developed with the aim of simplifying the procedures for cryopreservation and developing a systematic procedure that can be easily performed even by semi-skilled workers (Yamamoto *et al.* 2012b). In both protocols, the shoot tips/buds attached to the cryo-plates are dehydrated after osmoprotection in plant vitrification solution 2 (PVS2, Sakai 1990) and under the air current of a laminar flow cabinet, respectively.

1.4.5 Optimization of the D cryo-plate method for PoGRs

Our group developed both V cryo-plate and D cryo-plate methods using aluminum cryo-plates of *in vitro* potato (Yamamoto *et al.* 2015). In this work, we showed that the V cryo-plate and the D cryo-plate protocols are efficient and practical methods for cryopreservation of potato germplasm.

However, this method needs to be modified to be a simple and easy procedure without any shoot tips dropping from the cryo-plate at any step in cryopreservation and regeneration. Also, when a technique is transferred to other country, it is important to adapt the technique to the circumstances of each laboratory, because variations in responses to cryopreservation may arise from differences in operator skills and competence, culture systems, differences in equipment and minor technical details of cryopreservation, the environment of the laboratory room and so on (Reed *et al*. 2004; Keller *et al.* 2008). For the implementation of cryostorage, proper cryo-techniques should be determined for each laboratory parameter such as environment, infrastructure, cost, staff skills, and plant material available.

The Mexican government recently established a National Genetic Resources Center (CNRG) under the administration of the National Forestry, Crop and Livestock Research Institute (INIFAP) as a key component of its long-term strategy for conservation and sustainable use of genetic resources (Machida-Hirano *et al*. 2014). A key element of CNRG is the long-term storage of GRs using a cryopreservation technique at super-low temperature. Hence, it is imperative to establish a long-term storage method using cryopreservation to provide a steady backup supply of potato GRs at the CNRG in Mexico. With this study, at first, I investigated optimization of D cryo-plate procedure using *in vitro* grown potato shoot tips and adjusting the cryopreservation of CNRG.

After the development of the cryopreservation protocols for a wide range of genotypes is imperative to have a deeper understanding of the mechanisms in charge the survival under cold stress conditions. To have a further understanding of the effect of cold stress in cryopreserved potato genotypes, low temperature related mechanisms responsible for acclimation and survival to cold stress. Other works have generated information on the cold stress.

The potato (*Solanum tuberosum* L.) cultivars exposed to low temperature treatment showed differential response among accessions regrowth rates, due to cold-hardening exposure (5º C) prior cryopreservation procedure (Valle-Arizaga *et al.* 2017a). Therefore, evaluation of both D and V cryoplate methods, in three cultivars with different geographic distributions was performed in *Solanum tuberosum* (cvs. Atzimba, Desiree and Sayaka) to evaluate and compare the cryopreservation methodologies.

1.5 Hypotheses

A. The D cryoplate will have a high regrowth rate in various *S. tuberosum* genotypes reducing cost, time and performance budget.

B. The cold-hardening process increases the survival rates after cryopreservation between *S. tuberosum* L. cvs. Atzimba, Desiree and Sayaka will show a significative different.

1.6 Objectives

Develop a dehydration based protocol adaptable for a wide range of latin american *S. tuberosum* L. genotypes with high regrowth levels and low cost.

C. Evaluate the survival rates after cryopreservation between *S. tuberosum* L. cvs. Atzimba, Desiree and Sayaka using cold hardening.

1.7 Thesis structure and plan

The thesis structure and plan is summarized in Figure 1.1. This study aims to develop a simple and practical D cryo-plate method based on optimization of procedures of D cryo-plate method. The study consists as follows:

1) Improvement to the D cryo-plate protocol applied to practical cryopreservation of *in vitro* grown potato shoot tips in Chapter 2.
2) Comparison of the survival rates after V cryo-plate and D cryo-plate shoot tips of potato (*S. tuberosum* L. cvs. Atzimba, Sayaka and Desiree) in Chapter 3.
3) Comparison of the survival rates after cryopreservation between cold hardened and non-cold hardened shoot tips of potato (*S. tuberosum* L. cvs. Atzimba, Sayaka and Desiree) in Chapter 3.

1.8 Expected Outcome

By the results of this study we can promote the genebank long term standardization and establishment of a low-tech and low-budget cryobank with high efficiency for the implementation of this methodology in various genotypes (Figure 1.2).

1.9 Figures and Tables

Table 1.1. Status of main cryo-storage in the world except potato germplasm

Institute, Country	Total Accessions	Number of accessions				Cryo-storage accessions (No.)	Reference
		Field preservation	Seed Storage	*In vitro* storage	Cryost orage		
IPK/GLKS, Germany	6,124 (2,846)	89	2,846 (2,846)	2,855	1,436	DMSO droplet vitrification	
CIP, Peru	6,768 (2,414)	3,931	6,125 (2,289)	4,062 (49)	869	Droplet vitrifivation & Vitrification	
Nothern Region 6, USA NCGRP, USA	5,808				247	Droplet vitrification	Niino and Valle-Arizaga 2015
NICS, RDA, Rep. Korea NAC, RDA, Rep. Korea	1,223	670		1,223	130	Droplet vitrification	
NARO/NIAS, Japan NCSS, Japan	1,964	1,964		20 130	20	V Cryo-plate	Yamamoto *et al.* 2013
KAES HRO, Japan CAES HRO, Japan	500	500			100	Encapsulatio n vitrification	Hirai *et al.* 2011

The ( ) means number of wild potato accessions. IPK (Leibniz Institute of Plant Genetics and Crop Plant Research); GLKS (The Groß Lüsewitz Potato Collection); CIP (International Potato Center); NR6 (The US Potato Center); NCGRP (National Center for Genetic Resources Preservation); NICS, RDA (National Institute of Crop Science, Rural Development Administration); NAC, RDA (National Agrobiodiversity Center, Rural Development Administration); NIAS (National Institute of Agrobiological Sciences); NCSS ((National Center of Seeds and Seedlings); KAES, HRO (Kitami Agricultural Experiment Station, Hokkaido Research Organization); CAES, HRO (Central Agricultural Experiment Station, Hokkaido Research Organization).

Table 1.2. Current cryo-storage status of *in vitro* shoot tips of PoGRs in the world (Niino and Valle-Arizaga 2015)

Institute	Materials	Plant Spp.	Cryo-storage accessions (No.)	Cryopreservation Methods
NCGRP, USA		10% seeds of accession over 37,000	over 37,000	
NBPGR, India	Orthodox seeds	50 species	1,200	Desiccation
NAB, RDA, Rep. Korea		*Panax gingseng*	400	
IRD, France	Non orthodox seeds	*Coffea* spp.	500	
NCGRP, USA		*Malus* spp.	2,200	
NIAS, Japan	Dormant buds	*Morus* spp.	1,236	Cryopreservation using dormant buds (Slow freezing)
NBPGR, India		*Morus* spp.	329	
AFOCEL, France		*Ulmus* spp.	440	
NCGRP/NCGR, USA	*In vitro* shoot tips	*Pyrus* spp.	100	Slow frezzing
NCGRP/NCG, USA		*Rubus* spp.	57	Slow frezzing
CIAT, Colombia		*Manihot esculenta*	480	Droplet vitrification
INIBAP, Belgium		*Musa* spp.	700	Droplet vitrification
NICS, RDA, Rep. Korea	Shoot from cloves	*Allium sativum* L.	300	Droplet vitrification
IPK, Germany	*In vitro* shoot tips	*Allium sativum* L.	101	Vitrification
IPK, Germany		*Mentha* spp. L.	86	Droplet vitrification
SARC, Japan		*Wasabi japonica* M.	40	Vitrification
NIAS, Japan		*Juncus effusus*	50	D Cryo-plate

These information obtained in 2nd International Symposium on Plant Cryopreservation (Aug. 2013), Fort Collins, Colorado, USA, except seeds. NCGRP (National Center for Genetic Resources Preservation); NCGR (National Clonal Germplasm Repository); NBPGR (National Bureau of Plant Genetic Resources); NAC, RDA (National Agrobiodiversity Center, Rural Development Administration); IRD (Institute de Recherche pour le Développement); NCGR (National Clonal Germplasm Repository); NIAS (National Institute of Agrobiological Sciences); AFOCEL (Associa- tion Forêt Cellulose); CIAT (International Center for Tropical Agriculture); INIBAP (International Network for the Improvement of Banana and Plantain); NICS, RDA (National Institute of Crop Science, Rural Development Administration); IPK (Leibniz Institute of Plant Genetics and Crop Plant Research); SARC (Shimane Agriculture Research Center).

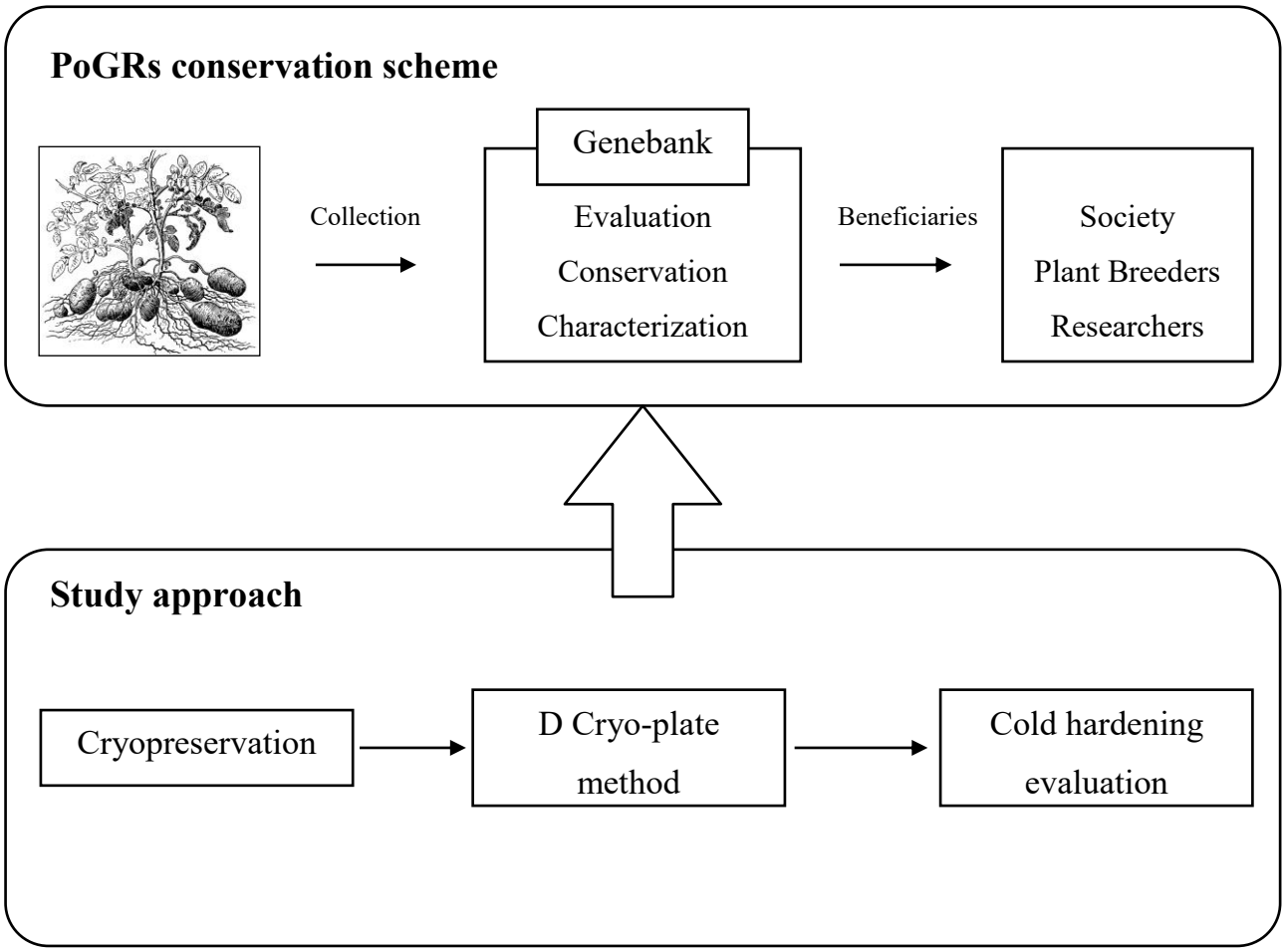

Figure 1.1. Structure of this study. This study consists of development of cryopreservation method for potato genetic resources. Potato Image Copy Right: Joseph Y. Bergen AM (1896) Elements of Botany, Boston MA, Ginn and Company.

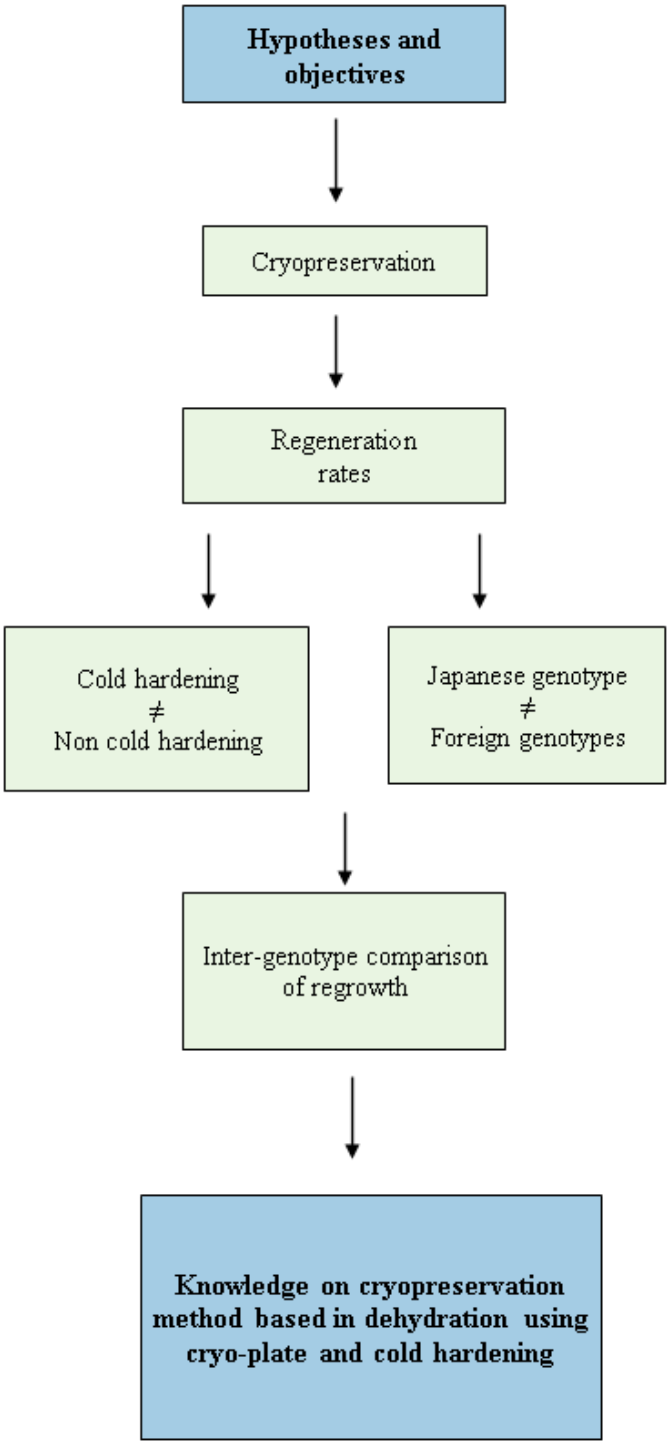

Figure 1.2. Expected outcome from this study.

CHAPTER 2

Improvement to the D cryo-plate method applied to practical cryopreservation of *in vitro* grown potato shoot tips

2.1 Introduction

Cryopreservation is increasingly used for long-term storage of plant genetic resources, requiring minimal space and low maintenance (Niino and Valle-Arizaga 2015). In our previous study, V cryo-plate and D cryo-plate methods were proposed as cryopreservation techniques for preservation of potato genetic resources (Yamamoto *et al.* 2015). The D cryo-plate method has been reported for *in vitro* shoot tips of the mat rush and shoot tips of the persimmon from dormant buds (Niino *et al.* 2013; Matsumoto *et al.* 2015). However, this method needs to be modified to be a simple and easy procedure without any shoot tips dropping from the cryo-plate at any step in cryopreservation and regeneration. Also, when a technique is transferred to other country, it is important to adapt the technique to the circumstances of each laboratory, because variations in responses to cryopreservation may arise from differences in operator skills and competence, culture systems, differences in equipment and minor technical details of cryopreservation, the environment of the laboratory room and so on (Reed *et al.* 2004; Keller *et al.* 2008). For the implementation of cryostorage, proper cryo-techniques should be determined for each laboratory parameter such as environment, infrastructure, cost, staff skills, and plant material available.

The Mexican government recently established a National Genetic Resources Center (CNRG) under the administration of the National Forestry, Crop and Livestock Research Institute (INIFAP) as a key component of its long-term strategy for conservation and sustainable use of genetic resources (Machida-Hirano *et al.* 2014). The national potato program of INIFAP in Mexico maintains approximately 1,500 accessions of potato genetic resources *ex situ*, and approximately 500 in an *in vitro* medium-term collection (López-Delgado *et al*. 1998). Hence, it is imperative to establish a long-term storage method using cryopreservation to provide a steady backup supply of potato genetic resources at the CNRG in Mexico.

This study investigated modification of the D cryo-plate procedure by paper mounting on an aluminum plate to avoid shoot tips dropping from the cryo-plate and easy handling of cryopreservation and rewarming procedures. We investigated optimization of this revised D cryo-plate procedure using *in vitro* grown potato shoot tips and adjusting the cryopreservation of CNRG. We subsequently tested the optimized protocol on “B-71-240-2” and 12 additional potato genotypes, and stable storage in LN for 1 year. As a result, we indicate a new technique for CNRG in Mexico.

2.2 Materials and Methods

2.2.1 Plant materials

The potatoes (*Solanum tuberosum*) materials were obtained from the *in vitro* Collection at Campo Experimental Toluca, INIFAP, Mexico. Experiments to apply the D Cryo-plate method to potato shoot tips were performed under accession number "B-71-240-2". The optimized procedure was tested on an additional 12 genotypes. Almost all experiments were performed in CNRG in Mexico. Cultured plants were subcultured every 3 months on solid Murashige and Skoog (MS) medium (Murashige and Skoog 1962) with 3.0% (w/v) sucrose, 0.03% calcium chloride, and 0.3% gellan gum (Yamamoto *et al.* 2015). Shoots were incubated at 18° C ± 2° C with a 24-h light under a light intensity of 32 μmol:m^{-2}:s^{-1} provided by white fluorescent tubes (standard condition). Apical shoots (about 5 mm) were cut, plated on 20 mL solid MS medium in Petri dishes (90 × 20 mm), and cultured for 2 - 3 weeks under standard conditions. Shoot tips were dissected from the shoots and pre-cultured overnight on solid MS medium containing 0.3 M sucrose at 24° C. For modification and optimization of the D cryo-plate protocols, we used 2.0 - 2.5 mm long explants consisting of the apical meristem with two young leaves.

2.2.2 Base D Cryo-plate method

The aluminum cryo-plates used for the D Cryo-plate method were 7 × 37 × 0.5 mm, containing 10 oval wells with a length of 2.5 mm, a width of 1.5 mm, and a depth of 0.75 mm (Figure 2.2 A1). The base D Cryo-plate method was derived from the protocol developed by Niino *et al*. (2014) and Yamamoto *et al.* (2015) as shown in the Fig. 2.1 and described as follows:

1. Droplets (approximately 4 μL) of 2% (w/v) sodium alginate solution with 0.4 M sucrose in MS basal solution were poured into the cryo-plate wells.
2. Pre-cultured shoot tips were transferred one by one into the wells with a scalpel blade and covered with droplets (approximately 3 μL) of the sodium alginate solution.
3. The calcium chloride solution (0.1 M calcium chloride in MS basal solution with 0.4 M sucrose) was poured drop wise onto a cryo-plate until it was covered completely. Polymerization was complete after 15 min at room temperature (RT) around 25° C, and the calcium chloride solution was removed by tapping it gently on filter paper in a Petri dish.
4. The shoot tips on the cryo-plate were treated for 45 min at RT with loading solution (LS) containing 2.0 M glycerol and 1.0 M sucrose in MS basal solution.
5. Cryo-plates were removed from the LS, tapped gently on filter paper in a Petri dish and dehydrated in a Petri dish with filter paper and 35 g silica gel; shoot tips attached to the cryo-plates were desiccated for 90 min at 24° C (Figure 2.2 E).

6. After dehydration, the cryo-plates were plunged directly into LN and transferred into 2 mL cryotubes held on a cryo-cane and filled with LN. The cryo-plates were kept for at least 60 min in LN.

7. For regeneration, the cryotubes were retrieved from LN. The cryo-plates were immersed and rewarmed in cryotubes containing 2 mL 1 M sucrose solution with MS basal medium in which they were incubated for 15 min at RT. The shoot tips were then removed from alginate gel, plated onto fresh solid MS medium, and cultured under standard conditions at 24° C.

2.2.3 Modification of the base D Cryo-plate method

One step was added to the base D cryo-plate steps between 2 and 3, because dropping of shoot tips was noted occasionally during cryopreservation treatment and/or rewarming steps, becoming a possible problem in terms of damage or loss of shoot tips. A disinfected sheet of BEMCOT paper (7 × 30 mm) (Figure 2.2 A2) was used to cover the cryo-plates, taking care to avoid dropping the shoot tips (Figure 2.2 C). BEMCOT (Clean wipe-P) is a cellulose wiper produced by Ozu Co., Japan. The BEMCOT paper has qualities such as high absorption, easy dehydration, no damage after LN storage, and low impurities contents. After step 2, the sodium alginate solution was poured on the above and below parts of cryo-plate (Figure 2.2 B). Next dried paper was mounted on an aluminum plate with the sodium alginate solution and shoot tips (Figure 2.2 C) followed by the calcium chloride treatment on the paper (Figure 1.2 D). The regeneration was done by step 7. The cryo-plates retrieved from LN were immersed in cryotubes containing 2 mL 1 M sucrose solution with MS basal medium in which they were incubated for 15 min at RT. Then the paper-attached shoot tips were removed from the cryo-plates and plated onto solid MS medium (Figure 2.3 F). The shoot tips were then removed from alginate gel and plated onto fresh solid MS medium. Effect of BEMCOT paper mounting was tested on the regeneration of cryopreserved shoot tips.

2.2.4 Optimization of the revised D Cryo-plate method

Various steps of the revised D Cryo-plate method were optimized or reconfirmed, and the effect of these optimizations on the regeneration of shoot tips was studied. Optimizations were as follows:

1. Effect of cold hardening and preculture: after apical culture for 2 weeks, the shoots were cold hardened at 5° C for 1 week under an 8 of light photoperiod with intensity of 16 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Hirai and Sakai 1999). Cold-hardened shoot tips were then pre-cultured overnight on solid MS medium containing 0.3 M sucrose at 24° C.

2. Comparison of LS sucrose concentrations: sucrose concentrations in LS were 0.6, 0.8, 1.0, 1.2, and 1.4 M.

3. Effect of duration of exposure to LS and duration of dehydration in Petri dish with silica gel: durations of exposure to LS were 30, 45, 60, and 90 min. Dehydration periods in a Petri dish with silica gel were 30, 60, 90, and 120 min at 24° C.

4. Effect of three initial light conditions after rewarming: standard light conditions (32 μmol:m^{-2}·:s^{-1}), 3 days dim condition (16 μmol:m^{-2}·:s^{-1}), and 3 days dark condition.

5. Effect of rehydration condition and removal of alginate gel: after rewarming in 1.0 M sucrose solution for 15 min, shoot tips with alginate gel were plated on the medium for 0, 1, and 16 h, and then shoot tips were removed from the alginate gel.

For large-scale cryostorage, the optimized D cryo-plate procedures with and without the cold hardening were assessed on "B-71-240-2" and 12 additional potato genotypes. For safety long-term storage, the 1 month, 6 months, and 1 year storage in LN were tested using these procedures. Also, effect of different media including ascorbic acid (Vitamin C) and/or polyvinyl pyrrolidone (PVP) on regrowth of cryopreserved shoot tips was tested.

2.2.5 Regeneration assessment and statistical analyses

Post-LN regrowth was evaluated after 4 weeks of culture under standard conditions at 24° C by counting the number of shoots that developed into normal shoots. Three replicates for each of the 10 samples were tested in each experimental treatment. Statistical analysis was performed using Tukey's test or one way ANOVA through the program on the web site (http://www.gen- info.osaka-u.ac.jp/MEPHAS/tukey.html) to compare the means and determine significant differences ($P < 0.05$) (Yamamoto *et al*. 2015).

2.3 Results

2.3.1 Modification of the D Cryo-plate method by paper mounting on cryo-plate

When using the D cryo-plate method, it is important to adhere the shoot tips firmly to the cryo-plates throughout the whole procedure for efficient performance. To avoid dropping the shoot tips, it is easy way to cover the paper on the cryo-plate. Also, after rewarming, the shoot tips attached on the paper by alginate gel, are easily transferred to the media by removing the paper. The effect of mounting the BEMCOT paper on the aluminum plate on the regrowth of cryopreserved shoot tips was evaluated (Table 2.1).

There were no significant differences in regrowth of cryopreserved shoot tips between paper mounting and no paper mounting, regardless of the dehydration time by silica gel. We observed no negative effect on regrowth of cryopreserved potato shoot tips after rewarming for paper mounting. The paper mounting increased the performance of each step, preventing detachment of explants during cryopreservation manipulations, because shoot tips were firmly fastened between the paper and cryo-plate with the alginate gel. We also observed no dropping of shoot tips during regeneration steps. In this study, we adapted the paper mounting procedure (revised D Cryo-plate method) in all other experiments.

2.3.2 Optimization of the revised D Cryo-plate method

Several steps in preconditioning are necessary to achieve high regrowth after cryopreservation, not only a making uniform and vigorous shoots but also cold-hardening of shoots and preculture of shoot tips on medium with high sucrose, for acquisition of osmoprotection and dehydration tolerance. When the shoot tips were cold-hardened and pre-cultured, regrowth after cryopreservation was highest (90%), whereas the regrowth (80%) was lower after only preculture. By contrast, shoot tips without preculture, the regrowth rate showed very low, regardless of cold-hardening (Table 2.2). In potato shoot tips that were cold hardened at 5° C for 1 week and pre-cultured overnight on solid MS medium containing 0.3 M sucrose at 24° C, regrowth after cryopreservation was highest (90%). Cold hardening following preculture is effective and necessary to obtain high regrowth in many potato genotypes.

LS treatment and dehydration in a Petri dish with silica gel are crucial to obtain vitrification of shoot tips and high regrowth using the D Cryo-plate method (Engelmann *et al*. 2008; Niino *et al*. 2013). High regrowth of cryopreserved shoot tips was obtained after 90 min silica gel dehydration reaching 73.3% to 86.7% regardless of the LS treatment duration (Table 2.3). The highest regrowth was obtained after 45 min of LS treatment and 90 min dehydration. Differences in sucrose concentration (0.6, 0.8, 1.0, 1.2, and 1.4 M) resulted in no significant difference in regrowth (Table 2.4). To standardize the dehydration step, the use of silica gel must be considered as the dehydration rate can fluctuate between laboratories due to differences in RT, relative humidity (RH), and laminar air flow speed. The optimal dehydration time for the potato shoot tips on cryo-plates by silica gel was 1.5 - 2.0 hrs.

2.3.3 Post-warming regrowth

To achieve high recovery after LN exposure, an efficient tissue culture procedure and post-rewarming handling system are necessary (Rafique *et al.* 2015). When cryopreserved shoot tips were placed in dim light or dark for 3 days, their regrowth was significantly higher than that of

shoot tips that were kept in standard light conditions (Table 2.5). Plating the shoot tips on medium for 1 h or overnight before removal of the gels was more effective in achieving higher regrowth than without plating. Dehydrated shoot tips encapsulated in alginate gel were rehydrated and swollen on the medium for more than 1 h, resulting in high regrowth due to the ease of handling during gel removal, preventing damage to the shoot tips (Table 2.6). Removal of alginate gel from shoot tips was important to obtain high regrowth as seen by comparison to shoot tips encapsulated in alginate gel (Table 2.6). The standard medium and the medium containing 1 mM Vitamin C were produced high regrowth of cryo-preserved shoot tips (Table 2.7).

2.3.4 Optimized procedure for cryopreservation

Based on the above results, we developed an optimized procedure as follows for efficient application of the D Cryo-plate method to Mexican potato genetic resources.

Cold-hardened shoot tips were excised to 2.0 - 2.5 mm in length, pre-cultured for 16 h at 24° C on MS with 0.3 M sucrose, attached on the cryo-plates by alginate gel with paper, treated with 2.0 M glycerol and 1.0 M sucrose solution for 45 min at 24° C, and dehydrated in a Petri dish with 35 g silica gel at 24° C for 90 min before direct immersion in LN. For regeneration, the cryo-plates were immersed in 2 mL cryotubes containing 2 mL 1 M sucrose solution with MS basal medium and incubated for 15 min at RT. The BEMCOT paper with cryopreserved shoot tips attached was removed from the cryo-plate, and plated on the medium for 1 h. Shoot tips were then removed from the alginate gel, plated on fresh solid MS medium, and cultured for 3 days under dim light and then under standard light conditions.

This optimized procedure was applied to shoot tips of “B-71-240-2” and 12 additional potato genotypes (Table 2.8). Regrowth on D cryo-plates was high for all genotypes, ranging from 70.0% to 93.3%, with an average of 82.8%. However, regrowth of some genotypes without cold hardening was lower than those with cold hardening. Shoot tips resumed growth within 5 days after plating and developed normal shoots without initial callus formation, growing into complete normal plantlets with roots (Figure 2.2). There were no significant differences in regrowth after LN storage ranging from 1 h to 1 year (Table 2.9).

2.4 Discussion

2.4.1 Potato cryopreservation in CNRG, Mexico

Preservation of *in vitro* shoot tips at cryogenic temperatures is considered a suitable alternative that can ensure the long-term security of vegetatively maintained germplasm. Once

stored in LN, germplasm can be kept for apparently unlimited periods, and as a result are cryopreservation is most appropriate for long-term storage of base collections (Niino and Valle-Arizaga 2015). The large scale cryo-storage of *in vitro* potato shoot tips has been accomplished at several Institutes by optimizing cryopreservation protocols (Kaczmarczyk *et al.* 2011; Niino and Valle-Arizaga 2015). In Mexico, CNRG was established on 2012 as a center of its long-term strategy for conservation and sustainable use of genetic resources under INIFAP (Machida-Hirano *et al.* 2014). We started research to establish long-term storage method using cryopreservation as a safe backup of PoGRs at CNRG from 2013. Keeping in mind the summarized practical issues that need to be resolved before initiation of a cryo-bank from Reed (2008). These issues include the plant materials to be preserved, storage records, storage forms, quantity to store, protocol testing, storage controls and recovery, as well as to say nothing of facilities and equipment. To start an experiment of potato cryopreservation in CNRG, we should decide a protocol suitable to this laboratory condition of environment, equipment, tools and obtainment of materials. Finally, we adopted D cryo-plate method after minor modifications, because its practicality and efficiency. In this study, some steps such as cold-hardening, paper mounting, silica gel dehydration and post-rewarming handling system were modified or adapted D cryo-plate procedure for the revised procedure.

2.4.2 Effect of paper mounting on cryo-plate by D cryo-plate

In D cryo-plate method, it is important to adhere the shoot tips steadily in the cryo-plates throughout the whole procedure for efficient performance. Niino *et al.* (2014) indicated the need to avoid some shoot tips detaching themselves from the cryo-plates during manipulations. The addition of 1 M glycerol and 0.4 M sucrose to the sodium alginate gel prevented the dropping of mat rush buds from the cryo-plates (Niino *et al.* 2014). However, this was not enough to avoid dropping the shoot tips from the cryo-plate perfectly, especially just after taking it out from the LN for rewarming and LS treatment. In this chapter, we observed no dropping of shoot tips during regeneration steps without negative effect on regrowth of cryopreserved potato shoot tips after rewarming for paper mounting. The paper mounting increased the performance of each step, preventing detachment of explants during cryopreservation manipulations, because shoot tips were firmly fastened between the paper and cryo-plate with the alginate gel. In addition, after rewarming, the shoot tips attached to the paper by the alginate gel were easily transferred to the medium by removing the paper. The paper-attached shoot tips can be used for transferring to the medium for rehydration (Figure 2.2 F). The modification step of paper mounting may allow the use of D and V

cryo-plate procedures for other plant species, larger materials, and longer treatments using an osmoprotective solution.

2.4.3 Optimization of the revised D Cryo-plate method

Cold acclimatization and preculture were effective and necessary for the high regrowth level (Yamamoto *et al.* 2011). When the potato shoot tips were cold-hardened at 5° C for 1 week and pre-cultured overnight on solid MS medium containing 0.3 M sucrose at 24° C, regrowth after cryopreservation was higher than the ones with no cold-hardened shoot tips. The DMSO droplet method of potato cryopreservation is currently being applied of cold accumulation of shoots under 8-h photoperiod at 21/8° C day/night temperature for 7 days (Kaczmarczyk *et al.* 2008). In droplet vitrification method, cold hardening of potato germplasm had the positive effect for the regrowth after cryopreservation, especially for varieties with low regrowth ability (Folgado *et al.* 2014; Panta *et al.* 2014). The response to cryopreservation is strongly determined by genotype and species-specific, limiting the use of the current protocols to large diverse collections such as the CIP's potato collection. To overcome this issue, it is crucial not only to optimize the protocol for different genotypes, but also to make a uniform, healthy and robust shoot tips able to tolerate cryopreservation procedures and the regeneration system (Panta *et al.* 2014). Cold acclimatization with following preculture is effective and necessary to obtain the high regrowth for many potato genotypes. However, if potato shoots were cultured under strong light intensity more than 100 $\mu mol{:}m^{-2}{:}s^{-1}$, cold acclimatization might be able to skip from the procedure (Kim *et al.* 2006; Yamamoto *et al*. 2015).

In D cryo-plate method, it is important to adhere the shoot tips steadily in the cryo-plates throughout the whole procedure for efficient performance. The addition of 1 M glycerol and 0.4 M sucrose to the sodium alginate gel was involved in avoiding that mat rush buds dropped from the cryo-plates if there was no negative effect on regrowth after rewarming (Niino *et al*. 2014). In this chapter, a sheet of BEMCOT covered the shoot tips mounted on the cryo-plate for efficient performance and avoiding that some explants detach themselves during manipulations. There is no negative effect on regrowth of cryopreserved potato shoot tips after rewarming by the paper mounting. This paper can be used for transferring the attached shoot tips after rewarming to the medium for rehydration (Figure 2.2 D). Using this technique, the possibility of using no well cryo-plate for V and D cryo-plate methods was indicated, while needed further improvement.

To standardize the dehydration step, the use of silica gel must be considered, because the dehydration rate can fluctuate between laboratories due to differences in room temperature, air RH and laminar air flow speed. Dehydration in D cryo-plate method could be performed using silica gel

if an air conditioner is not in-stalled in the transfer room (Niino *et al.* 2013). There was no different regrowth after rewarming of cryo-preserved mat rush buds dehydrated by both laminar air flow and silica gel (Niino *et al.* 2014). Due this concern, we adopted silica gel as a dehydration step in laboratory in CNRG, resulting high regrowth. Optimal dehydration time of the potato shoot tips on cryo-plate by silica gel was 1.5 - 2.0 hrs. But optimal dehydration time of the mat rush buds on cryo-plate by silica gel was 2.0 - 2.5 hrs (Niino *et al.* 2014). This difference might be due to the volume of shoot tips.

2.4.4 Post-warming regrowth

An efficient tissue culture procedure and post rewarming handling system are vital to achieve high regrowth after LN. In the cryopreservation of *in vitro* sugarcane shoot tips using V cryo-plate, removing the alginate gel, keeping the cultures in the dark for 7 days after LN and optimal medium with growth regulators improved re-growth (Rafique *et al.* 2015). In the cryopreservation of *in vitro* potato shoot tips by droplet vitrification, post culture is kept in the dark for a week on a medium with progressive decrease in sucrose levels from 0.3 M to 0.07 M finally (Panta *et al*. 2014). Also, cryopreserved potato shoot tips are post-cultured on semi solid MS with 0.05 mg/L IAA, 0.3 mg/L zeatin, 0.05 mg/L GA3, 30 g/L sucrose and 1.8 mg/L phytagel at 24° C under low light intensity for 7 days (Kim *et al*. 2006). In this revised method, the BEMCOT sheet with cryopreserved shoot tips attached is removed from cryo-plate after rewarming, plated on the medium for 1 h, the shoot tips were re-moved from alginate gel and paper and placed on the medium in dim or dark condition for 3 days and then incubated at 24° C under standard conditions. This optimized protocol was successfully applied to 13 potato genotypes, resulting 70.0 to 93.3% regrowth (Table 2.8) and stable LN storage in1 year.

In case of potato where huge diversity exists (Machida-Hirano 2015), the genotype needs to be considered in proper steps of cryopreservation protocol. Especially, preconditioning step for a weak genotype to cryopreservation could be positive effects on regrowth after it, which could be due to increased membrane stability (Kaczmarczyk *et al.* 2012). In the rewarming step, regrowth might be increased due to the removal of photo-oxidative stress, reducing oxidative stress, decrease the formation of reactive oxygen and quenching free radical species (Kaczmarczyk *et al.* 2012). Adding antioxidants in the cryoprotectant or the recovery media that counteract these reactions may improve recovery (Uchendu *et al*. 2010a, 2010b).

2.4.5 Conclusion

The differences in cryopreservation results between laboratories arise because of culture systems including facility, technical procedures and operator skills (Reed *et al.* 2004). For making a

cryostorage routine work in gene bank, a procedure for cryopreservation should be transferred easily (Keller *et al.* 2008). The procedure of cryo-preservation should be simple, efficient and practical and are also easily optimized after minor modifications of the procedure (Yamamoto *et al.* 2015). It is important to have many choices of protocol for cryopreservation, because there are many types of plant propagules and plant species to be cryopreserved (Niino *et al.* 2015). Choice of the protocol may depend on laboratory situation, cost, and sensitivity of explant to PVS and/or staff skills. To establish cryo-banking of potato genetic resources in CNRG, INIFAP, Mexico, we decided to adopt the D cryo-plate method and indicated how we modified the procedures to optimize in CNRG. The revised D cryo-plate procedure developed produced high regrowth in 13 potato genotypes shown in Table 2.8 which have maintained by slow growth method in INIFAP. This procedure appears as an efficient and practical method for cryopreservation of potato shoot tips in CNRG and facilitates to implement the cryobanking of potato germplasm in Mexico. Also, this technique could be useful to cryopreservation research of other plant species that are established tissue culture techniques.

2.5 Figures and Tables

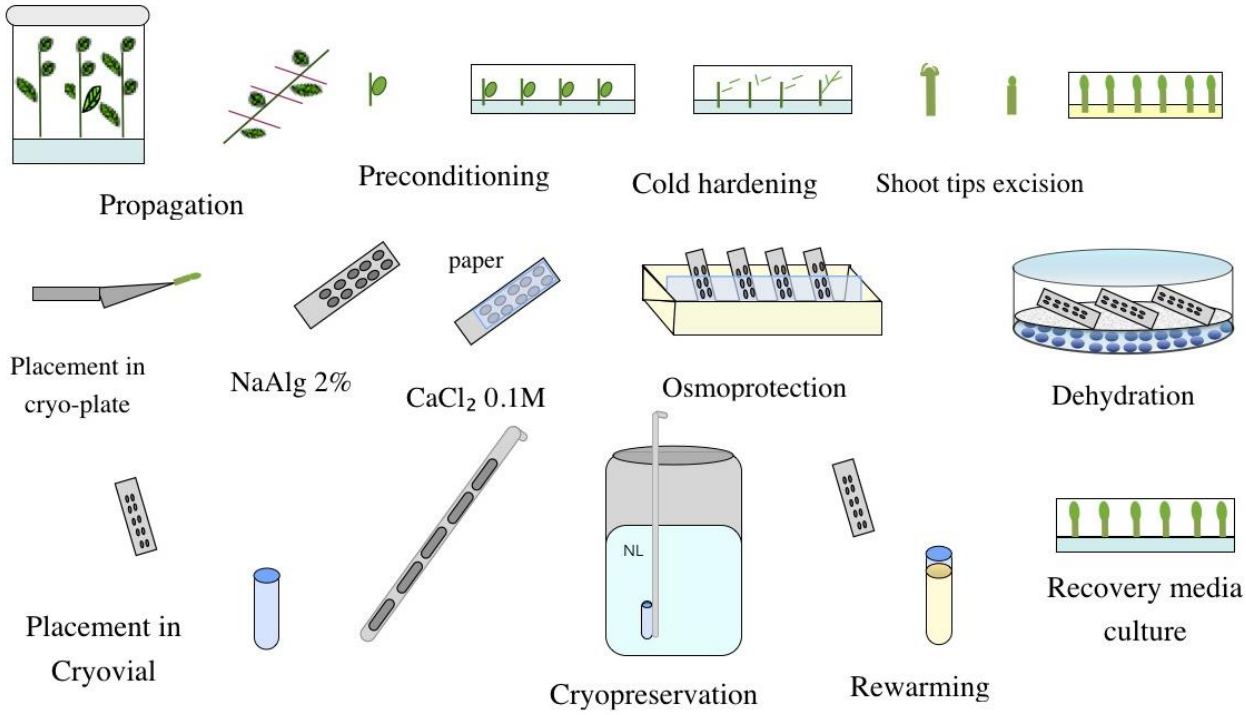

Fig 2.1. Scheme of the D cryo-plate method full procedure to standardize a genotype independent methodology.

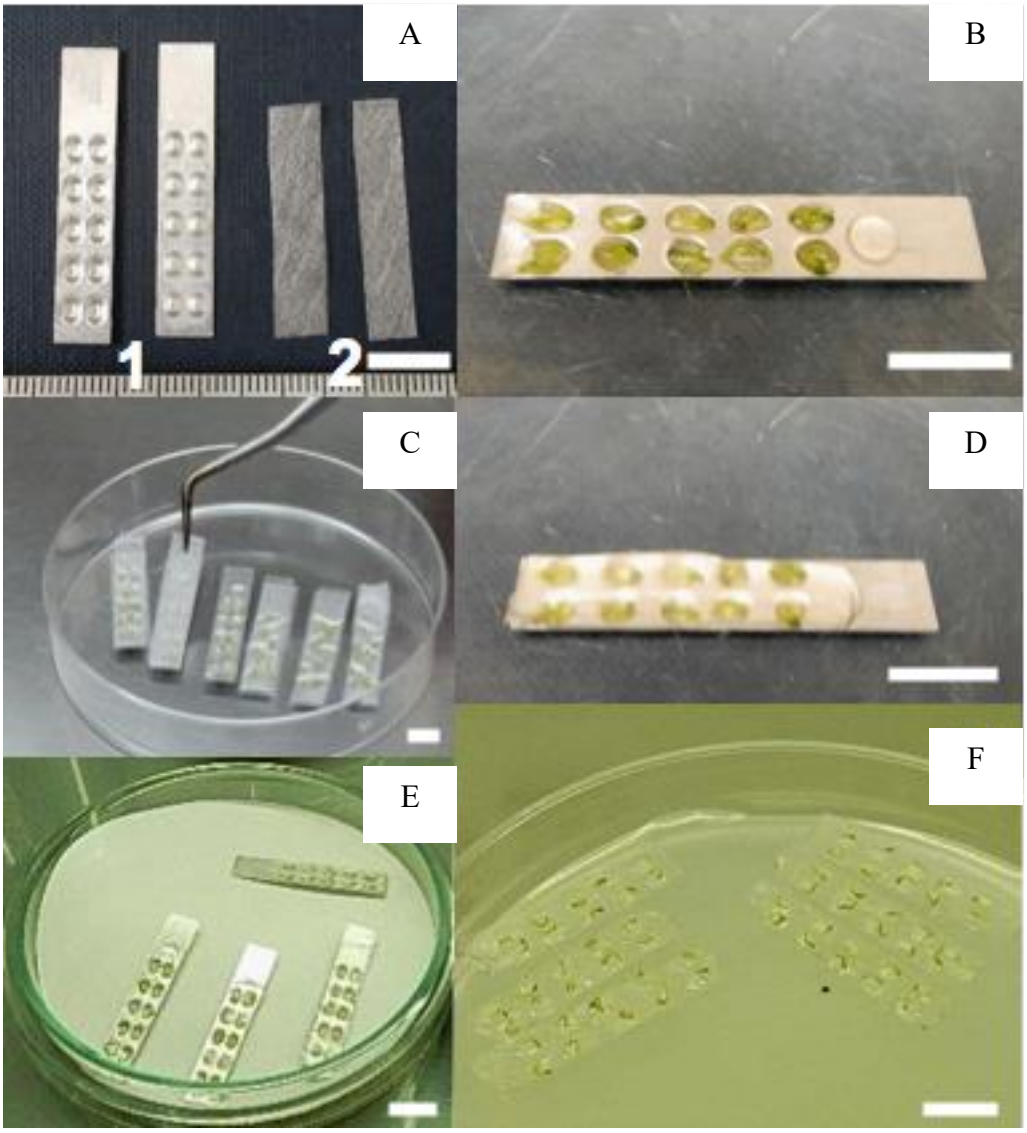

Figure 2.2. Overview of the revised D cryo-plate method procedures for cryopreservation of *in vitro* grown potato shoot tips. (A) 1: Cryo-plate with wells, 2: A sheet of BEMCOT paper; (B) Alginate solution on a cryoplate; (C) Mounting the paper on the cryo-plates; (D) Pouring calcium chloride solution on the paper; (E) Desiccation of shoot tips in a Petri dish with silica gel; (F) Rehydration of cryopreserved shoot tips attached to the paper on the medium. Bars indicate 10 mm.

Figure 2.3. Regenerated potato shoots of accession “B-71-240-2” by the optimized D cryo-plate method. A, B, C, D, E, and F refer to regenerated shoots from cryopreserved shoot tips at 3 days, 3 weeks 2 months, 3 months, 5 months, and 6 months after plating, respectively. Bar indicates 10 mm.

Table 2.1. Effect of BEMCOT paper mounting on the aluminum plate with the sodium alginate solution and shoot tips on regrowth of cryopreserved shoot tips of potato accession "B-71-240-2" using the D cryo-plate method.

Dehydration time (min)	Regrowth (% ± SE)	
	Non paper	Paper
60	73.3 ± 12.0	73.3 ± 3.3
90	86.7 ± 6.7	90.0 ± 0.0
120	86.7 ± 3.3	83.3 ± 3.3

Table 2.2. Effect of Cold Hardening (CH) and preculture on regrowth of cryopreserved shoot tis of potato accessions “B-71-2140-2” using the revised D cryo-plate method.

Preconditioning		Regrowth (% ± SE)	
Cold Hardening	Preculture	- LN	+ LN
+	+	96.7 ± 3.3	90.0a ± 5.7
+	-	90.0 ± 0.0	16.7b ± 3.3
-	+	96.7 ± 3.3	80.0a ± 5.8
-	-	90.0 ± 0.0	6.7b ± 3.3

Shoot tips were excised to 2.0 - 2.5 mm in length, pre-cultured for 16 h at 24° C on MS with 0.3 M sucrose, treated with 2.0 M glycerol and 1.0 M sucrose solution for 45 min at 24° C, and dehydrated in a Petri dish with 35 g silica gel at 24° C for 90 min. Others procedures were followed by the base protocol. Values in each column followed by different letters indicate significant differences between treatments ($P < 0.05$).

Table 2.3. Effect of LS treatment and dehydration durations on regrowth (% ± SE) of cryopreserved shoot tips of potato accessions “B-71-240-2” using the revised D cryo-plate method.

LS treatment	Dehydration duration (min)			
(min)	30	60	90	120
30	16.7b ± 3.3	36.7b ± 3.3	73.3 ± 3.3	50.0b ± 5.7
45	13.3b ± 3.3	63.3a ± 3.3	86.7 ± 3.3	80.0a ± 5.7
60	46.7a ± 3.3	46.7ab ± 3.3	83.3 ± 3.3	76.7a ± 3.3
90	56.7a ± 3.3	50.0ab ± 5.8	80.0 ± 5.7	70.0ab ± 5.7

Shoot tips were excised to 2.0 - 2.5 mm in length, pre-cultured for 16 h at 24° C on MS with 0.3 M sucrose, treated with 2.0 M glycerol and 1.0 M sucrose solution for 45 min at 24° C, and dehydrated in a Petri dish with 35 g silica gel at 24° C for 90 min. Others procedures were followed by the base protocol. Values in each column followed by different letters indicate significant differences between treatments ($P < 0.05$).

Table 2.4. Effect of sucrose concentration in LS on regrowth of cryopreserved shoot tips of potato accession “B-71-240-2” using the revised D cryo-plate method.

Sucrose concentration in LS	Regrowth (% ± SE)	
	- LN	+ LN
0.6 M	86.7 ± 3.3	80.0 ± 0.0
0.8 M	90.0 ± 0.0	80.0 ± 5.8
1.0 M	90.0 ± 0.0	83.3 ± 3.3
1.2 M	86.7 ± 3.3	80.0 ± 5.8
1.4 M	80.0 ± 0.0	73.3 ± 3.3

Shoot tips were excised to 2.0 - 2.5 mm in length, pre-cultured for 16 h at 24° C on MS with 0.3 M sucrose, treated with 2.0 M glycerol and 0.6 - 1.4 M sucrose solution for 45 min at 24° C, and dehydrated in a Petri dish with 35 g silica gel at 24° C for 90 min. Other procedures were followed using the base protocol.

Table 2.5. Effect of light conditions during the first 3 days after rewarming on regrowth of cryopreserved shoot tips of potato accession "B-71-240-2" by the D cryo-plate method.

Initial light condition after rewarming	Regrowth (% ± SE)	
	- LN	+ LN
Light	90.0a ± 0.0	55.0b ± 4.3
Dim for 3 days	93.3a ± 3.3	83.3a ± 2.1
Dark for 3 days	93.3a ± 3.3	85.0a ± 2.1

Shoot tips were excised to 2.0 - 2.5 mm in length, pre-cultured for 16 hrs at 24° C on MS with 0.3 M sucrose, loaded in 2.0 M glycerol and 1.0 M sucrose solution for 45 min at 24° C, dehydrated in Petri dish with 35 g silica gel at 24° C for 90 min Other procedures were followed by the base protocol. Different letters in columns indicate significant differences between treatments ($P < 0.05$). Ten shoot tips were tested for each of the three replicates.

Table 2.6. Effect of rehydration method and alginate gel removal on regrowth of cryopreserved shoot tips of potato accession “B-71-240-2” by the D cryo-plate method

Rehydration and gel removal		Regrowth (% ± SE)	
Rehydration	Gel	-LN	+ LN
-	Remain	43.3b ± 8.8	33.3b ± 3.3
-	Removal	90.0a ± 5.7	83.3a ± 2.1
1hr on the medium	Removal	96.7a ± 3.3	85.0 ± 2.1
Overnight on the medium	Removal	96.7a ± 3.3	90.0a ± 0.0

Shoot tips were excised to 2.0 - 2.5 mm in length, precultured for 16 hrs at 24° C on MS with 0.3 M sucrose, loaded in 2.0 M glycerol and 1.0 M sucrose solution for 45 min at 24° C, dehydrated in Petri dish with 35 g silica gel at 24° C for 90 min. Other procedures were followed by the base protocol. Different letters in columns indicate significant differences between treatments ($P < 0.05$). Ten shoot tips were tested for each of the three replicates.

Table 2.7. Effect of composition of the regrowth medium on regrowth of cryopreserved shoot tips of potato accession "B-71-240-2" by the D cryo-plate method.

Regrowth medium	Regrowth (% ± SE)	
	-LN	+ LN
Vitamin C (1mM)	93.3a ± 3.3	86.7a ± 3.3
PVP (6mM)	93.3a ± 3.3	66.7ab ± 8.8
VC and PVP	90.0a ± 0.0	40.0b ± 5.8
Control	93.3a ± 3.3	86.7a ± 6.7

Shoot tips were excised to 2.0 - 2.5 mm in length, pre-cultured for 16 hrs at 24° C on MS with 0.3 M sucrose, loaded in 2.0 M glycerol and 1.0 M sucrose solution for 45 min at 24° C, dehydrated in Petri dish with 35 g silica gel at 24° C for 90 min. Other procedures were followed by the base protocol. Different letters in columns indicate significant differences between treatments ($P < 0.05$). Ten shoot tips were tested for each of the three replicates.

Table 2.8. Regrowth percentage of shoot tips of 13 potato genotypes cryopreserved using the optimized D cryo-plate method.

Accession No.	Name	Country of Origin	Regrowth (% ± SE)	
			Non cold hardening	Cold hardening
575031	CRUZA-27	MEXICO	50.0 ± 5.8	86.7 ± 3.3
676004	CFR-69-1	MEXICO	73.3 ± 3.3	90.0 ± 5.8
676064	CRUZA-118	MEXICO	43.3 ± 0.0	90.0 ± 0.0
676070	CRUZA-155	MEXICO	83.3 ± 3.3	83.3 ± 3.3
676171	1-822 PAOLA	-	46.7 ± 3.3	73.3 ± 3.3
720083	ICA-GUANTIVA	COLOMBIA	20.0 ± 5.7	70.0 ± 5.7
720088	B-71-240-2	ARGENTINA	86.7 ± 3.3	90.0 ± 0.0
720132	MINEIRA	PERU	63.3 ± 8.8	93.3 ± 3.3
720139	YAMGUANAINTA	ARGENTINA	73.3 ± 3.3	90.0 ± 0.0
750660	ADELA	MEXICO	50.0 ± 5.8	76.7 ± 3.3
800097	RUSSET-BURBANK	U.S.A	86.7 ± 3.3	90.0 ± 0.0
573272	MICHOACAN	MEXICO	60.0 ± 5.8	73.3 ± 3.3
676007	CIP67007	MEXICO	66.7 ± 3.3	70.0 ± 5.8
		Av.	61.8 ± 5.4	82.8 ± 2.4

Shoot tips were excised to 2.0 - 2.5 mm in length, pre-cultured for 16 hrs at 24° C on MS with 0.3 M sucrose, loaded in 2.0 M glycerol and 1.0 M sucrose solution for 45 min at 24° C, dehydrated in Petri dish with 35 g silica gel at 24° C for 90 min. Other procedures were followed by the base protocol. Ten shoot tips were tested for each of the three replicates.

Table 2.9. Effect of long-term storage in LN on regrowth of cryopreserved shoot tips of potato accession "B-71-240-2" by the D cryo-plate method.

Storage in LN	Regrowth (% ± SE)
Initial (1day)	83.3a ± 3.3
1 month	86.7a ± 3.3
6 months	90.0a ± 0.0
1 year	86.7a ± 3.3

Shoot tips were excised to 2.0 - 2.5 mm in length, pre-cultured for 16 hrs at 24° C on MS with 0.3 M sucrose, loaded in 2.0 M glycerol and 1.0 M sucrose solution for 45 min at 24° C, dehydrated in Petri dish with 35 g silica gel at 24° C for 90 min. Other procedures were followed by the base protocol. Different letters in columns indicate significant differences between treatments ($P < 0.05$). Ten shoot tips were tested for each of the three replicates.

CHAPTER 3

Comparison of V cryo-plate and D cryo-plate method and cold hardening conditions in *S. tuberosum* L. cvs. Atzimba, Desiree and Sayaka

3.1 Introduction

Since the first reports of potato cryopreservation methodologies (Schäfer-Menuhr *et al.* 1997) several core changes had been made to improve regrowth rate and healthy development of the cryopreserved tissues after storage in liquid nitrogen. The vitrification method (Golmirzaie and Panta 2000), encapsulation vitrification method (Hirai and Sakai 1999), droplet vitrification method (Kim *et al.* 2006; Panta *et al*. 2014) and gelled droplet vitrification (Hirai 2011) are all based as stated, on the principle of vitrification. There are two types of liquid-solid phase transition in aqueous solutions:

(a) Ice formation where the phase transition is from liquid to ice crystals;

(b) Vitrification where phase transition is from a liquid to amorphous glass that avoids crystallization (Sakai *et al*. 2008).

Genebanks worldwide have implemented vitrification methodologies (Niino and Valle-Arizaga, 2017a). However, in order to implement vitrification protocols as a routine preservation method skillful manipulation is required and cumbersome steps involving the osmoprotective, vitrification and dehydration treatments during the processing of samples (Yamamoto *et al*. 2011b). This limits the potential of vitrification protocols being adopted as a customary part of the preservation process in genebanks and research institutes. Therefore, a standard cryopreservation protocol that is not laborious or fraught with problems associated with untimely exposure and over-manipulating the tissue in various solutions is needed (Yamamoto *et al.* 2015).

Cryopreservation methods using aluminum cryo-plates have been reported for several species including strawberry, Dalmatian chrysanthemum, mint, mulberry, carnation and mat rush shoot tips/buds (Sekizawa *et al.* 2011; Yamamoto *et al.* 2011a, b, 2012a, b; Niino *et al*. 2013). The efficiency of aluminum cryo-plates for dehydration (D cryo-plate method) and vitrification (V cryo-plate) methodologies using various vitrification solutions has been demonstrated.

The V cryo-plate and D cryo-plate protocols were developed to simplify and standardize procedures so that they can be easily performed by semi-skilled personnel (Yamamoto *et al.* 2012b). In the V cryo-plate method the tissue is attached and encapsulated to the cryo-plate then osmoprotected and vitrified by PVS2 (Sakai 1990). For the D cryo-plate method the vitrification

step is replaced by dehydration using an air current of a laminar flow cabinet or silica gel (Yamamoto *et al*. 2015; Valle-Arizaga *et al.* 2017a).

Solanum tuberosum L. is one of the main crops consumed globally. More than 4,500 varieties of *Solanum tuberosum* L. are held in genebanks (Hils and Pieterse 2009). Due to their allogamous nature potato genetic resources are preserved in genebanks mainly by vegetative propagation either by tissue culture or in field collections (Niino and Valle-Arizaga 2015).

The objectives of this study was to determine the efficiency of D cryo-plate and V cryo-plate methodologies on three *S. tuberosum* L. genotypes: Atzimba (Mexico), Desiree (Netherlands) and Sayaka (Japan). The principal objective was to compare the selected methodologies efficiency in these cultivars in relation to their geographic origins. Both methods were applied to *in vitro* grown shoot tips of potato following the procedure described by Yamamoto *et al.* (2012b) and Valle-Arizaga *et al*. (2017a).

3.2 Materials and Methods

The regrowth rates were evaluated and compared between *S. tuberosum* L. cvs. Atzimba, Desiree and Sayaka under D and V cryo-plate cryopreservation methods with and without two weeks cold-hardening (5º C).

3.2.1 Biological material

The biological materials for this work were from the collections of the Gene Research Center at the University of Tsukuba.

3.2.2 Plant material and growth conditions

The initial plantlets of potato cultivars (cvs. Atzimba, Desiree and Sayaka) were cultured in phytohormone-free MS medium (Murashige and Skoog Plant Salt Mixture, Wako, Japan) containing 25 g/L sucrose, 9 g/L agar, 5.8 pH under photoperiod conditions of 16 h light/8 hrs darkness cycle at 25º C. After 4 weeks of *in vitro* tissue cultivation, the two stress treatments (with and without cold hardening) were applied to the three genotypes. After liquid nitrogen treatment samples were placed in regrowth conditions as in the initial culture conditions.

3.2.3 Temperature treatments: Cold hardening, Cryopreservation and Regrowth evaluation

The apical shoots (2 - 2.5 cm) were sub-cultured in MS phytohormone-free media and divided into two groups where the first group was exposed to 5º C (cold-hardening), and the second

one (no cold-hardening) at 25º C both with standard photoperiod. After 2 weeks, both groups were cryopreserved following Yamamoto *et al.* (2012b) V cryo-plate method and Valle-Arizaga *et al. (*2017a) D cryo-plate method. The cryopreserved meristems where rewarmed and placed in MS media for regeneration at 25º C and evaluated every 24 hours for 3 weeks, recording survival and morphological characteristics of each sample.

The experimental materials was divided into three groups: N1 (*S. tuberosum* cv. Atzimba), N2 (*S. tuberosum* cv. Desiree) and N3 (S. *tuberosum* cv. Sayaka) which were exposed to the following treatments; T1 (without cold-hardening) and T2 (with cold-hardening). The number of samples per treatment were 10 units with 3 replications.

For the D cryo-plate method, potato plantlets were exposed to the treatments T1 (without cold hardening) and T2 (2 weeks cold hardening at 5º C). Cold-hardened shoot tips were excised to 2.0 - 2.5 mm in length, precultured for 16 h at 24° C on MS with 0.3 M sucrose, attached on the cryo-plates by alginate gel with paper, treated with 2.0 M glycerol and 1.0 M sucrose solution for 45 min at 24° C, and dehydrated in a Petri dish with 35 g silica gel at 24° C for 90 min before direct immersion in LN. For regeneration, the cryo-plates were immersed in 2 mL cryotubes containing 2 mL 1 M sucrose solution with MS basal medium and incubated for 15 min at room temperature. The BEMCOT paper with cryopreserved shoot tips attached was removed from the cryo-plate, and plated on the medium for 1 h. Shoot tips were then removed from the alginate gel, plated on fresh solid MS medium, and cultured for 3 days under dim light (90% of of the Petri dish was covered with aluminum foil) and then under standard light conditions. After two weeks of exposure to the temperature treatments (T1 and T2), the explants used for V cryo-plate were excised into 1.0 to 1.5 mm, following the standard encapsulation with alginate solution contains 2% (w/v) sodium alginate in calcium-free MS basal medium with 0.4 M sucrose and polymerization with calcium solution contains 0.1 M calcium chloride in MS basal medium with 0.4 M sucrose; the shoot tips were treated with Loading Solution (LS) 0.8 M sucrose for 30 min followed by the vitrification in PVS2 for 60 min. The cryoplates where immersed in LN for 90 min and rewarmed in 1 M sucrose solution for 15 min. For regrowth the shoots where placed carefully in MS free hormone media and left in dim light for 3 days.

Survival rate data was recorded after cryopreservation at 25º C for 3 week of recovery. Statistical analysis was performed using Tukey's test or one way ANOVA using the program at the web site (http://www.gen- info.osaka-u.ac.jp/MEPHAS/tukey.html) to compare the means and determine significant differences ($P < 0.05$)

3.3 Results and Discussion

3.3.1 Cold hardening treatments, D and V cryopreservation

The regrowth rates are presented (Table 3.1 and Figure 3.1). The regrowth of cryopreserved shoot tips by the D cryo-plate method was 50% (Atzimba), 76.7% (Desiree) and 83.3% (Sayaka) after two weeks of cold hardening. The cold hardening effectiveness was shown by the higher post-LN regrowth compared to the absence of the 5º C temperature treatment. All three cultivars showed higher post LN regrowth due to cold hardening.

The regrowth rates are provided (Table 3.2 and Figure 3.2). Regrowth percentages of the shoot tips using V cryo-plate method were 50% (Atzimba), 70% (Desiree) and 80% (Sayaka) after two weeks of cold hardening. The cold hardening was effective in obtaining higher recovery rates after the LN exposure, compared to the treatment without cold hardening. Especially in cvs. Desiree and Sayaka higher regrowth rates occurred after the 5º C cold hardening treatment.

The result of regrowth level of cryopreserved shoot tips showed no significant difference in recovery post LN by either the D or V cryo-plate methods (Table 3.3). This indicates that the V cryo-plate or D cryo-plate procedures can be successfully performed in a wide range of potato genotypes and can be easily adopted as an alternative protocol for preservation of potato genetic resources. Cold hardening 5º C treatment for two weeks should be applied to ensure success in cryopreservation of potato genotypes.

3.4 Conclusions

The cryopreserved shoot tips grown under cold hardening conditions for two weeks showed consistent regrowth rates with both cryo-plate based methods with vitrification (PVS2) and dehydration (silca gel). The D cryo-plate procedure appears to be efficient and practical for cryopreservation of *S. tuberosum* L. shoot tips without using PVS, facilitating the implementation of low-budget and low-tech methodology while maintaining the regrowth rates as high as the previously reported methods. Cold hardening at 5º C for two weeks improved cryopreservation by both the D cryo-plate and V cryo-plate methods.

The cold hardening process is effective in obtaining higher post LN regrowth in cryopreservation techniques. Further research is needed to determine why cold hardening has the effect it does to improve the regrowth rates among potato genotypes.

3.5 Figures and Tables

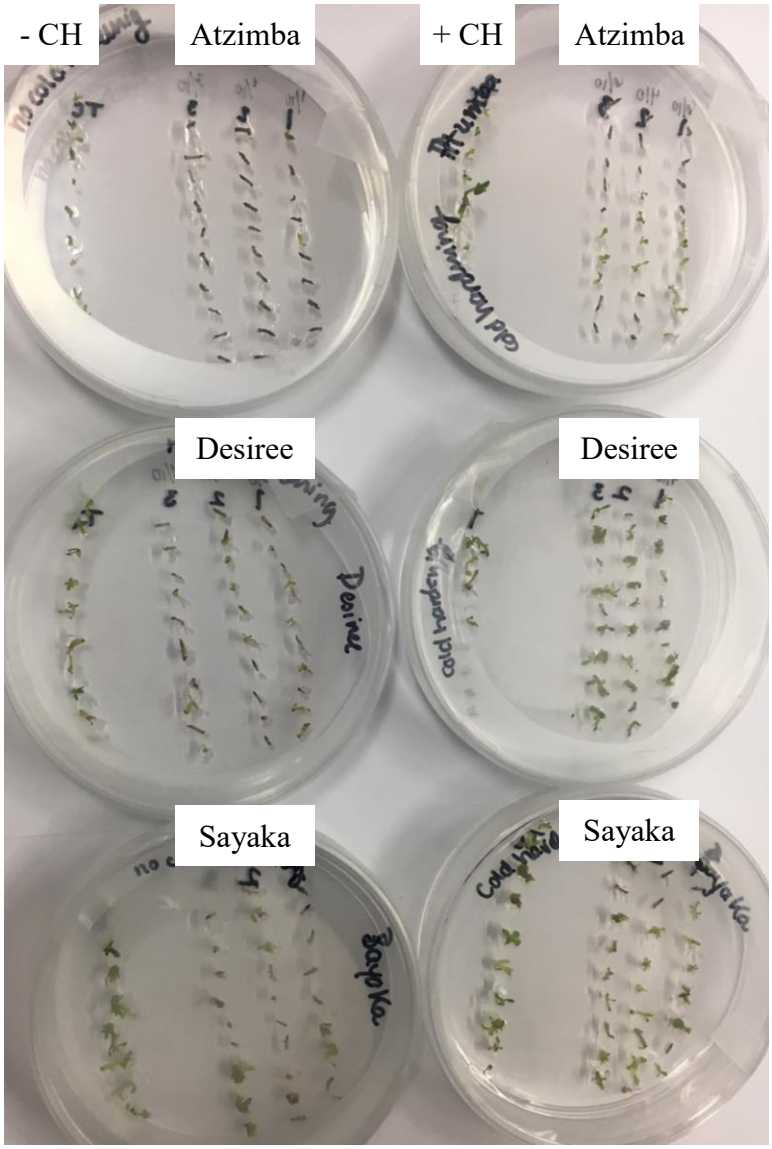

Figure 3.1. D cryo-plate method regrowth after 3 weeks cryopreservation treatment without cold-hardening (right column) and with cold-hardening (left column); potato cultivars, Atzimba, Desiree and Sayaka.

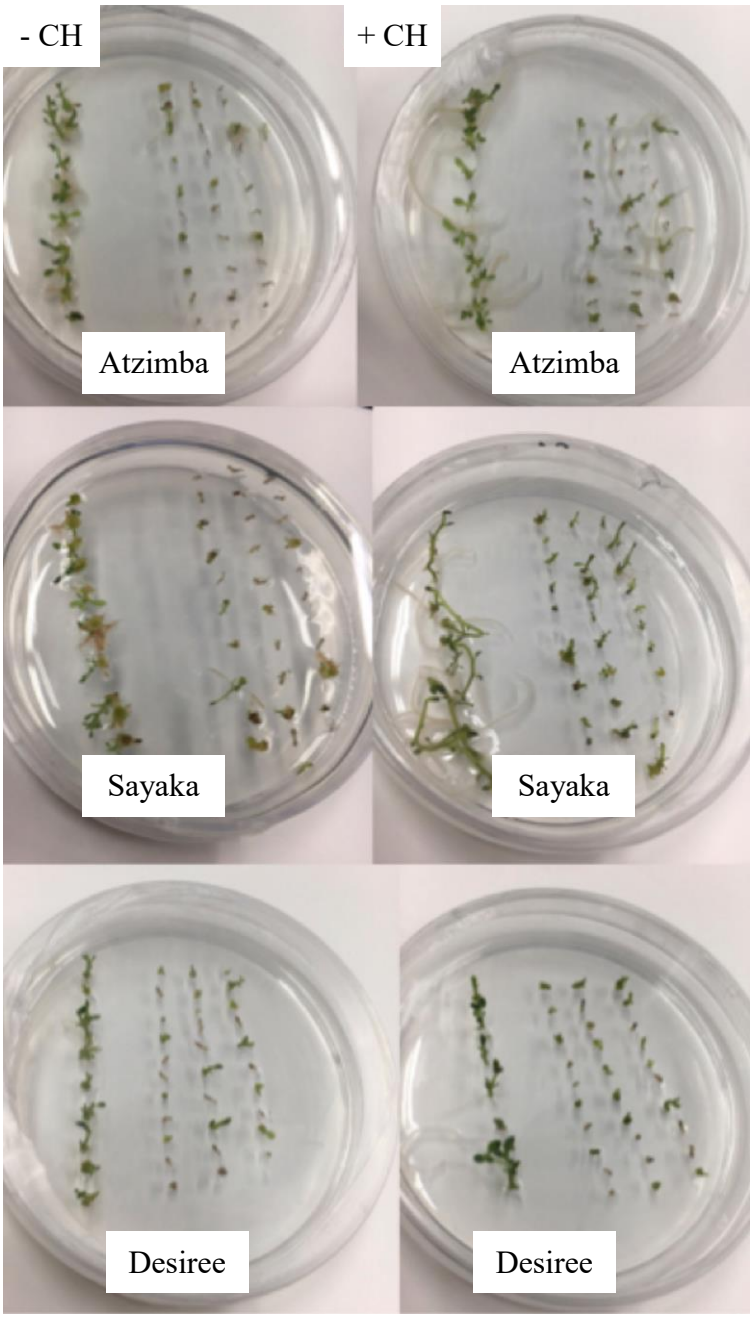

Figure 3.2. V cryo-plate method regrowth after 4 weeks cryopreservation treatment without cold-hardening (right column) and with cold-hardening (left column); potato cultivars, Atzimba, Desiree and Sayaka.

Table 3.1. Regrowth of shoot tips of *S. tuberosum* (cvs. Atzimba, Desiree and Sayaka) cryopreserved using the optimized D cryo-plate method.

Name	Country of Origin	Regrowth (% ± SE)	
		Non cold-hardening	Cold-hardening
Atzimba	MEXICO	20.7 ± 0.0	50.0 ± 3.3
Desiree	NETHERLANDS	49.0 ± 3.3	76.7 ± 0.0
Sayaka	JAPAN	58.0 ± 3.3	83.3 ± 0.0

In the D cryo-plate method, cold-hardened shoot tips were excised to 2.0 - 2.5 mm in length, precultured for 16 h at 24° C on MS with 0.3 M sucrose, treated and 1.0 M sucrose solution for 45 min at 24° C, and dehydrated in a Petri dish with 35 g silica gel at 24° C for 90 min. Other procedures were followed using the base protocol. The treatments (between Non cold-hardening and Cold-hardening) indicated significant differences ($P < 0.05$) by one-way ANOVA with the dependent case. Ten shoot tips were tested for each of the three replicates

Table 3.2. Regrowth of shoot tips of *S. tuberosum* (cvs. Atzimba, Desiree and Sayaka) cryopreserved using the optimized V cryo-plate method.

Name	Country of Origin	Regrowth (% ± SE)	
		Non cold-hardening	Cold-hardening
Atzimba	MEXICO	43.3 ± 0.0	50.0 ± 3.3
Desiree	NETHERLANDS	46.7 ± 3.3	70.0 ± 3.3
Sayaka	JAPAN	61.8 ± 3.3	80.0 ± 0.0

In the V cryo-plate method, cold hardened shoot tips (1.5 mm long) were excised, pre-cultured for 16 h at 25° C on MS with 0.3 M sucrose, loaded in 2.0 M glycerol and 0.8 M sucrose solution for 30 min at 25° C and exposed to PVS2 for 30 min at 25° C. The treatments (between Non cold-hardening and Cold-hardening) indicated significant differences ($P < 0.05$) by one-way ANOVA with the dependent case. Ten shoot tips were tested for each of the three replicates

Table 3.3. Regrowth of shoot tips of *S. tuberosum* (cvs. Atzimba, Desiree and Sayaka) cryopreserved using the optimized D and V cryo-plate methods.

Name	Country of Origin	Regrowth (% ± SE)	
		V cryo-plate	D cryo-plate
Atzimba	MEXICO	50.0 ± 3.3	50.0 ± 3.3
Desiree	NETHERLANDS	70.0 ± 3.3	76.7 ± 0.0
Sayaka	JAPAN	80.0 ± 0.0	83.3 ± 0.0

The treatments (vitrification and dehydration cryo-plate protocol) indicated significant differences ($P < 0.05$) by one-way ANOVA with the dependent case but not between them.

Chapter 4
General discussion

4.1 General discussion

Based on the results of this study, the D cryo-plate method showed high regrowth rate in fifteen genotypes of *S. tuberosum* L. after cryopreservation resulting in reducing cost and time. This research has shown results of cryopreservation experiments for *S. tuberosum* L. cultivars from Latin America, the Netherlands and Japan. A low cost protocol with high regrowth rates suitable for genebank application for potato germplasm that can be utilized for future potato breeding and contribute to the food security has been developed (Figure 4.1).

4.1.2 D Cryo-plate method for potato shoots

Developing cryopreservation protocols that can reduce cost and time substantially with a high regrowth performance is important for genebanks. Previous reports revealed the differences in cryopreservation results between laboratories can be attributed to tissue culture systems including facilities, technical procedures and operator skills (Reed *et al.* 2004). In order to develop a method suitable to be included in the cryo-storage routine work of a genebank, the cryopreservation procedure should be transferred easily (Keller *et al.* 2008).

The revised D cryo-plate procedure presented here produced high regrowth in thirteen distantly related potato genotypes (Table 2.7), which have been maintained by slow growth of INIFAP, Mexico. This procedure appears to be efficient and practical method for cryopreservation of potato shoot tips in CNRG and facilitates the implementation of cryobanking of potato genetic resources in Mexico and could be easily transferred to developing countries. This technique could be applied for cryopreservation of other major and minor crops that are currently maintained in field genebanks or tissue culture.

4.1.3 D cryo-plate and V cryo-plate method comparison for potato shoots

D and V cryo-plate method protocols are efficient and practical protocols for cryopreservation of potato germplasm. In both protocols regrowth rates were optimized by the application of a cold hardening treatment of 5º C for two weeks to genotypes from different geographic regions. By comparing the efficiency of the currently utilized cryopreservation methodology using PVS2 and dehydration by silca gel (Chapter 3), it was confirmed that D cryo-plate method can be performed in a wide range of potato genotypes and easily be adopted as a low-cost and environmentally friendly protocol for preservation of potato genetic resources due to the

omission of DMSO. Results presented in chapters 2 and 3 show that adding the cold hardening treatment for two weeks should be considered to ensure success in cryopreservation of plant materials tissue before being cryopreserved.

4.2 Future applications

Temperature stress is a limiting abiotic factor for the development of plant species. Due to the current climate change variable temperature conditions threaten plant genetic resources (Akhtar *et al*. 2012). Long term conservation strategies should be stable, low-cost, efficient and easily transferable to developing countries where plant diversity is high. Valuable traits in crops and their wild relatives will be needed to breed future stress tolerant varieties and disease resistant food crops (Reed *et al*. 2004; Keller *et al.* 2008).

The D cryo-plate method provides all the requirements to ensure a backup of potato genetic resources with a user and budget friendly methodology that can be applied in the routine of Cryobanks around the world. In addition, research on DRE/CT *cis*-elements transcription factor detection and their impact on cold temperature tolerance could contribute to the identification of genotypes with useful traits for breeding and development of cold stress tolerant cultivars and food security in a near future (Shinozaki and Yamaguchi-Shinozaki 2000; Chinnusamy *et al*. 2003; Sakuma *et al.* 2006, 2007; Cramer *et al.* 2011; Shi *et al.* 2014). To identify cold stress influence in the conservation process, the gene expression of the selected genes shown in the DRE/CRT pathway (Figure 4.2) should be evaluated using the potato cultivars used in this study. The present study has demonstrated the improvement of regrowth rates after cold hardening treatment. It would be helpful to have a further understanding of the impact of cold temperature accumulation leading to a higher regrowth rate in cryopreservation methodologies.

The D cryo-plate method has been shown to be efficient and flexible enough to be applied to other minor crops. The methodology was successfully applied to *Ullucus tuberosus* Cal. (Valle-Arizaga *et al.* 2017b).

To further investigate of cold stress genes standardize cryopreservation protocols need to be developed and using these protocols identify cultivars with affinity of this methodology that may express cold tolerance related genes (Figure 4.2). This proposal could broaden knowledge of the genes response to cold hardening and its impact on the process of cryopreservation. Eventually it could speed up the process of development and standardization of long-term conservation. Moreover this research could identify cold stress tolerant cultivars that could be used for breeding purposes.

4.3 General Conclusion

The implementation of a D Cryo-plate method for potato lowers physical damage to the tissues, including the apical meristem that is exposed after excision; chemical toxicity of PVS2 (Plant Vitrification Solution 2) and osmotic stress due to exposure for treatment is reduced by replacing the vitrification treatment with dehydration using silca gel in a small controlled environment that is provided by an enclosed container, such as a petri dish. Excising strong cold hardened shoot tips and avoiding vitrification solutions, can limit the damage and increase the regrowth rate of potato cultivars. These methods can easily be implemented in conservation institutions. Successful custom-made cryo-plate protocols for preservation of potato cultivars with a user-friendly methodology as well as reduce cost of conservation of *in vitro* potato collections have been demonstrated.

4.4 Figures and Tables

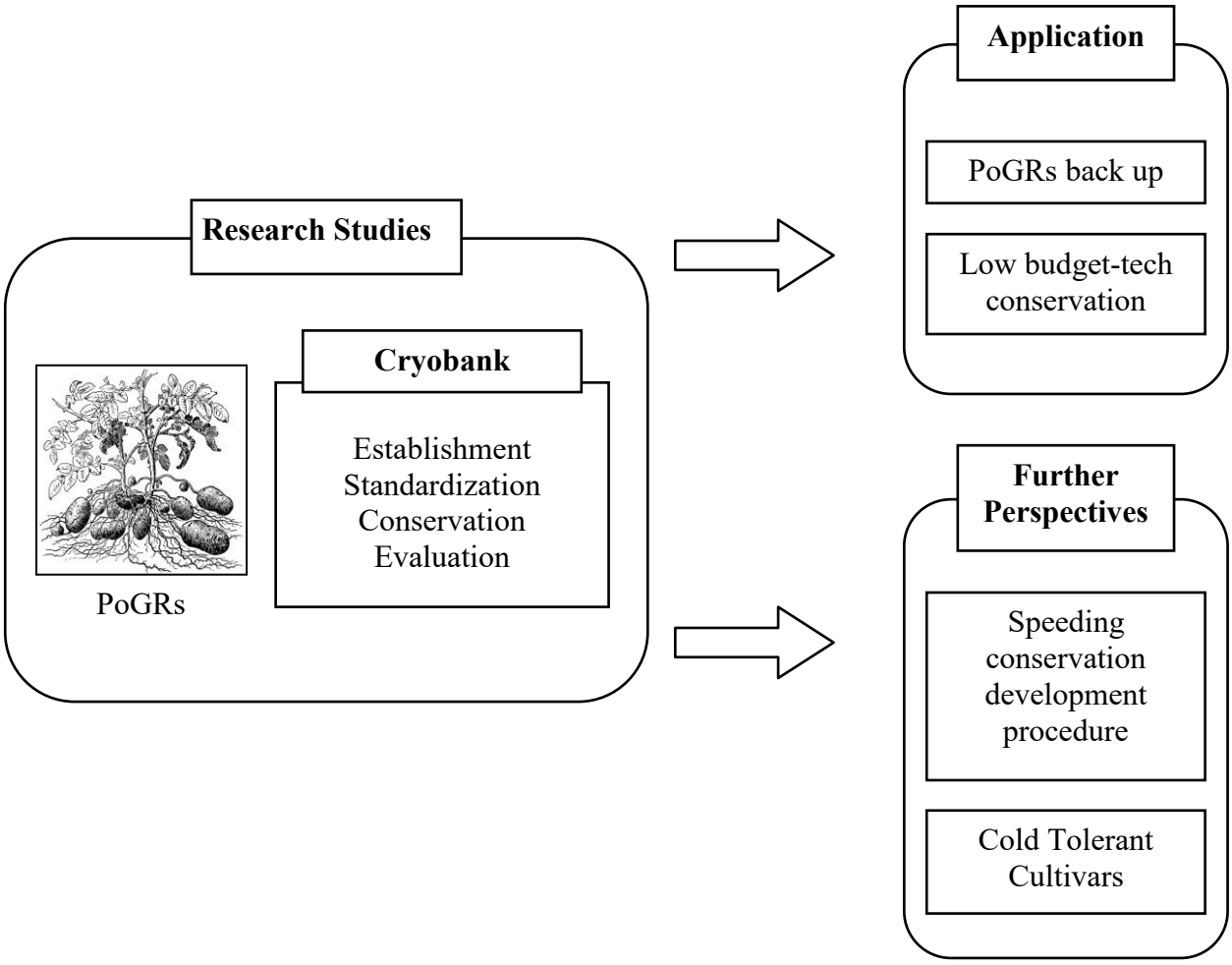

Figure 4.1. Application of the research studies from this thesis for cryobanking currently and future application perspectives.

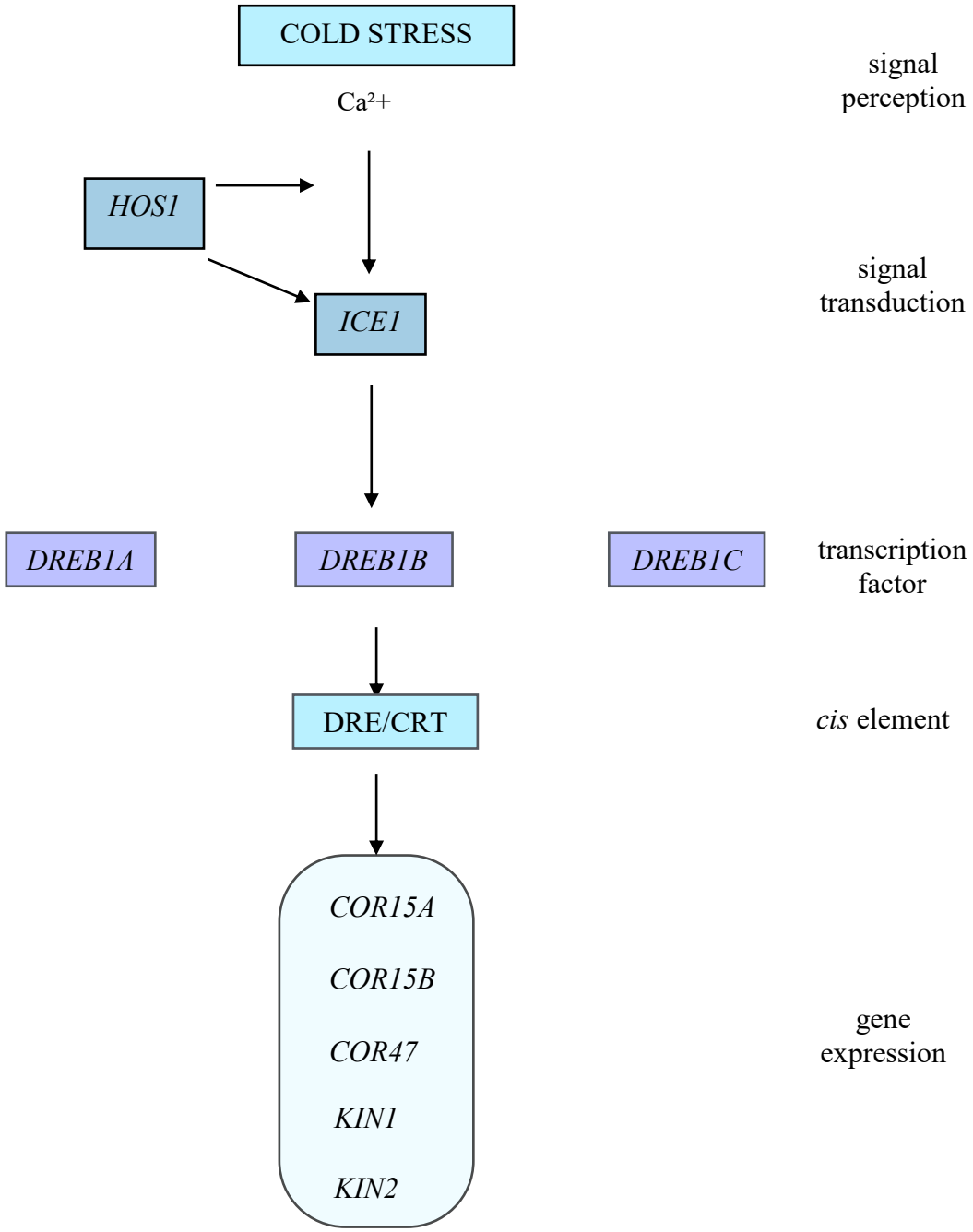

Figure 4.2. Simplified working model of cold stress signaling network pathway of target genes related with the DRE/CRT dehydration response element binding protein/C-repeat binding factor (Shinozaki and Yamaguchi-Shinozaki 2000; Chinnusamy *et al*. 2003; Sakuma *et al.* 2006, 2007; Cramer *et al.* 2011; Shi *et al.* 2014).

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要約：バレイショにおける遺伝子型非依存の超低温保存法の応用

環境ストレスは植物の成長に影響し、作物の生産性を極端に低下させる。その一つは極端な温度変化である。生育期における環境ストレスの変化は作物の生存にも影響し、野生植物においては植物の生息可能な地理的分布にも影響する重要な原因である。

生息域外保全において、フィールドコレクションでは、環境変動の影響を直接受け高温や低温で、遺伝資源が死滅する可能性もある。また、温度ストレスは、組織培養等での低温保全法の開発に影響することもある。液体窒素を用いた超低温保存(cryoconservation)も昨今では、植物に適用可能になってきているが、技術的な改善や多様な植物種の生理特性を踏まえた手法確立の必要がある。超低温保存は、植物遺伝資源の長期保存にとって重要なツールとなり、効率的な凍結保存プロトコールが多くの植物種に対して開発されているが、多くの事例は遺伝子型非特異的手法にいたっていず限られた系統での手法構築でしかない。また、超低温への低温化への過程の温度変化の調整も研究が必要な要因である。

バレイショ（*Solanum tuberosum* L.）は世界中で消費される主要作物であるが、現在の気候変動によって影響を受けることが往々にある。メキシコでは、疫病抵抗性やウイルス病抵抗性を備えた多数遺伝資源が存在し、これらが歴史的に、世界諸方でバレイショ抵抗性品種育種に貢献してきている。一方、これら系統の安定した保全は、グローバルな視点での品種改良の材料の確保に必要であるが、圃場での維持は、上に挙げたように温度変化等による脆弱性のため、遺伝資源を失うような事態になりかねない。

遺伝資源の長期安定保全のために、培養でのバレイショ遺伝資源保全が世界各所で試みられてきているが、培養だけでは、頻繁な継代培養が必要で、労力と資材管理に大きな負担がかかる。液体窒素での超低温保存が、効率化としては考えられるが、バレイショでは、多様な遺伝子型に適用できる超低温保存は、未だ世界的に共通体系ができていな

い。また、大量の茎頂芽について多数保存する必要もあるが、茎頂導入処理は効率的ではない。そこで、筆者は、効率的かつ安価な超低温保存プロトコールの開発を検討し、新規の技術体系の提案を試みた。

超低温保存は、組織培養への導入、茎頂の成長を抑制する低温での長期保存法を経て、超低温保存法を適用することが多い。超低温保存法には、DMSO ドロップレット法、ドロップレット（ガラス化）法、ビーズガラス化法、クライオプレートガラス化法などがあるが、液体窒素浸漬後の茎頂の再生率の問題がある。総じて、超低温導入過程の煩雑性や作業者への技術的に高い要求度が課題となっている。使用する凍結保護剤は、再生に影響することも報告されており、超低温導入の手間やコスト面での課題とも関連している。

筆者は、遺伝的に多様なバレイショ品種群を体系的に組織培養に誘導し、培養促進後、低温順化誘導法と多様な遺伝子型に対応できる超低温保存プロトコールを整備した。筆者は、超低温誘導前の氷温前後での低温順化が効果あることを検討した。異なる凍結保護剤の使用も、plant vitrification solution (PVS)の構成成分について効果と効率において顕著な有効性が認められた。これら検討事項は、液体窒素（LN）に保存された後の再生及び増殖率を確実に向上させた。

筆者は、超低温導入されたジャガイモの遺伝子型における事前低温順化および脱水の方法の効果についてさらなる理解を得るために、いくつかの浸透圧保護剤濃度、その処理時間、低温順化培地培養等について条件を検討し、さらなる改善を検討した。異なる低温順化温度で再増殖率の差が認められた。液体窒素浸漬されたバレイショ品種の場合、凍結保存手順として低温順化（5℃）が必須であることがわかった。

これらの一連の流れは、遺伝的に多様なバレイショ遺伝資源について、新規の遺伝子型の系統の茎頂を大量に超低温誘導できる手法としてクライオプレート乾燥（D

cryo-plate）法を独自改変し、適用した。筆者の提唱手法は、先行研究の既知の手法と比べ簡易であり容易に多数の系統を導入でき、かつ浸透圧保護剤の使用等を極力控えたためにコスト削減になった。日本の農研機構遺伝資源センターやメキシコ国立遺伝資源銀行等において、幅広く世界中の異なるジーンバンクで利用されつつある。さらに当該手法は、異なる作物種にも適用できることが報告されており、メキシコ国立遺伝資源銀行等で在来作物種の保全にも適用されつつあることが知られている。

筆者は今後の展望として、２つの提案をした。バレイショの異なる遺伝子型でのD cryo-plate 法は、液体窒素浸漬後の再生率が十分であり適用できることがわかったが、遺伝子発現において恒常的に発現する遺伝子群の発現について、液体窒素浸漬前後で、顕著な差異がないか検討することの必要性を指摘した。また、低温順化及び超低温誘導過程で機能する遺伝子群を特定することで、超低温導入後の再生率の指標化の可能性もあることを提案した。

キーワード：超低温保存、クライオプレート乾燥法、DMSO ドロップレット法、ドロップレット（ガラス化）法、ビーズガラス化法、バレイショ遺伝資源、クライオプレートガラス化法、*Solanum tuberosum* L.

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