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REVIEW PAPER

Biobanking of vegetable genetic resources by in vitro conservation and cryopreservation

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Abstract

Today, application of in vitro culture by means of slow growth storage of shoot cultures and cryopreservation of organs, tissues and cells in liquid nitrogen presents a remarkable strategic tool to support medium- and long-term conservation of plant genetic resources. Over the last 30 years, considerable progresses have been made in the development of both methods that are currently considered as ex situ conservation strategies, complementary to traditional seed banks and in-field clonal collections. Efficient protocols were developed for the conservation of a large number of crops, including strategically-important vegetables, such as garlic, artichoke, asparagus, cassava, Jerusalem artichoke, mint, potato, sweet potato, chicory, taro, thyme and yam. As a consequence, more than 45,000 accessions of vegetable crops are maintained in 22 genetic resources conservation centers (biobanks), located in 16 countries and 6 continents (Europe, Asia, Africa, Oceania, North and South America). Approximately 4/5 of these accessions are maintained in vitro by means of slow growth storage of shoot cultures, but cryopreservation is also constantly growing, with almost 8300 vegetable accessions being stored in liquid nitrogen at − 196 °C.

Keywords Biobanks · Cryopreservation · Cryobanks · In vitro banks · Slow growth storage

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This article belongs to the Topical Collection: Ex-situ conservation.

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Abbreviations

ARC	Agricultural Research Council; South Africa
CAAS	Chinese Academy of Agricultural Sciences; China
CAES	Central Agricultural Experiment Station; Japan
CePaCT	Centre for Pacific Crops and Trees; Republic of Fiji
CIAT	International Center of Tropical Agriculture; Colombia
CIP	International Potato Center; Peru
CPRI	Central Potato Research Institute; India
CRI	Crop Research Institute; Czech Republic
EMBRAPA	Brazilian Agricultural Research Corporation; Brazil
ICAR - NBPGR	National Bureau of Plant Genetic Resources; India
IITA	International Institute for Tropical Agriculture; Nigeria
InHort	Research Institute of Horticulture; Poland
IPK	Leibniz Institute of Plant Genetics and Crop Plant Research; Germany
IRD	Institut de Recherche pour le Developpement; France (ex-ORSTOM, Office de la Recherche Scientifique et Technique Outre-Mer).
NAC	National Agrobiodiversity Center; South Korea
NARO	National Agriculture and Food Research Organization; Japan
NCSS	National Center of Seed and Seedlings; Japan
NIAS	National Institute of Agrobiological Sciences; Japan
NICS	National Institute of Crop Science; South Korea
PFR	New Zealand Institute of Plant and Food Research; New Zealand
PRI	Potato Research Institute; Czech Republic
USDA-ARS	US Department of Agriculture-Agricultural Research Service; United States
USPG	US Potato Genebank; United States
VIR	N.I. Vavilov Institute of Plant Genetic Resources; Russia

Introduction

The actual worldwide erosion of plant genetic variability is attribute to many important factors, including partial degradation of the natural habitat, changes in land use, replacement of traditional varieties with modern cultivars, intensification of agriculture, population increase, poverty, land degradation and climate change (FAO 2010). Over time, there has been a dramatic depletion of genetic heritage and an increase in the number of threatened species. Recent assessments indicate that more than 75,000 species of the estimated 391,000 total number of plant species are at risk of extinction (Pimm and Raven 2017) which has caused global alarm. Vegetable biodiversity is an important genetic resource for food supply, which can play an important role in ensuring adequate levels of nutrition but is subjected to worrying genetic erosion.

The conservation of plant germplasm can be carried out in situ and ex situ. In the first case, the species are kept in their natural environment through conservation practices of habitat and ecosystem management, or conservation is pursued by promoting farming landraces and ancient crop varieties. However, inevitable damage and transformation of natural environments may cause declines in the variability of species, populations and

ecosystems, with consequent loss of biodiversity. In situ strategies alone are rarely sufficient to guarantee the conservation of the plant genetic heritage. Therefore, it is essential to integrate plant biodiversity conservation programs through additional approaches using ex situ strategies which keep the biological materials in artificial environments, with the possibility of reintroducing them to their natural habitats. In addition to the traditional approaches of seed-banking and clonal orchards for the ex situ conservation of genetic resources, there are today some recently developed and rapidly evolving techniques that can be validly considered as integrated and complementary to plant biodiversity conservation programs, providing a further guarantee against accidental loss of genetic resources.

In this context, in vitro culture is a useful tool for the collection, multiplication and conservation of plant germplasm. Plant, species and genetically improved germplasm (such as elite genotypes or cell lines with peculiar characteristics) can be safely maintained in vitro at low costs. Although the risk of contamination and somaclonal variation cannot be neglected (Larkin and Scowcroft 1981; Bairu et al. 2011), yet the reduction of the frequency of periodic subculture susceptible to human errors, as well as the very low proliferation during conservation, minimize those risks significantly. In vitro systems allow plants to be reproduced in an asexual manner with high rates of proliferation, exploiting the intrinsic potential of a cell to regrow into an entire organism (totipotency), in a sterile artificial environment with defined nutritional conditions (Normah et al. 2013). In vitro culture technology has the distinct advantage of being able to conserve cells and organized tissue such as shoots, cells, meristems, and zygotic or somatic embryos (Mycock et al. 2004). The miniaturization of plants due to in vitro culture allows the creation of large collections in small spaces, through either the medium-term conservation by slow growth storage of shoots (Lambardi and Ozudogru 2013), or the long-term cryopreservation (usually in liquid nitrogen, at − 196 °C) of tissues and organs excised from in vitro material (Panis and Lambardi 2006). Both these approaches reduce the cost of labor for maintenance and encourage the establishment of biobanks. Moreover, the nature of in vitro cultures, uncontaminated by bacteria and fungi, facilitates the exchange of germplasm across international borders (Sharma and Sharma 2013).

This review illustrates biobanking by in vitro conservation and cryopreservation techniques available today for the conservation of vegetable genetic resources and discusses their effectiveness.

In vitro conservation as a form of medium-term conservation of vegetable genetic resources

Micropropagation

The in vitro propagation method, commonly known as micropropagation, is of great importance to multiply the collected genetic material and is fundamental in the case of species in danger of extinction. Micropropagation allows rapid and continuous production of propagules of plants, starting from primary explants, with an exponential increase in the number of individuals (shoots) in a controlled micro-environment, independent of seasonal changes. The classical micropropagation, based on the repeated proliferation of axillary buds, is flanked by two other forms of regeneration, organogenesis and somatic embryogenesis which refer to morphogenetic processes that can be initiated directly by non-meristematic organ and tissue explants (for example portions of leaves or stem), or

indirectly through the formation of an undifferentiated tissue, i.e., the callus. Organogenesis refers to the formation of uni-polar structures (shoots, roots) that may arise directly from the differentiated adventitious meristems or indirectly from the callus. Somatic embryogenesis is the formation of bi-polar structures (somatic embryos), similar to organogenesis, initiated directly from the explants or indirectly by the establishment of callus, and can develop into whole plantlets without undergoing the process of sexual fertilization (George 1996; Oseni et al. 2018). Direct morphogenesis produces low events of genetic alteration, while indirect morphogenesis (i.e., through callus) allows very high rates of multiplication, but with greater risks of genetic alterations. However, as they cannot guarantee absolute genetic responsiveness, none of these regeneration systems are used in in vitro clonal propagation.

The advent of in vitro propagation and its commercial application have expanded the available methods of plant germplasm conservation. The maintenance of the crops under standard growth conditions implies periodic transfers (i.e., subculture of shoot cultures every 4–6 weeks, depending on the species) on fresh media for several years, depending upons the species. However, the limit of this approach is the loss of the crop that may occur due to aging of the shoots (when in multiplication for a long time), or accidental contamination. A good example of in vitro maintenance of crops under standard growth conditions is the ICAR-NBPGR of New Delhi, India, where a large germplasm (i.e., more than 15,800 accessions of 'difficult-to-conserve' plant species) is maintained in in vitro banking (Pandey et al. 2015) by means of micropropagation. Standard growth of in vitro preservation has the advantage of allowing the germplasm to be readily available for distribution when required. However, this approach is limited by high hand labor, the frequent losses of material due to contamination, somaclonal variation and loss of regenerative capacity over time. For this reason, the development of in vitro banks has, over time, been directed towards medium-term conservation by slow growth storage which has its starting point with standard micropropagation.

Slow growth storage

Slow growth storage (also called minimal growth storage) is a low-cost method that allows preservation of a large number of healthy in vitro shoots, by slowing their cellular metabolism and consequently reducing the growth rate of plant cultures. This method allows a significant expansion of subculture intervals, with a reduction in maintenance costs and risk of contamination during the handling of the shoots.

This form of conservation is based on the reduction of plant metabolism through modifications of the physical conditions of the cultured shoots, and the chemical composition of the nutritive medium. Among the physical factors, low temperature plays an important role in reducing growth. Temperatures used for medium-term conservation of temperate species are usually between 2 °C and 12 °C, 4–5 °C being the most frequently applied temperature ranges (Lambardi and Ozudogru 2013). On the other hand, tropical species are very sensitive to low temperatures in vitro and may incur physiological damage, called cooling lesions (Graham and Patterson 1982), which cause changes in metabolism, protein content, composition and functioning of the membranes. These changes, directly proportional to the reduction of the temperature, are generally reversible only if they are short-lived. Therefore, tropical species must rather be kept at temperatures of 15–22 °C, depending on the sensitivity of the crop. For instance, plantlets of coffee (*Coffea arabica* and *Coffea racemosa*) are best conserved at 27 °C, cassava (*Manihot*

esculenta) plantlets have to be stored at temperatures higher than 20 °C and oil palm (*Elaeis guineensis*) somatic embryos are not able to resist to temperatures lower than 18 °C (Engelmann 1991). In combination with the low temperature, the shoot cultures are kept in total darkness. However, many species have benefitted from the culture maintenance at low light intensity, in terms of both photoperiod (12 or 8 h of light), and light intensity, reduced sometimes up to 1.5 µmol m^{-2} s^{-1} (Benson et al. 2011). Low temperature and darkness (or low light intensity) act in harmony with the physiology of the plant, reducing respiration, water loss, drying and ethylene production (Lambardi and Ozudogru 2013). Furthermore, they are an excellent solution to limit maintenance energy costs (Koo et al. 2003).

Modifications to the culture medium may include the modification of the carbon source content, the concentration in mineral salts (macro and microelements), the variation in levels of plant growth regulators (quality and concentration) and the inclusion of growth retardants. Nutrient limitation is usually imposed by reducing sucrose and nitrogen in the medium, to half or quarters of the standard concentration (Benson et al. 2011). However, the application of a high sucrose concentration (40 g L^{-1} and over) is also occasionally reported (Table 1). Of particular interest is the addition to the medium of osmotically active substances which cause a slowing of growth due to the limitation of the available water, associated with osmotic shrinkage through the semi-permeable membranes. Osmotic agents can be applied individually or in combination with other additives. The substances most frequently used are mannitol and sorbitol (at the concentration of 15–60 g L^{-1}), in combination with each other, and/or with sucrose (20–60 g L^{-1}), depending on the species or genotype (Lambardi and De Carlo 2003). In some cases, the increase in the concentration of gelling agent may also be useful.

Other compounds that may be added are growth retardants or inhibitors, such as abscisic acid (ABA), compounds of the anti-gibberellin group, ancymidol, acetylsalicylic acid as an alternative to mannitol (Cha-um and Kirdmanee 2007), and activated charcoal, although the effects induced by such a category of chemicals are often discordant. To alleviate the effects of stress induced by the use of these substances (e.g. hyperhydricity, in vitro senescence and phenolic production), as well as the onset of morphological abnormalities caused by the consequent accumulation of ethylene, it is possible to resort to the use of culture vessels that allow gaseous exchange with the outside atmosphere, or limit the production of gas through the application of silver thiosulphate and other inhibitors of the ethylene pathway. However, it is prudent to use minimum growth regimes that do not compromise the nutritional status of the crop, e.g. by optimizing the availability of iron by means of the inclusion of chelating agents, adding calcium, and altering the composition of macronutrients (Cha-um and Kirdmanee 2007).

Other factors that can play an important role in achieving the maximum time of shoot conservation (i.e., the maximum lengthening of the subculture cycle), without compromising the regrowth once the standard culture conditions have been restored, include the characteristics of the explants, the quality of the crop lines and the genotype (Kulus 2018). Shoot cultures are undoubtedly the most used material for conservation, however it is worth mentioning that these should not come from too many cycles of periodic standard subculturing, and should not present any symptoms of in vitro pathologies, such as hyperhydricity, apex necrosis and browning. Thermal shocks can also induce the appearance of latent bacteria that cause strong contamination, both during storage and reintroduction to standard culture conditions. Moreover, since the cuts produced at the time of subculture produce a pronounced stress in the sprouts, which, depending on the species, last for 7–10 days, it is advisable to maintain shoot cultures at standard

Table 1 Slow growth storage of vegetable species: summary of storage conditions and recovery after maximum time of conservation

Species	Material	Temperature (°C)	Osmotic compound (concentration)	Light condition[a]	Medium	Conservation time (month)	Recovery (%)[b]	References
Artichoke								
Cynara cardunculus var. *scolymus*	Shoots	6 °C	–	Dark	MS + 5 mg L^{-1} Kin	12	30	Bekheet and Usama (2007)
Cynara cardunculus var. *scolymus*	Shoots	4 °C	–	Dark	NR	4	100	Benelli et al. (2010)
Cynara cardunculus var. *scolymus*	Shoots	18 °C	SUC (20 g L^{-1}) + SOR (20 g L^{-1})	16 h (37.5 µmol m^{-2} s^{-1})	Gik modified	12	100	Tavazza et al. (2015)
Cynara cardunculus var. *scolymus*	Shoots	5 °C	–	8 h (25 µmol m^{-2} s^{-1})	½ MS modified	6	100	Ruta et al. (2016)
Cassava								
Manihot esculenta	Shoots	23–24 °C	SUC (20 g L^{-1})	12 h (18.5 µmol m^{-2} s^{-1})	MS + 0.02 mg L^{-1} BA + 0.1 mg L^{-1} GA_3 + 0.01 mg L^{-1} NAA + 10 mg L^{-1} $AgNO_3$	24	NR	Mafla et al. (2000)
Manihot esculenta	Nodal microcuttings	NR	SOR, MAN	NR	MS + 0.1 µM BA + 0.1 µM GA_3 + 0.1 µM NAA + 0.001 µM $AgNO_3$ + AC (1 mg L^{-1})	12	NR	Unnikrishnan et al. (2002)
Manihot esculenta	Secondary embryos	16 °C	MAN (20 g L^{-1})	Dark	MS	16	74	Opabode et al. (2016)
Chicory								
Cichorium intybus	Shoots	4 °C	–	Dark	MS	9	95	Lambardi et al. (2006)
Cichorium intybus	Shoots	5 °C	–	8 h (25 µmol m^{-2} s^{-1})	½ MS modified + 0.05 mg L^{-1} BA	6	100	Ruta, *unpublished data*

Table 1 continued

Species	Material	Temperature (°C)	Osmotic compound (concentration)	Light condition[a]	Medium	Conservation time (month)	Recovery (%)[b]	References
Garlic, Shallot, Onion								
Allium cepa	Microbulbs	-1 °C	SUC (100 g L^{-1})	10 h (20 µmol m^{-2} s^{-1})	MS	12	100	Kästner et al. (2001)
Allium spp.	Shoots	2–10 °C	–	NR	MS	12	NR	Keller and Senula (2002), Keller et al. (2006)
Allium sativum	Shoots	Low (NR)	SUC (30–40 g L^{-1})	8 h	B5 + 1.3–2.2 µM BA + 0.5–1.6 µM NAA + 38-115 µM ABA	25	100	Xu et al. (2005)
Allium sativum	Bulb portions	4 °C	SUC (0.4 M)	Dark	MS	15	90	Hassan et al. (2007)
Allium sativum	Microbulbs	4 °C	–	Dark	MS + 0.3 mg L^{-1} BA	9	100	Previati and Benelli (2009)
Allium sativum	Microbulbs	25 °C	SUC (45 g L^{-1})	16 h (13 µmol m^{-2} s^{-1})	¼ MS	7	100	Pardo et al. (2014)
Mint								
Mentha spp.	Shoots	2 °C	SUC (30 g L^{-1})	16 h (33–55 µmol m^{-2} s^{-1})	MS	6	98	Islam et al. (2003)
Mentha spp.	Shoots	2–10 °C	SUC (30 g L^{-1})	16 h (2–4 µmol m^{-2} s^{-1})	MS	24	NR	Keller et al. (2006); Martin et al. (2013)
Potato								
Solanum tuberosum	Shoots	6 °C	SUC (60 g L^{-1})	16 h (20 µmol m^{-2} s^{-1})	MS + 10 mM ancymidol	16	79	Sarkar et al. (2001)

Table 1 continued

Species	Material	Temperature (°C)	Osmotic compound (concentration)	Light condition[a]	Medium	Conservation time (month)	Recovery (%)[b]	References
Solanum tuberosum	Synseeds	6 °C	SUC (40 g L^{-1}) + MAN (20 g L^{-1})	16 h (20 µmol m^{-2} s^{-1})	MS + 0.5–1.0 mM STS	16	92	Sarkar et al. (2002)
Solanum tuberosum	Shoots	24 °C	SUC (20 g L^{-1}) + SOR (40 g L^{-1})	16 h (40 µmol m^{-2} s^{-1})	MS	12	78	Gopal et al. (2002)
Solanum tuberosum	Microtubers	6 °C	SUC (60–80 mg L^{-1})	24 h (20 µmol m^{-2} s^{-1})	MS + 44.38 µM BA	12	NR	Gopal et al. (2004)
Solanum tuberosum	Microtubers	4 °C	NR	Dark	NR	18	NR	Keller et al. (2006)
Solanum tuberosum	Microcuttings	8–10 °C	–	16 h (6000 lx)	MS + 50 mg L^{-1} Alar + 50 mg L^{-1} ABA	12	80	Dimitrova and Marcheva (2009)
Solanum tuberosum	Uni-nodal microcuttings	7 °C	SUC (40 g L^{-1}) + SOR (20 g L^{-1})	16 h (20 µmol m^{-2} s^{-1})	MS	18	58	Gopal and Chauhan (2010)
Solanum tuberosum	Shoots	8–10 °C	SOR (30 g L^{-1})	16 h (25 µmol m^{-2} s^{-1})	MS	18	NR	Bamberg et al. (2016)
Sweet potato								
Ipomoea batatas	Uni-nodal microcuttings	18 °C	SUC (30 g L^{-1})	12 h (60 µmol m^{-2} s^{-1})	1/2 MS	9	74	Arrigoni-Blank et al. (2014)
Ipomoea batatas	Uni-nodal microcuttings	26 °C	–	16 h (60 µmol m^{-2} s^{-1})	MS + 0.5- 1.0 mg L^{-1} ABA + 0.18 mg L^{-1} NAA + 0.22 mg L^{-1} BA	4	70	Bazán-Zafra et al. (2014)
Ipomoea batatas	Uni-nodal microcuttings	27 °C	SUC (20 g L^{-1})	16 h (25 µmol m^{-2} s^{-1})	MS	6	100	Vettorazzi et al. (2017)
Ipomoea batatas	Uni-nodal microcuttings	23 °C	MAN (20 g L^{-1}) + SOR (20 g L^{-1})	16 h (25 µmol m^{-2} s^{-1})	MS modified	16	100	Ruta (*unpublished data*)

Table 1 continued

Species	Material	Temperature (°C)	Osmotic compound (concentration)	Light condition[a]	Medium	Conservation time (month)	Recovery (%)[b]	References
Thyme								
Thymus vulgaris	Shoots	4 °C	SAC (30 g L^{-1})	Dark	MS + 1.0 mg L^{-1} Kin + 0.3 mg L^{-1} GA_3	12	78	Ozudogru et al. (2011)
Thymus moroderi	Shoots	4 °C	SAC (15 g dm^{-3}) + MAN (15 g dm^{-3})	Dark	MS	7	NR	Marco-Medina and Casas (2012)

Main papers published on international journals from 2000 onwards are reported (*SUC* sucrose, *SOR* sorbitol, *MAN* mannitol, *AC* activated charcoal, *BA* 6-benzyladenine, *NAA* α-naphthalene acetic acid, *ABA* abscissic acid, *Kin* kinetin, GA_3 gibberellic acid, *STS* silver thiosulfate, *NR* not reported)

[a]In parenthesis: light intensity applied with fotoperiod (when specified)

[b]The data reported refers to the maximum percentage of recovery in post-conservation of the accessions or species of that specific report

temperature and light conditions during the first 7–10 days after subculturing, and then transfer them to cold storage (Lambardi and Ozudogru 2013).

It is common for conservation centers to apply the same storage conditions to a wide range of species and genera, preserved in one large climatic chamber. Nonetheless, it should be considered that, when storing different crop lines in the same chamber, quite different results can be obtained in terms of maximum conservation time. Another factor that should be carefully considered in slow growth storage is the type of container. A wide range of in vitro propagation containers are now available, differentiating by shape, material (plastic or glass), volume and gas permeability. As for this last feature, the new generation of polystyrene boxes allows a partial exchange of the main internal gases (CO_2 and ethylene) with the outside, produced during the in vitro culture. This avoids the accumulation that can be detected in the traditional gas-impermeable glass jars. A very low accumulation is detected inside the polypropylene boxes that allow the exchange of gas through the filter strip placed on the lid, or by means of a special closing system that allows exchange of gases. For example, boxes with medium-sized filters allow about 10 gas exchanges per day. However, during a long in vitro conservation, careful control is required, as a more rapid dehydration of the medium can be observed inside such containers in comparison to the use of classic glass jars (Lambardi and Ozudogru 2013).

From what has been pointed out so far, it is clear that several factors contribute to the maximum conservation time of shoot cultures, i.e. the maximum time that the cultures can be maintained in slow growth storage before being re-introduced in standard culture conditions. The time is very much variable, ranging from a few months up to 2–3 years, depending on the species and the quality of the starting in vitro culture line. Conservation by slow growth storage must guarantee the unaltered maintenance of the functionality of the plant tissues which, once the standard in vitro culture conditions have been restored, resume normal metabolic activity and can be subjected to the multiplication, rooting and acclimatization phases of micropropagation.

The advantage of the slow growth storage method lies in the use of the basic structures used for micropropagation and in the adoption of conservation schemes based on the modification of the previously established conditions for rapid in vitro multiplication. However, the problems related to labor costs remain, albeit reduced when compared to culture in standard conditions, together with the need for space and the potential risk of somaclonal variation in some species. In vitro germplasm conservation biobanks should therefore carefully consider the economic and effective benefits of the optimization of slow growth regimes, especially for the storage of recalcitrant accessions such as in *Coffea* spp. (Dussert 1997). Low post-conservation regrowth in stress-sensitive genotypes may make slow growth storage uneconomical. This is the case of the organ dormancy, occurred in species as *Dioscorea* spp. forming organs during in vitro storage, or in some accessions of *Allium* spp. for in vitro-derived bulblets (Keller 2005). However, numerous studies show that, today, in vitro conservation is a method of great importance also for several vegetable species, as reported below (i.e. *Allium sativum, M. esculenta, Solanum tuberosum*).

Biobanks around the world have already conserved vegetable genetic resources by in vitro slow growth storage. Efficient protocols for slow growth vegetable storage are reported in Table 1, while the species and their consistency already stored in the biobanks of the main international conservation centers are listed in Table 3.

Artichoke (*Cynara cardunculus* var. *scolymus*)

Early studies highlighted the possibility of preserving in vitro the vitality and genetic integrity of local artichoke varieties by slow growth storage to complement the in-field collections where they are traditionally maintained. Shoots of an Egyptian local variety ('Balady'), showed 30% regrowth after its storage for 12 months at 6 °C and in the dark (Bekheet and Usama 2007). Low temperature (4 °C) and darkness were found to be the best conditions for two 'Violetto' local varieties of the Veneto region, in Italy, with a capacity of 100% regrowth after 4 months of preservation (Benelli et al. 2010). Another study involved 7 'Romanesco' artichoke genotypes, established in vitro from Italian cultivated varieties and kept in conservation at 18 °C, under osmotic stress produced by a high carbohydrate content in the storage medium; the carbohydrate level in the medium was increased by replacing sucrose (30 g L^{-1}) with a combination of sucrose (20 and 30 g L^{-1}) and mannitol (40 g L^{-1}) or sucrose (20 and 30 g L^{-1}) and sorbitol (20 and 40 g L^{-1}). The optimal responses, evaluated after 12 months of preservation and also supported by molecular and phenotypic studies, were obtained with a modified Gik medium, enriched with 20 g L^{-1} of sucrose and 20 g L^{-1} of sorbitol (Tavazza et al. 2015).

The suitability of Artichoke biodiversity from the Apulia region, in Italy to in vitro slow growth storage was investigated. The best storage conditions were temperature 5 °C, photoperiod 8 h, light intensity 25 µmol m^{-2} s^{-1} and half-strength modified MS (Murashige and Skoog 1962) medium (Morone Fortunato et al. 2005) and allowed the maintenance in vitro of 6 autochthonous varieties up to 6 months, among which 'Centofoglie di Rutigliano' is in danger of extinction (Ruta et al. 2016). Subsequently, the conservation time was safely extended up to 12 months (Ruta *unpublished data*).

Cassava (*Manihot esculenta*)

Cassava is a principle food source in Africa, Latin America and Asia, offering a cheap source of calories from its starchy, thick, tuberous roots, which can be consumed either fresh or processed (FAO 2018). Angel et al. (1996) were the first who investigated the DNA rearrangements of cassava plants after 10 years of in vitro storage (through subcultures in every 18 months, at the maximum) at 22–24 °C, with a 12 h photoperiod and 1500 lx illumination, on MS medium containing 20 g L^{-1} sucrose, 0.05 µM 6-benzyladenine (BA), 0.02 µM gibberellic acid (GA_3) and 0.05 µM α-naphthaleneacetic acid (NAA). These authors demonstrated the genetic stability of the preserved germplasm by applying Restriction Fragment Length Polymorphism (RFLP) and Random Amplified Polymorphic DNA (RAPD) fingerprinting methods.

Cassava germplasm is traditionally maintained by vegetative cultivation in the field. However, various diseases (Cassava Bacterial Blight, African Cassava Mosaic Virus, Super- Elongation Disease, Frog-skin Disease) resulted in great losses in the in-field collections (Lozano and Nolt 1989). Thus in vitro storage of the species under slow growth storage conditions was developed in the mid'70 s. The CIAT (Colombia) has an in vitro bank of the species which contains 6700 accessions, as a complementary strategy to the in-field collections. The protocol of slow growth developed at CIAT for cassava uses semi-solid MS medium, containing 20 g L^{-1} sucrose, 0.02 mg L^{-1} BA, 0.1 mg L^{-1} GA_3, 0.01 mg L^{-1} NAA and 10 mg L^{-1} silver nitrate ($AgNO_3$), which allows the shoots to remain viable for 18–24 months (Mafla et al. 2000). Other large in vitro cassava collections are held by IITA, Nigeria, (2469 accessions), and EMBRAPA, Brazil (262) that

support the in-field collections, as well as low numbers collected in other minor centers in the world. However, the exact number of unique accessions in in vitro conservation can only be estimated, since there has not been an attempt to identify duplicate accessions across biobanks. Hershey (2008) estimated more than 26,000 unique cassava landrace varieties, meaning that there are still many landrace varieties uncollected and not conserved ex situ.

Unnikrishnan et al. (2002) induced slow growth storage of in vitro nodal cultures of diverse genotypes with the osmotic retardants sorbitol and mannitol. Addition of $AgNO_3$ (0.001 μM) and activated charcoal (1 g L^{-1}) was also found to help in reducing leaf shedding and preventing root browning in cultures stored for longer periods. Subculture cycles of cassava could be extended up to 12 months.

Adding mannitol (20 g L^{-1}) to the culture MS medium retained for up to 16 months the regeneration ability of cassava secondary embryos stored in the dark at low temperature (16 °C). The genetic uniformity of somatic embryos, both among themselves and in respect to the genome of the mother plant, was confirmed by RAPD analysis (Opabode et al. 2016).

Chicory (*Cichorium intybus*)

Two typologies of red chicory ('Treviso precoce' and 'Chioggia') have been the subject of an extensive investigation with the aim of selecting superior lines to be propagated in vitro and used for controlled crossings in greenhouses, and to produce high-quality seeds. To allow the in vitro maintenance of the lines, different combinations of low temperature, presence or absence of light, and the addition of mannitol to the medium were tested to induce slow growth. After 9 months of storage at 4 °C, independently of the light conditions applied, and without the application of mannitol, 95% of recovery was obtained, with a limited difference between the two typologies of red chicory (Lambardi et al. 2006).

To achieve a quality production and conserve the genetic resource of the traditional Apulian (Italy) chicory varieties, such as 'Galatina' and 'Molfetta', after developing an effective micropropagation protocol and ascertaining their morphological and phenotypic uniformity in the field (Ruta et al. 2017), plantlets were stored at 5 °C and 8 h photoperiod with reduced light intensity (25 μmol m^{-2} s^{-1}), on half-strength modified MS medium, enriched with 0.05 mg L^{-1} BA. After six months of conservation, a total regrowth (100%) of the shoots was achieved (Ruta *unpublished data*).

Garlic (*Allium sativum*), shallot (*A. ascalonicum*) and onion (*A. cepa* spp.)

The species belonging to the genus *Allium* show some problems with regard to in vitro culture. For instance, explants are often taken from bulbs or cloves developed at or above the ground, thus require careful and efficient surface decontamination, which does not always result in success (Previati A, *personal communication*). Another limitation is determined by the internal position of the bud apex, containing the meristem, which requires a relatively longer time to prepare a suitable number of explants. Furthermore, often endogenous bacterial infection remains in the latent state and appears under stress, such as the passage from low temperature storage to the standard culture conditions (Keller et al. 2006). Nevertheless, due to the disadvantages related to the difficulties of traditional conservation and especially to the reduction of material caused by the attacks of pathogens in the in-field collections, there are numerous studies demonstrating the possibility of

applying innovative conservation methods, such as slow growth storage, to the genus. These studies take into account different factors such as the genotype, the components of the culture medium and the light and temperature conditions of the growth chambers.

Regarding the impact of genotype, Xu et al. (2005) defined a medium that allowed extension of the storage time up to 25 months, with a 100% survival rate of garlic (*Allium sativum*) shoots in two of the six tested Chinese genotypes. Another example of different treatment response, dependent on the genotype, is reported by Hassan et al. (2007) which kept two Egyptian garlic varieties in slow growth at 4 °C, comparing different concentrations of sorbitol and sucrose (0.1, 0.2 and 0.4 M for each one). After 15 months, one of the two varieties exhibited 90% recovery in the presence of sucrose at the highest concentration tested, while for the other a maximum percentage of recovery of 36% was obtained with the highest concentration of sorbitol. The positive influence of an increase in sucrose concentration when combined with the reduction of the content in nutrients on recovery was also reported by Pardo et al. (2014) for the conservation of a Venezuelan garlic clone.

An effective protocol to slow down the growth of garlic (*A. sativum*) shoot cultures was developed for the Italian cv. Avorio. The protocol allowed excellent preservation of microbulbs for 9 months at low temperature (4 °C) and in darkness (Previati and Benelli 2009). Research carried out at the IPK in Gatersleben (Germany) on various species of the genus *Allium* (garlic, onion, shallot, spontaneous species) reported a shelf life of 12 months of the shoots at temperatures ranging from 2 to 10 °C with intermediary warm culture phases of two months at 20–25 °C (Keller et al. 2006). As reported by Keller and Senula (2002), cold storage of *Allium* spp. must take place on a medium free of plant growth regulators to avoid hyperhydricity and reduce the risk of somaclonal variation. However, if hyperhydricity is detected in the early stages, it can be reversed simply by modifiying the culture conditions, especially the osmotic potential of the medium used and the type and concentration of the growth regulators.

A study on slow growth storage of onion (*A. cepa*) has highlighted the possibility of its preservation for one year at a temperature of − 1 °C and 10 h photoperiod, with a low light intensity (20 µmol m^{-2} s^{-1}), by adding a high concentration of sucrose (100 g L^{-1}) to the storage medium. This procedure permitted maximum regrowth of the bulbs (100%), once the standard in vitro growth conditions were re-established (Kästner et al. 2001). However, it should be noted that medium-term in vitro storage of the genus *Allium* is conditioned by the existence of endophytic microorganisms that can consistently reduce the storage time of the clones (Keller et al. 2011).

Mint (*Mentha* spp.)

An experiment carried out on four accessions of mint (with a different ploidy level and origin) showed the possibility of preserving shoots in vitro via micropropagation for 6 months at 2 °C and allowed a survival from 38 to 98% depending on the genotype (Islam et al. 2003). The storage at 2 °C is also applied at the IPK of Gatersleben with the majority of accessions (93%) having a conservation time of over 18 months, while only 7% of the total collection requires a higher storage temperature (i.e., 10 °C) (Keller et al. 2006; Martin et al. 2013). The study also demonstrated genetic stability of the collections after a preservation time of over 10 years. The study of Islam et al. (2005) investigated the possibility of reducing the atmospheric space of containers to induce slow growth of shoot cultures. With containers of 53 mL the number of mint leaves was 1.3 times less than in

the control containers (380 mL), thus allowing the storage of a higher density of plants. The interval between subcultures was also higher, thus reducing the cost of storage.

Potato (*Solanum tuberosum*)

The biotic and abiotic factors which limit potato tuber conservation in the field have stimulated researchers, from the early 80's, to study the in vitro culture method as a tool to support traditional conservation approaches. The influence of several factors on slow growth storage of shoots have been tested, among which are osmotic stress, growth inhibitors, temperature, light intensity and photoperiod, either alone or in combination (Westcott 1981; Mix 1982).

Some of the developed protocols use different temperatures and/or osmotic compound combinations for in vitro biobanking of potato germplasm. At the NICS, Republic of Korea, 1223 in vitro accessions are maintained at 25 °C (Niino and Valle Arizaga 2015) (Table 3). The same temperature is used for 130 accessions at the NCSS, Japan, carrying out subcultures every 3–4 months (Niino and Valle Arizaga 2015). A study carried out by Gopal et al. (2002) on shoots of six potato genotypes, showed the possibility to extend the time between subcultures up to 12 months by including 20 g L^{-1} of sucrose and 40 g L^{-1} of sorbitol to the MS medium, which resulted in a maximum regrowth after storage of 56% to 78%, depending on the genotype. This approach allowed the use of normal temperature conditions (24 °C), avoiding the energy costs associated with low temperatures. However low temperature (7 °C) and 16 h photoperiod are used at the CPRI of Shimla, India, to preserve for 18 months over 1,500 parental lines and varieties of potato on MS medium, containing 40 g L^{-1} of sucrose and 20 g L^{-1} of mannitol (Gopal and Chauhan 2010). With this carbohydrate combination, it was possible to obtain a maximum survival of 58%. This is 8% higher than the value established as the intervention limit imposed by the CPRI to avoid the risks of germplasm loss. The same osmotic components and concentrations have been adopted by the CIP, Peru, whose in vitro collection consists of 8,354 accessions maintained in slow growth storage on MS medium at 6–8 °C, 16 h photoperiod and 22 µmol m^{-2} s^{-1} light intensity (Niino and Valle Arizaga 2015). This allowed the shoot cultures to be stored in vitro for about 2 years. Sorbitol is used in MS medium to conserve in vitro 624 clones at the USPG, located in Sturgeon Bay, Wisconsin, belonging to the USDA-ARS of the United States. The shoots, obtained following in vitro multiplication of axillary buds and their stabilization for 2 weeks at 20–22 °C and 16 h photoperiod, were transferred to 8–10 °C for medium-term conservation, maintaining the same photoperiod at a light intensity of 25 µmol m^{-2} s^{-1}. These conditions allowed a safe storage for 18 months (Bamberg et al. 2016). A total of 2257 accessions of the genus *Solanum* represents the potato germplasm stored in vitro at 10 °C and 10 h photoperiod at the PRI of Czech Republic, on MS medium enriched with 6% sucrose (Faltus et al. 2011; Acker et al. 2017).

The use of growth inhibitors or retardants can induce slow growth in vitro. Alar (also known as Daminozide) and ABA, for instance, were tested at 50 mg L^{-1} on 2 cultivars maintained for 12 months at low temperature (8–10 °C) and 16 h photoperiod, showing a slight decrease in vitality (80%) during the recovery (Dimitrova and Marcheva 2009). Significant interaction was detected between the ancymidol, a growth retardant, and other factors, such as sucrose, temperature and genotype, on survival and regrowth of shoots in post-conservation. The ancymidol (at 10 mM concentration) showed a growth inhibition effect that persisted for 16 months, especially when the shoots were placed at

6 °C on MS medium enriched with a high sucrose (60 g L^{-1}) (Sarkar et al. 2001). In another study, the inclusion of shoot tips in alginate capsules, containing the silver thiosulphate anionic complex (STS, $[Ag(S_2O_3)_2]^{3-}$), reduced ethylene-induced growth abnormalities during prolonged in vitro storage (16 months) in MS medium, added of sucrose (40 g L^{-1}) and mannitol (20 g L^{-1}) (Sarkar et al. 2002).

Another important form of in vitro preservation for potato germplasm is the use of microtubers. IPK maintains 2932 in vitro potato accessions at 4 °C as microtubers. The conservation cycle involves a phase at 20 °C of 2–3 months with a 16 h long day, a phase of induction of microtubers with a short day (8 h) at 9 °C for 2–4 months, and a final period in which the microtubers are stored at 4 °C for 16–18 months (Keller et al. 2006; Niino and Valle Arizaga 2015).

A study carried out to better understand the effect of ABA on microtuber dormancy, as well as its interaction with sucrose and the possible implications with the genotype, showed that the best storage conditions were obtained on a medium without ABA, but with high concentrations of sucrose (60–80 g L^{-1}) and BA (44.38 µM), under continuous light at intensity of 20 µmol m^{-2} s^{-1} and 6 °C of temperature (Gopal et al. 2004). Under these conditions, the microtubers were safely stored for about 12 months.

Sweet potato (*Ipomoea batatas*)

Studies conducted on sweet potato, a highly heterozygous hexaploid species (2n = 6x = 90), have highlighted the possibility to maintain microcuttings in slow growth storage by adjusting the concentration of the medium, the use of growth regulators and/or osmotic substances. An experiment conducted at a temperature of 18 °C on four different genotypes, tested on three concentrations of the MS medium (1, 3/4, 1/2) and three sucrose concentrations (10, 20, and 30 g L^{-1}), highlighted the different capacity of survival of the tested genotypes (Arrigoni-Blank et al. 2014). The best conservation (6–9 months, depending on the genotype) was obtained with half-strength MS, enriched with 30 g L^{-1} sucrose. In a previous experiment, different concentrations of ABA added to the conservation medium gave unsatisfactory results in terms of survival and recovery. In contrast, another study found the use of ABA in combination with NAA and BA, played a significant role in slowing the growth of two Peruvian genotypes which showed 70% maximum survival after 4 months of conservation (Bazán-Zafra et al. 2014). A study of the in vitro preservation of 30 Brazilian accessions allowed a doubling of the time required between two subcultures (from 90 to 180 days) by reducing sucrose concentration of the MS medium to 20 g L^{-1} (instead of 30 g L^{-1}) and keeping the culture vessels at a temperature of 27 °C (Vettorazzi et al. 2017).

The results obtained by the use of osmotic substances, such as sorbitol and mannitol, are particularly interesting. In a pre-2000 study, the combined use of both compounds at a concentration of 20 g L^{-1}, tested on 30 accessions, allowed a conservation of 16 months, with a maximum survival of 82% (Golmirzaie and Toledo 1998). The same combination and concentration, applied to uni-nodal segments with an apical or axillary bud of 2 accessions of sweet potato from the Salento area of Italy, resulted in a 90% recovery after slow growth storage of 16 months at 23 °C with a photoperiod of 16 h and under light intensity of 50 µmol s^{-1} m^{-2} (Ruta *unpublished data*).

Thyme (*Thymus* spp.)

To preserve thyme germplasm, in vitro shoots of *Thymus vulgaris* were stored for over 13 months in the dark at 4 °C on MS medium enriched with kinetin and GA_3 (Ozudogru et al. 2011). Monthly monitoring showed an increasing loss of vitality that affected 75% of the shoots at 13 months of conservation; therefore, the maximum acceptable storage period was assessed to be 12 months. A subsequent study (Marco-Medina and Casas 2012) evaluated the role of polyamines in the slowing of growth imposed by the combined action of osmotically-active compounds (sucrose and mannitol) with the physical environment (4 °C and darkness). Under these conditions, shoots survived for seven months without subcultures, showing an increase in free putrescine, a reduction in soluble conjugated putrescine (during the first weeks of storage), and a constant increase in the conjugated spermidine.

Yam (*Dioscorea* spp.)

Thanks to its high morpho-ecological and physiological adaptation ability, yam is found in all continents (Degras 1993). Like cassava, yam is also an important species for root and tuber production, with a global annual production estimated at 70 million tons (FAOSTAT 2017). This crop is a major source of food for more than 50 million people in West and Central Africa (Asiedu and Sartie 2010). In West Africa, it occupies an important place in food security and accounts for 95% of production. Thus, it is an important source of income for farmers (Sedami et al. 2017). However, as a vegetatively propagated crop, yam is seriously affected by an accumulation of pathogens. When this is the case, establishing in vitro germplasm collection not only provides disease-free plants, but also enables a good control on the preservation of genetic resources, also facilitating international exchanges of healthy plants (Malaurie et al. 1998).

In vitro conservation of yam has a story that initially developed much before the 2000, when the necessity of establishing large in vitro collections became urgent. In 1986, Hanson suggested to subculture the clones every two years and to use growth retardants (such as mannitol) as additives to the culture medium. At that time, few species were maintained in vitro, and their medium-term maintenance, without a loss of their genetic stability, was still uncertain. Afterwards, a collection of 14 species from Africa and Asia were introduced successfully to slow growth storage conditions, by keeping in vitro shoot cultures at 28 °C, under 16 or 12 h photoperiod (depending on the species), with a light intensity of 120 µmol m^{-2} s^{-1}. They were subcultured, every 6 to 12 months, to maintain their physiological development stages, to fresh medium containing Knop's mineral salts, MS vitamins, 30 g L^{-1} sucrose, 8 g L^{-1} agar, 2 g L^{-1} activated charcoal and 200 mg L^{-1} glutamin. The study demonstrated that an in vitro biobanking was feasible to preserve yam nodal microcuttings, under minimal growth conditions (Malaurie et al. 1993). After the introduction and maintenance of 14 yam species, the same group continued to enrich the collection by introducing new genotypes and reached 20 species and over then 280 accessions in collection at IRD (ex-ORSTOM of Montpellier, France) by the year 1998 (Malaurie et al. 1998). The consistency of yam conservation in slow growth storage amounted to almost 2,000 accessions in the 2017 (Table 3) at IITA, Nigeria.

Cryopreservation as a form of long-term conservation of vegetable genetic resources

Cryopreservation, i.e., the storage at ultra-low temperatures such as the temperature of liquid nitrogen (LN; − 196 °C), is the most innovative method which enables long-term conservation of plant genetic resources. The method preserves organs and tissues, from in vitro culture and the field, by means of an ultra-fast cooling process that, if properly developed and well-adapted to the specific plant specimen, arrests almost all metabolic processes in the cell, while preserving its structure and biological functionality. In fact, at a cryogenic temperature very few biological reactions and significant variations of the physiochemical properties remain active. Moreover, below − 150 °C a state of "vitrification" of cell cytosol is induced and protects the plant samples from damages which may occur as a result of freezing and rewarming procedures (Mazur 1964). Nitrogen, is a cryogenic gas that, at the liquid phase, is easily available, has a limited cost, and is used universally in cryobanks where it ensures the maintenance of temperatures ranging from approximately − 150 °C (for samples stored in the space of the container filled with the gas vapors) to − 196 °C (for samples immersed in the liquid phase of LN). The total absence of subcultures and the arrest of cellular metabolism produced by cryogenic temperatures make cryopreservation a safe method in terms of the genetic stability of the stored material. Proof of this is found in the numerous experimental works that, particularly in the last 30 years have evaluated the phenotypic, cytological, biochemical and molecular stability aspects of the material subjected to conservation in LN, never showing significant stable alterations (Harding 2004; Volk 2010; Coste et al. 2015). Even the few reports of genetic alterations do not seem to cause excessive concern in the use of cryogenic conservation (Martin and González-Benito 2005; Kulus 2020). In this sense, cryopreservation is even more secure than slow growth storage of in vitro shoot cultures, where there is a risk, albeit limited, of the onset of somaclonal variability in the medium-term.

The first scientific evidence of the possibility of applying the cryogenic technology to the conservation of plant material dates back to 1960, when Akira Sakai, whose innovative studies would later become the reference point of the entire scientific community involved in the sector, authored a note in 'Nature' that demonstrated the possibility of conserving microcuttings (twigs) of *Salix* spp. (willow) and *Populus* spp. (poplar) by means of the application in sequence of slow cooling and ultra-fast cooling in LN (Sakai 1960). Since then, numerous methods and variations in procedures have been proposed and applied to a broad pool of plant species (see, e.g., Benson 1995; Sakai and Engelmann 2007; Benelli et al. 2013; Romadanova et al. 2017; Kulus 2019). After 60 years, the cryopreservation of plant genetic resources is today a reality that effectively supports, together with slow growth storage, traditional conservation approaches in seed banks and in-field clonal collections (Acker et al. 2017). Today, the number of accessions stored in cryobanks is significative (Table 3) and constantly growing.

Among the main advantages of cryopreservation is the possibility of exploiting the technology of in vitro culture to select a wide range of organs and tissues for storing, i.e. apical and axillary buds, nodal segments, somatic embryos, bulbils, cell lines and hairy roots (Lambardi and De Carlo 2003; Lambardi and Shaarawi 2017). For instance, the use of shoot tips as conservation units makes possible storage of 7000 to over 30,000 samples in small- and medium-sized LN dewars (i.e., from 35 to 200 L), with low conservation costs (in practice, only those necessary for the control of the cryobank and maintenance of

the appropriate level of LN). However, with regard to conservation costs, a study has shown that the introduction and maintenance of one accession in cryopreservation is more expensive than, not only traditional conservation in seed banks and clonal collections, but also slow growth storage of in vitro cultures. On the other hand, in the long term (over 20 years), the maintenance of genetic resources in cryobank becomes significantly cheaper than all other forms of conservation, especially when operating with a high number of accessions (Acker et al. 2017).

Overview of cryopreservation theory

In nature, plants undergoing prolonged periods of exposure to low temperatures (below 0 °C for temperate species) trigger frost defense mechanisms that are essentially based on the synthesis of protective substances (di- and oligosaccharides, polypeptides, proline, polyamines, antioxidants) and changes in lipid composition of membranes that limit osmotic and oxidative stress, stabilize membranes, promote recovery from sublethal damage (Pearce 2004). On the other hand, these mechanisms are not able to counteract the transformation of cytoplasmic water molecules into intra-cellular ice crystals when the cells undergo rapid ultra-fast cooling produced by direct exposure of the cytoplasm to the temperature of liquid and vapor phase of nitrogen. At ultra-low temperatures, intra-cellular ice crystals grow inside the cells and destroy membranes and organelles. In short, cells immersed in LN without any preparation die instantly upon freezing. Cryopreservation procedures counteract the formation of intra-cellular ice crystals, partly through direct mechanisms of physical and osmotic dehydration, and partly by replacing part of the water molecules with cryoprotective substances that have colligative properties. When the intra-cellular water molecules consistently decrease and an appropriate cytoplasmic concentration is reached, cell vitrification is induced. The term "vitrification" refers to the solidification of a liquid without crystallization. This physical state is triggered when an aqueous solution is highly concentrated and ultra-cooled rapidly to increase its viscosity and prevent its molecular re-organization in a crystalline form. During progressive accentuated cooling, the viscosity of the aqueous solution increases to a point where it assumes a consistent and amorphous "glassy" state (Taylor et al. 2004). Although Luyet (1937), the pioneer of the idea of vitrification, initially emphasized the use of relatively dilute solutions, in later years the use of highly concentrated vitrification solutions became the mainstream practice.

In cryopreservation, vitrification of explants is essentially induced in three ways:

- by 'cryodehydration' of the cells which is induced by applying a slow and controlled cooling of the explants up to the temperature of − 30/− 40 °C. As a consequence, the formation of extra-cellular ice crystals (not harmful) followed by the migration of the water molecules to extracellular spaces to maintain the osmotic balance between intra- and extra-cellular solutions increases the concentration of the cytoplasmic solution. If an appropriate cooling rate is applied and cell dehydration is not pushed beyond the tolerance limit of the cell (producing cell plasmolysis), vitrified cells maintain their vitality during the subsequent immersion of the explants into LN;
- by 'osmodehydration' which is induced through the treatment of explants with concentrated mixtures of cryoprotectants. Cryoprotectants are substances that are widely used in cryopreservation for their cell protection features against ultra-cooling damage. Among the most widely used substances are glycerol and dimethyl sulphoxide (DMSO or Me_2SO), which permeate the cell wall and cell membranes, and

polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP), which do not permeate. Thanks to their colligative properties, they lower the freezing temperature of the cell cytoplasm, increase its viscosity (thus reducing the quantity of ice crystals), maintain the concentration of solutes at non-toxic levels, limit the cellular wrinkling, and thus protect cells from the damage due to excess dehydration;

- by 'physical dehydration' (evaporation) of cells which is induced by exposure of explants (generally encapsulated in an alginate matrix to form "synthetic seeds") to a sterile air flow or silica gel.

Procedures of cryopreservation of plant cells, tissues and organs

Several procedures are available today for the cryopreservation of plant cells, tissue and organs. They are briefly described here.

Treatment with a vitrification solution

Often referred as "vitrification technique", it is based on the treatment of explants (in general, shoot tips from in vitro apical or axillary microbuds) with a vitrification solution (PVS, plant vitrification solution), with a combination of highly concentrated cryoprotectants. The two most widely used solutions in vegetable species are: (i) the PVS2 solution (Sakai et al. 1990), consisting of 30% (v/v) glycerol, 15% (v/v) ethylene glycol, 15% DMSO (v/v) and 0.4 M of sucrose, and (ii) the PVS3 solution (Nishizawa et al. 1993) which consists of 50% glycerol and 50% sucrose (v/v) optionally in liquid MS medium. Shoot tips (obtained from axillary and apical microbuds of in vitro proliferating shoots) are the most used explants when a vitrification cryo-method is applied. Here, the standard procedure involves the following steps: (i) removal of microbuds from in vitro proliferating shoots by using a stereomicroscope, and reduction of their size to 1–2 mm; (ii) preculture of the excised shoot tips on media containing high sucrose concentration (other osmotic agents, such as proline and ABA, may also be used): this phase has a variable duration (one or more days) and is carried out at a sucrose concentration of 0.1–1 M (sucrose concentration can be constant during the whole preculture period, or it can be increased gradually to reach the maximum concentration eventually); (iii) osmoprotection, carried out for 20–30 min with a solution composed of glycerol (2.0 M) and sucrose (0.4 M). This step increases the osmolarity of the cells, minimizing the possible osmotic damage that may arise with the subsequent immersion in PVS2 or PVS3 solutions; (iv) transfer of treated explants to cryovials (2-mL cryotubes, resistant to the temperature of LN) containing PVS2 or PVS3, with time and temperature of treatment to be selected experimentally; (v) ultra-rapid cooling to − 196 °C by direct immersion in LN of cryovials, containing the shoot tips and about 0.6 ml of fresh vitrification solution; (vi) rapid rewarming of explants (see "Rewarming and regrowth of explants in post-cryopreservation"); (vii) washing of the explants for about 20 min by substituting the vitrification solution with a washing solution, consisting of liquid MS medium containing 1.2 M of sucrose; (viii) plating of explants on an appropriate optimized regrowth medium.

Droplet freezing, droplet vitrification, cryo-plates

Over 20 years ago the method of "droplet freezing" was proposed for the cryopreservation of potato buds (Schäfer-Menuhr et al. 1997), and later became one of the most widely

used cryo-procedure. It is based on the inclusion of explants in microdroplets (generally 2.5–6 µL) of the cryoprotectant substance DMSO, placed in groups of 4–5 drops over aluminum strips. This procedure is commonly named droplet method. When PVS2 or PVS3 solutions are used to form the microdroplets, the method is named droplet vitrification. Following the treatment period (generally shorter than that used with PVS2 vitrification in cryovials), the aluminum strip with drops and explants is placed in LN and then inserted into a cryovial, or directly into a cryovial filled with LN; after closure, the cryovial is immersed in the LN dewar for storage. The main characteristic of the method is that it allows cooling and rewarming speeds of the explants much highers than in other "one-step cooling" methods, thanks to the small quantity of cryoprotectant in which the explants are immersed, the direct contact of the explants, immersed in vitrification solution, with LN, as well as the high thermal conductivity of the aluminum strip. Similar to aluminum strips, "cryo-plates" are rigid aluminum bars, about 1 mm thick and of such size that they fit inside the cryovials. The cryo-plates have two rows of micro-trays in which the explants are placed. They are then covered with a sodium alginate film which is subsequently hardened by treatment with calcium solution. The cryo-plates are then treated with vitrification solution (V-cryo-plate) or subjected to dehydration in sterile air flow or in silica gel (D-cryo-plate). For a recent review on the droplet and cryo-plate techniques, see Wang et al. 2020.

Cryopreservation of explants encapsulated in synthetic seeds

Synthetic seeds (also known as artificial seeds or synseeds) are a form of in vitro culture and conservation, originally developed by Steward et al. (1958) for somatic embryos, then extended to other types of explants, such as apical and axillary buds, uni-nodal segments, zygotic embryos and bulbs (Standardi and Micheli 2013). The preparation technique is based on the immersion of explants in a sodium alginate solution (usually 2 or 3%) in water or nutrient medium. Drops of alginate including explants are then poured in a complexing solution, generally calcium chloride at the concentration of 100 mM, where they remain for 20–45 min. During this time a Na^{+}–Ca^{2+} ion exchange occurs which produces the formation of solid calcium alginate capsules.

After its first proposal in the early'90 s (Fabre and Dereuddre 1990), the synthetic seed technique has found wide application in cryopreservation. In the procedure of "encapsulation-dehydration", vitrification of explants during ultra-rapid cooling is promoted by the pre-treatment of seeds in a medium with high sucrose concentration and subsequent dehydration in sterile air flow of a laminar flow hood or on silica gel for a sufficient time to significantly reduce its water content, to values of 30% or less. At the end of the dehydrating treatment, the capsules are inserted in cryovials and these are immersed directly in LN. A variant, named "encapsulation-vitrification", provides the treatment of synthetic seeds with PVS2 or PVS3 solution, thus combining the practicality of the explants included in synthetic seeds with the vitrification effectiveness of PVS2 and PVS3 (Sakai and Engelmann 2007).

Slow cooling

The method is based on the gradual and controlled cooling of the samples down to a temperature of − 40 °C, generally conducted at a rate of − 0.5 °C to − 2 °C min^{-1}, more frequently − 1 °C min^{-1}, followed by the immersion of explants in LN. Controlled

cooling of the samples is achieved by the use of a complex equipment that is connected to and controlled by a computer to regulate the cooling rate accurately, or with a freezing container called Nalgene® Mr. Frosty, developed by Sigma, that provides − 1 °C min^{-1} temperature reduction when filled with isopropyl alcohol and placed at − 70 °C. The controlled cooling technique (also named "two-step cooling") makes extensive use of DMSO, both as a pre-treatment of explants, and as a cryoprotectant during the whole process of cooling and immersion in LN. DMSO is used at concentrations between 5 and 15% (Reed and Uchendu 2008). DMSO is sometimes used in combination with other cryoprotectants, such as glycerol, sucrose, PEG, sorbitol and proline. Although largely replaced over time by the "one-step cooling" procedures, the slow cooling method continues to be the most used approach for the cryopreservation of embryogenic callus cultures (Ozudogru and Lambardi 2016).

Rewarming and regrowth of explants in post-cryopreservation

The formation of intra-cellular ice crystals may also occur during rewarming of explants due to a phenomenon called "migratory recrystallisation": the first molecules of water, formed from nuclei of condensation, attract other water molecules and evolve into large ice crystals (Wesley-Smith et al. 2015). This effect is undesirable like the formation of crystals in the ultra-rapid cooling phase, and can be avoided or largely limited by a rapid heating of the cryovials containing the samples, obtained by immersing them in a water bath at a high temperature, generally 38–40 °C which determines a heating rate of about 250 °C min^{-1}. This way, in less than a minute, the explant temperature increases from − 196 to 20–25 °C. Alternatively, rewarming is performed at room temperature, generally by immersing the explants in MS solution, sometimes with a high concentration of sucrose, usually for up to 30 min in the case of vegetables (see Table 2).

The regrowth of the explants that survived the ultra-cooling and rewarming processes takes place in gelled media, often developed to induce a prompt recovery of the cellular metabolic activity, limit the formation of undifferentiated tissue (callus) and stimulate the shoot tip regrowth (or the conversion of synthetic seeds into plantlets), while simultaneously favouring the "repair" of micro-damages that may have arisen in the meristem.

Application of cryopreservation to the conservation of vegetable genetic resources

Cryopreservation has been successfully applied to various vegetable species. The applied procedure and the type of cryopreserved explant vary according to the species. Nonetheless, the use of PVS2 and PVS3 vitrification solutions with shoot tips from in vitro culture has been the most widely applied approach. Table 2 summarizes the main outcomes of the studies, published in international journals from 2000 onwards, and reports complete and efficient procedures of cryopreservation of explants coming from in vitro culture. Moreover, the present consistency of cryobanks maintaining germplasm of vegetables is reported in Table 3.

Artichoke (*C. cardunculus* var. *scolymus*)

The possibility of efficiently cryopreserving shoot tips from shoot cultures of artichoke was demonstrated in the 2013 by two Italian working groups, using the PVS2-vitrification

Table 2 Synthesis of the cryopreservation procedures applied to vegetables of the temperate climate

Species	Explant	Cryotechnique	Vitrification procedure	Rewarming °C (min)[a]	RPC (%)[b]	References
Artichoke						
Cynara cardunculus var. *scolymus*	Shoot tips (3–4 mm)	Vitrification	PVS2, 90 min at 0 °C → LN	40 (1)	36	Tavazza et al. (2013)
Cynara cardunculus var. *scolymus*	Shoot tips (1–1.5 mm)	Vitrification	PVS2, 55 min at 0 °C → LN	40 (1.5)	95	Taglienti et al. (2013)
Asparagus						
Asparagus officinalis	Shoot tips (0.5–3.5 mm)	Droplet method	10% DMSO, 2 h at RT → drops (5 µL) on AS → LN	RT (30)	96	Mix-Wagner et al. (2000)
Cassava						
Manihot esculenta	Shoot tips	Encapsulation-vitrification	0.3 M SUC, 16 h → encapsulated → 2 M glycerol and 0.6 M SUC, 90 min at RT → PVS2, 4 h at 0 °C → LN	NR	80	Charoensub et al. (2004)
Manihot esculenta	Embrogenic calli	Slow cooling	0.3 or 0.5 M SUC, 2d (on gelled MS) → laminar flow hood, 6 h → 1 °C min^{-1} up to -40 °C → LN	45 (1.5)	60	Danso and Ford-Lloyd (2011)
Manihot esculenta	Uni- and bi-nodal segments	Dehydration	MS + 1 M SOR, 0.1 M SUC, 0.1 M DMSO, 3 d → laminar flow hood, 10 min → MS + 1 M SOR, 0.1 M SUC, 1.28 M DMSO, 20 min	37 (2)	66	Diantina et al. (2016)
Garlic						
Allium sativum	Apices of bulbs	Droplet vitrification	PVS2, 30 min at 0 °C → drops on AS → LN	25 (20)	75	Volk et al. (2004)
Allium sativum	Shoot tips	Vitrification	PVS3, 120 min at RT → LN	40 (2)	70	Keller (2005)
Allium sativum	Apices of bulbs (1–2 mm)	Droplet vitrification	PVS2/PVS3, 15–20 min at 0 °C → drops on AS → LN	PVS2: 25 (20) PVS3: 40 (2)	100	Ellis et al. (2006)

Table 2 continued

Species	Explant	Cryotechnique	Vitrification procedure	Rewarming °C (min)[a]	RPC (%)[b]	References
Allium sativum, A. grayi, A. proliferum	Aereal bulbs (primordia)	Droplet vitrification	PVS3, 150 min at 24 °C → drops (5 µL) on AS → LN	40 in 0.8 M SUC, RT (0.5)	85	Kim et al. (2006)
Allium spp.	Immature inflorescenses[c], apices of bulbs, aereal bulbs	Droplet vitrification	PVS3, 150 min at 24 °C → drops (5 µL) on AS → LN	40 in 0.8 M SUC, RT (0.5)	77[d]	Kim et al. (2007)
Mint						
Mentha spp.	Shoot tips	Droplet vitrification	Drops (2 µL) on AS → PVS2, 20 min at RT → LN	40 (3–5 s) + 1,2 M SUC, RT (20)	89	Senula et al. (2007)
Mentha spp.	Shoot tips (1 mm)	Slow cooling	Polyethylenglicol, glucose and DMSO, 10% each, in liquid MS → -0.1 °C min^{-1} up to -40 °C → LN	45 (1) + 25 (1)	95	Uchendu and Reed (2008)
Mentha spp.	Shoot tips (1–1.5 mm × 1 mm)	V-cryo-plate	Bud, encapsulated in alginate on alluminium bar → PVS2, 20 min at 25 °C → LN	1 M SUC, RT (15)	100	Yamamoto et al. (2012)
Potato						
Solanum tuberosum	Microtubers (≤ 2 mm)	Dehydration	Silica gel for 3–6 h	45 (1)	100	Uchendu et al. (2016)
Solanum spp.	Shoot tips (1.8–2.5 mm)	Droplet vitrification	PVS2, 50 min at 0 °C → drops (10- 15 µL) on AS → LN	Liquid MS, RT (20)	59	Panta et al. (2014, 2015); Vollmer et al. (2016)
Solanum spp.	Shoot tips (1–2 mm)	Droplet vitrification	PVS2, 20 min with agitation → drops (2,5 µL) on AS → LN	0.8 M SUC, RT (30)	94	Kim et al. (2006)
Solanum spp.	Shoot tips (1.5–2 mm)	V-cryo-plate, D-cryo-plate	V-cryo-plate: PVS2, 30 min at 25 °C → LN D-cryo-plate: sterile air flow, 2 h at 25 °C, RH 40–50% → LN	RT (15)	100	Yamamoto et al. (2015)

Table 2 continued

Species	Explant	Cryotechnique	Vitrification procedure	Rewarming °C (min)[a]	RPC (%)[b]	References
Solanum tuberosum	Shoot tips (5–6 mm)	Droplet vitrification	Drops (4 µL) on AS → PVS2, 20 min at RT → LN	Liquid MS, RT (NR)	57	Halmagyi et al. (2005)
Solanum tuberosum	Shoot tips (2–2.5 mm)	D-cryo-plate	Dehydration on silica gel, 90 min at 24 °C → LN	Liquid MS + 1 M SUC, RT (15)	90	Valle Arizaga et al. (2017)
Solanum spp.	Shoot tips (0.6–1.3 mm)	Droplet vitrification	Drops on AS → PVS2, 20 min at RT → LN	SUC 0.8 M, RT (30)	100	Bamberg et al. (2016)
Sweet potato						
Ipomoea batatas	Shoot tips	Droplet vitrification	PVS2, 16 min at 22 °C → drops on AS → LN	22 (20)	62	Pennycooke and Towill (2000)
Ipomoea batatas	Shoot tips (1 mm)	Encapsulation-vitrification	Encapsulation in 2% alginate → PVS2, 60 min at 25 °C → LN	38 (2)	80	Hirai and Sakai (2003)
Ipomoea batatas	Shoot tips	Droplet vitrification	PVS3, 60 min at RT → drops (5 µl) on AS → LN	30% SUC, 40 (40)	19	Park and Kim (2015)
Ipomoea batatas	Shoot tips (1 mm)	Encapsulation-vitrification	Encapsulation in alginate → PVS3, 2 h at 25 °C → LN	40 (2)	91	Yi et al. (2016)
Red chicory						
Cichorium intybus	Shoot tips (1–2 mm)	Slow cooling Encapsulation-dehydration	DMSO 15%, 1 h → -0.5 °C min^{-1} up to -40 °C → LN Encapsulation in alginate 3% → preculture on SUC 0.3 and 0.75 M, 24 h each → Dehydration in air flow, 7 h → LN	40 (2–3)	83	Vandenbusshe et al. (2002)
Cichorium intybus	Shoot tips (2–3 mm)	Vitrification	PVS2, 60 min at 0 °C → LN	40 (1)	76	Lambardi et al. (2006); Benelli et al. (2011)

Table 2 continued

Species	Explant	Cryotechnique	Vitrification procedure	Rewarming °C (min)[a]	RPC (%)[b]	References
Thyme						
Thymus vulgaris	Shoot tips	Droplet vitrification	0.25 M SUC, 48 h → Drops (4 µL) on AS → PVS2, 90 min at 0 °C → LN	Liquid MS + 1 M SUC, RT (NR)	80	Ozudogru et al. (2011); Ozudogru and Kaya (2012)

Main papers published on international journals from 2000 onwards are reported (RPC, explant regrowth in post-cryopreservation; AS, aluminum strips; LN, liquid nitrogen; SUC, sucrose; RT, room temperature; RH, relative humidity; NR, not reported)

[a]The substrate used during rewarming of the explant (when reported), the temperature and the time (minute) of rewarming

[b]The data presented refers to the maximum percentage of recovery in post-conservation of the accessions or species of that particular report

[c]Portions of immature inflorescenses, containing bulbil primordia and floral buds

[d]The average regeneration obtained from a total of 252 accessions of *A. sativum, A. cepa, A. macrostemon, A.* x *proliferum*

Table 3 Consistency of accessions of vegetables, preserved in biobanks (in vitro and cryobanks)

Species Conservation Center*	Accessions in vitro (n°)	Accessions in liquid nitrogen (n°)	Total accessions (no.)	Material in conservation
Cassava (*Manihot esculenta*)				
CIAT, Colombia	6700	2100	8800	Shoots (V), Shoot tips (C)
EMBRAPA, Brazil	262	1	263	Shoots (V), Shoot tips (C)
IITA, Nigeria	2469	–	2469	Shoots (V)
Total	9431	2101	11,532	
Garlic (*Allium sativum*)				
CRI, Czech Republic	–	127	127	Shoot tips (C)
InHort, Polond	–	168	168	Shoot tips (C)
IPK, Germany	18	230	248	Shoots (V), shoot tips, inflorescenses (C)
NICS, South Korea	–	300	300	Shoot tips (C)
USDA-ARS, United States	–	100	100	Shoot tips (C)
Total	18	925	943	
Jerusalem artichoke (*Helianthus tuberosus*)				
CAAS, China	27	4	31	Shoots (V), Shoot tips (C)
Total	27	4	31	
Mint (*Mentha* spp.)				
NARO, Japan	–	16	16	Shoot tips (C)
IPK, Germany	146	148	294	Shoots (V), Shoot tips (C)
USDA-ARS, United States	–	43	43	Shoot tips (C)
Total	146	207	353	
Potato (*Solanum* spp.)				
ARC, South Africa	1200	–	1200	Shoots (V)
CIP, Perù	8354	2500	10,854	Shoots (V), Shoot tips (C)
CRI, PRI, Czech Republic	2257	74	2331	Shoots (V), Shoot tips (C)
NARO, NIAS, NCSS, CAES, Japan	150	188	338	Shoots (V), Shoot tips (C)
CAAS, China	224	24	248	Shoots (V), Shoot tips (C)
IPK, Germany	2932	1625	4557	Microtubers (V), Shoot tips (C)
NICS, NAC, South Korea	1223	130	1353	Shoots (V), Shoot tips (C)
PFR, New Zealand	180	87	267	Shoots (V), Shoot tips (C)
CPRI, India	1500	–	1500	Shoots (V)

Table 3 continued

Species Conservation Center*	Accessions in vitro (n°)	Accessions in liquid nitrogen (n°)	Total accessions (no.)	Material in conservation
USDA-ARS, United States	–	393	393	Shoot tips (C)
USPG, United States	624	–	624	Shoots (V)
VIR, Russia	300	–	300	Shoots (V)
Total	18,944	5021	23,965	
Sweet Potato (*Ipomea batatas*)				
CIP, Perù	5328	34	5362	Shoots (V), Shoot tips (C)
EMBRAPA, Brazil	26	1	27	Shoots (V), Shoot tips (C)
Total	5354	35	5389	
Taro (*Calocasia esculenta*)				
CePaCT, Republic of Fiji	1165	–	1165	Shoots (V)
CAAS, China	7	3	10	Shoots (V), Shoot tips (C)
Total	1172	3	1175	
Yam (*Dioscorea* spp.)				
IITA, Nigeria	1976	–	1976	Shoots (V)
Total	1976	–	1976	

Data from: Ellis et al. (2020), Pathirana et al. (2019), Ebert and Waqainabete (2018), Acker et al. (2017), Jenderek and Reed, (2017), Bamberg et al. (2016), Niino and Valle Arizaga (2015), Myeza and Visser (2013), Gavrilenko (2008); (V, in vitro banks with slow growth storage; C, cryobanks)

*ARC, Agricultural Research Council; CAAS, Chinese Academy of Agricultural Sciences; CAES, Central Agricultural Experiment Station; CePaCT, Centre for Pacific Crops and Trees; CIAT, International Center of Tropical Agriculture; CIP, International Potato Center; CPRI, Central Potato Research Institute; CRI, Crop Research Institute; EMBRAPA, Brazilian Agricultural Research Corporation; IITA, International Institute for Tropical Agriculture; InHort, Research Institute of Horticulture; IPK, Leibniz Institute of Plant Genetics and Crop Plant Research; NAC, National Agrobiodiversity Center; NARO, National Agriculture and Food Research Organization; NCSS, National Center of Seed and Seedlings; NIAS, National Institute of Agrobiological Sciences; NICS, National Institute of Crop Science; PFR, Plant and Food Research; PRI, Potato Research Institute; USDA-ARS, US Department of Agriculture-Agricultural Research Service; USPG, US Potato Genebank; VIR, N.I. Vavilov Institute of Plant Genetic Resources

technique. It is important to note that, in both cases, the optimized procedures have proved to be effective in producing virus-free plantlets (Tavazza et al. 2013; Taglienti et al. 2013). This technique, called "cryotherapy", is a very promising application of cryogenics for remediation from virus, viroid, phytoplasma and bacteria, with important results in some economically-important vegetables, such as artichoke, potato and sweet potatoes (Wang et al. 2009).

Asparagus (*Asparagus officinalis*)

Asparagus cryopreservation was proposed at the beginning of the'90 s (Uragami et al. 1990), based on the dehydration of uni-nodal segments on silica gel to a water content of less than 20% before direct immersion in LN. High percentages of recovery (up to 96%) were obtained by applying the droplet method with DMSO to shoot tips from eight asparagus cultivars (Mix-Wagner et al. 2000). However, to date there are no reports of the inclusion of asparagus germplasm in cryobanks.

Cassava (*Manihot esculenta*)

Charoensub et al. (2004) successfully cryopreserved shoot tips by the encapsulation-vitrification method. The shoot tips were precultured on MS medium containing 0.3 M sucrose for 16 h, encapsulated and osmoprotected with a mixture of 2.0 M glycerol and 0.6 M sucrose for 90 min at 25 °C before dehydration with PVS2 at 0 °C for 4 h, then plunged into LN. Successfully vitrified shoot tips resumed growth within 3 days of recovery, without callus formation, and developed shoots. The post-rewarming survival rate of vitrified shoot tips depended on the day of excision, with shoot tips excised from two day-old plantlets producing the highest survival result. The protocol was successfully applied to four cultivars of cassava with a post-rewarming survival level of up to 80%. In another study, shoot tips, nodal cuttings and embryogenic calli of cassava were cryopreserved by slow cooling and vitrification methods. Shoot tips, recovered from storage in LN, showed post-rewarming callus formation only, which did not produce any shoots. Similarly, nodal segments also failed to develop into whole plantlets, although they produced vestigial shoots. In contrast, embryogenic calli, cryopreserved by slow cooling, survived storage in LN, resulting in a higher post-rewarming recovery (60%) after being precultured on 0.3 M sucrose instead of 0.5 M (Danso and Ford-Lloyd 2011). More recently, Diantina et al. (2016) precultured uni- and bi-nodal segments of two in vitro-grown cassava accessions on MS medium supplemented with 1 M sorbitol, 0.1 M sucrose, 0.1 M DMSO, 0.05 mg L^{-1} BA, 0.05 mg L^{-1} GA_3 and 0.01 mg L^{-1} NAA for 3 days in dark. Pre-cultured explants were dehydrated in laminar air flow for 10 min at 25 °C, and then treated with liquid MS medium supplemented with 0.1 M sucrose, 1.0 M sorbitol, 1.28 M DMSO for 20 min at 25 °C. Explants were then directly plunged into LN. Following rapid rewarming at 37 °C for 2 min, they were transferred onto recovery medium (MS medium supplemented with 0.02 mg L^{-1} NAA, 0.05 mg L^{-1} BA, 0.05 mg L^{-1} GA_3), kept in dark for 1 week and then gradually transferred to the light and kept at 25 °C with a 16 h photoperiod. They regenerated into plantlets two months after rewarming, and the maximum recovery rate was 66%.

Garlic (*Allium* spp.)

Numerous works demonstrate the possibility of cryopreserving various types of garlic explants, such as immature inflorescences, primordia and apices of clove, bulbils and shoot tips excised from in vitro shoots. The most recent procedures that have been produced for *A. sativum* and other garlic species always resort to the use of the PVS2 and PVS3 vitrification solutions, mainly with the droplet vitrification method. The effectiveness of the methods developed is evidenced by the high survival and regrowth of the explants in post-cryopreservation (70–100%, Table 2). The droplet method with PVS2 applied to

immature inflorescences of numerous accessions of garlic (Kim et al. 2007) is very interesting because from each single immature inflorescence it was possible to obtain, through in vitro culture, more than 20 microbulbs which were generally virus-free or bore an extremely low viral load. In general, the high post-rewarming survival and regrowth rates that could be achieved with optimized procedures have allowed the development of an effective strategy for the worldwide conservation of the *Allium* germplasm in cryobanks (Table 3). It should be noted that, at the IPK in Gaterslaben, the cryopreservation method is being extended to a large pool of species of the genus *Allium*, with results that are not always equally satisfactory. For example, with shallot it is not possible to obtain percentages of survival and regrowth of the shoot tips comparable to other species of *Allium* and, therefore, this species cannot yet be introduced into the cryobank (Keller and Senula 2016).

Mint (*Mentha* spp.)

The three main post-2007 studies investigating mint cryopreservation resorted to three different methods: droplet vitrification, slow cooling and the V-cryo-plate. The last method, in particular, developed at the NIAS in Japan, has proved to be very effective when applied to a large pool of species (*Mentha arvensis, M.* × *piperita, M. arvensis* var. *piperascens, M. spicata, M. japonica, M.* × *rotundifolia*), allowing from 70 to 100% of regrowth of cryopreserved material (Yamamoto et al. 2012).

Potato (*Solanum* spp.)

Potato is by far the most cryopreserved vegetable, allowing cryobanking of a large number of accessions of *Solanum* spp. (Table 3). Effective droplet freezing in DMSO (droplet method) was developed in the'90 s (Schäfer-Menuhr et al. 1997) using shoot tips of about 2–3 mm in length, treated in drops of DMSO on strips of aluminum, and then directly ultra-cooled by inserting the strips into cryovials containing LN. After rewarming in MS liquid medium at room temperature and reintroduction to in vitro culture, an average shoot tip regrowth of 40% was obtained. From this basis, research groups worldwide have dedicated their activity to the improvement of the technology, with the aim of increasing the growth rate in post-cryopreservation and broadening the applicability of the process to the genetic resources of the species. Most of the reports are based on the application of the droplet vitrification method to shoot tips of various sizes, from a maximum of 6 mm (Halmagyi et al. 2005) to a minimum of 0.6 mm (Bamberg et al. 2016). It is noteworthy that, in the latter report, the optimized protocol produced 100% of shoot tip regrowth in post-cryopreservation, even using so small explants. Using shoot tips as starting explants, other recent studies have been based on the use of the V-cryo-plate (Yamamoto et al. 2015) and D-cryo-plate (Yamamoto et al. 2015; Valle Arizaga et al. 2017) with very high post-cryopreservation survival and regrowth (90–100%). Also microtubers grown in vitro (up to 2 mm in size) could be effectively cryopreserved, after simple dehydration in silica gel for 3–6 h before direct immersion in LN, with 100% survival and regrowth (Uchendu et al. 2016). Detailed reviews on cryopreservation of potato genetic resources have been provided by Kaczmarczyk et al. (2011) and Niino and Valle Arizaga (2015).

Sweet potato (*Ipomoea batatas*)

Since 2000, four studies have optimized two different procedures for the cryopreservation of shoot tips (around 1 mm in size) taken from in vitro shoot cultures of sweet potato: droplet vitrification and encapsulation-vitrification, using either PVS2 or PVS3 as vitrification solutions. It should be noted that, in these studies, the rewarming procedure applied to the explants recovered from LN was quite different, as well as the percentages of shoot tip regrowth, ranging from a minimum of 19% (Park and Kim 2015) to a maximum of 91% (Yi et al. 2016). However, this variability in the outcomes of the procedures did not prevent the introduction of the species into conservation in some cryobanks (Table 3).

Red chicory (*Cichorium intybus*) and thyme (*Thymus* spp.)

For these two species, efficient recently-developed cryopreservation procedures are available. Yet, there are no reports of entry of accessions into cryobanks.

The cryoconservation of red chicory shoot tips has been reported in three studies (Vandenbussche et al. 2002; Lambardi et al. 2006; Benelli et al. 2011). In the most recent report, explants (2–3 mm long shoot tips) were transferred to cryovial and treated with PVS2 for 60 min at 0 °C and then the cryovials were directly immersed in LN. Rewarming was carried out in a water bath at 40 °C for 1 min, and regrowth was obtained on MS medium containing 0.5 μM BA. The procedure proved to be very effective, with a maximum survival of 76%. After 3 months of regrowth, the shoots were rooted in vitro, acclimatized in vivo and transferred to the field, demonstrating an absolute morphological and molecular correspondence (by ISSR/Inter Simple Sequence Repeat) to the original stock plant (De Carlo et al. 2007).

Shoot tips of *Thymus vulgaris* were cryopreserved by droplet vitrification through the treatment of the explants with PVS2 solution for 90 min at 0 °C (Ozudogru and Kaya 2012). The highest post-rewarming recovery (80%) was achieved by comparing several treatments; (i) cold hardening of in vitro shoot cultures at 4 °C in darkness for 1, 2, 3 or 4 weeks, (ii) sucrose pre-culture of the excised shoot tips at 4 °C in darkness on MS medium, supplemented with 0.12, 0.25, 0.50, 0.75 or 1.00 M sucrose, for 24, 48 or 72 h, and (iii) comparison of PVS2-based "one-step cooling" methods, i.e., PVS2 vitrification, encapsulation-vitrification and droplet vitrification. The optimized protocol (2-week cold hardening of in vitro shoot cultures, 48 h preculture of shoot tips on MS medium supplemented with 0.25 M sucrose, and a 90 min PVS2 treatment in droplets) was then applied also to *T. cariensis*, resulting in 25% post-rewarming recovery, i.e., a very promising result for the long-term storage of this endemic and endangered species.

Biobanking (in vitro and in LN) of vegetable species in the world

Table 3 shows the situation, updated to 2017, of the preservation of vegetable species in in vitro banks and cryobanks. A total of more than 45,000 accessions are maintained in 22 conservation centers, located in 16 countries and 6 continents (Europe, Asia, Africa, Oceania, North and South America). About four-fifths of accessions are preserved in vitro as shoots in slow growth storage, but germplasm maintained in cryopreservation is in constant growth and currently involves almost 8300 accessions.

The highest number of accessions in biobanks are of potato and cassava. The preserved germplasm of *Solanum* spp., in particular, represents over 51% of the total accessions

of vegetable species maintained in in vitro banking, and over 60% of those preserved in cryobanking (Fig. 1). The main conservation centers for potato are in Peru, at CIP in Lima, and in Germany, at the IPK in Gaterslaben. A large amount of accessions (over 1,000) maintained as shoots in slow growth storage are also reported from the Czech Republic (CRI and PRI), India (CPRI), South Korea (NICS and NAC) and South Africa (ARC). As for cryobanking, low numbers of accessions are preserved in USA, Japan, South Korea, New Zealand, Czech Republic and China, in comparison to the centers in Peru and Germany. Cassava is mainly preserved as shoots in Colombia (6700 accessions in slow growth storage and 2100 in cryopreservation), and Nigeria (about 2500 accessions in slow growth storage).

Among vegetables, the genetic resource biobanking of sweet potato and garlic is also relevant, using the different available methods. Sweet potato is mainly preserved in vitro, in slow growth storage, at the CIP in Lima, Peru, where there are more than 5300

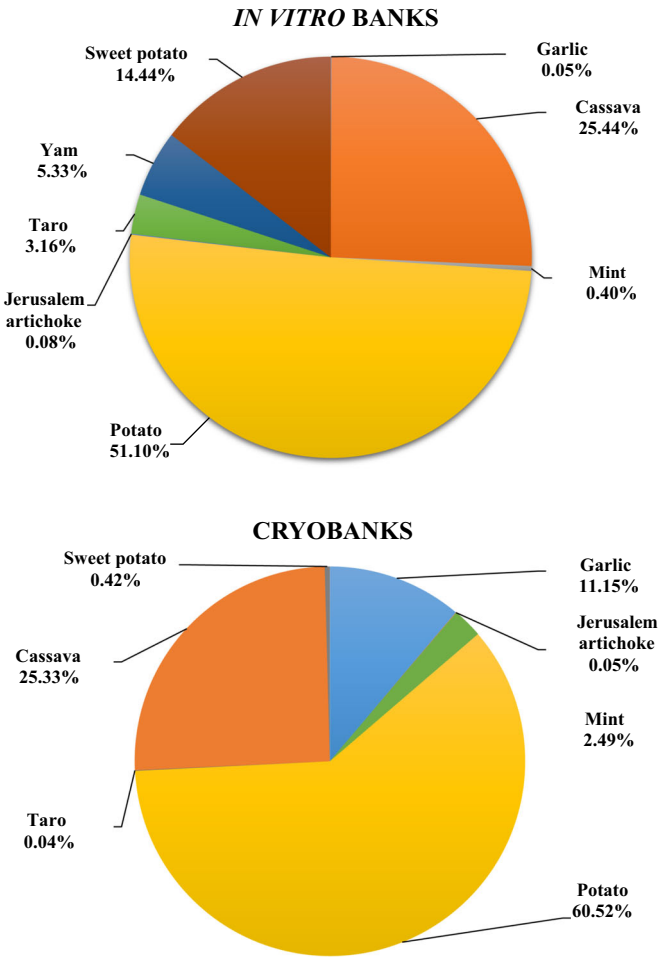

Fig. 1 Vegetable crops, preserved in in vitro banks and cryobanks. Data from: Ellis et al. (2020), Pathirana et al. (2019), Ebert and Waqainabete (2018), Acker et al. (2017), Jenderek and Reed (2017), Bamberg et al. (2016), Niino and Valle Arizaga (2015), Myeza and Visser (2013), and Gavrilenko (2008)

accessions, representing more than 14% of the total genetic resources of vegetables stored in vitro in the world. The germplasm of garlic is almost entirely cryopreserved (925 accessions), divided between five cryobanks (in the Czech Republic, Poland, South Korea, Germany and USA) over three continents.

Mint is another vegetable species already preserved both in in vitro banks, and in cryobanks, with a more limited number of accessions (353 in total), considering the comparatively minor economic importance of the species compared to above-mentioned genetic resources. Mint biobanks are located in Japan, USA and Germany.

Almost 2000 accessions of yam are conserved in slow growth storage of shoots in Nigeria, at the IITA, representing more than 5% of the total vegetable genetic resources. There are, however, no cryobank accessions yet.

The CAAS center, in China, maintains a limited number of accessions of Jerusalem artichoke (31) and taro (10). For both species, protocols of slow growth storage of shoots, and cryopreservation of shoot tips have been developed and used for the conservation in the Chinese biobank. However, the main taro in vitro conservation center is the CePaCT in the Republic of Fiji, where almost 1200 accessions are preserved as shoots in slow growth.

Conclusions

In vitro culture represents a strategic tool to support medium- and long-term conservation of plant genetic resources by means of slow growth storage of shoots and cryopreservation of organs and tissues in LN. In the last 30 years, considerable progress has been made in the development of both biobanking approaches. In particular, knowledge of cryogenic technology has made the process of vitrification of explants more efficient. This is indispensable to the acquisition of cell tolerance to ultra-rapid cooling in LN, as determined by the application of "one-step cooling" methods. The species that can be effectively preserved in LN are constantly growing, including a large number of economically-important germplasm of vegetables. The experimental work carried out in various research centers across the world has produced important applicative impact, with the establishment of in vitro and in LN conservation centers, spread across 16 countries and 6 continents. This conservation strategy should be regarded as complementary to the traditional approach of preservation in seed banks and in field clonal collections, in order to achieve to an effective back-up of this world germplasm. It is important to note that the activities of conservation of vegetable germplasm in in vitro banks and in cryobanks is not yet adequately supported by a detailed exchange of information between institutions to minimize the unnecessary maintenance of the same genetic resources in different biobanks. The development of a network system was recently undertaken under the aegis of Bioversity International, the Global Crop Diversity Trust and the International Potato Center. This led, in July 2017, to a report containing a partial census on plant genetic resources in in vitro banks and cryobanks alongside numerous indications and directives on the organization and cost of these forms of conservation (Acker et al. 2017). This has been the first important step towards a global coordination of conservation actions in vitro and in LN that will soon become a fundamental strategy to efficiently counteract the erosion of plant genetic resources.

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