CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Tropical AgriSciences

Department of Crop Sciences and Agroforestry

Conservation of Andean root and tuber crops by novel biotechnological methods

DOCTORAL THESIS

Author: Ing. Stacy Denise Hammond Hammond

Supervisor: Ing. Iva Viehmannová, Ph.D.

Co-supervisor: Ing. Jiří Zámečník, CSc.

Prague 2021

Declaration

I declare that I have worked on my dissertation thesis titled "Conservation of Andean root and tuber crops by novel biotechnological methods" by myself and I have used only the sources mentioned at the end of the thesis. As the author of the dissertation thesis, I declare that the thesis does not break the copyrights of any third person.

Date and time: _____

Stacy Hammond

Acknowledgements

I would like to express my sincere gratitude to the people who devoted their time to help me during the elaboration of this thesis and throughout my entire Ph.D. studies.

First and foremost, I extend special thanks to my thesis supervisor Ing. Iva Viehmannová, Ph.D. for her unconditional and continuous support and patience. For sharing her immense knowledge during the elaboration of my research, her guidance was the pillar of my studies and the writing of this thesis. I could never imagine having a better mentor!

I also extend my sincere gratitude to my co-supervisor Ing. Jiří Zámečník, CSc. for his indispensable information, help and support in various aspects of my research and for guiding me with my experimental work on cryopreservation.

Additionally, I express my gratitude to Dr Ir. Bart Panis for giving me not one but two internship opportunities with his team at Bioversity International in KU Leuven and for always being a continuous and reliable part of my research team.

Likewise, I would like to thank Ing. Miloš Faltus, Ph.D. and the technicians from the Crop Research Institute in Prague for allowing me to carry out my experimental work in this institution and for welcoming me as a part of their team.

To my colleagues and the staff at the Faculty of Tropical AgriSciences (FTZ), I extend a massive thank you for opening your doors and welcoming me to your tropical family in Europe. As I finish this stage and embark on new adventures, I will always carry with me the name of the Czech University of Life Sciences (CZU) with pride.

I dedicate this thesis to my family and close friends who despite the ups and downs their advice, moral support and encouragement never failed. Thank you!

The research was financially supported by the Internal Grant Agency of the Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague, IGA (Project No. 20165014, 20175015, 20185015, 20195002 and 20205006), the Czech Ministry of Agriculture (Project No. QJ1630301 and RO0418) and the institutional project of the Crop Research Institute (Project No. MZE-RO0418).

Table of Contents

List of articles

Hammond SDH, Viehmannova I, Zamecnik J, Panis B, Hlasna Cepkova P. 2019. Efficient slow-growth conservation and assessment of clonal fidelity of *Ullucus tuberosus* (Caldas) microshoots. Plant Cell Tiss. Organ Cult. 138:559-570.

List of manuscripts

Hammond SDH, Viehmannova I, Zamecnik J, Panis B, Faltus M. Droplet-vitrification method for shoot tip cryopreservation of yacon [*Smallanthus sonchifolius* (Poepp. and Endl.) H. Rob.]: effects of PVS2 and PVS3 on survival and regrowth. Plant Cell Tiss. Organ Cult. Submitted for consideration in Plant Cell Tiss. Organ Cult.

List of conference contributions

Hammond HSD, Viehmannova I, Hlasna Cepkova P, Duong H. 2016. Assessment of new yacon (*Smallanthus sonchifolius*) genotypes obtained via indirect somatic embryogenesis. Poster presentation at TROPENTAG conference 2016 in Vienna, Austria.

Hammond HSD, Viehmannova I, Zamecnik J. 2017. Slow-growth *in vitro* Conservation of *Ullucus tuberosus* (Loz.), an Andean tuber crop. Poster presentation at TROPENTAG conference 2017 in Bonn, Germany.

Hammond HSD, Viehmannova I, Zamecnik J, Panis B, Hlasna Cepkova P. 2018. Development of a PVS2 droplet vitrification method for yacon (*Smallanthus sonchifolius*) Cryopreservation. Poster presentation at TROPENTAG conference 2018 in Ghent, Belgium.

Hammond HSD, Viehmannova I, Zamecnik J, Panis B, Hlasna Cepkova P. 2019. *In vitro* Conservation and Recovery of *Ullucus tuberosus* (Loz.) after Reduced Growth of Microshoots. Poster presentation at TROPENTAG conference 2019 in Kassel, Germany.

List of projects

IGA Project 2016: 20165014 - Micropropagation and genotyping *in vitro*; proteomics and molecular analyzes in selected plant species. Financial donor: Faculty of Tropical AgriSciences, CZU, Prague. Co-researcher.

IGA Project 2017: 20175015 - Application of plant explants in plant propagation and breeding; molecular and proteomic analyzes of selected crops. Financial donor: Faculty of Tropical AgriSciences, CZU, Prague. Co-researcher.

IGA Project 2018: 20185015 - Application of biotechnological and proteomic processes in selected crops. Financial donor: Faculty of Tropical AgriSciences, CZU, Prague. Co-researcher.

IGA Project 2019: 20195002 - Biotechnology as a tool in plant breeding and conservation of genetic resources. Financial donor: Faculty of Tropical AgriSciences, CZU, Prague. Co-researcher.

Project 2019-2020: č. 7/2020/02, Enhancement of the education and scientific research in the field of biotechnologies, agricultural production and extension at Hawassa University, Ethiopia. Financial donor: Czech Development Agency. Co-researcher.

Project 2020-2021: Strengthening scientific capacities and cooperation of Ukrainian universities in agricultural sciences. Financial donor: Ministry of Foreign Affairs, Czech Republic. Co-researcher.

List of tables

List of figures

List of abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic acid
AFLP	Amplified fragment length polymorphism
ARTC	Andean root and tuber crop
BA	N^6-Benzyladenine
BSA	Bovine serum albumin
CCC	Chlorcholinchlorid
CIP	International Potato Center (Centro Internacional de la Papa)
CTAB	Cetyltrimethylammonium bromide
CZU	Czech University of Life Sciences Prague
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSC	Differential scanning calorimetry
EtBr	Ethidium bromide
FGN	Fully regenerated plant
FOS	Fructooligosaccharide
FTA	Faculty of Tropical AgriSciences
HH	Hyperhydricity
IRAP	Inter retrotransposon amplified polymorphism
ISSR	Inter-simple sequence repeat
LN	Liquid nitrogen
LS	Loading solution
MH	Maleic hydrazide
MS	Murashige and Skoog (1962) medium
MT	Microtuber
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGR	Plant growth regulator
PVP	Polyvinylpyrrolidone
PVS	Plant vitrification solution

Abstract

In the present study, we developed reliable and efficient methods of *in vitro* conservation for the vegetatively propagated Andean root crop yacon [*Smallanthus sonchifolius* (Poepp. et Endl.) H. Robinson] and tuber crop ulluco (*Ullucus tuberosus* Caldas). In the case of yacon, a cryopreservation protocol was developed using a genotype originated from Ecuador classified as ECU 41. Osmotic dehydration of apical shoot tips (2-3 mm long) was carried out by testing plant vitrification solution (PVS) 2 (15, 30, and 60 min) at 0 °C and PVS3 (30, 45, 60, and 75 min) at 22 °C. After cryopreservation, the shoot tips were thawed and placed on MS (Murashige & Skoog 1962) medium ± 0.1 mg l^{-1} N^6-Benzyladenine (BA). The optimized protocol was then applied to four other yacon genotypes originated from Bolivia and Peru classified as BOL 22, BOL 23, PER 12 and PER 14 to determine its applicability. The results using ECU 41 showed that MS without 0.1 mg l^{-1} BA as regrowth medium and PVS2 and PVS3 60 min treatment duration was most effective in providing the highest survival (80-93.3%) and regeneration (up to 73%) rates with no morphological abnormalities post cryopreservation. When applied to the other yacon accession the survival and regeneration rates were also high ranging between 79.7-94.1% and 66.3-75.4% respectively. In the case of ulluco, a medium-term conservation protocol was developed using slow-growth *in vitro* method. Nodal segments were placed on half-strength MS with mannitol (10-30 g l^{-1}), sorbitol (10-30 g l^{-1}), sucrose (10-120 g l^{-1}), chlorcholinchlorid (CCC; 300-700 mg l^{-1}), abscisic acid (ABA; 1-3 mg l^{-1}), or maleic hydrazide (MH; 0.1-0.5 mg l^{-1}) and at two cultivation temperatures (either 5 °C or 17 °C). These treatments were assessed for an 18-month slow-growth period. Results showed that the most suitable slow-growth treatment was mannitol at 30 g l^{-1} concentration at 17 °C cultivation temperature. Regenerated plantlets post conservation were genetically identical as proved by ISSR markers and showed no signs of morphological abnormalities. The development of these conservation techniques not only ensures the conservation of yacon and ulluco but also contributes to both general and specific knowledge on conservation of Andean root and tuber crops germplasm and will provide suitable biotechnological tools for gene banks maintaining these species.

Keywords: biotechnological methods, cryopreservation, *in vitro* conservation, slow-growth, *Smallanthus sonchifolius, Ullucus tuberosus*

Spanish abstract

En el presente estudio, desarrollamos métodos confiables y eficientes de conservación *in vitro* para cultivos de raíces andinas propagadas vegetativamente yacón [*Smallanthus sonchifolius* (Poepp. et Endl.) H. Robinson] y tubérculos ulluco (*Ullucus tuberosus* Caldas). En el caso del yacón, se desarrolló un protocolo de criopreservación utilizando un genotipo originario de Ecuador clasificado como ECU 41. La deshidratación osmótica de la punta apical del brote (2-3 mm de largo) se llevó a cabo probando solución de vitrificación vegetal (PVS) 2 (15, 30 y 60 min) a 0 ºC y PVS3 (30, 45, 60 y 75 min) a 22 ºC. Después de la criopreservación, las yemas se descongelaron y se colocaron en medio MS (Murashige & Skoog 1962) ± 0.1 mg l^{-1} N^6-Benzyladenina (BA). El protocolo optimizado se aplicó a otros cuatro genotipos de yacón originados en Bolivia y Perú clasificados como BOL 22, BOL 23, PER 12 y PER 14 para determinar su aplicabilidad. Los resultados usando ECU 41 mostraron que MS sin 0.1 mg l^{-1} BA como medio de rebrote y PVS2 y PVS3 60 min de duración del tratamiento fue más efectivo para proporcionar las tasas más altas de supervivencia (80-93.3%) y regeneración (hasta 73%) sin anomalías morfológicas tras la criopreservación. Cuando se aplicó a las otra accesiónes de yacón, las tasas de supervivencia y regeneración también fueron altas, oscilando entre 79.7-94.1% y 66.3-75.4% respectivamente. En el caso de ulluco, se desarrolló un protocolo de conservación a medio plazo mediante el método *in vitro* de crecimiento lento. Los segmentos nodales se colocaron en MS de concentración media complementado ya sea con manitol (10-30 g l^{-1}), sorbitol (10-30 g l^{-1}), sacarosa (10-120 g l^{-1}), cloruro de clorcolin (CCC; 300-700 mg l^{-1}), ácido abscísico (ABA; 1-3 mg l^{-1}) o hidrazida maleica (MH; 0.1-0.5 mg l^{-1}) y a dos temperaturas de cultivo (5 °C o 17 °C). Estos tratamientos se evaluaron durante un período de crecimiento lento de 18 meses. Los resultados mostraron que el tratamiento de crecimiento lento más adecuado fue el manitol a una concentración de 30 g l^{-1} a una temperatura de cultivo de 17 °C. Las plántulas regeneradas después de la conservación fueron genéticamente idénticas, como lo demuestran los marcadores ISSR, y no mostraron signos de anomalías morfológicas. El desarrollo de estas técnicas de conservación no solo asegura la conservación del yacón y ulluco, sino que también contribuye al conocimiento general y específico sobre la conservación del germoplasma de cultivos de raíces y tubérculos andinos y proporcionará herramientas biotecnológicas adecuadas para los bancos de genes que mantienen estas especies.

Palabras clave: conservación *in vitro,* crecimiento lento, criopreservación, métodos biotecnológicos, *Smallanthus sonchifolius, Ullucus tuberosus*

Czech abstract

Tato studie byla zaměřena na optimalizaci protokolů pro *in vitro* uchování andských okopanin jakonu [*Smallanthus sonchifolius* (Poepp. et Endl.) H. Robinson] a meloku hlíznatého (*Ullucus tuberosus* Caldas). U jakonu byla kryoprezervace optimalizována s použitím genotypu původem z Ekvádoru, označeného ECU 41. Pro osmotickou dehydrataci u apikálních pupenů byly testovány vitrifikační roztoky PVS2 (15, 30, 60 min.) při 0 °C a PVS3 (30, 45, 60, 75 min.) při 22 °C. Po kryoprezervaci byly pupeny rozmrazeny a umístěny na MS kultivační médium (Murashige & Skoog 1962) s přídavkem 0,1 mg l^{-1} N^6-Benzyladeninu (BA) či bez cytokininu. Optimalizovaný protokol byl následně aplikován na další genotypy jakonu (BOL 22, BO L 23, PER 12, PER 14) za účelem ověření efektivity tohoto postupu při kryoprezervaci jakonu. Při použití vitrifikačních roztoků PVS2 a PVS3 po dobu 60 min. u genotypu jakonu ECU 41 a následném dopěstování vyrostny vrcholna kultivačním médiu bez obsahu BA bylo dosaženo nejvyššího procenta přežití pupenů (80-93,3 %) po kryoprezervaci a následného obnovení růstu (až 73%) bez náznaku morfologických změn. Při použití tohoto protokolu u dalších genotypů jakonu přežilo 79,7-94,1% pupenů a růst obnovilo 66,3-75,4% z nich. U meloku hlíznatého byl optimalizován postup středně dlouhodobého uchování rostlinného materiálu s využitím metody pomalého růstu (tzv. „slow-growth method“). Nodální segmenty byly umístěny na MS kultivační médium s poloviční koncentrací živin, s přídavkem mannitolu (10-30 g l^{-1}), sorbitolu (10-30 g l^{-1}), sacharózy (10-120 g l^{-1}), chlorcholinchloridu (CCC; 300-700 mg l^{-1}), abscisové kyseliny (ABA; 1-3 mg l^{-1}), nebo hydrazidu maleinové kyseliny (MH; 0,1-0,5 mg l^{-1}). Kultury byly pěstovány při 5 °C nebo 17 °C a byly hodnoceny po dobu 18 měsíců. Nejlepší výsledky byly zaznamenány u rostlin pěstovaných na médiu s přídavkem 30 g l^{-1} mannitolu při kultivační teplotě 17 °C. Rostliny z této variant byly následně podrobeny analýze ISSR, která neprokázala žádné genetické změny v průběhu uchování rostlin na médiu s mannitolem. Rovněž u těchto rostlin nebyly zaznamenány žádné morfologické abnormality. Výsledky této studie přinášejí nové poznatky v oblasti uchování rostlinných genetických zdrojů *ex situ*. Protokoly vyvinuté pro uchování jakonu a meloku hlíznatého mohou nalézt uplatnění při uchování těchto druhů v genobankách.

Klíčová slova: biotechnologické metody, kryoprezervace, pomalý růst, *Smallanthus sonchifolius*, uchování *in vitro, Ullucus tuberosus*

1. Introduction

Yacon [*Smallanthus sonchifolius* (Poepp. et Endl.) H. Robinson] and ulluco (*Ullucus tuberosus* Caldas) are root and tuberous crops native to the Andean mountain region (Malice & Baudoin 2009). These crops belong to the so-called Andean Root and Tuber Crops (ARTCs), a group of crops mainly grown for their edible underground organs used by local people as cash crops or for subsistence (Hermann & Heller 1997; Zardini 1991; Malice & Baudoin 2009).

In the past, there has been a rapid erosion of the Andean tubers' genetic diversity (King & Gershoff 1987) due to anthropogenic selection for food purposes and crop husbandry (Malice & Baudoin 2009), habitat destruction, land degradation and environmental changes (Rao & Sthapit 2013). These conditions along with the risk of pest and diseases in conventional conservation practices such as field collection and on-farm conservation management make conventional methods of conservation difficult to perform and have therefore placed these species at risk of genetic erosion (Panta et al. 1999; Tay 2013). Additionally, seed collections of these species do not present a suitable conservation system due to the low reproductive capacity of these species. This creates the need to use advanced biotechnological approaches as an alternative to preserving the species' genetic material and their biodiversity (King & Gershoff 1987; Engelmann 2011).

So far, long-term cryopreservation (preservation at ultra-low temperatures) protocol for ulluco genetic resources was developed with the use of a PVS2 droplet-vitrification method (Sanchez et al. 2011). Zamecnikova et al. (2011) developed a cryopreservation protocol for ulluco using the preparation of shoot tips with sucrose and dehydration pre-treatment, and Arizaga et al. (2017) developed a cryopreservation protocol for ulluco using the D cryo-plate system.

In the case of yacon, the optimization of medium-term conservation using plant growth inhibitors under *in vitro* conditions has already been reported by Skalova et al. (2013), who tested the effect of mannitol, sorbitol and cytokinin benzyl adenine (BA) on the growth reduction. CIP also reported the medium-term conservation of yacon by using semi-solid MS (Murashige & Skoog 1962) culture medium and temperatures of 6-8 °C, with low light intensity, and using mannitol as an osmotic agent (Panta et al. 1999).

On the other hand, there have not been any reported studies on long-term conservation of yacon using cryopreservation methods with liquid nitrogen (LN), or slow-growth methods for ulluco medium-term.

Thus, this PhD thesis aimed at developing a protocol for medium-term conservation of ulluco microshoots by testing various culture media supplements such as mannitol, sorbitol, sucrose, maleic hydrazide (MH), chlorcholinchlorid (CCC), BA and two cultivation temperatures (5 and 17 °C). Genetic fidelity of ulluco after *in vitro* plantlets slow-growth conservation was assessed using ISSR molecular markers. Furthermore, we optimized a protocol for the cryopreservation of yacon using various osmoprotectants such as PVS2 or PVS3 plant vitrification methods and microshoots as explants. The optimization and development of efficient *in vitro* conservation techniques for these species are paramount in contributing to the overall knowledge of the species genetic resources conservation.

2. Literature review

2.1. The Andes and Andean root and tuber crops (ARTCs)

The Andes is the longest and widest cool region in the tropics extending over 1.5 million km^2, from 11º N to 23º S with elevations ranging from 600-800 m up 6,000 m above sea level. The area has characteristic features such as deep gorges, wide valleys, a vast mountain plain and steep slopes (Cuesta et al. 2011). The Altiplano (high tableland of Central South America) elevations extend above 3,500 m across western Bolivia and much of southern Peru. The Northern and Central Andes are mountainous areas that cover only a minor fraction of the continent's landmass but is of great altitudinal and climatic variation resulting in floristic richness and abundance in the diversity of domesticated tuber crops (Hermann & Heller 1997; Josse et al. 2009).

Apart from the seven species of cultivated potatoes (*Solanum* genus) from which *Solanum tuberosum* is the only one known worldwide, there are nine lesser-known species of root and tuber crops native to the Andes including oca (*Oxalis tuberosa* Molina), ulluco (*Ullucus tuberosus* Caldas), mashua (*Tropaeolum tuberosum* R. & P.), arracacha (*Arracacia xanthorrhiza*), yacon [*Smallanthus sonchifolius* (Poepp. et Endl.) H. Robinson], achira (*Canna edulis* Ker-Gawler), mauka (*Mirabilis expansa* R. & P.), ahipa [*Pachyrhizus ahipa* (Weddell) Parodi] and maca (*Lepidium meyenii* Walpers). These species are commonly referred to as Andean Root and Tuber Crops (ARTCs) (Hermann & Heller 1997; Tay 2013). They are all mainly grown for their edible underground organs used by local people as cash crops or for subsistence (Hermann & Heller 1997).

2.2. Ecology, propagation, and cultivation of ARTCs

The ARTCs can naturally cope and adapt to extreme environments and marginal lands with limitations such as poor soil conditions, waterlogging salinity, and long periods of drought (Tay 2013). The Andean farmers took advantage of these unique features to domesticate the species and so grow crops on the steep slopes of the Andes, which are prone to extreme fluctuations in rainfall and temperature, constant erosion and relatively poor soils (Flores & Flores 1997).

To grow crops under these unfavourable conditions, farmers combine their cultivation with crop rotation, natural pesticides and a complex system of irrigation canals (Flores et al. 2003). In most cases, once the plant has started to set storage roots and tubers, there is no need to wait for a one-time maturation, harvesting can be carried out periodically (Tay 2013).

Regarding the form of propagation, the ARTCs are quite diverse. Though most of the ARTCs have retained sexual fertility, they are mostly propagated vegetatively. The exception is of maca, ahipa, and achira being propagated by seeds. In the case of yacon, mauka, and arracacha the edible root is not suitable for propagation (does not contain buds), however, the plants in addition to the edible tuber, also produce rootstocks with fleshy offshoots that serve as propagules. Similar to potato, oca, ulluco, and mashua are propagated by the use of tubers which have the dual purpose of food and seed (Hermann & Heller 1997).

The cropping cycle for highland tubers such as oca, ulluco, and mashua starts at the onset of the rainy season and end with a senescent plant. Aerial plant parts die after tuber bulking is completed, leaving an underground stolon that has produced dormant tubers used for food and seed. Due to the need for a cropping duration between 6 to 9 month and the fact that the tuber formation occurs only during days shorter than 13-14 hours, the cultivation of these species at extra-tropical latitudes is a serious constraint (Hermann & Heller 1997).

Higher ecological plasticity is displayed in yacon and ulluco with the first being introduced to Italy, New Zeeland, Japan, Korea, Brazil, Czech Republic, China, United States, Paraguay, and Taiwan (Seminario et al. 2003; Calvino 1940) proving that this crop can be cultivated under climatic conditions outside of the Andean region, though, due to climatic factors such as spring and autumn frosts there are limitations in the vegetation period in certain areas (Fernandez 2007). Ulluco, on the other hand, has been introduced to New Zealand where fifteen accessions from Bolivia and Argentina are being cultivated (Busch et al. 2000).

2.3. Conservation of ARTCs

The biodiversity of root and tuber crops within the Andean region has been enhanced by the varied ecosystem and the cultural inheritance of conservation from the Inca civilization (Panta et al. 1999; William et al. 2007).

However, there has been a rapid erosion of the tubers' genetic diversity (King & Gershoff 1987). The species are placed at risk due to crops being grown under increasingly changing environments such as changes in farming and land use caused by rural development (Panta et al. 1999), anthropogenic selection for food purposes and crop husbandry (Malice & Baudoin 2009).

Such conditions create the need to use advanced biotechnological approaches to preserve the species' genetic material and their biodiversity in the Andean region (King & Gershoff 1987). Hence, actions in safeguarding the Andean tuber and roots crops such as potato

and the other nine ARTCs species are being undertaken. The conservation scheme of these crops is based on achieving complete germplasm conservation of wild and cultivated species either by *in situ* collections (field genebank in natural habitat), on-farm conservation or by *ex situ* conservation which encompasses *in vitro* genebank (cryopreservation or slow growth), seed genebank or field genebanks (Panta et al. 1999; Tay 2013).

2.3.1. *In situ* and on-farm conservation

In situ conservation is regarded as the conservation strategy that maintains the species populations in the habitats where they naturally occur (Brush 2010; Bellon et al. 1997). This method preserves the ecosystems and natural habitats and recovers viable populations of species in their natural surroundings. This includes the areas where domesticated or cultivated species have developed their distinctive properties (Rao & Sthapit 2013). Through this method, traditional agricultural, horticultural, or agri-silvicultural systems carry out sustainable management of genetic diversity of the species and associated wild relatives (Maxted et al. 1997).

On-farm conservation is the maintenance of cropping systems and traditional crop landraces by farmers within the natural habitats where they occur in farmers' fields and uncultivated plant communities (Altieri & Merrick 1998).

Genetic erosion is caused by various factors such as habitat destruction, increased human population, land degradation, environmental changes, poverty, government subsidies to plantation crops and the introduction of modern crop varieties. Hence, it is necessary to complement the on-farm conservation method with *ex situ* conservation approaches (Rao & Sthapit 2013).

Various ARTCs are being preserved. In the case of ulluco, oca and mashua the *in situ* conservation is carried out in the diversity microcenter of Huanuco, Peru, (mainly in farmers' fields) where they are planted and maintained with the collaboration of farmers as reported by Malice et al. (2010).

Additionally, Ignacio et al. (2017) reported that the *in situ* conservation of yacon, arracacha and their wild relatives is being carried in the Huacrachuco and San Pedro de Coholon district, in the province of Maranon and Huanaco region of Peru. The *in situ* conservation of these species is complemented by on-farm conservation as they are also grown on farmers plots were they preserve landraces and wild varieties. CIP also maintains *in situ* collection of the ARTCs and some of their wild relatives (Hermann & Heller 1997; Panta et al. 1999).

2.3.2. *Ex situ* conservation

Ex situ conservation methods when focusing on living plant conservation includes seed collection, cryopreservation, field (including in greenhouse) and *in vitro* collections. Each method has its advantages and disadvantages, hence, in most genebanks, a combination of them are employed to safeguard the collection (Tay 2013; Engelmann 2011; Hermann & Heller 1997).

Pollen genebank can also be considered as *ex situ* conservations as plant pollen can be preserved well at freezing temperatures and under cryopreservation. DNA samples can also be frozen at -70 °C for many years. CIP has initiated a DNA bank for potato and ARTCs. CIP also has a herbarium collection ARTCs which is also a form of DNA preservation (Tay 2013; Hermann & Heller 1997).

2.3.2.1. Seed conservation

Most cultivated and related wild species of root and tuber crops, for example, arracacha and other *Arracacia* species, ahipa and maca are seed propagated crops that produce orthodox seeds (Arbizu 2009). At low seed moisture content of around 5% (wet weight basis) the seeds can be stored for a long period at -20 °C (Tay 2013; Walker 2013).

Arbizu (2009) reported the conservation of achira, mauka and oca as well as ahipa and maca in form of seed genebanks, in the case of achira, mauka and oca, the seed conservation was established to complement their clonal conservation of both the cultivated and wild material. The others are maintained in a long-term conservation program by CIP.

This method of conservation may not apply to vegetatively propagated plants such as yacon or ulluco as due to continuous vegetative propagation these plants are prone to lose the ability to produce viable seed as is the case with yacon which has a low flower and viable seed production and high pollen sterility considered as an effect of vegetative propagation (Meza 1995).

2.3.2.2. Field conservation

Field conservation is the most common method used for the storage of ARTCs. The collections are either grown in the field or a container, usually maintained in greenhouses. This method is usually used in smaller genebanks where there is no tissue culture laboratory and is mostly

suitable for vegetatively propagated plants, in which seed collection is not possible (Tay 2013; Hermann & Heller 1997).

Advantages of this method include the use of field collection to carry out characterization and genetic identity monitoring of the conserved crops as well as the having the planting materials readily available for field trials (Tay 2013). However, field collections are susceptible to pest and diseases (Panta et al. 1999). Large collections are costly to plant and manage, they are also subjected to the elements of natural disasters such as drought, flood, and frost (Tay 2013).

Field conservation of oca, ulluco, and mashua is carried out by CIP, the conservation strategy of the species implies clonal conservation of each accession and takes place at 3,500-3,800 m above sea level as at lower altitudes they are prone to be affected by bacteria, virus, and fungi (Arbizu 2009). At CIP, the field conservation of each accession within their collection of yacon, arracacha, achira and mauka are clonally maintained, they are planted and harvested once a year (Arbizu 2009). In the case of achira and its wild relatives, the field collection can also be grown for up to 10 years if the plants are pruned, fertilized, and watered. Some accessions of yacon have also shown similar features of being able to be conserved for several cropping periods without harvesting (Arbizu 2009).

2.3.2.3. *In vitro* genebank

In vitro conservation techniques offer ways to overcome problems in the conservation of crop genetic resources such as the risk of pest and diseases in field collection and on-farm conservation management (Panta et al. 1999; Tay 2013) as well as in vegetatively propagated species that have lost their reproductive capacity and ability to produce viable seeds (King & Gershoff 1987; Engelmann 2011). This conservation method is also considered a reliable alternative to cope with genetic erosion, reinforce *ex situ* collections and ensures long and medium-term conservation of vegetatively propagated species (Izquierdo & Roca 1997; Engelmann 1997).

The technique primarily involves the use of *in vitro* slow-growth for medium-term conservation with the aim to reduce growth and to increase the intervals between subcultures. Additionally, cryopreservation in liquid nitrogen is also considered a feasible *in vitro* technique for the long-term storage of plant germplasm. This form of conservation also entails important applications in other areas, including multiplication, exchange and germplasm collecting (Engelmann 1997; Tay 2013; Panta et al. 1999).

Slow-growth

Slow-growth conservation strategies are used in several institutions throughout the world for the preservation and distribution of clonally propagated plant germplasm. It encompasses medium-term storage under *in vitro* conditions (Engelmann 2011; Reed et al. 2012; Ashmore 1997).

In vitro, slow-growth collections are mainly carried out through meristem culture in a well-controlled environment. The meristem culture method is mainly chosen because of its reduced risk of somaclonal variation (Tay 2013; Engelmann 1997; D'amato 1985).

In vitro, slow-growth conditions provide a secondary storage method for clonal field collections, a reserve of germplasm for plant distribution or a viable storage mode for experimental material. The method for this form of storage must meet two main criteria 1) minimal maintenance for an extended period and 2) maintain the stability of the genetic integrity of the stored material (Engelmann 2011; Reed et al. 2012).

To develop a well-defined storage method using this technique, various aspects of experimental work need to be taken into consideration to reduce the growth of the stored *in vitro* plantlets. These aspects could include: medium alterations such as a reduction in mineral or sucrose concentrations, chemical additions in the growth medium (plant growth inhibitors) which may include, maleic hydrazide (MH), paclobutrazol, chlorcholinchlorid (CCC), abscisic acid (ABA) etc. and reducing osmotic pressure in the growing medium which can be achieved by the use of osmotic agents such as sucrose, mannitol, sorbitol etc. (Noor et al. 2011; Panta et al. 1999; Tay 2013; Engelmann 1997; Ashmore 1997). Temperature reduction, alginate encapsulation or oxygen reduction are parameters that can also be modified to reduce the growth of *in vitro* plantlets (Dulloo et al. 1998; Reed 2002; Rai et al. 2008; Sarasan 2010; Zee & Munekata 2019).

The slow-growth storage technique varies with the crop, the facility, and its mandate but once developed, it is a versatile tool that can provide readily available, and diseases free plants for distribution to scientists or farmers (Reed et al. 2012).

In the case of yacon, slow-growth has been achieved by Panta et al. (1999) using MS media containing 4% mannitol and 3% sucrose.

Skalova et al. (2013) also developed a reliable slow-growth conservation method for yacon using half-strength MS (Murashige & Skoog 1962) medium and media supplemented by 10 or 20 g l^{-1} mannitol or sorbitol. However, the effect of plant growth inhibitors and osmotic agents has not been reported so far on ulluco plant species.

In vitro cultures are sometimes associated with a somaclonal variation which is not restricted to but is especially linked to *in vitro* propagation system using a dedifferentiated phase of callus (Engelmann 1997; Bhatia & Sharma 2015).

This type of variation can also occur from stresses imposed by the conservation procedure (Engelmann 1997; Bhatia & Sharma 2015). Explant source, the period length duration of *in vitro* culture, physical parameters, as well as chemical agents used for *in vitro* reduced growth cultivation are all potential determinants of somaclonal variation (Bordallo et al. 2006).

Somaclonal variation can be genotypic or phenotypic and can in the latter case be either genetic or epigenetic in origin. Genetic variations are typically changes in chromosome numbers (polyploidy and aneuploidy), changes in the chromosome structure by translocations, deletions, insertions, and duplications and changes in the DNA sequence (base mutations). An epigenetic variation, on the other hand, is related to events such as gene amplification and gene methylation (Leva et al. 2012; Bhatia & Sharma 2015, Martinez 2018).

Somaclonal variation can be beneficial in some cases as it can lead to new cultivars that may contain desirable traits, increased pest resistance or ornamental characteristics. However, in the case of *in vitro* slow growth conservation, somaclonal variation is usually undesirable (Engelmann 1997; Bhatia & Sharma 2015). Hence, genetic fidelity assessment after conservation is needed in some cases (Bhatia & Sharma 2015).

The detection of potential somaclonal variation of *in vitro* plantlets is most commonly carried out by the use of molecular markers (Jin et al. 2008; Koc et al. 2014; Martínez 2018; Bradaï et al. 2019). Inter simple sequence repeat (ISSR) and sequence-related amplified polymorphism (SRAP) (Peng et al. 2015), as well as, random amplified polymorphic DNA (RAPD) (Nayak et al. 2011; Kamińska et al. 2018), inter retrotransposon amplified polymorphism (IRAP) and amplified fragment length polymorphism (AFLP) (Koc et al. 2014) have all been used in assessing the genetic stability of conserved *in vitro* germplasm using *in vitro* slow-growth method.

In vitro storage of tuberous crops also entails the induction of tuberization; however, this method has mainly been developed for potato germplasm (Kwiatkowski et al. 1988; Gopal et al. 2004; Naqvi et al. 2019).

Cryopreservation

Cryopreservation is the conservation of plant germplasm at an ultra-low temperature usually at -196 °C and stored for indefinite periods without genetic erosion (Panta et al. 1999; Engelmann 2011). The meristems are usually stored in liquid nitrogen (LN). The general belief is that meristems frozen at this ultra-low temperature will remain alive for hundreds of years or more (Tay 2013; Engelmann 2011). At this freezing temperature, all cellular divisions and metabolic processes are at a standstill, hence the plant material can be stored without alteration or modification. There has been no report of modification at the biochemical, phenotypic or chromosomal level in cryopreserved plant germplasm (Engelmann 2004). Hence, it is considered that this preservation technique can ensure the greatest possible maintenance of the integrity of the cryopreserved samples (Engelmann 1997; Engelmann 2004). However, freezing plant tissue can result in intracellular ice nucleation and subsequent ice crystallization leading to cell damage during cooling and/or rewarming of the samples (Hammond et al. 2019a; Zamecnikova et al. 2011; Zamecnik & Faltus 2009). Low water content minimizes ice crystallization. Hence, the objective status for cryopreservation is to reduce the water content in the plant tissue to obtain a glassy state and to avoid the formation of lethal intracellular ice crystals to obtain optimal recovery and regeneration after cryopreservation. This can be achieved by the induction of vitrification status which is a glass induction by dehydration, the addition of cryoprotectants, and a very fast decrease in temperature (Hammond et al. 2019a).

The reduction in water content and achieving a glassy state is the main stage to carry out successful cryopreservation of plant material (Hammond et al. 2019a; Zamecnik et al. 2011). Methods based on dehydration include osmotic dehydration, air desiccation and freeze dehydration (Benson et al. 1996; Grospietsch et al. 1999; Hirai & Sakai 1999). In cryopreservation, cryoprotectants help to replace some of the water to avoid ice crystals formation in plant tissues when frozen. The intracellular ice formations can be avoided by vitrification (production of a glossy state that behaves like a solid due to increased viscosity without any crystallization) (Berk 2018).

Various plant vitrification solutions (PVS) can be used for osmotic dehydration. These are all labelled with a number according to the specific mixture of basic cryoprotectants and their concentrations. The most common cryoprotective substances for plant vitrification are dimethylsulfoxide (DMSO), polyethylene glycol (PEG) and sucrose. These substances have osmotic actions (a fluid that can pass through a semipermeable membrane into a solution where the solvent concentration is higher, thus equalizing the concentrations of substances on either

side of the membrane) and induce glass transition (Kalaiselvi et al. 2017). The main PVSs are PVS1 (Uragami et al. 1989; Towill 1990), PVS2 (Suzuki et al. 2008), PVS3, PVS4, PVS5 (Nishizawa et al. 1993), VS6 (Liu et al. 2004a), PVSL (Liu et al. 2004b), and VSL (Suzuki et al. 2008), with different concentration and combination of four main components: DMSO, sucrose, glycerol and ethylene glycol, these compounds have the dual effect of reducing the water content through their osmotic dehydration function and acting as cryoprotectants (Hammond et al. 2019a; Gonzalez-Arnao et al. 2008; Sestak & Zamecnik 2007).

Thawing is also a critical stage of the recovering plants after cryopreservation in LN. The rewarming of samples is carried out as quickly as possible to avoid devitrification, which would also lead to the formation of ice crystals detrimental to cellular integrity. Samples are immersed in a water-bath or liquid medium held at 20-40 °C (Engelmann 1997).

In addition to the need of reaching a glassy state before directly immersing the tissue in LN and accurate thawing of samples, there are other constraints in using this conservation method, such as the cost of implementing a cryobank (cryo-equipment, technician costs, laboratory space, LN and cryo-storage tanks), tedious protocols and the need for accuracy as well as the fact that developing a protocol is time-consuming. However, once the samples are successfully in LN the maintenance cost is low in comparison with the other conservation methods (Tay 2013).

The development of cryopreservation protocols has been used for several plant species i.e. *Solanum tuberosum* L. ssp. *Tuberosum* (Kaczmarczyk et al. 2011), *Ullucus tuberosus* Cal. (Zamecnikova et al. 2011), *Oxalis tuberosa* Mol. (Gonzalez-Arnao et al. 2008) among others (Benson et al. 1996; Sestak & Zamecnik 2007; Zamecnik & Faltus 2009; Zamecnik et al. 2007). Cryopreservation protocol has been developed for potato as the first and most important ARTC using meristems with leaf primordia and PVS treatment (Golmirzaie & Panta 1997). In the case of other ARTCs, research on cryopreservation has only been carried for ulluco and oca (Arizaga et al. 2017; Sanchez et al. 2011; Zamecnikova et al. 2011; Panta et al. 1999).

In the case of ulluco, Arizaga et al. (2017) tested the V and D cryo-plate methods and subsequently the D cryo-plate method was selected for ulluco cryopreservation. Additionally, Zamecnikova et al. (2011) also developed a long-term conservation protocol for ulluco using the preparation of shoot tips with sucrose and dehydration pre-treatment of ulluco shoot tips for cryopreservation. Sanchez et al. (2011) also developed cryopreservation protocols for ulluco and oca using PVS2 droplet-vitrification protocol tested on excised shoot tips.

Each cryopreservation protocol is genotype and species-specific and needs to be optimized accordingly. Hence the standardization of the protocol is necessary to ensure and facilitate an effective cryostorage of the plant germplasm (Zamecnik & Faltus 2009).

2.4. *Smallanthus sonchifolius* (Poepp. et Endl.) H. Robinson

2.4.1. Taxonomic classification

Smallanthus sonchifolius (Poepp. & Endl.) H. Rob. is a plant species belonging to the kingdom Plantae, the order Asterales, and family Asteraceae. Originally it was placed in the genus *Polymnia* (Compositae, Helinate, sub-tribe Melampodinae) (Wells 1967), which was founded by Linnaeus (1751) but was then re-established in the genus *Smallanthus* by Robinson (1978) who divided the *Polymnia* genus into two generas the *Smallanthus* and the *Polymnia*. Both genera are included in the sub-tribe Melampodinae.

Thus, the species was previously known as *Polymnia sonchifolia* (Poeppig and Endlicher 1845) and *Polymnia edulis* (Weddell 1857), however, the newer classification according to Robinson (1978), *Smallanthus sonchifolius* [(Poeppig & Endlicher) Robinson 1978], is currently preferred while the old one suggested by Poeppig & Endlicher (1845), is considered as a synonym (Robinson 1978).

The name yacon comes from the Quechua word ´yakun referring to its sweet juicy storage roots of some 70% water content (Campos et al. 2012). Though the common name yacon is mostly known the plant has various common names in English it is called yacon or leafcup, in Spanish it is called yacon, yacuma, jicama, in countries such as Ecuador and Bolivia, in Colombia, it is called arboloco (Lizarraga et al. 1997) other names such llacon, and strawberry jicama is also used (Michaels & Michaels 2007).

2.4.2. Botanical description

Yacon is a perennial herb that grows as a compact herbaceous clump reaching 2-2.5 m in height. Its stems are green to purplish, cylindrical or subangular, the diameter can range between 10 and 28 mm and can reach up to 1.5-3 m in height (Lachman et al. 2005; Valentova & Ulrichova 2003).

The plant can produce 13-16 leaf pairs at its full flowering stage, producing only small leaves afterwards. The leaves are ovate-lanceolate or broadly ovate the upper and lower surfaces are dark green (Fig. 1), densely pubescent, and with trichomes that are 0.5-1.5 mm long and

measure 0.05 mm in diameter on both epidermises (Seminario et al. 2003; Valentova & Ulrichova 2003; Herman et al. 1990).

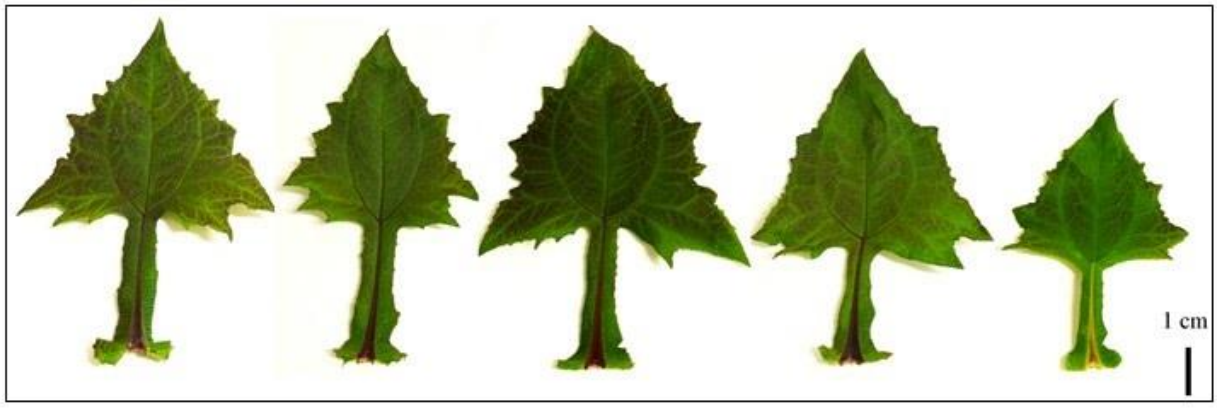

Figure 1. Yacon leaves

Source: Author

Yacon produces stem tubers called rhizomes, growing directly on the basal part of the main stem. They are branched and irregular in shape often with a white, creamy, and purple colour, influenced by the soil type and the genotype and contains many buds in the upper part. These fleshy rhizomes are harvested from the fully developed plant and are used for vegetative propagation or as a rich source of polyphenolic antioxidants (Seminario et al. 2003; Lachman et al. 2005).

The yacon root system is also composed of an extensive system of thin fibrous roots and 4-20 fleshy tuberous storage roots, reaching up to 25 cm in length and 10 cm in diameter, weighing between 0.5 and 4.5 kg, depending on the size, ecotype, thickness, and environmental conditions (Lachman et al. 2005; Lachman et al. 2003; Leon 1964; Meza 1995). The fibrous pulpy and bulky storage roots are found on the bottom of the rhizomes (Fig. 2). The storage roots water content usually exceeds 70% of the fresh weight while a major portion of the dry matter consists of fructooligosaccharides (FOS) (Campos et al. 2012; Lachman et al. 2004). The roots are used by the plant as saccharides storage and by humans for food consumption (Robinson 1978).

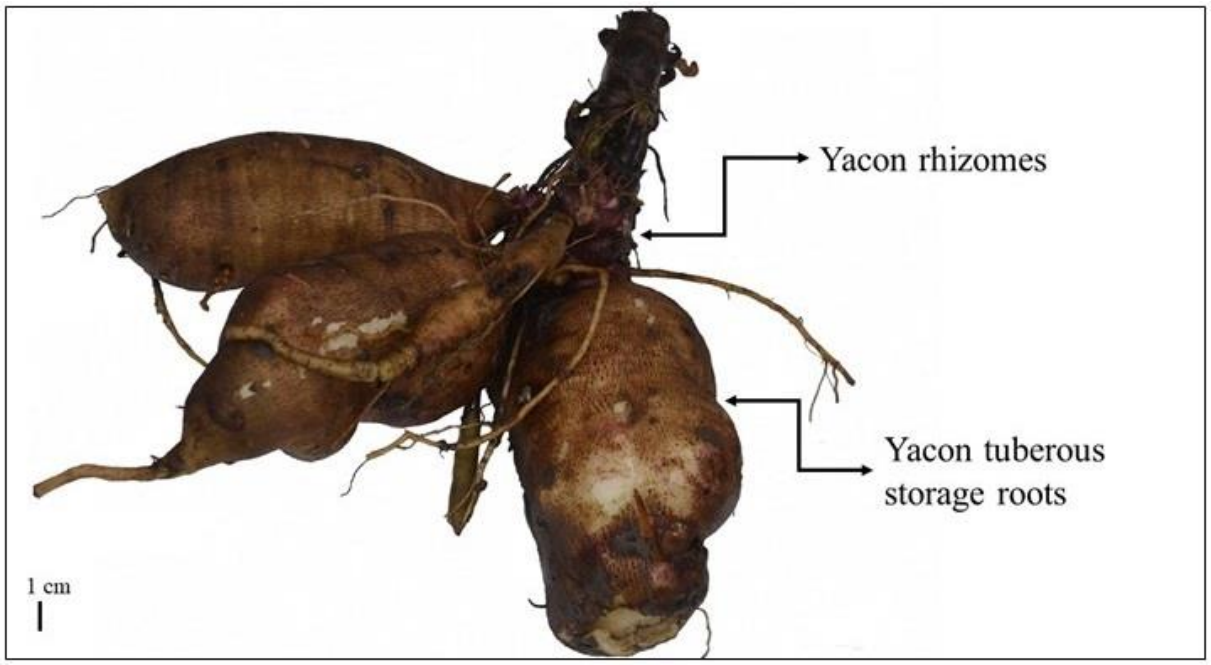

Figure 2. Tuberous roots and rhizomes of yacon

Source: Author

2.4.3. Reproductive biology

During its evolution, yacon was continuously vegetatively propagated even in the wild it is known to be propagated by rhizomes. The cultivated landraces are considered to produce a more reduced number of flowers compared to the wild relatives of the *Smallanthus* species. The selection for root yield may have impaired the flowering and fruit set, which is a common feature in clonally propagated species of tuber and root crops (Robinson, 1978).

In some areas, the flowering is abundant, however, it is strongly dependent on the environmental conditions of the place of cultivation and growth. The produced seed sets are also largely nonviable or show low vigor. Additionally, the production of seeds is frequently poor or none existent, causing high levels of pollen sterility causing difficulty in obtaining viable seeds even under greenhouse conditions and seeds produced show inadequate germination rates, hard coat and dormancy (Robinson 1978; Meza 1995; Lizarraga 1997; Grau & Slanis 1996; Rea 1995; Grau & Slanis 1996; Fisher & Wells 1962; Wells 1971).

2.4.4. Origin and geographic distribution

The species is originated in the northern and central Andean regions. Its wild relatives were discovered to show preference to disturbed habitats in this area. It is considered that the strategy of yacon to colonize areas free of vegetation such as roadsides, landslides and river banks has allowed the species to be discovered by the early Andean inhabitants who converted the weed state of yacon to a managed plant and later to a cultivated agricultural crop (Herman & Heller 1997).

Yacon is considered to be cultivated in 18 of the 22 regions of Peru with a total area of cultivation of around 600 ha (Seminario et al. 2003). It is mainly grown in the Peruvian valleys around Cusco and Puno with a maximal concentration in the northern and southern mountains of Cusco, between altitudes of 2,000 and 3,000 meters above sea level (Seminario et al. 2003). It can also be found in some areas of South America at medium altitudes in various localities throughout the Andes, from Ecuador to north-western Argentina, in most cases cultivated for family consumption and rarely grown as a cash crop (Hermann & Heller 1997). Fig. 3 illustrates the distribution of yacon thought the Andes and the areas of high diversity of the species.

The species was introduced in Europe in the 20th century as part of an exhibition but did not evoque interest in regards to its cultivation at the time (Arbelaez 1956), it was again introduced in Italy in 1939 but by the agronomist Mario Calvino who aimed at introducing the species as a profitable crop, however, his efforts faded due to the II World War (Calvino 1940). Then in the 1990s, it was again introduced in Europe as plant whit fertility-enhancing properties many nutritive values (Valentova et al. 2001).

Yacon is now distributed in Brazil, New Zealand, Japan, Korea, United States, China, Paraguay, and Taiwan (Seminario et al. 2003) and was also introduced to the Czech Republic in 1993 (Valentova & Ulrichova 2003) showing that yacon can be grown successfully outside its natural habitat (Lebeda et al. 2003).

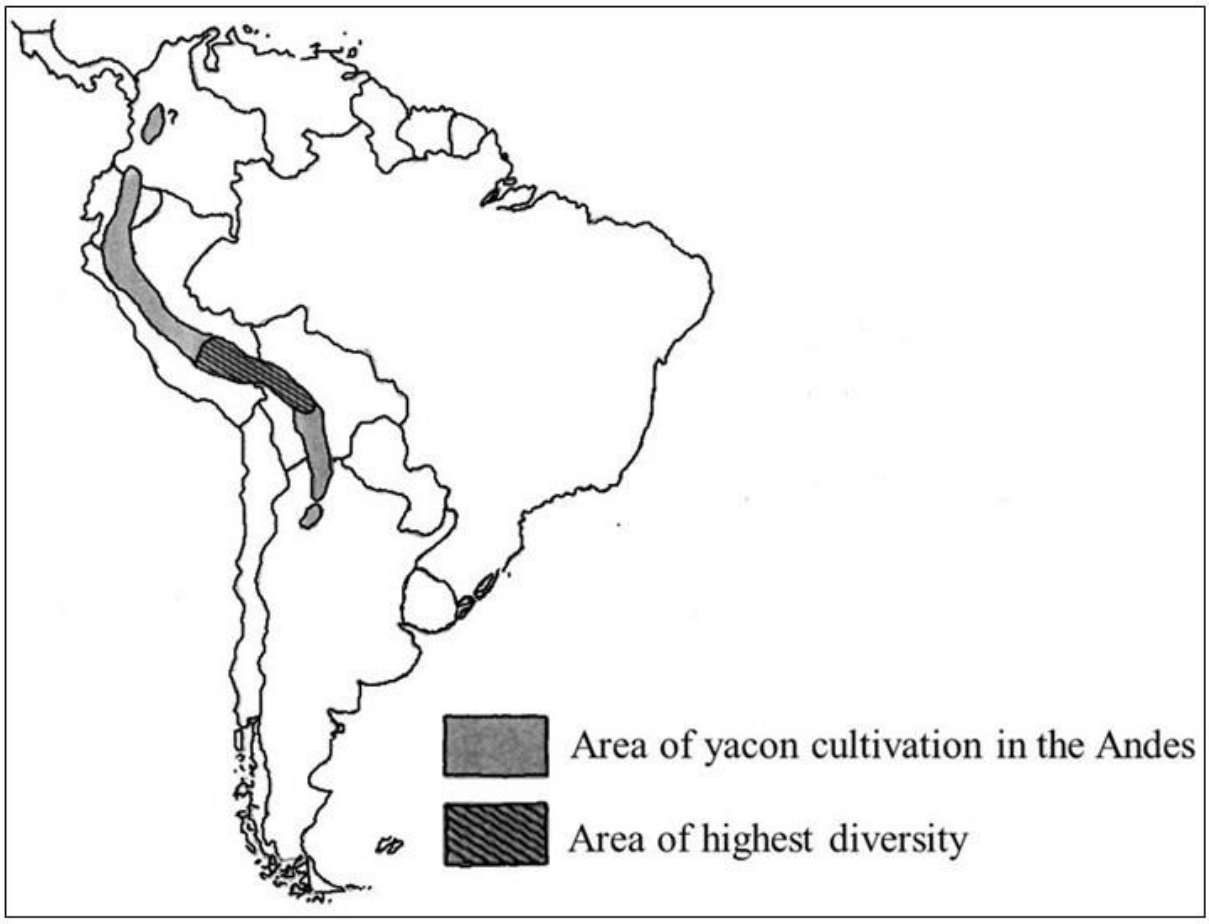

Figure 3. Yacon distribution in the Andean region

Source: Hermann and Heller (1997)

2.4.5. Uses and nutritional value

Yacon as is the case with other root or tuber crops is mainly grown for its edible underground organ (Hermann & Heller 1997). Its tuberous rhizomes are eaten raw, cooked, dried or transformed into syrup, chips, juice, salads, pickled or stewed (Herrera 1943; Kakihara et al. 1996; Ishiki et al. 1997; Hernandez Bermejo & Leon 1994). The fresh tuberous rhizome contains up to 20% saccharides (FAO 1989) and about 70-80% of their dry weight in the form of inulin, fructose, glucose and sucrose (Lachman et al. 2004).

Yacon's tuberous rhizomes provide a good source of fructooligosaccharides with low caloric value (Manrique et al. 2005). It is considered to be the highest plant source of fructooligosaccharides. These have health benefits such as low caloric value (4.2-6. kJ g^{-1}), reduces the risk of colon cancer, reduces the triglycerides and cholesterol levels reduces

constipation, promotes the synthesis of folic acid and B-complex vitamins, does not increase the glucose level, improves calcium assimilation and strengthens the immune system (Manrique et al. 2005). Fructooligosaccharides can be considered as a good sweetener for diabetic patients that suffer from digestive problems, as a substitute for table sugar (Manrique et al. 2005).

The main by-product of yacon is a syrup that can be obtained by concentrating the juice of its tuberous rhizomes at low pressure, the obtained syrup has a significantly lower energy level for humans as compared to that of sugar cane (Chaquilla 1997).

The leaves are also edible and are mainly used to make tea for medicinal purposes due to their high content of antibiotic properties (Kakihara et al. 1996; Lachman et al. 2005). Yacon leaves contain monoterpenes, sesquiterpenes and diterpenes, which have a physiological role in the pest-resistant and antimicrobial activities of this plant (De-Qiang et al. 2012). The leaves contain considerable amounts of caffeic acid, chlorogenic acid, ferulic acid, myricetin, rutin, and p-coumaric acid (Khajehei et al. 2017). Along with the rhizomes, the leaves are a rich source of polyphenolic antioxidants (Kakihara et al. 1996; Lachman et al. 2005).

2.4.6. Genetic background and variability within the species

Yacon is a species with a high ploidy level (Frias et al. 1997). Heiser (1963) was the first to publish a report on yacon's chromosome number through his work carried out on material from Ecuador with which he published the number 2n=60. Leon (1964), later used material from the Molina University in Peru and reported the material to have a chromosome number of 2n=32. Talledo & Escobar (1996) later suggested that yacon is a tetraploid based on the research also carried out on material from Peru in which they also discovered a chromosome number 2n=60.

Later more detailed research was carried out by Salgado (1996) and Ishiki et al. (1997) based on 1,256 cell counts on 15 clones in which 14 had the value of 2n=58 and 1 had a somatic number of 87. Out of the clones that had 2n=58, 8 were from Peru, 1 from Ecuador, 1 from Argentina and 4 from Bolivia. Grau & Slanis (1996) suggested that *Smallanthus sonchifolius* was allotetraploid along with *Smallanthus macroscyphus*, one of yacon's assumed parents along with *Smallanthus riparius*. The results obtained by Ishiki et al. (1997) were also consistent with allopolyploid suggesting a yacon karyotype composed of two genomes. They suggested an allooctoploid 6A+2B as the dominant in the yacon clones that have a chromosome number of 2n=58, and an allododecaploid 9A+3B for the one with 2n=87 (Fig. 4). Various studies carried out on yacon ecotypes published a chromosome number of 2n=58 (Fernandez & Kucera 1997; Frias et al. 1997; Salgado 1996).

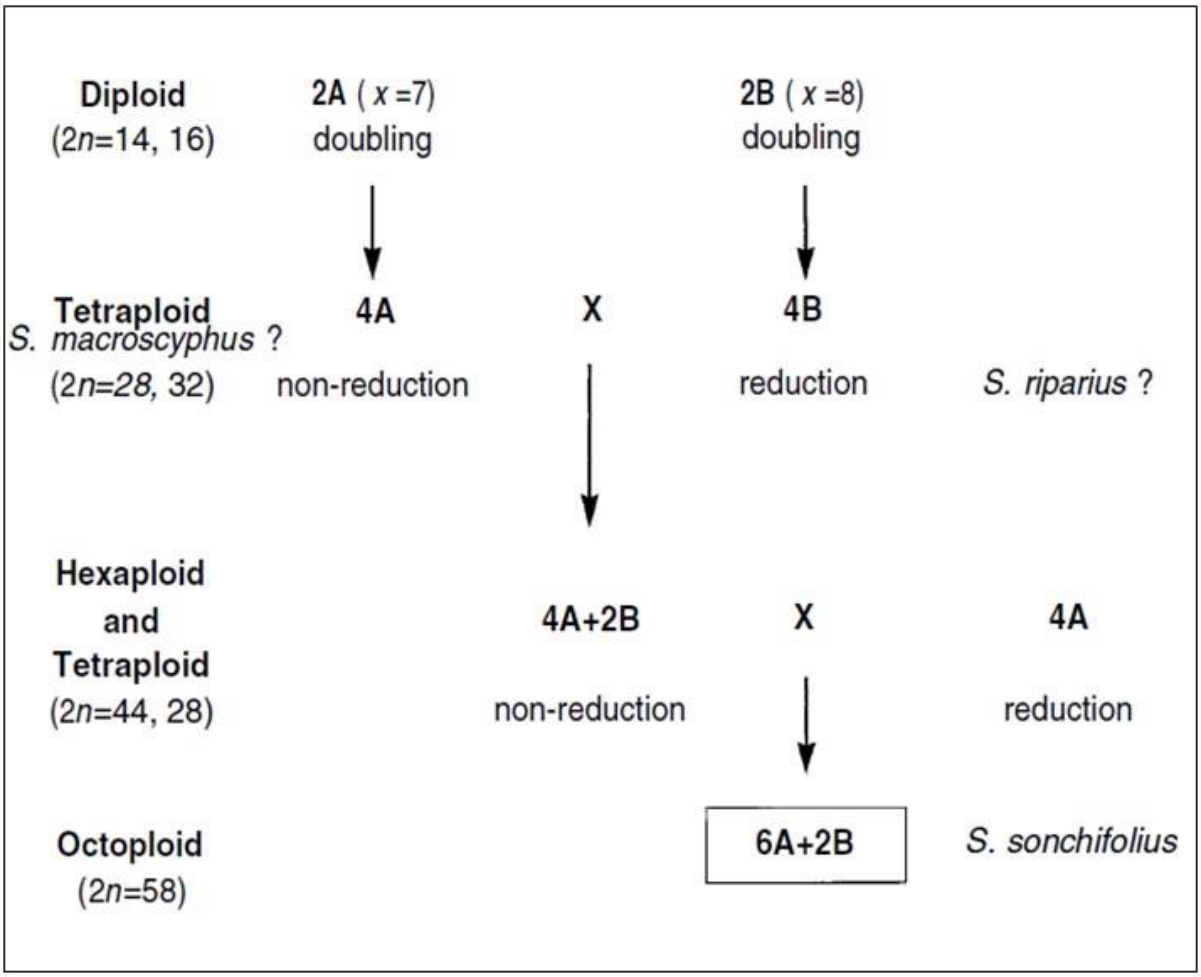

Figure 4. Scheme of a hypothetical evolution of *Smallanthus sonchifolius*

Source: Ishiki et al. (1997)

2.4.7. Biodiversity conservation practices

As is the case of most ARTCs, yacon is mainly conserved in its area of origin in field or on-farm genebanks (Tay 2013; Panta et al. 1999). In the countries where it has been distributed it is also maintained in field conditions by vegetative means of propagation e.g. in the Czech republic (Grau & Rea 1997; Lebeda et al. 2004; Fernandez et al. 2006), New Zeeland, Japan, Korea, United States, China, Paraguay, and Taiwan (Seminario et al. 2003).

Optimization of medium-term conservation of yacon was reported by Skalova et al. (2013), who used half-strength MS medium and media supplemented by 10 or 20 g l^{-1} mannitol or sorbitol to conserve *in vitro* plantlets.

There have also been various reports on the medium-term conservation of yacon by maintaining the *in vitro* plants in test tubes, in semi-solid MS culture medium under temperatures of 6-8 °C, with low light intensity, and using osmotic agents to reduce the plant growth (Roca et al. 1978; Espinoza et al. 1992; Lizarraga et al. 1992; Golmirzaie & Panta 1997; Golmirzaie et al. 1999). On the other hand, cryopreservation methods with LN as a long-term conservation technique for yacon has not yet been optimized, hence, it is not being used in genebanks, though it may represent an alternative long-term method to safeguard field and on-farm collections.

2.5. *Ullucus tuberosus* Caldas

2.5.1. Taxonomic classification

Ulluco (*Ullucus tuberosus* Caldas) belongs to the Basellaceae family which comprises 19 species grouped into four genera: *Basella*, *Anredera*, *Boussingaultia*, and *Ullucus* (Eriksson 2007), being the ulluco (*Ullucus tuberosus*) the only species within the *Ullucus* genus. Within the genus, only two subspecies are recognized: subsp. *tuberosus*, which includes all cultivated forms; and subsp. *aborigineus*, which groups all wild forms (Sperling 1987).

According to the country and the region of origin, the ulluco receives different names. Hence, in different areas of Bolivia and Peru, it is known by the names of olluco, papa lisa, olloco, ulluco, ullush, ilaco, lisas, and ulluma, while in Ecuador it is known as rubas and melloco (Arbizu 2004; NRC 1989).

2.5.2. Botanical description

Ulluco grows as an erect, compact low-growing herb with very variable elongation of stems which can be light yellowish green with the pigmentation of edges or angles, all parts are succulent and mucilaginous (Fig. 5a). The wild varieties are prostrate while the cultivated varieties are ranges from prostrate or semi climbing vines to compact, dense, bushlike mounds growing up to 50 cm tall (Manrique et al. 2017; National Research Council 1989).

The alternate cordate or heart-shaped leaves are formed on the long petioles from the angular stem, frequently yellowish-green and sometimes light yellowish-green in colour depending on the cultivar (National Research Council 1989). The plants scarcely produce flowers but when it does, they form inflorescence or spine, light yellowish-green (Fig. 5b) to reddish (Fig. 5d) in colour, the plant has the tendency to producer flowers with more than five

petals which form in clusters arising from the forks of the branches (National Research Council 1989).

The plant produces spherical tubers on long stolons both below and above the ground, though most are formed below ground from the mass of fibrous roots. The tuber shape is mostly spherical though in some cultivated forms they are curved or elongated (2-15 cm). The tuber colour can vary depending on the cultivar, it can range from lemon yellow to red, white, pink, orange or magenta. A common form has the predominant colour of tuber surface being pale orange with a reddish-purple secondary colour of tuber surface which is distributed irregularly on the tuber surface (Fig. 5c). The inside of the tuber is yellow/white (National Research Council 1989; Manrique et al. 2017).

Ulluco tubers have high water content (72-87%). The main components (in dry base) are in the form of carbohydrates (74-94%), proteins (8-16%), ashes (<6%) and lipids (<1%). Within carbohydrates, starch is the most abundant component, but there is also a significant amount of mucilage, a heterogeneous and complex polysaccharide that is recognized as a type of soluble fiber (King & Gershoff 1987, Espín et al. 2004, Busch et al. 2000), it is also considered to be a good source of vitamin C (Jean 2015).

Figure 5. General aspects of ulluco: a. plant, b. yellowish-green flowers, c. tubers d. reddish flowers

Source: Adopted from Manrique et al. (2017)

2.5.3. Reproductive biology

The reproduction of the species is vegetative and the production of botanical seeds and their germination is very rare (Pietilä & Jokela 1990; Lempiainen 1989; National Research Council 1989). Cytological studies of ulluco indicate that abnormal meiosis resulting in incomplete microsporocyte formation may account for the rarity of seed formation (Sperling & King 1990). However, the most probable cause for the poor seed set of ulluco could be the reduction of its general sexual fertility due to a long period of vegetative reproduction (Pietilä & Jokela 1990).

2.5.4. Origin and geographic distribution

The wild forms of ulluco, for instance, *Ullucus tuberosus* subsp. *aborigineus* Brucher has origin in Peru, Bolivia, and northern Argentina (National Research Council 1989). Various archaeological evidence suggests that ulluco was already being consumed 9,000-8,500 BC. and that it was an important crop for many pre-Inca civilizations (Leon 2013; Morales 2007). The natural distribution of the habitat of domesticated species of ulluco extends from southern Venezuela to northern Chile and Argentina between 2,800 and 4,000 masl, but its cultivation is most important in Peru, Bolivia, and Ecuador (Arbizu 2004; National Research Council 1989). Fig. 6 depicts the distribution of ulluco in its area of origin and cultivation in the Andean region according to CIP (Manrique et al. 2017).

Due to its photoperiod restrictions, along with sensitivity to summer heat and early fall frosts, the cultivation of ulluco outside of the Andean region is considered difficult, however, overcoming these challenges may prove that ulluco tubers may follow the now-universal potato in popularity (Jean 2015).

Figure 6. Geographical distribution of ulluco accessions conserved in the germplasm bank of the CIP. Blue markers show the accessions that have morphological characterization data. Red markers are accessions that are lacking morphological characterization data

Source: Manrique et al. (2017)

2.5.5. Uses and nutritional value

Ulluco is mainly grown for its edible tubers, it is estimated that around 30 million people in the Andes consume ulluco. Outside the Andes, the commercial value/sale of ulluco has been successful in New Zealand (Busch et al. 2000; Svenson et al. 2008; Martin et al. 2005; Hermann & Heller 1997). After the potato, ulluco is the tuber with the largest cultivation area in the high

Andes and the one that has best managed to articulate to the market, thus becoming a source of monetary income for thousands of families within this region (Barrera et al. 2004; INEI 2013).

Within the Andes, before being consumed, the tubers are soaked in water to remove as much mucilage as possible. In several areas of Peru, the tubers are processed to obtain ´lingli´ (National Research Council 1989), a product obtained by an ancestral technology which consists of exposing the tubers to the night frosts and dehydrate them during the day taking advantage of high solar radiation and dry weather prevailing in the high Andean areas (Manrique et al. 2017).

Ulluco also contains betalains, a type of pigments that stains the tubers causing them to have varied colours, such as cream, yellow, orange, fuchsia, red and purple (Campos et al. 2006). Scientific evidence suggests that the betalains are very stable in ulluco tubers, hence, the plants present a potential source of natural pigments for the industry (Campos et al. 2006; Manrique et al. 2017).

The leaves of ulluco are succulent and can be used as a vegetable they present a similar texture as that of spinach. The leaves are known to contain high levels of calcium, carotene, and protein (Hermann 1992).

2.5.6. Genetic background and variability within species

Most of the accessions are diploids (2n=24), although triploids have also been found (2n=36) and very sporadically tetraploids (2n=48) (Mendez et al. 1994). The CIP has collected 548 accessions of ulluco originating in various parts of Peru and some locations in Argentina, Bolivia, Colombia, and Ecuador (Manrique et al. 2017). Based on 18 morphological descriptors, these accessions have been classified into 179 morphotypes (Malice & Baudoin 2009; Manrique et al. 2017). This shows that morphological diversity in ulluco is high. However, to achieve a better understanding of its genetic diversity it is necessary to develop a deeper level of analysis that also includes a more detailed molecular marker-based analysis of the DNA (Manrique et al. 2017).

2.5.7. Biodiversity conservation practices

The world collection of ulluco is maintained at CIP mainly under *in situ* or on-farm conservation through managed agricultural fields (Manrique et al. 2017).

A long-term storage protocol for ulluco conservation under *in vitro* conditions has been developed by Sanchez et al. (2011). The storage scheme consists of long-term storage by

cryopreservation with the use of a PVS2 droplet-vitrification method. Zamecnikova et al. (2011) also reported a long-term conservation protocol for ulluco using a preparation of shoot tips with sucrose and dehydration pre-treatment of ulluco shoot tips for cryopreservation.

A primary study on medium-term conservation of ulluco has been reported by Cruz et al. (1995). In this study, only the effect of sorbitol and temperatures between 28.5 °C and 22.3 °C were tested. Other culture medium supplements such as mannitol, sucrose, ABA, MH and CCC, has not been tested so far.

2.6. *In vitro* conservation techniques and their application in root and tuber crops

Seed collections in gene banks is the most common and cost-effective traditional method of conservation. Unfortunately, this method is not applicable to conserve clonal crops that are propagated vegetatively and do not produce seeds, or for those species which produce short-lived recalcitrant or none viable seeds, as is the case of most root and tuber crops (Reed, 2001a; Tay 2013). Hence, a commonly used storage option for vegetatively propagated species are field genebanks or cold storage conservation in vegetative genebanks for species that produce dormant vegetative propagules (Reed, 2001a; Maryam et al. 2014). However, these conservation approaches have a number of limitations regarding their costs, security, efficiency, quality and long-term maintenance (Benson et al. 2011; Grout, 1990). Therefore, *in vitro* conservation in a pathogen-free sterile environment is preferential as it ensures safe germplasm maintenance under-regulated phytosanitary controlled conditions for medium to long-term storage (Reed 2001a). This modern conservation technique involves two methods, 1) *in vitro* slow-growth method for medium-term conservation and 2) cryopreservation for long-term conservation (Chauhan et al. 2019; Engelmann, 2011).

2.6.1. Application of slow-growth in root and tuber crops

In vitro slow-growth conservation method is used to reduce the interval of sub-culture of *in vitro* plantlets to a critical level without causing any deleterious effect on the quality and stability of the regenerated/regrown germplasm (Chauhan et al. 2019; Benson et al. 2011). Within this conservation technique, minimal growth storage is achieved by using several physical (low light/restricted photoperiod, low temperature, minimal O2, osmotic stress, etc.) and chemical (growth regulator or growth inhibitors) growth limitations, as well as minimal nutrition supply (low macro and micronutrients level), applied individually or in combination (Reed, 2004; Engelmann, 2011). Germplasm conserved under slow-growth for medium-term storage is considered to be cost-effective as sub-culture intervals are extended which increases

the efficient use of staff time and resources and also offsets selection risks and contamination (Chauhan et al. 2019; Benson et al. 2011; Mafla et al. 2009; Reed, 2001a). This method has been applied to various root and tuber crops.

Muñoz et al. (2019) reported the development of an *in vitro* slow-growth method for the conservation of three Chilean genotypes of potato (*Solanum tuberosum* L.). In their study they tested MS media supplemented with osmotic agents, such as sucrose, mannitol or sorbitol at various concentrations and reported that sorbitol at 20 g l^{-1} was most effective in reducing the growth of the tested potato genotypes. Gopal and Chauhan (2010) also reported the development of a slow-growth *in vitro* conservation method for three potato cultivars using MS medium supplemented by 20 g l^{-1} sucrose in addition to 40 g l^{-1} sorbitol. Gopal et al. (2002) also reported the development of a slow-growth conservation protocol for potato using 20 g 1^{-1} sucrose in combination with 40 g 1^{-1} sorbitol. Sarka et al. (2001) also reported the development of an *in vitro* slow-growth conservation protocol for two potato genotypes after assessing the effects of various concentrations of ancymidol and sucrose as MS media supplements. In their study, they reported that 2.3 ml l^{-1} ancymidol in combination with 60 g l^{-1} sucrose at a cultivation temperature of 6±1 °C can also be used to conserve potato under slow-growth conditions for a 16-month culture period.

A slow-growth conservation protocol has also been developed for six yam (*Dioscorea alata* L.) varieties and 4 genotypes as reported by Villaluz et al. (2012) who used MS media supplemented with 40 mg l^{-1} mannitol. Likewise, Garcia et al. (2011) also reported the development of a slow-growth conservation protocol for yam using MS media supplemented by 30 g l^{-1} sucrose and 0.1 mg l^{-1} BA. Additionally, Malaurie et al. (1998) also developed a medium-term conservation protocol for twenty yam species by using a medium with low mineral nutrient and a low sucrose concentration as their conservation media.

Bessembinder et al. (1993) reported that at 9 °C in total darkness on MS medium the root crop taro (*Colocasia esculenta* L.) can be stored under slow-growth conditions for more than eight years with an approximate transfer interval of three years ensuring a 90% survival rate. A slow-growth conservation protocol has also been developed for two varieties of the tuberous root crop of cassava (*Manihot esculenta* Crantz) as reported by Roca et al. (1992), who managed to conserve the genotypes for 8-9 months on MS medium supplemented by active charcoal and mannitol in combination with a storage temperature of 22 °C to 24 °C. Jarret et al. (1991) also reported the development of a 12-month slow-growth conservation protocol for

the tuberous root crop sweet potato (*Ipomoea batatas* L.) after assessing the effect of abscisic acid (ABA) on growth reduction of six sweet potato varieties.

2.6.2. Application of cryopreservation in root and tuber crops

Cryopreservation is becoming the method of choice for the long-term storage of *in vitro* cultures for species that have recalcitrant seeds or for those that are vegetatively propagated. This method is considered to be cost-effective and safe, ensuring the long-term storage of genetic resources of vegetatively propagated crops. The cryo-storage is usually based on conservation in liquid nitrogen which can ensure a constant storage temperature of -196 °C. At this temperature, all metabolic processes and cell divisions in the tissue of the stored sample are arrested (Reed, 2001a; Benson et al. 2011; Dussert et al. 2003). This method of conservation has been developed for various root and tuber crops.

Potato cryo-banking is one of the most well developed, as various institutions have placed their focus on ensuring the long-term conservation of this globally important tuber crop (Niino and Arizaga, 2015). Institutions such as the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) reported to have 1,436 potato accessions under cryopreservation by the use of DMSO droplet vitrification (Keller 2011), the International Potato Center (CIP) conserves 869 potato accessions under cryostorage by the use of Droplet vitrification & Vitrification (Niino and Arizaga, 2015), the National Agrobiodiversity Center, Rural Development Administration (NAC RDA) conserves 130 potato accessions by the use of Droplet vitrification (Kim 2006a), the National Institute of Agrobiological Sciences (NIAS) conserves 20 potato accessions under cryostorage by the use of V cryo-plate cryopreservation method (Yamamoto, 2013) and the Central Agricultural Experiment Station, Hokkaido Research Organization (CAES HRO), reported to have 100 potato accessions under cryo-storage using Encapsulation vitrification (Hirai 2011).

However, the development of cryostorage for other root and tuber crops has also been reported. The International Center for Tropical Agriculture (CIAT) has reported that in this center 480 accessions of manihot (*Manihot esculenta* Crantz) are under cryostorage using the droplet vitrification method (Niino and Arizaga, 2015). Ming-Hua & Sen-Rong (2010) reported the successful cryopreservation of yam (*Dioscorea bulbifera* L.) using encapsulation-vitrification. Leunufna & Keller (2005) also reported the development of a cryopreservation protocol for four yams species (*Dioscorea* spp.) using a modified droplet method in combination with cold acclimation in an alternating 5 °C and 28 °C, 12 h thermo-photo-period

and two sucrose concentrations as a pre-culture medium. Likewise, Li et al. (2009) reported the development of a cryopreservation protocol for five *Dioscorea opposita* Thunb. species using DMSO based vitrification methods such as PVS2. Wilms et al. (2020) have reported the development of a PVS2 based cryopreservation protocol for ten sweet potato cultivars as did Towill and Jarret (1992) who also reported the development of a cryopreservation protocol for sweet potato by the use of verification pre-treatment.

3. Aims of the thesis

The research aims to develop novel biotechnological methods for long-term conservation of genetic resources of *Smallanthus sonchifolius* and medium-term conservation of *Ullucus tuberosus* genetic resources.

The main aims of the research are:

- Development of a cryopreservation protocol for long-term conservation of *Smallanthus sonchifolius* using PVS2 or PVS3 vitrification methods.
- Application of the cryopreservation protocol for five genotypes of *Smallanthus sonchifolius* originating from Ecuador, Peru and Bolivia and determining which protocol will ensure the long-term conservation of the species' genetic material.
- Optimization of the protocol for medium-term *in vitro* conservation of *Ullucus tuberosus* using mannitol, sorbitol, sucrose, chlorcholinchlorid, abscisic acid, or maleic hydrazide as culture medium supplements, and cultivation temperatures of either 5 °C or 17 °C.

The specific aims of the research are:

- Determine the optimal osmotic dehydration and regeneration conditions of *Smallanthus sonchifolius* shoot tips for cryopreservation.
- Determine what culture media is optimal to ensure the survival and regeneration of plants after submersion in LN (liquid nitrogen).
- Determining which culture medium supplement is optimal for medium-term conservation of *Ullucus tuberosus*.
- Determining the optimal temperature for medium-term conservation of *Ullucus tuberosus*.

4. Hypotheses

Four hypotheses were adopted:

1. The use of PVS2 or PVS3 vitrification solutions will enable cryopreservation of *Smallanthus sonchifolius* and the determination of an optimal protocol.
2. The optimized cryopreservation protocol will be applicable for *Smallanthus sonchifolius* genotypes originated from Ecuador, Peru, and Bolivia.
3. Application of plant inhibitors in the culture medium will reduce the growth of *Ullucus tuberosus* plantlets and will enable the development of a medium-term conservation technique for the species.
4. Low temperature during cultivation will increase the efficiency of slow-growth conservation of *Ullucus tuberosus*.

5. Materials and Methods

5.1. *Smallanthus sonchifolius*

5.1.1. Plant material

In the case of *Smallanthus sonchifolius* (Poepp. et Endl.) H. Robinson, the initial plant material, was taken from the field and greenhouse collection at CZU, Prague. The accessions used for the experiments included one allooctoploid (2n=8x=58) genotype from Ecuador classified as ECU 41, two allooctoploids from Bolivia classified as BOL 22 and BOL 23 and two dodecaploids (2n=12x=87) from Peru classified as PER 12 and PER 14. The cryopreservation protocol was optimized using the accession ECU 41. The optimized protocol was then applied to the other four genotypes.

5.1.2. Establishment of *in vitro* culture and *in vitro* propagation

To establish *in vitro* cultures of yacon, apical shoots (top shoot of plants comprising of the meristematic dome plus 2-4 primordial leaves) were collected from yacon plants maintained in the field collection of the Botanical Garden at the Faculty of Tropical AgriSciences (FTZ), Czech University of Life Sciences Prague (CZU). These were then surface-sterilized using 70% ethanol for 1 min, and 2% NaClO for 15 min. They were then rinsed three times in sterile distilled water and transferred to MS medium, containing 30 g l^{-1} sucrose, 100 mg l^{-1} *myo*-inositol, 1 mg l^{-1} thiamine, and pH adjusted to 5.7. Before being used, the medium was autoclaved at 121 °C in 100 kPa for 20 min. The *in vitro* cultures were maintained in a culture room at 25/23±1 °C under a 16/8 h light/dark regime, and at a photosynthetic photon flux density of 35 μmol m^{-2} s^{-1} l^{-1} provided by cool-white fluorescent tubes. After sprouting of shoots from meristems, the plants were regularly sub-cultured every 28 days using segments with axillary or apical meristems on the same medium, until sufficient plant material for the experiment was obtained.

5.1.3. Microshoot preparation and pre-culture

Apical shoot tips (0.5 cm) (Fig. 7c) were excised from 2-3-week old *in vitro* plantlets (Fig. 7a). The leaves were removed, and the stem was cut off until an apical shoot tip (2-3 mm long) comprising the meristematic dome plus 2 primordial leaves remained (Fig. 7c). The plant material was prepared under a binocular microscope. A total of 180 shoot tips were excised

which were then placed on pieces of sterile filter paper on solid MS medium containing 103 g l^{-1} sucrose, and placed in a cultivation chamber in dark conditions for 12 hrs as a pre-culture before exposure to the osmoprotective treatments.

Ten shoot tips were used as the main control (pre-cultured shoot tips on MS+103 g l^{-1} sucrose with no exposure to osmoprotectants or LN).

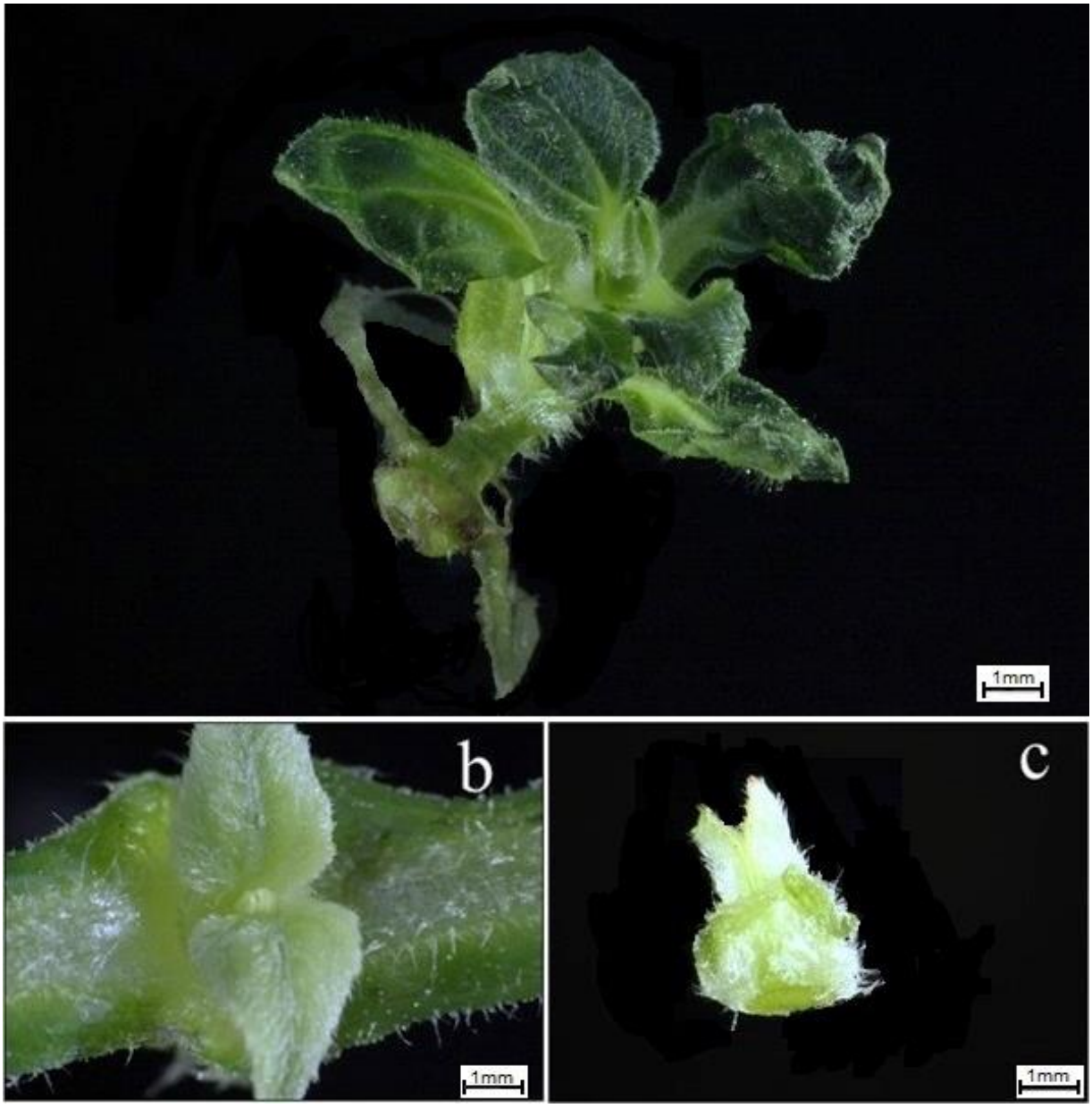

Figure 7. Images of yacon shoot tips excised under a binocular microscope for cryopreservation: a. yacon plantlet, b. birds view of apical meristem placement on the plant apex and c. excised (1.8-2.5 mm) apical shoot tip.

Source: Author

5.1.4. PVS2 and PVS3 treatments and cryopreservation

Pre-cultured shoot tips were placed in 5 ml loading solution (LS) composed of MS medium supplemented with 184 g l^{-1} glycerol and 137 g l^{-1} sucrose and filter-sterilized before being used. These were incubated for 20 min at 22±1 °C. Ten shoot tips were used as LS control (shoot tips only exposed to LS) and 20 were used for each PVS2 and PVS3 treatment time exposure. The PVS2 solution consisted of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) dimethyl sulfoxide (DMSO), dissolved in MS salts with 137 g l^{-1} sucrose, pH adjusted to 5.8 (Sakai et al. 1990). The PVS3 solution consisted of 50% (w/v) glycerol and 50% (w/v) sucrose (Nishizawa et al. 1993).

To place the shoot tips in the PVS2 solution, the LS was removed with the use of a Pasteur pipette, and replaced with 5 ml ice-cooled (0 °C) filter-sterilized PVS2 solution. The shoot tips were maintained for either 15, 30, or 60 min on ice to determine what PVS2 time duration would be optimal for yacon cryopreservation. At the end of each PVS2 treatment, 10 shoots were randomly chosen and transferred to a PVS2 drop on a sterile aluminium foil strip (0.5-2 cm). All manipulations of the strips were done in a Petri dish placed on ice to maintain a temperature of around 0 °C, following the method according to Panta et al. (2006) and Panis et al. (2005).

Once on the ice, the aluminium foil strips containing the shoots were then cryopreserved by rapidly being plunged in LN using forceps and were placed in cryotubes filled with LN for 1 hour. The remaining 10 shoot tips were used as control. These were directly placed in recovery media (5 shoots on MS medium and 5 shoots on MS medium with 1 mg l^{-1} BA) and were maintained in a cultivation room under complete dark conditions for 2 weeks and were then placed in 24 h light at 25±1 °C cultivation conditions to use as control.

In the case of the PVS3 treatment, all steps of the experiments were carried out as in the case of the PVS2 treatments with slight differences; i) the experiments were carried out at 22±1 °C, ii) the shoot tips were maintained in PVS3 for up to 75 min (30, 45, 60, or 75 min).

Both PVS2 and PVS3 experiments were carried out in 3 repetitions.

5.1.5. Explant thawing, unloading, and regeneration

In both plant vitrification solution methods (PVS2 and PVS3), two recovery/regeneration media were tested; MS with the addition of no plant growth regulators (PGRs) and MS supplemented with 1 mg l^{-1} BA containing 30 g l^{-1} sucrose, 100 mg l^{-1} *myo*-inositol, 1 mg l^{-1} thiamine, and pH

adjusted to 5.7. Before being used, the medium was autoclaved at 121 °C in 100 kPa for 20 min.

The unloading solution for thawing of PVS2 cryo treatments consisted of 411 g l^{-1} sucrose dissolved in MS medium (pH 5.8). The frozen shoot tips on aluminium foil strips were taken out of LN and were rinsed in 10 ml unloading solution at 22±1 °C for 15 minutes. The thawed meristems were then placed on a filter paper on top of a semi-solid hormone-free MS medium containing 103 g l^{-1} sucrose and were placed in dark conditions for 24hrs, thereafter, they were then transferred directly into the above-mentioned recovery media (5 in MS and 5 in MS+1 mg l^{-1} BA) and were again placed in dark conditions for an additional 6 days after which they were then placed in a cultivation room and maintain in a 24 h light at 25±1 °C cultivation conditions. Post-thaw survival and regeneration of shoot tips were determined within 8 weeks following cryopreservation.

PVS3 unloading solution consisted of a liquid solution of water containing 102.69 g l^{-1} sucrose. A rapid rewarming of the cryopreserved samples was carried out by quickly plunging the opened cryotubes filled with LN containing the aluminium foil strips with frozen shoot tips in the PVS3 unloading solution at 40 °C for 30 sec to 1 min. The thawed shoot tips were then placed directly in the above-mentioned recovery media (5 in MS and 5 in MS+1 mg l^{-1} BA) and were then placed in a cultivation room under complete dark conditions for two weeks and were then placed in a 24 hrs light at 25±1 °C cultivation conditions. Post-thaw survival and regeneration of shoot tips using PVS3 solution were also determined within 8 weeks following cryopreservation.

5.1.6. Survival and recovery evaluation after cryopreservation

In both PVS2 and PVS3 methods, shoot tip survival after cryopreservation was defined as shoot tips remaining green 3 weeks after thawing, while white shoot tips were considered as dead with no potential for regrowth.

Regrowth was defined as further development of apices into shoots up to 8 weeks after rewarming. Survival and regrowth rates were expressed relative to the total number of shoot tips treated per treatment. During the regrowth phase parameters such as callus formation, hyperhydricity (HH), shoot+callus formation (live shoot with callus base), colour and full growth normal (FGN=a fully regenerated plant with well-developed roots and leaves and no morphological abnormalities), full growth+callus (fully grown plant with callus base) was also

evaluated to determine the effect of PVS2 and PVS3 solutions on the quality of regeneration of cryopreserved shoot tips.

These parameters were also evaluated on control samples for both PVS2 and PVS3 treatments during survival and regrowth phases.

The developed cryopreservation protocol for PVS2 and PVS3 vitrification solutions on the yacon genotype ECU 41 was then applied to apical shoot tips of the other four genotypes of yacon classified as BOL 22, BOL 23 PER 12 and PER 14 to determine which vitrification method applies to the species and can be used as a tool for the genetic resources' conservation of yacon by cryopreservation. To carry out these experiments a total of 160 apical shoot tips (0.5 cm) were excised from 2-3-week old *in vitro* plantlets of each accession (40 belonged to each genotype of which 20 was for the PVS2 treatment and 20 for the PVS3). The regeneration of these shoot tips was carried out on the optimal regeneration media defined in the optimization phase using the ECU 41 genotype. Only the most optimal PVS2 and PVS3 treatment time duration were used. The methodological application of the PVS2 and PVS3 treatment on the BOL 22, BOL 23 PER 12 and PER 14 genotypes was the same as the ECU 41 genotype. All experiments were carried out in three repetitions.

5.2. *Ullucus tuberosus*

5.2.1. Plant material

As initial plant material for *Ullucus tuberosus* Caldas experiments, a diploid clone of ulluco (2n =2x=24) also maintained at the Botanical Garden of the FTA, CZU, Prague, classified according to the morphological descriptors proposed by the International Potato Center (CIP) (Manrique et al. 2017) as morphotype No. 156, was used (Fig. 8).

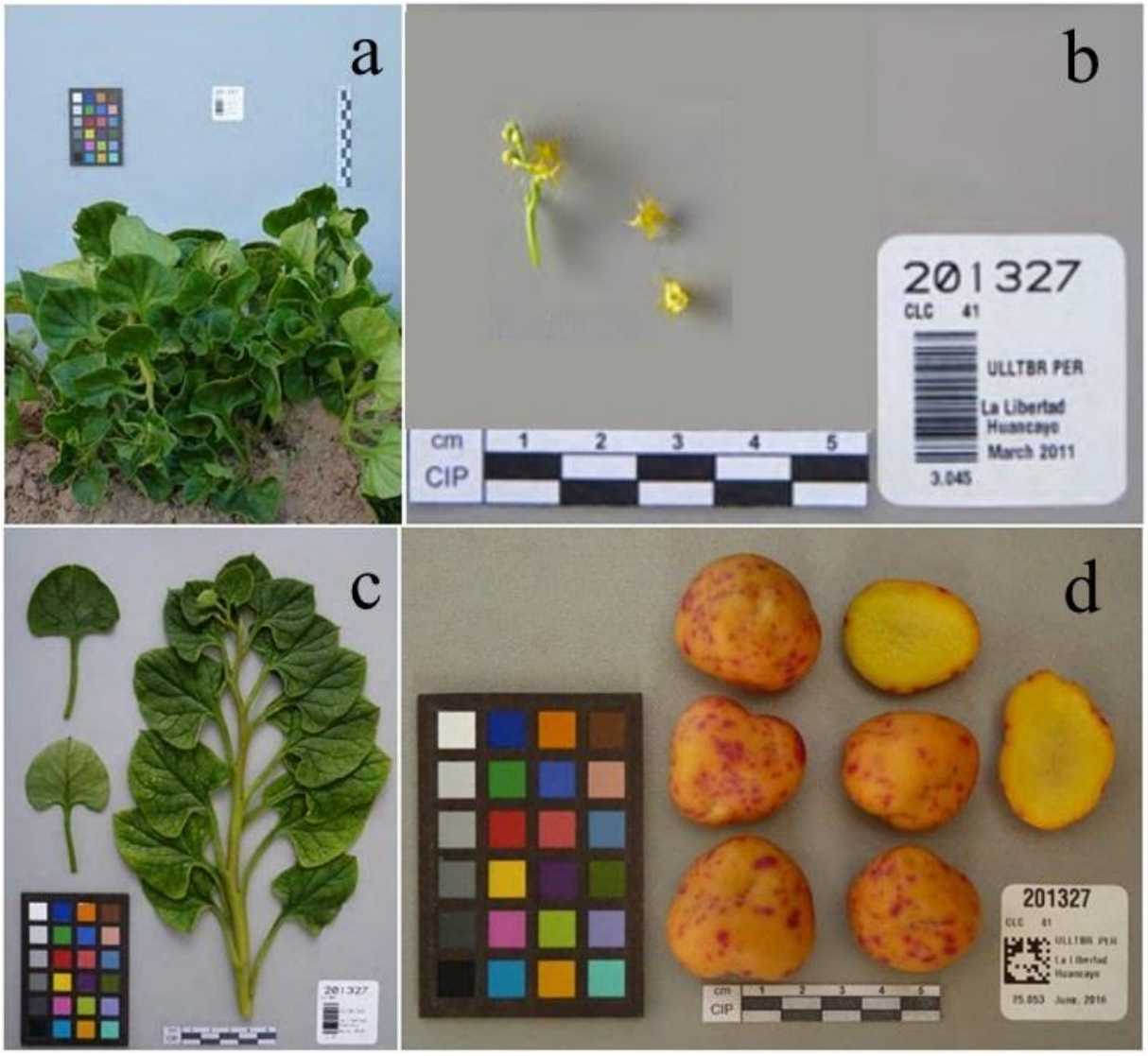

Figure 8. Ulluco morphotype No. 156. a. pant, b. flowers, c. leaves and stem, d. tubers.

Source: Manrique et al. (2017)

5.2.2. Establishment of *in vitro* culture and *in vitro* propagation

To establish *in vitro* culture, nodal segments were collected from clonally propagated ulluco plants grown under greenhouse conditions and were surface-disinfected using 70% ethanol for 1 min, and 1% NaClO for 10 min. The nodal segments were then rinsed three times in sterile distilled water and transferred to half-strength MS medium, containing 30 g l^{-1} sucrose, 100 mg l^{-1} *myo*-inositol, 1 mg l^{-1} thiamine, and pH adjusted to 5.7. Before being used, the medium was autoclaved at 121 °C in 100 kPa for 20 min. The *in vitro* cultures were maintained in a culture room at 25/23±1 °C under a 16/8 h light/dark regime, and at a photosynthetic photon flux

density of 35 µmol m^{-2} s^{-1} provided by cool-white fluorescent tubes. After sprouting of shoots from meristems, the plantlets were regularly sub-cultured every 28 days using segments with axillary or apical meristems on the same medium, until sufficient plant material for the experiment was obtained (Hammond et al. 2019b).

5.2.3. Slow growth treatments for medium-term conservation

For the medium-term conservation of ulluco, nodal segments about 2 cm in length containing one axillary bud were used. To standardize the experiment, all segments were collected from the same position on plants; the second nodal segment from the top of the plant was always selected. Slow-growth storage of ulluco was optimized using a half-strength MS medium containing 30 g l^{-1} sucrose (except for sucrose treatments, where specific sucrose concentrations were used), and supplemented with mannitol at a concentration of 10, 20 and 30 g l^{-1}, sorbitol at 10, 20 and 30 g l^{-1}, sucrose at 10, 20, 60, 90 and 120 g l^{-1}, CCC at 300, 500 and 700 mg l^{-1}, ABA at 1, 2 and 3 mg l^{-1}, or MH at a concentration of 0.1, 0.3 and 0.5 mg l^{-1}. A half-strength MS medium without any plant growth regulators was used as the control.

Culture media with pH adjusted to 5.7 were autoclaved at 121 °C and 100 kPa for 20 min. ABA, CCC, and MH were filter sterilized and added to media after autoclaving. Twenty millilitres of culture medium was always dispensed into test tubes (25×150 mm). In each treatment, 40 plants were used out of which 20 were placed in constant cultivation temperatures of 5 °C and twenty at 17±1 °C.

The experiment was conducted in two repetitions. The *in vitro* cultures were maintained in a culture room under a 16/8 h light/dark regime, and at a photosynthetic photon flux density of 35 µmol m^{-2} s^{-1} provided by cool-white fluorescent tubes. Plant height was measured once a month for a total of 18 months. The number of meristems per plant was counted at the end of the conservation period.

After 18-month conservation, the survival form of the plants was evaluated (on a visual 0-3 scale, 0=dead shoot, 1=dead/dried shoot+live microtuber (MT), 2=live shoot+live MT, 3=only live shoot without MT), as well as the survival percentage (as the sum of grade 1-3). Dying/drying stem in combination with MT development was regarded as a plant senescence sign and potentially less suitable for further ulluco conservation. In this experiment, microtubers were not considered for ulluco conservation, due to their size variability, different numbers of vegetative buds, and potential dormancy.

5.2.4. Post-storage regrowth of shoots

After 18 months of ulluco conservation, the regrowth potential of surviving microshoots was tested. For post-storage regrowth, four conservation treatments were selected based on the following criteria: survival percentage after conservation (preferably with the highest shoot and/or shoot+MT survival rate), and treatments with superior morphological characteristics; i.e., mannitol 17 °C, sorbitol 17 °C, sucrose 17 °C and MH 17 °C, all of them being represented by one concentration (30 g l^{-1}, 30 g l^{-1}, 60 g $l^{-1,}$ and 0.3 mg l^{-1}, respectively). As a control treatment, plantlets from the half-strength MS medium 17 °C were used for post-storage regrowth.

Apical shoots were used from microplants that survived in form of either shoots or shoots+MT. Shoots about 2 cm in length collected from these plantlets were transferred to a half-strength MS medium containing 30 g l^{-1} sucrose, 100 mg l^{-1} *myo*-inositol, and 1 mg l^{-1} thiamine (pH of 5.7). The cultures were maintained in an incubator at 25/23±1 °C under a 16/8 h light/dark regime for 28 days. Thereafter, survival percentage, plant height (cm), number of shoots, meristems and roots were evaluated. Based on the results from the post storage regrowth experiment, plantlets from the best treatment (30 g l^{-1} mannitol) were selected for *ex vitro* transfer and genetic fidelity assessment.

5.2.5. *Ex vitro* transfer to greenhouse conditions

Twenty rooted plantlets after post-storage regrowth from a selected treatment, 30 g l^{-1} mannitol, and control plantlets were transferred to a greenhouse. Microplants 3-5 cm high, with a minimum of 6 leaves and a minimum of 2 well-developed roots (min. 2 cm long), were selected for *ex vitro* transfer. They were removed from flasks; the roots were rinsed with tap water to remove adhering medium and the plantlets were transferred into flower pots (0.4 dm^3) containing a sterile mixture of garden substrate and perlite (1:1 v/v) and covered with plastic perforated foil to maintain a microclimate with 80-85% humidity. A temperature of 25/19 (day/night)±1 °C was maintained in the greenhouse. The plastic covers were gradually removed from the pots after 2-3 weeks of cultivation. The survival percentage of plants was evaluated after an 8-week period.

5.2.6. DNA isolation and ISSR analysis

Leaves of 10 plants from 30 g l^{-1} mannitol treatment, collected in the greenhouse after plant acclimation were used for molecular analysis for the assessment of genetic stability post

conservation as medium-term conservation by slow-growth can be linked to somaclonal variation. As a control, the initial mother plant/field control was used. Additionally, samples from 10 control plants obtained after post-storage regrowth, and after *ex vitro* transfer, were also analyzed.

Genomic DNA from fresh leaves was isolated by the DNA extraction method with cetyltrimethylammonium bromide (CTAB) (Doyle and Doyle 1989) with a few drops of polyvinylpyrrolidone (PVP). The extracted DNA was used for ISSR analysis with twelve selected ISSR primers (Integrated DNA Technologies, Belgium; Table 1). Polymerase chain reaction (PCR) amplification was done on the individual samples in two repetitions. The DNA samples were used in 50 ng μl^{-1} concentration. The PCR amplification was done in a total reaction volume of 20 µl containing 2 µl template DNA, 0.5 µl of primer at a concentration of 0.1 mM, 10 PPP Master Mix (Top-Bio, Czech Republic), 0.2 µl bovine serum albumin (BSA) of concentration 20 mg ml^{-1} (Thermo Scientific, Lithuania), and 7.3 µl PCR water. Amplifications were carried out in cycler (Bio-Rad, USA) with modification of the annealing temperature depending on which primer was being used for ISSR (Table 1).

The specifications for PCR cycling were initial denaturation for 5 min at 94 °C, then 40 cycles of 1 min each at 94 °C, 1 min at specific annealing temperature, 2 min at 72 °C for an extension, and then a final extension cycle for 10 min at 72 °C. The PCR amplification of the DNA was done in duplicates. Electrophoresis was done with 7 µl of the amplified DNA on 2% agarose with the final concentration of ethidium bromide (EtBr) in the gel 0.5 µg ml^{-1}.

The gels were run for 3-3.5 hours at 60 V and 120 mA. Bands were visualized and isolated from the gel under UV light (Cleaver Scientific, UK). The size of amplicons was estimated using Thermo Scientific Gene Ruler 100 bp Plus DNA Ladder (Thermo Scientific, Lithuania).

ISSR fragments were scored for the presence (1) or absence (0) of bands in the gel profile. Only clear and distinct bands were evaluated**.**

Table 1. List of ISSR primers, annealing temperatures, number and sizes of amplified fragments

Nº	Primer Code UCB	Sequence (5´- 3´) [a]	Annealing Tem. (°C)	Total Nº of bands amplified	Nº of scorable bands per primer	Nº and frequency of polymorphic bands per primer	Range of amplifications (pb)
1	UCB807	$(AG)_8T$	52	105	5	0	300-1,150
2	UCB809	$(AG)_8G$	52	168	8	0	200-950
3	UCB810	$(GA)_8T$	50.8	105	5	0	500-1,000
4	UCB812	$(GA)_8A$	51.5	147	7	0	200-900
5	UCB813	$(CT)_8T$	50.8	105	5	0	350-1,150
6	UCB826	$(AC)_8C$	49.1	147	7	0	450-1,200
7	UCB828	$(TG)_8A$	47.1	105	5	0	950-1,500
8	UCB834	$(AG)_8YT$	49.1	147	7	0	500-1,500
9	UCB836	$(AG)_8YA$	47.1	189	9	0	300-1,600
10	UCB856	$(AC)_8YA$	49.1	147	7	0	450-,200
11	UCB859	$(TG)_8RC$	46.4	168	8	0	300-1,500
12	UCB866	$(CTC)_6$	46	126	6	0	800-1,200
Total				1,659	79	0	

[a] Single letter abbreviation for mixed-base positions: Y=(C, T), R=(A, G)

5.3. Statistical analysis

Data from the slow-growth of ulluco and cryopreservation of yacon experiments and post-storage evaluation were analyzed using analysis of variance (ANOVA) and the least significant ($P<0.05$) differences between mean values were estimated using Tukey´s HSD test [STATISTICA 12.0, StatSoft, Inc. USA]. A two-tailed t-test using [STATISTICA 12.0, StatSoft, Inc. USA] was carried out to compare survival and regeneration quantity between PVS2 and PVS3 treatment for each tested genotype. All results presented in the form of graphs were taken from statistical analysis.

6. Results

6.1. *Smallanthus sonchifolius*

6.1.1. Shoot tip survival after cryopreservation using PVS2 and PVS3

The LS treatment did not have a significant influence on the survival of the shoot tips before exposure to the PVS2 and PVS3 osmoprotectants and LN. In comparison to the control shoot tips (no exposure to LS, PVS treatments, and LN) that showed 100% survival, there was only a 1.7% decline in survival after 20 min in LS (Fig. 9a, b).

Overall, the shoot tip survival percentage after cryopreservation ranged from 37 to 90% within all treatments (Fig. 9c). After cryopreservation, the survival of shoots for both PVS2 and PVS3 treatments in many cases showed no significant differences to those of control treatments with no exposure to LN (Fig. 9c).

In the case of PVS2, the longest tested treatment time duration (60 min) at 0 °C provided the highest shoot tip survival percentage (83%) post cryopreservation (Fig. 9a, c). Shorter PVS2 treatment durations (15 min and 30 min) at 0 °C led to more reduced shoot tips survival with only a 53% and 70% post-exposure to LN, respectively (Fig. 9a, c), and did not provide results as promising as PVS2 60 min post cryopreservation (Fig. 9a, c).

In the case of PVS3 treatments, 60 min treatment duration also proved to be most effective in comparison to other PVS3 treatments, providing the highest shoot tip survival of 90% after cryopreservation. Shorter exposure of shoot tips (30 min and 45 min) as well as longer (75 min) provided lower survival percentage when compared to control treatments and PVS3 60 min, reaching maximally 77% (Fig. 9b, c).

When comparing the differences among treatments between the PVS2 and PVS3 treatments, there was no statistical difference in survival between PVS2 at 30 min and 60 min treatment durations and PVS3 at 45 min and 60 min treatment duration (Fig. 9c).

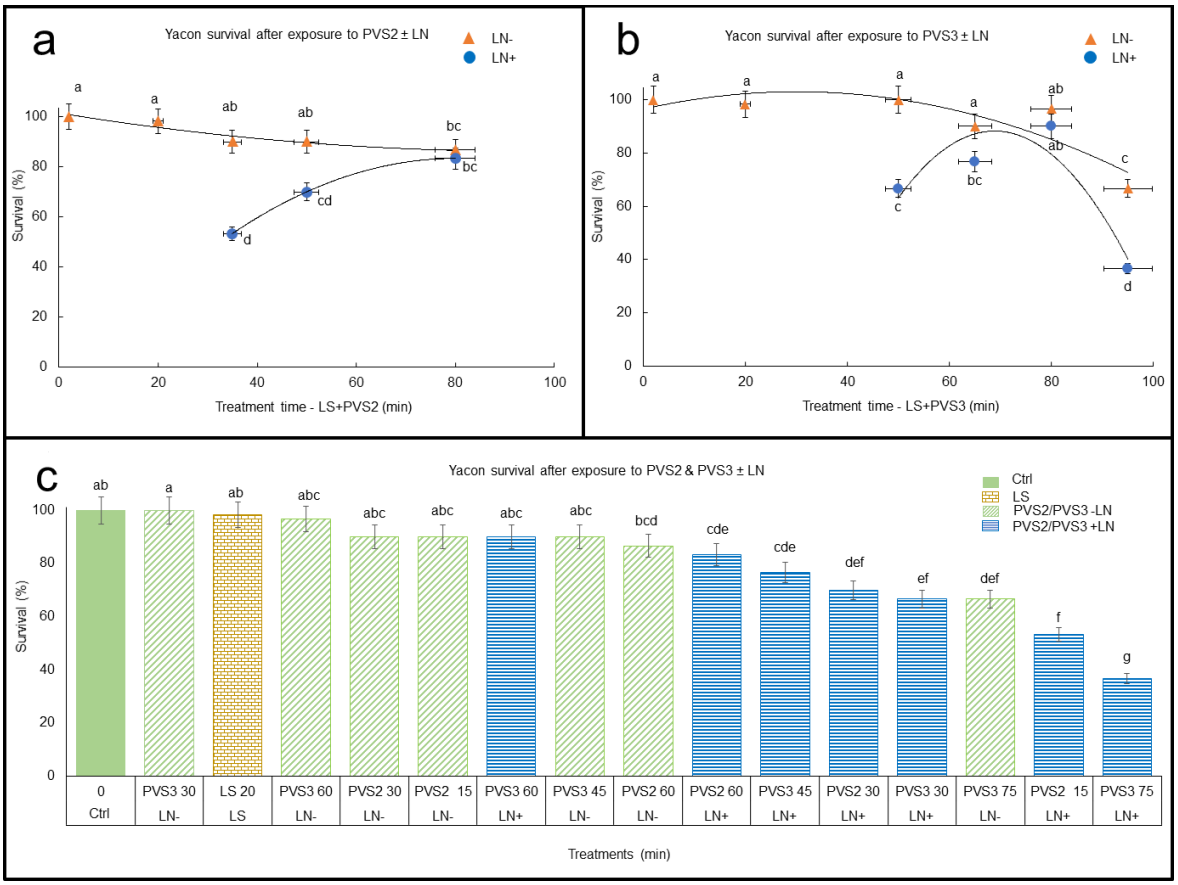

Figure 9. Survival of yacon shoot tips post-exposure to LN and vitrification solutions. a. influence of PVS2 on survival. b. influence of PVS3 on survival. c. comparison of both PVS2 and PVS3 treatments. Shoot tips -LN as controls and +LN as cryopreserved. Ctrl=general control with no exposure to vitrification solutions and LN, LS=after 20 min loading solution, -LN=shoot tips exposed to LS and either PVS2 or PVS3 vitrification solutions but not to LN, +LN=shoot tips exposed to LS and either PVS2 or PVS3 vitrification solutions and LN. Values represent the mean of three determinations. Differences in mean values of survival with different letters are statistically significant ($p<0.05$) in each treatment.

Source: Author

6.1.2. Effect of PVS2 and PVS3 on shoot tip regrowth

Control treatments not exposed to LS, PVS2 or PVS3, and LN did not exhibit any damage (Fig. 10a). However, shoot tips exposed to both PVS2 and PVS3 treatments and LN appeared to have surface damage, exhibiting a dark/black colour. This, however, did not affect shoot development and regrowth (Fig. 10b).

After exposure to PVS2 or PVS3 treatments and cryopreservation, most surviving shoot tips regenerated into FGN within 12 weeks. During the first two weeks on both types of recovery media, the cryopreserved shoot tips started to regrow. During further cultivation (week 4±2 days) they started forming new leaves (Fig. 10c), and by week 8 (±2 days) the shoots had formed into *in vitro* plantlets with 2-3 pairs of well-developed leaves (Fig. 10d). These plants had 0, 1, or only 2 underdeveloped roots. By week 12 (±2 days) the regenerated plantlets were well-rooted, had at least 3 pairs of leaves, and 2-3 well-developed roots (Fig. 10e). Only plantlets with these characteristics and no morphological abnormalities were considered as fully regenerated into FGN. Some surviving shoot tips showed signs of HH: these were excluded from the group of FGN.

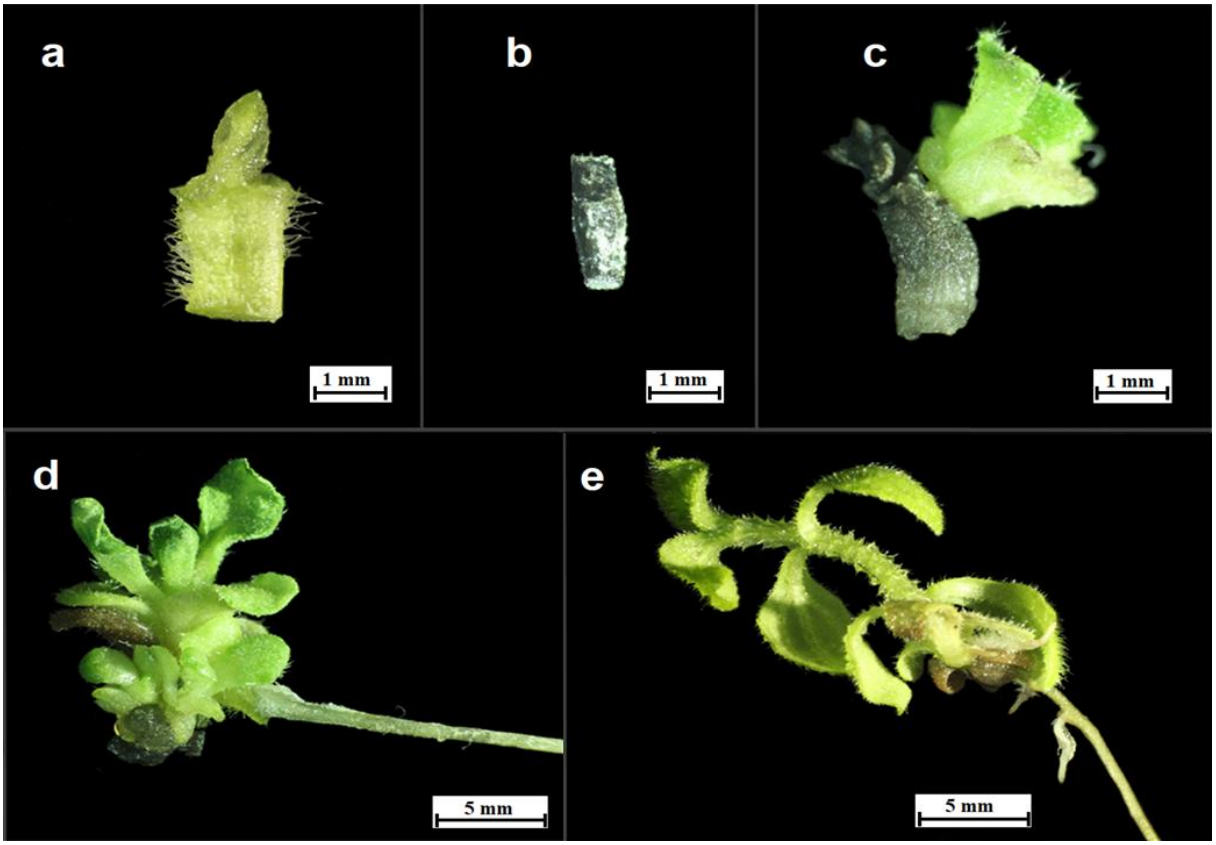

Figure 10. A view of yacon shoot tip regrowth after cryopreservation, under a binocular microscope. a. excised yacon shoot tip with no exposure to LN. b. excised yacon shoot tip after exposure to LN. c. shoot tip regrowth after 4 weeks of cultivation post cryopreservation on MS medium. d. yacon plantlet 8 weeks after cultivation post cryopreservation. e. yacon shoot tip regrowth 12 weeks post cryopreservation.

Source: Author

Most shoot tips regenerated in the form of FGN post cryopreservation in both PVS2 and PVS3 treatments and on both recovery media (MS medium and on MS supplemented with 0.1 mg l^{-1} BA) (Fig. 11). This type of regrowth was mostly observed on MS media within the 12th week of the cultivation period post cryopreservation (Fig. 11c). PVS2 and PVS3 applications for 60 min proved to be most effective in comparison to other PVSs treatments. Regrowth of plants in these treatments on MS medium reached 73% FGN respectively (Fig. 11c).

Shorter PVS2 treatment durations (15 min and 30 min) were not as effective on MS media as the longer treatment (60 min), reaching a maximum FGN regrowth of only 20% and 60%, respectively, after cryopreservation (Fig. 11c). The shorter PVS3 treatment time duration (30 min and 45 min), as well as the longer treatment (75 min), also provided lower regrowth percentages in comparison to 60 min PVS3 treatment reaching a maximum FGN regrowth percentage of 67%, post cryopreservation on MS regrowth media (Fig. 11c).

PVS3 60 min treatment also provided the highest regrowth percentage in the form of FGN (67%) on MS medium supplemented by 0.1 mg l^{-1} BA among other treatments (Fig. 11b, d). The other PVS3 and the PVS2 treatments did not exceed 53% FGN regrowth on this media post cryopreservation (Fig. 11b).

Shoot hyperhydration represented the most frequent form of morphological abnormalities observed within the regenerating shoot tips on both regrowth media tested in control treatments and those post cryopreservation (Fig. 11a, b, c, d). The hyperhydrated plants were characterized by having translucent leaves and stem, undeveloped trichomes, as well as leaf veins (Fig. 12b).

The highest rate of HH was observed in plants on MS medium supplemented by 0.1 mg l^{-1} BA regardless of their exposure to LN and vitrification treatment (Fig. 11b, d). The phenomenon of HH on this medium was presented in regenerated plants from both PVS2 and PVS3 treatments before and after cryopreservation, in control shoot tips, and shoot tips regenerated post-exposure to the vitrification solutions and LN (Fig. 11b, d). The level of HH on medium supplemented with BA ranged from 5 to 48% in treatments before and after cryopreservation (Fig. 11b, d).

On this medium, HH started appearing within the first week of regrowth. By the end of the regrowth period (8-12 weeks), the plants could not fully recover.

Hyperhydration of plantlets on MS medium was also detected; however, it only ranged from 0 to 30% and was only observed in some treatments before and after exposure to LN (Fig. 11a, c).

Callus formation was also observed within the shoot tip regrow phase, as well as fully grown plants with callus developed on the shoot tip base (FGN+callus) after cryopreservation. These forms of regrowth were recorded only in shoots cultivated on MS medium supplemented by 0.1 mg l^{-1} BA.

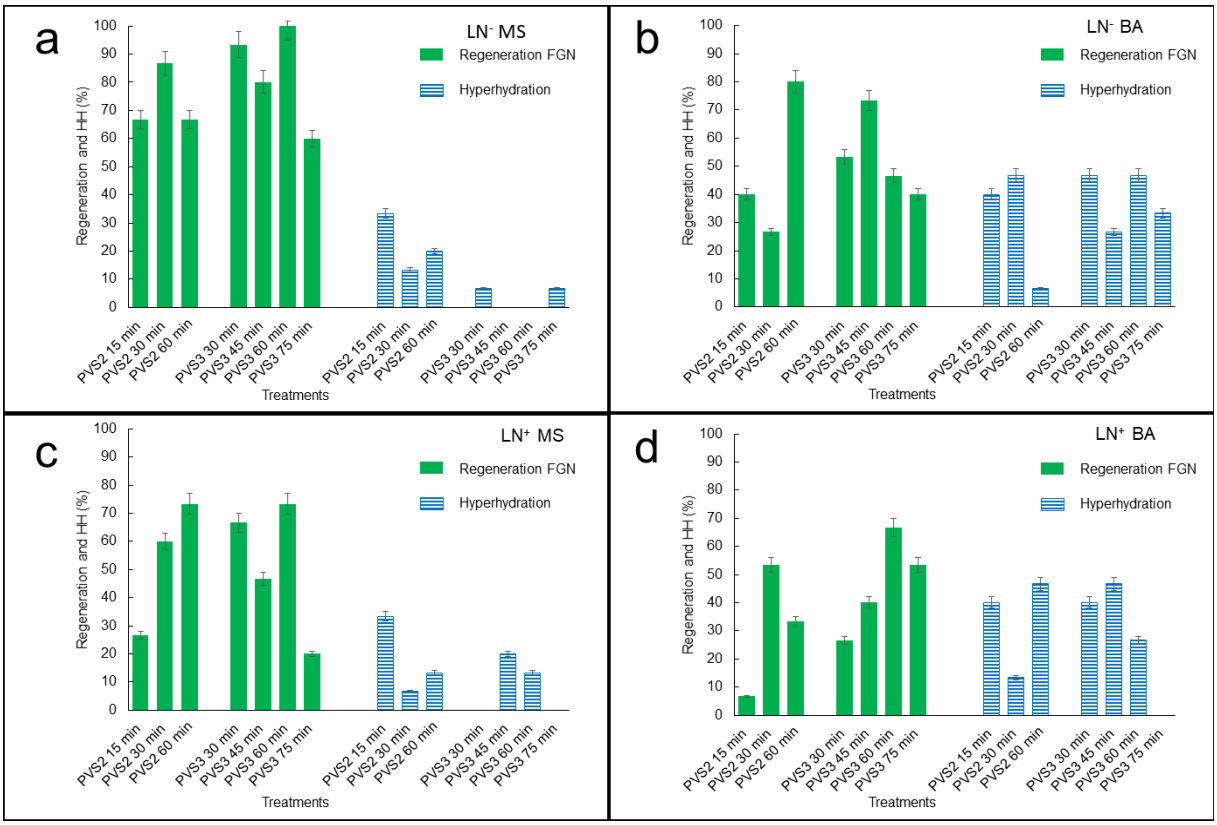

Figure 11. Effect of PVS2 and PVS3 treatments and recovery media on yacon shoot tip regrowth and hyperhydration occurrence before and after cryopreservation. a) control shoot tips exposed to PVS2 and PVS3 and their recovery on MS medium with no exposure to LN (LN^- MS). b) control shoot tips treated with PVS2 and PVS3 and their recovery on MS+0.1 mg l^{-1} BA medium with no exposure to LN (LN^- BA). c. shoot tips treated with PVS2 and PVS3 on MS medium and exposed to LN (LN^+ MS). d. shoot tips treated with PVS2 and PVS3 on MS+0.1 mg l^{-1} BA medium and exposed to LN (LN^+ BA). PVS2=plant vitrification solution number 2, PVS3=plant vitrification solution number 3, HH=hyperhydration, LN^+=Treatments exposed to LN, LN^-=control treatments not exposed to LN, LN=liquid nitrogen. All values are presented as mean ± standard deviation (SD).

Source: Author

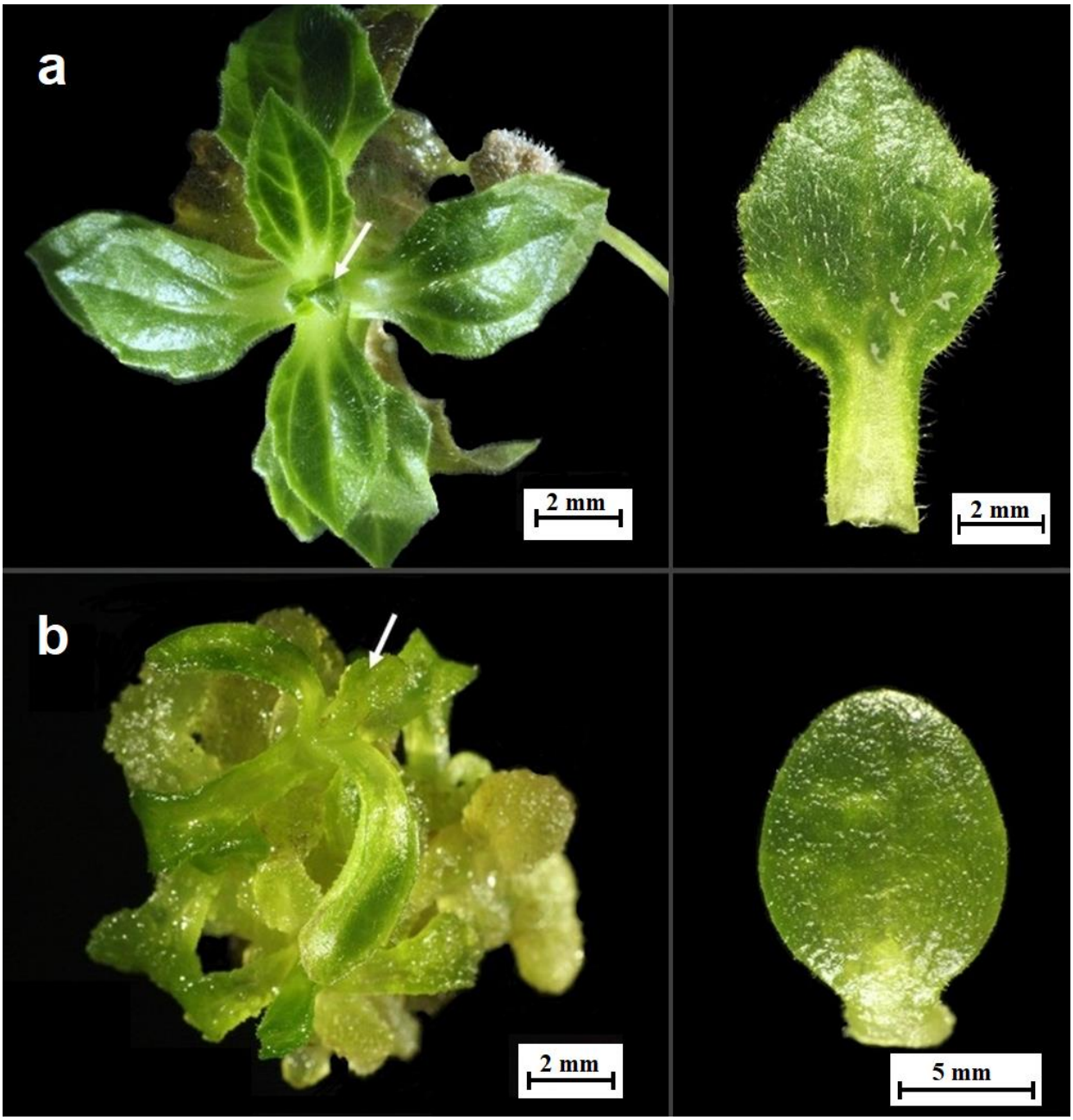

Figure 12. Comparison of HH occurrence in regenerated yacon plantlets on regrowth media post cryopreservation. a. plantlets on MS medium with magnified leaves without morphological abnormalities. b. yacon plantlets on regrowth MS medium containing 0.1 mg l^{-1} BA and showing signs of HH (translucent leaves and stem, undeveloped trichomes, and leaf vein). Arrows indicate the position of the magnified leaves on the right.

Source: Author

6.1.3. PVS2 and PVS3 treatments on various yacon genotypes

All control treatments (no exposure to LS, PVS treatments, and LN) showed a 100% survival and LS treatment also did not have a significant influence on the survival of the shoot tips of the other four yacon genotypes (BOL 22, BOL 23, PER 12 and PER 14) as was the case with the ECU 41 genotype before exposure to the PVS2 and PVS3 osmoprotectants and LN.

Overall, the shoot tip survival percentage of the four yacon genotypes after cryopreservation ranged from 79.7 to 94.1% (Fig. 13a,). When comparing both PVS2 and PVS3 treatments in terms of survival there were no significant differences in survival percentage for most genotypes except for the genotype BOL 22 which had a significantly higher survival rate on PVS2 in comparison to the PVS3 treatment as shown in figure 13a. When it comes to regeneration, the comparison between the PVS2 and PVS3 regeneration rates showed that the genotype BOL 23 AND PER 12 had a significantly higher regeneration rate in the PVS2 treatment, all other accessions had comparable regeneration rate in both treatments (Fig.13b)

In the case of PVS2 at 0 °C, the highest shoot tip survival percentage (90.2%) post cryopreservation was shown in the BOL 23 genotype followed by the PER 14 genotype with 90%, PER 12 with 89.1% and BOL 22 with an 87.6% survival, when compared to the ECU 41 genotype (86.7%) the survival rates were comparable (Fig. 13a, c, d).

In the case of PVS3 60 min treatment duration, the BOL 22 genotype provided the highest survival percentage (94.1%) in comparison to all other genotypes (including ECU 41), followed by BOL 23 which had a survival rate of 88.9%, PER 14 87.5%, the PER 12 accession provided the lowest survival rate in this treatment (79.7) (Fig. 13d).

When comparing the differences among treatments between the PVS2 and PVS3 treatments, there was no significant difference in survival between the genotypes (Fig. 13c, d).

The regeneration of surviving shoot tips after exposure to the PVS2 or PVS3 treatment and cryopreservation was mainly in the form of FGN (Fig. 13c, d), this was determined within 12 weeks post thawing. All shoot tip belonging to each genotype started germinating within the first two weeks on the MS recovery media as was also the case of the ECU 41 genotype (±2 days). Slight signs of morphological abnormalities mainly in the form of HH was detected in the regenerated shoot tips ranging from 1.2-18.2% (Fig. 13e, f), however, the main form of regeneration in all genotypes was FGN ranging from 75.4-62.3% (Fig. 13e, f).

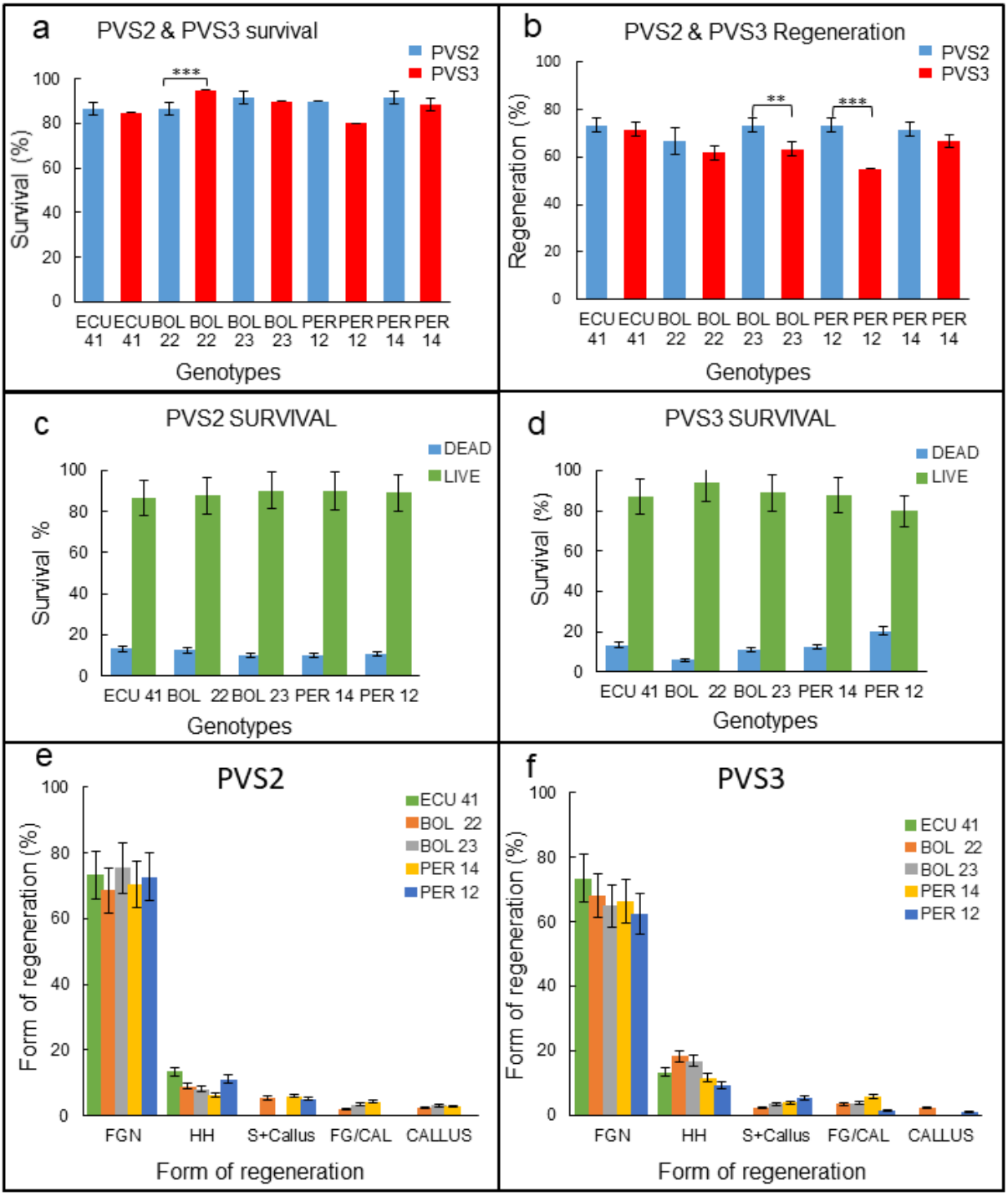

Figure 13. Survival and regeneration of shoot tips excised from five yacon genotypes post cryopreservation using the optimal PVS2 and PVS3 treatment time duration and regrowth media. a. comparison of PVS2 and PVS3 survival percentage. b. comparison of PVS2 and PVS3 treatments regeneration percentage. c. influence of PVS2 on survival. d. influence of PVS3 on survival. e. influence of PVS2 on the form of regeneration. f. influence of PVS3 on the form of regeneration. FGN=a fully regenerated plant with well-developed roots and leaves, HH=hyperhydricity, S+Callus=live shoot with callus base, FG/CAL=fully grown plantlet with

callus base, CALLUS= regrowth of shoot only in the form of callus, ECU 41=Ecuadorian yacon genotype, BOL 22 and BOL 23=Bolivian yacon genotype, PER12 and PER 14=Peruvian yacon genotypes. All values are presented as means ± standard deviation (SD). Figure a and b statistical significance on alpha level for *** significant difference 0.01, for ** significant difference 0.05 others are not significant.

Source: Author

6.2. *Ullucus tuberosus*

6.2.1. Effect of culture medium supplements on plantlet conservation at 5 °C

Conservation at 5 °C led to extremely reduced plant growth in all tested treatments. The differences in plant height (1.6-5.3 cm) (Fig. 14a, b, c, d, e, f) and the number of meristems between plants (0-7 meristems/plant) (Fig. 15) from various treatments were relatively small. Overall, shoots produced during the conservation period were thin and leaves were reduced or absent. During cultivation, usually, one MT per individual plant developed within 5-8 months in all treatments, which was followed by drying of the stem and, therefore, by the end of the conservation period, survival in the form of MTs on a dried shoot prevailed in the experiment, including plants from the control treatment. Survival in the form of a shoot without MT was not recorded in any of the treatments maintained at 5 °C. Overall, the survival percentage after 18-month plant cultivation varied from 0% to 100% between treatments (Fig. 16a).

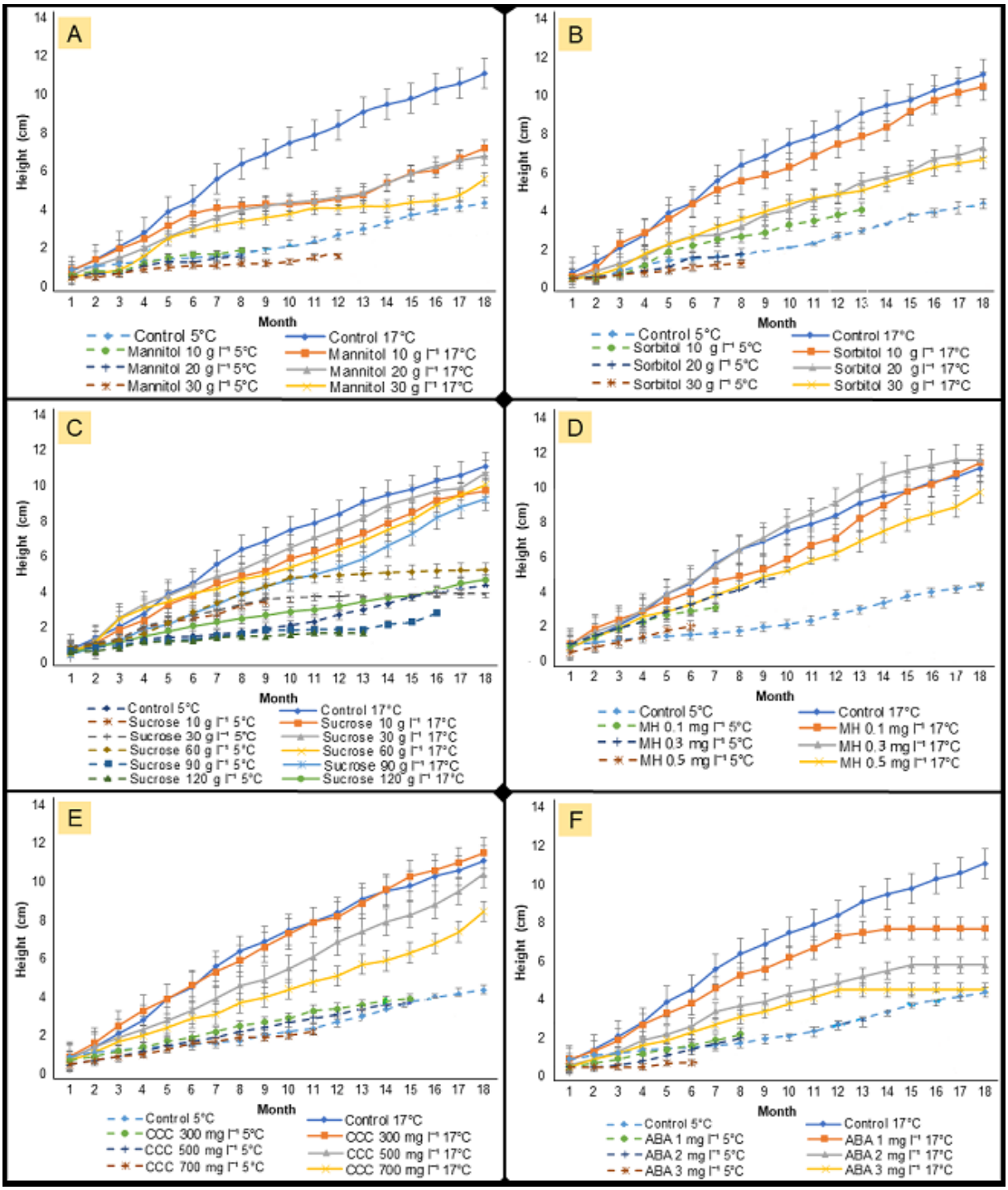

Figure 14. Effect of culture medium supplements and culture temperature on plant height during the 18-month conservation period. a. mannitol at 17 °C and 5 °C. b. sorbitol at 17 °C and 5 °C. c. ABA at 17 °C and 5 °C. d. MH at 17 °C and 5 °C. e. CCC 17 °C and 5 °C. f. sucrose at 17 °C and 5 °C). ABA=abscisic acid, CCC=chlorcholinchlorid, MH=maleic hydrazide. All values are presented as means ± standard deviation (SD).

Source: Data adopted from the published study of Hammond et al. (2019b)

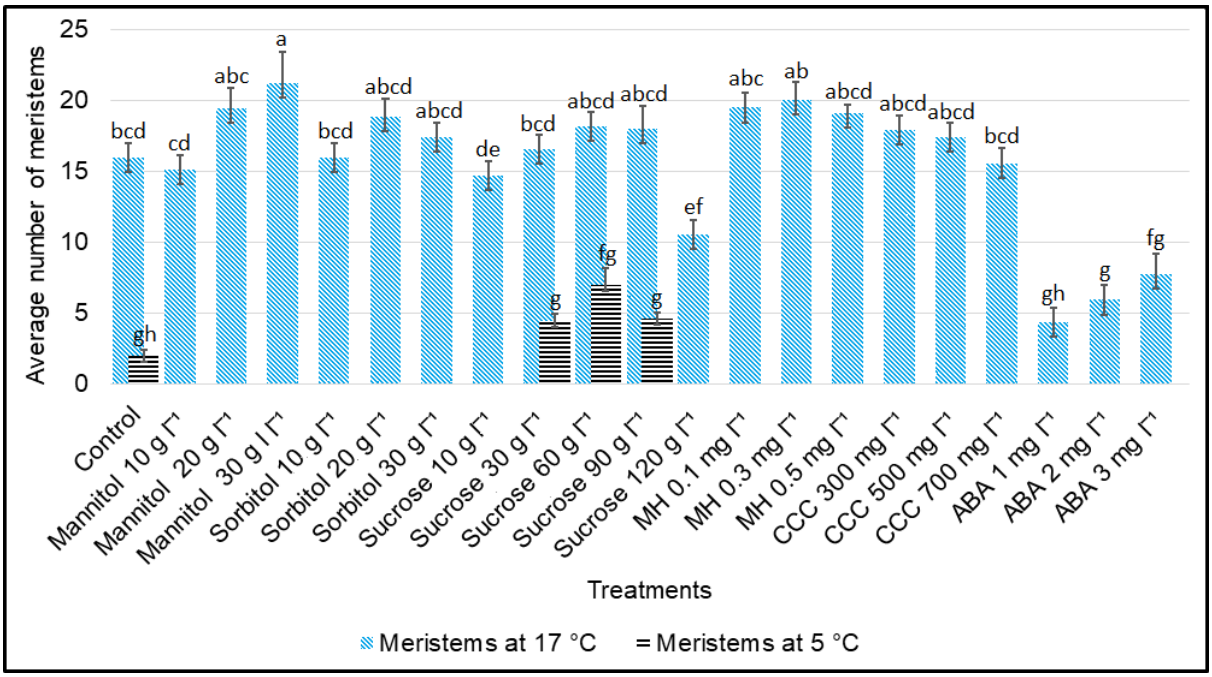

Figure 15. Effect of culture medium supplements and conservation temperatures on the average number of plant meristems in surviving explants after 18-month conservation period at 17 °C and 5 °C. ABA=abscisic acid, CCC=chlorcholinchlorid, MH=maleic hydrazide. All values are presented as means ± standard deviation (SD).

Source: Data adopted from the published study of Hammond et al. (2019b)

Mannitol and sorbitol (10-30 g l^{-1}) treatments showed similar tendencies in plant survival; at the end of the conservation period, the survival percentage of plants was 100%. All plants cultivated on media with both osmotic agents survived only in MT form. In both cases, MT formation at lower concentrations (10-20 g l^{-1}) started within the sixth month of conservation, while at higher osmotic concentration plants developed MTs two months later. In all cases, the shoot started drying within two months after MT formation, with the exception of treatment with 30 g l^{-1} mannitol, where shoots started dying within three months after MT development.

Supplementation of culture medium with sucrose showed a positive effect on conservation, providing a high survival rate at concentrations of 30, 60 and 90 g l^{-1}, with 100%, 90% and 80% survival respectively; this was not only in MT formation but also with a partial survival of shoots with developing MTs. The highest (120 g l^{-1}) and lowest (10 g l^{-1}) sucrose

concentration tested had a reduced effect on ulluco plant survival and led to survival exclusively in MT form (Fig. 16a).

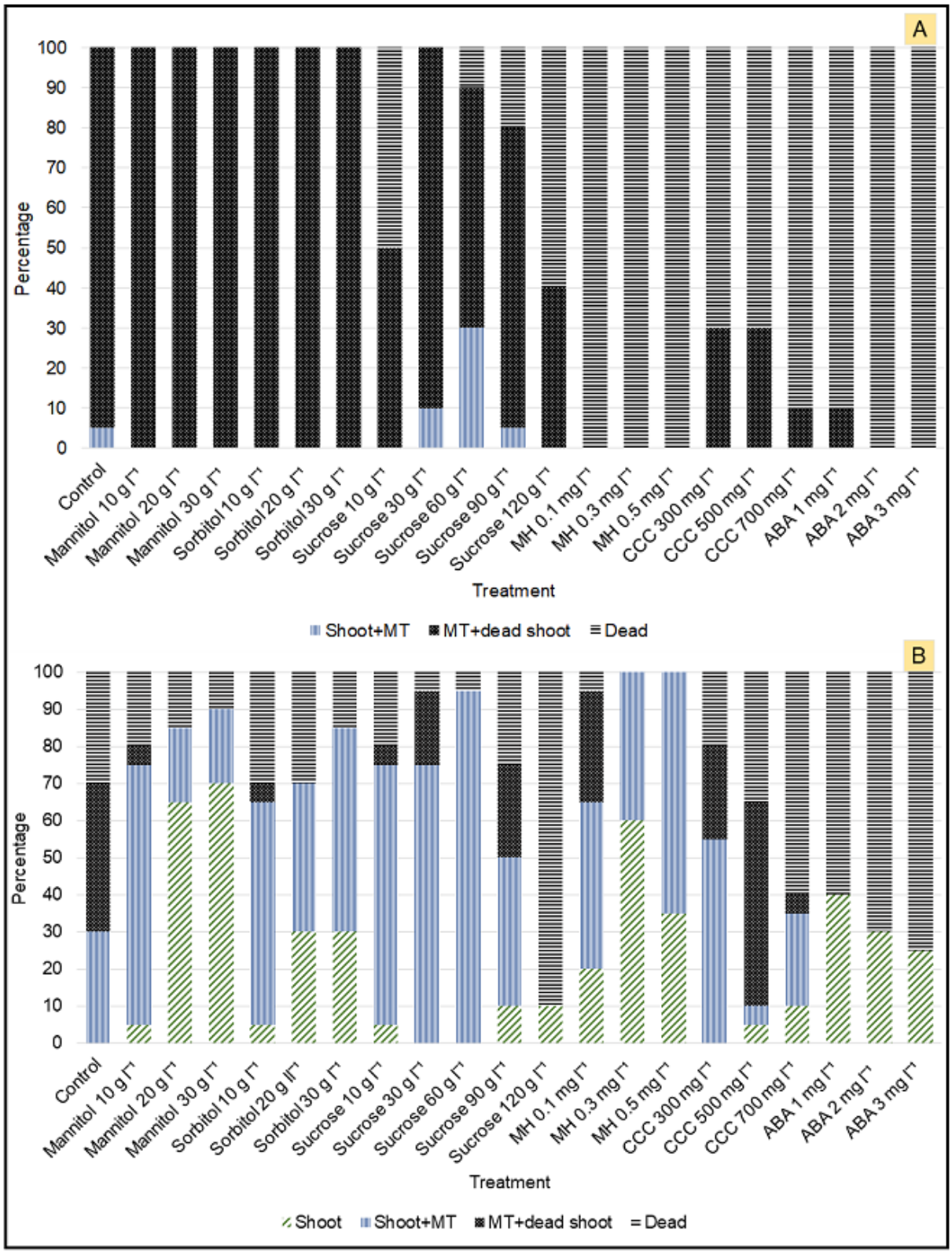

Figure 16. Effect of culture medium supplements and conservation temperature on plant survival after 18-month conservation period. a. plant survival at 5 °C. b. plant survival at 17 °C. Shoot=only live shoot without MT, Shoot+MT=dead/dried shoot+live microtuber (MT), Dead=dead shoot with no microtuber (MT), ABA=abscisic acid, CCC=chlorcholinchlorid, MH=maleic hydrazide. All values are presented as means ± standard deviation (SD).

Source: Data adopted from the published study of Hammond et al. (2019b)

MH (0.1-0.5 mg l^{-1}) addition in culture medium displayed a negative effect on ulluco plants at 5 °C, leading to poor growth and providing 0% survival in all treatments after the conservation period. During *in vitro* cultivation, the plants did not produce MTs and grew only in the form of shoots. At higher MH concentration (0.5 mg l^{-1}), shoots started dying within the first 8 months of conservation while shoots on media with lower MH concentrations (0.1-0.3 mg l^{-1}) started drying three months later.

In all CCC treatments (300-700 mg l^{-1}) the plants survived after 18 months only in the form of a dried shoot with MTs, and the survival did not exceed 30%. A similar negative effect on ulluco conservation showed ABA supplementation (1-3 mg l^{-1}) into the culture medium, leading to poor growth of the plants and their early dying at all concentrations tested.

Considering the fact that most plants under 5 °C conservation temperature displayed dwarfed growth, had minimal numbers of meristems, and plants after the development of microtubers tended to turn brown and dry, showing low potential for further conservation, these treatments were not considered optimal for ulluco plant maintenance.

6.2.2. Effect of culture medium supplements on plantlet conservation at 17°C

Ulluco conservation at 17 °C, on the other hand, led to more vigorous plant growth (height 4.0-11.6 cm) (Fig. 14a, b, c, d, e, f) and a higher number of developed meristems (4-21 meristems/plant) (Fig. 15) in comparison to plants maintained at a cultivation temperature of 5 °C. Plants of all treatments, including control, conserved at 17 °C generally displayed a higher percentage of survival in the form of plants still without MT formation, or of living shoots with MTs, indicating that at this temperature, ulluco plants could be maintained for an even longer period. In treatments where MTs developed, this process usually began in the ninth month of conservation and the main shoot started dying two or three months later.

In mannitol treatments, the height of the plants decreased with osmotic concentration (10-30 g l^{-1}), while the meristem number increased. Application of 30 g l^{-1} mannitol resulted in the highest survival percentage in the form of shoots without the formation of MT from all treatments in the experiment, namely 70%. Thus, plants after 18-month conservation on this medium displayed relatively slow growth, had numerous meristems, and mostly did not initiate MT formation and their total survival reached 90%. Similar tendencies were also recorded for sorbitol, where the highest concentration tested (30 g l^{-1}) also led both to a high total survival rate and a high proportion of plants surviving in the form of just shoots when compared to lower sorbitol concentrations. Supplementation of MH (0.3-0.5 mg l^{-1}) in the medium at 17 °C provided the best results in terms of total survival among all treatments at higher tested temperature. These conditions generally decelerated plant senescence and MT formation, resulting in a maximum survival in the form of shoots without MTs, especially at a concentration of 0.3 mg l^{-1}. Sucrose added to a medium at concentrations of 10-60 g l^{-1} gradually increased plant survival percentage as well as the number of meristems, while plant height among treatments was relatively comparable. In contrast, further increasing sucrose concentrations (120 g l^{-1}) negatively affected all measured parameters. The plants cultivated on a medium with CCC produced MTs at higher rates and, by the end of the conservation period, plant stems with MTs tended to dry, especially at 500 and 700 mg l^{-1} CCC concentrations. Application of ABA (1-3 mg l^{-1}) in culture medium provided low plant survival, not exceeding 40%. The plants did not produce tubers and they grew exclusively in the form of shoots.

Based on the results obtained from the plant storage experiment, four promising treatments for ulluco conservation were selected. Taking into consideration total survival percentage and a preferably high proportion of plants surviving in the form of shoots or living shoots with MTs, plants cultivated on media supplemented with 30 g l^{-1} mannitol, 30 g l^{-1} sorbitol, 0.3 mg l^{-1} MH and 60 g l^{-1} sucrose, all cultivated at 17 °C, were tested for regrowth ability.

6.2.3. Recovery of plantlets after conservation and *ex vitro* transfer

After 7 days of cultivation on regeneration medium, the shoots collected from the medium supplemented with 30 g l^{-1} mannitol started to regenerate first among all treatments and, within 28 days, they produced the highest number of meristems (approx. 16.33 meristems/plant). Plants from this treatment also grew significantly faster (approx. plant height 5.67 cm), produced a higher number of roots (approx. 6.38 roots/explant), and had a survival rate of 100%

at the end of the regeneration period. Plants from other treatments started to regrow on the regeneration medium 12 days (±2 days) after their transfer to this medium. The survival percentage also reached 100% in these treatments, except for plants conserved on a medium with the addition of 0.3 mg l^{-1} MH, where just 89% of plants survived. Relatively low regeneration capacity, as well as slow growth of plants from this treatment, resulted in excluding this variant from further experiments. Plants conserved on media supplemented with 30 g l^{-1} sorbitol and 60 g l^{-1} sucrose also did not reach optimal results obtained in plants from the medium with mannitol (Table 2, Fig. 17). Therefore, the conservation medium with 30 g l^{-1} mannitol was evaluated as the most suitable for ulluco conservation and subsequent plant regrowth, and the plants from this medium were used for *ex vitro* transfer.

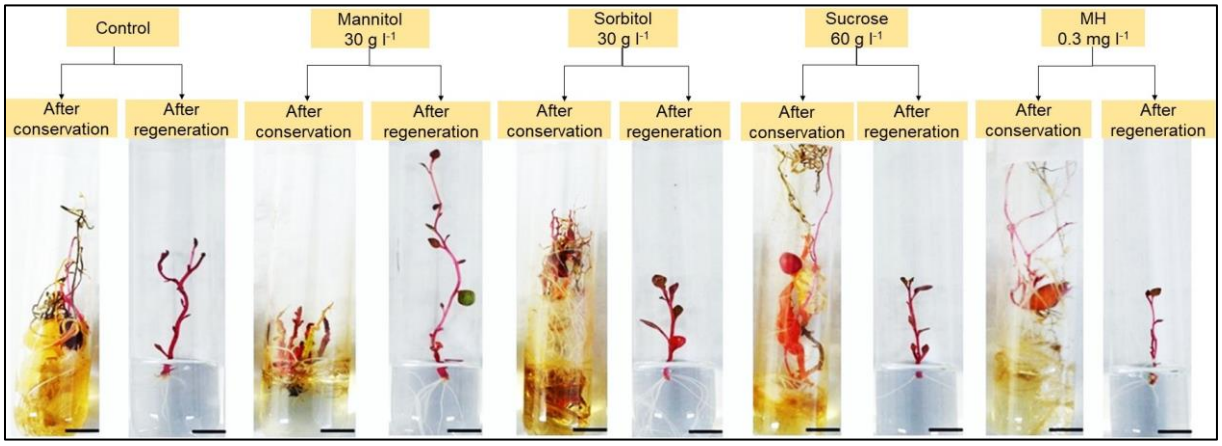

Figure 17. Plants of *Ullucus tuberosus* after 18-month conservation on half-strength MS medium supplemented with 30 g l^{-1} mannitol, 30 g l^{-1} sorbitol, 60 g l^{-1} sucrose, 0.3 mg l^{-1} MH cultivated at 17 °C (on the left) and plants after 28-day post-storage regrowth on half-strength MS medium (on the right) (*bar*=1 cm). ABA=abscisic acid, CCC=chlorcholinchlorid, MH=maleic hydrazide.

Source: Author

Shoots of this treatment rooted well on regeneration medium, and they were transferred to the greenhouse. Three weeks later, the plants began to regrow and, within further cultivation, they produced new leaves. No morphological abnormality was observed during greenhouse cultivation (Fig. 18a). The plants acclimated well and after 8 weeks of cultivation, 100% of plants survived.

6.2.4. ISSR analysis post *ex vitro* transfer

Plants originating from the 30 g l^{-1} mannitol treatment (17 °C), after their acclimation in the greenhouse, and plants from the control variant (17 °C) were subjected to ISSR analysis. Initial plant material was also included in the molecular analysis.

A total number of 1,659 amplified products were produced by the 12 ISSR markers and 79 amplification fragments were generated. Fragment sizes ranged from 200 to 1,600 bp. The number of bands per primer varied from 5 to 9, with an average of 6.6 (Table 1). All plants included in the molecular analysis had identical ISSR fingerprints and gave rise to monomorphic patterns. A representative monomorphic amplification pattern obtained with ISSR primer ‘UCB836’ is shown in Fig. 18b.

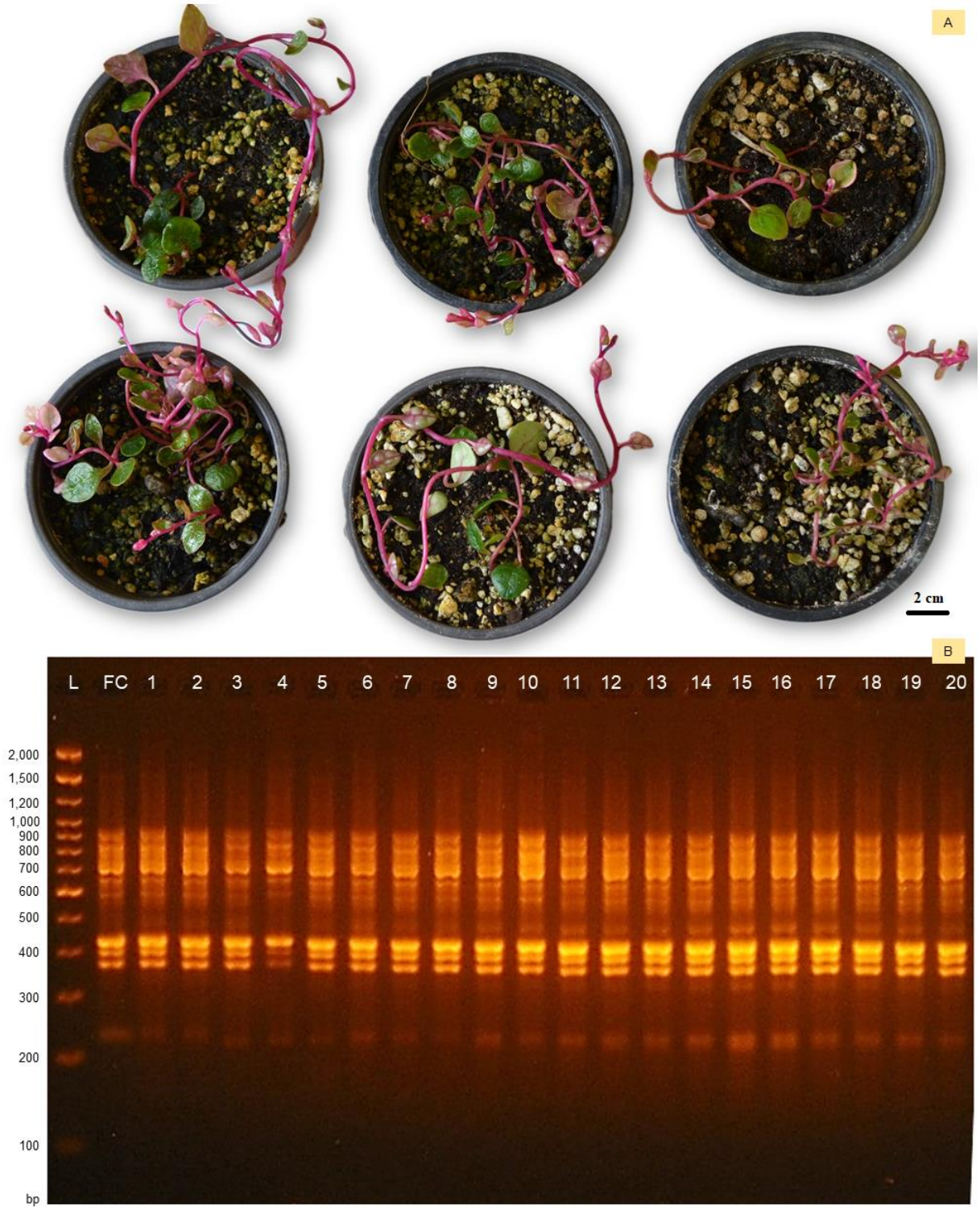

Figure 18. *Ullucus tuberosus* plantlets transferred *ex vitro* after conservation and results of their molecular analysis. a. ulluco plants 8 weeks after *ex vitro* transfer to the greenhouse (*bar*=2 cm). b. ISSR profile of ulluco field control (initial plant material), control plants from the

experiment (1-10) and plants conserved on medium supplemented with 30 g l^{-1} mannitol at 17 °C (11-20), using primer 'UCB836'. L=ladder, FC=field control, bp=base pair.)

Source: Author

Table 2. Effect of culture medium supplements on *in vitro* plant regrowth after 28 days of cultivation on regeneration medium

Treatment	**Height (cm)**	**Nº of meristems**	**Nº of roots**	**Survival %**
Control	3.12±0.44b	14 ±1.6ab	4.63±0.95ab	100
Mannitol 30 g l^{-1}	5.67±0.34a	16.±0.9a	6.38±0.74a	100
Sucrose 60 g l^{-1}	3.32±0.34b	12±0.7b	3.42±0.72b	100
Sorbitol 30 g l^{-1}	3.12±0.35b	14±1.1ab	4.76±0.77ab	100
MH 0.3 mg l^{-1}	2.80±0.39b	13±1.2ab	2.42±0.84b	89

*Mean values in a column, followed by different letters, are significantly different according to Tukey's HSD test ($P \leq 0.05$)

7. Discussion

7.1. *Smallanthus sonchifolius*

7.1.1. Effect of PVS2 and PVS3 on the shoot tip survival

When developing a vitrification-based cryopreservation method for plant tissues, the composition of vitrification solution, duration and temperature of exposure to the solution, and cooling and rewarming rates are five key elements that influence the success of the cryopreservation protocol (Kim et al. 2006b; Yin et al. 2014; Liu et al. 2017). Similarly, pre-culture and post thawing conditions of the plant tissue are considered crucial for successful long-term conservation (Niino et al. 2003; Harding et al. 2009).

Within our study on yacon, we focused mainly on the effects of PVS2 and PVS3 treatment duration and emphasized the post thawing conditions by testing two different regrowth media before and after cryopreservation.

Our study has shown that both PVS2 and PVS3 vitrification methods are effective for the cryopreservation of yacon. Overall, PVS2 60 min and PVS3 60 min treatments showed the highest shoot tip survival percentages in the optimization phase using the ECU 41 genotype. These findings show that even though PVS2 and PVS3 treatments are different in their temperature of implementation (PVS2 at 0 °C and PVS3 at 22 °C) and composition (different types and concentrations of cryoprotectants), they seemed to have similar cryoprotectant function in yacon, reaching similar rates of dehydration. The similarities in the desiccation rates between both treatments at similar time duration may be due to the presence of DMSO in PVS2. This solvent is widely considered as the main penetrating colligative cryoprotectant which causes higher desiccation rates. PVS3 is composed of none penetrating substances, hence the desiccation rate of this treatment is slower. Therefore, the shoot tip exposure of PVS2 treatments is implemented at 0 °C. This reduces or controls the dehydration rates of PVS2 during its application. Therefore, the presence of the penetrating substance DMSO in the PVS2 solution may be a possible justification as to why at 0 °C PVS2 60 min treatment duration reaches similar dehydration rates as those of PVS3 60 min implemented at 22 °C in yacon shoot tips. The presence of DMSO not only accelerates the desiccation rates but may also lead to cytotoxicity due to its penetrating properties. The possible toxic effects of the solution may compromise the viability of the plant cell; hence, the treatment exposure time and application temperature are fundamental parameters that must always be optimized when using PVS2 treatment (Bekheet et at. 2020; Kim et al. 2009; Martinez et al. 1998).

In addition to DMSO, PVS2 also includes ethylene glycol. Both are more membrane permeable than the components in PVS3 (glycerol and sucrose), as reported by Volk et al. (2017) who assessed the osmotic responses of sweet potato using PVS2 and PVS3 vitrification treatments. After treatment with PVS2 and PVS3 plant vitrification solutions, the results of their study showed that, contrary to our findings in yacon, the optimal dehydration time for PVS3 and PVS2 is different with PVS3 treatments requiring up to 3 hours.

PVS2 and PVS3 are among the most often used plant vitrification solutions and are applied to a wide range of plant species. However, the effectiveness of the protocol is very variable and strongly species-dependent and should, therefore, be optimized accordingly (Sakai and Engelmann 2007; Reed 2008). For example, cryopreservation protocols using the PVS2 method has been developed for 1,028 *Solanum* accessions at 50 min treatment time duration with a recovery rate ranging from 34 to 70% (Vollmer et al. 2016); for *Ribes aureum* cv. Red Lake at 20 min PVS2 treatment with a 30% recovery rate post cryopreservation (Reed et al. 2001b); for *Lycopersicon esculentum* Mill. at 30 min PVS2 treatment duration and a recovery rate ranging from 60 and 70% (Coste et al. 2015); for *Vitis* sp. at 20 min treatment duration and recovery rates ranging from 43 to 59% (Bi et al. 2018); and for *Ullucus tuberosus* Cal. and *Oxalis tuberosa* Mol. at 60 min PVS2 treatment time duration and a recovery rate reaching 35 and 15%, respectively, after cryopreservation (Sanchez et al. 2011). PVS3 method, on the other hand, has been optimized for species such as *Allium cepa* var. Aggregatum at 3 hours treatment duration, where this method resulted in 58% recovery rate (Wang et al. 2020), and *Lotus tenuis* INTA Pampa with at PVS3 treatment time duration of 1 hour and recovery rate post cryopreservation, reaching 79% (Espasandin et al. 2019). In the case of yacon, we found that both methods proved to be effective at the same treatment time duration (60 min) with up to a 73% recovery rate post cryopreservation.

As in the present study, a comparison of PVS2 and PVS3 vitrification solution was executed by Wang et al. (2020) who tested both treatments for shoot tip cryopreservation of shallot (*Allium cepa* var. Aggregatum). The findings also showed that in the case of PVS2 treatment, 40-60 min at 0 °C provided the highest rate of shoot tip survival (40%) among the treatments. This corroborates our findings in yacon where PVS2 60 min also provided the highest shoot tip survival rates; however, in the case of yacon, the rates were exponentially higher with an 83% survival (PVS2 60 min treatment). In the case of PVS3, Wang et al. (2020) reported that this vitrification solution was most effective in the survival of shallot after a 3-hour treatment duration at 22 °C with a 72% survival rate. In contrast, our findings showed that

the PVS2 60 min treatment in yacon had similar effectiveness as the PVS3 applied for 60 min with a survival rate of up to 93.3 and 86.7% respectively, showing that both methods can successfully be used for the cryopreservation of this species. Similar findings were also reported by Da Silva Cordeiro et al. (2015) for *Cleome rosea* Vahl, where both PVS2 and PVS3 methods proved to be effective for this species post cryopreservation, reaching high survival percentages of 97 and 70%, respectively.

As 60 min treatment time duration applied for PVS2 at 0 °C and PVS3 at 22 °C proved to be the overall best treatments in the optimization face using the ECU 41 clone, the treatments were then applied to the other yacon genotypes (BOL 22, BOL23, PER 12 and PER14). Our results showed both treatments provided high survival rates ranging from 86.7%-90.2% in the case of PVS2 and 79.7%-94.1% in the case of PVS3 with no significant differences in survival rates within clones and the PVS treatments showing that both methods are applicable as cryoprotectants for yacon cryopreservation. Similar findings were reported by Senula et al. (2018) who tested both PVS2 and PVS3 treatments on various *Mentha* spp. species, in their study they also found that both PVS2 and PVS3 can be used as cryoprotectants for *Mentha* spp. However, contrary to our findings in yacon which showed that both cryoprotectants provided comparable result, they reported that higher regrowth in *Mentha* spp. can be achieved when using PVS3 instead of PVS2. Ellis et al. (2006) also reported similar findings in garlic (*Allium sativum*), their study showed that both PVS2 and PVS3 treatments applied to various accession of garlic both ensuring more than 40% survival and regrowth. This shows that even though PVS treatments are strongly variable and species-dependent, optimization and assessment is of paramount importance as it can determine whether or not the cryoprotectants are applicable one, more or all accessions of the targeted species.

7.1.2. Regrowth post cryopreservation

In our study, the most frequent type of regrowth post cryopreservation for all genotypes was in the form of FGN; however, morphological abnormalities such as HH was also observed. Hyperhydration is a morphological and physiological disorder that affects plants regenerated under *in vitro* conditions (Fauguel et al. 2008). This disorder is associated with excessive hydration and abnormal shoot morphogenesis such as the glassy water-soaked appearance of shoots and leaves, its occurrence is also considered to be a response to stress, rich nutrient media or high relative humidity (Fauguel et al. 2008; Baker et al. 1999; Ziv 1991). Within the present research, HH was detected in the regenerated control shoot tips and shoot tips regenerated after

cryopreservation. Additionally, shoot tips showing signs of HH before and after cryopreservation were never able to recover and grown into FGN plants. Hyperhydration was mainly observed on MS media supplemented by 0.1 mg l^{-1} BA, regardless of exposure to LN and PVS treatments in the optimization phase. This shows that the form of regrowth was mainly influenced by the composition of recovery media rather than the vitrification solutions. Therefore, only MS without 0.1 mg l^{-1} BA was used for shoot tip regrowth post cryopreservation when applying the optimal PVS2 and PVS3 treatment time duration to the other yacon genotypes (BOL 22, BOL23, PER 12 and PER14). In the optimization phase using the ECU 41 genotype we also found that the vitrification solution influenced the overall survival of yacon shoot tips post cryopreservation because the variation in treatment duration caused different rates of dehydration, hence the most optimal PVS2 and PVS3 dehydration treatments were applied to the other yacon genotypes in other to obtain high survival and recovery rates post cryopreservation.

Da Silva Cordeiro et al. (2015) also investigated the effects of PVS2 and PVS3 as vitrification solutions for the cryopreservation of *Cleome rosea*, and MS medium supplemented various concentration of BA (0-0.5 mg l^{-1}) as recovery medium after cryopreservation. Their findings showed that for both PVSs the highest recovery percentage (100%) post cryopreservation was obtained in media supplemented either with 0.10 or 0.5 mg l^{-1} BA, while MS media without BA proved to be inefficient and only provided a maximum regrowth of 33%. These results are not in accordance with our study, in which we found that MS medium without the presence of BA proved to be optimal for the regrowth of yacon shoot tips post cryopreservation providing a 73% FGN regrowth rate in both PVS2 and PVS3 60 min treatment. Da Silva Cordeiro et al. (2015) also found that *Cleome rosea* shoot tips cultivated on MS media supplemented with BA formed morphological abnormalities, such as callus at the base of the regenerated plantlets after cryopreservation, which corroborates with our study in yacon. Comparable findings were reported in *Alnus glutinosa* (L.) Gaertn. where PVS2 was tested to develop a cryopreservation protocol for this species. A 50% regrowth percentage was obtained on MS regrowth medium supplemented with BA; however, the shoot tips that did not regenerate into FGN plantlets produced non-proliferating calli (San Jose et al. 2014).

As in the present study, similar results were also reported by Lambardi et al. (2000) in poplar (*Populus alba* L.) where the addition of 1.5 mg l^{-1} BA in MS regrowth medium led to callus formation and mainly HH of plants after cryopreservation. In the mentioned study, MS medium without supplements proved to be more effective for shoot tip regrowth. Medina et al.

(2010) also tested PVS2 at 0 °C for osmotic vitrification and MS with and without BA supplement as recovery media for *Thymus moroderi* Pau ex Martínez. In their study, it was found that PVS2 at 0 °C for 30 min in combination with one-week cultivation of shoot tips on MS medium supplemented with 0.06 mg l^{-1} BA led to the highest recovery rates after cryopreservation. However, prolonged cultivation of plants on recovery media and supplementation of media with BA concentrations above 0.2 mg l^{-1} BA resulted in high rates of HH. On the other hand, they found that MS without the addition of BA was not as effective showing that the use of a low concentration of BA could boost the post cryopreservation recovery rates in the case of *T. moroderi.*

This shows that the regrowth media influences the quality of the recovered plantlets post cryopreservation and the composition of the culture medium must be optimized for each species.

7.2. *Ullucus tuberosus*

Slow-growth is one of the most successful *in vitro* techniques for medium-term germplasm conservation (Peng et al. 2015), enabling plant storage for a prolonged period without plant manipulation while maintaining plant resources readily available (Cruz-Cruz et al. 2013). In our study, the effect of cultivation temperature and culture medium supplements on ulluco survival and plant re-growth after 18-month conservation was investigated.

7.2.1. Effect of cultivation temperature

Cold during storage has a significant impact on a tissue condition and it is known to increase the survival time of *in vitro* cultures (Peng et al. 2015; Kaminska et al. 2018). In our study, a significant reduction in plant growth at 5 °C was confirmed. However, overall, every growth inhibitor treatment group displayed superior results at 17 °C compared to 5 °C, despite ulluco's mountainous origin and its adaptation to lower temperatures. Many studies on slow-growth conservation show that optimal temperature is strongly species-dependent and very variable; for example, shoot cultures of *Prunus canescens* x *P. cerasus* were successfully maintained for 16 months at 4 °C (Ozudogru et al. 2017), *Tetrastigma hemsleyanum* microplants survived for 10 months at 10 °C (Peng et al. 2015), while shoot cultures of *Vanilla planifolia* were conserved at 24 °C for 6 months (Bello-Bello et al. 2015). This comparison shows that it is highly advisable to experimentally optimize conservation protocols using a range of various temperatures.

7.2.2. Effect of culture medium supplements on the medium-term conservation

Among medium supplements, the osmotically active compound mannitol at concentration 30 g l^{-1}, in combination with 17 °C conservation temperature, led to a high plant survival percentage with a major rate of plants surviving in the form of shoots without microtubers, indicating the potential of this treatment for use in an even longer conservation period than 18 months. The development of microtubers on shoots was associated with senescence as it was observed that this phase is followed by early drying of the plant and, therefore, survival in the form of a shoot without microtuber was considered superior. Mannitol addition to the medium led to reduced plant growth, confirming that mannitol, perceived by cells, acts as a chemical signal, suppressing growth (Steinitz 1999) while being metabolically inert (Yaseen et al. 2013). Our results corroborate those reported by Ozudogru et al. (2017) in cherry rootstock ´Gisela 5´, where mannitol inclusion in the storage medium was effective at reducing the metabolic activity of the shoot cultures during the storage period. However, in *Cannabis sativa*, mannitol considerably decreased the survival percentage as well as the plant re-growth ability of nodal cultures (Lata et al. 2012), proving not to be a universal medium supplement for the conservation of all species.

In our study, comparable to mannitol, sorbitol at 17 °C provided promising results for ulluco conservation and subsequent plant regrowth, though mannitol provided both slightly better plant survival and a higher rate of plants surviving without microtuber formation. Comparison between mannitol and sorbitol for conservation was also carried out in potato (Gopal and Chauhan 2010), resulting in 58% plant survival after 18 months with 40 g l^{-1} sorbitol compared to various mannitol treatments where just 30-48% plants survived. In *Asparagus racemosus* (Thakur et al. 2015) shoot culture, 100% of plants survived after a 6-month conservation period using 20 g l^{-1} sorbitol or mannitol as osmotics, both in combination with a reduced concentration of sucrose (15 g l^{-1}).

The effect on ulluco conservation of various sucrose concentrations in culture media was also investigated in our study, resulting in a high survival rate after the conservation at 17 °C using only 30 or 60 g l^{-1}; however, all plants within these treatments developed microtubers, indicating the end of vegetation period was approaching. In general, hydrolysis of sucrose in the culture medium produces high concentrations of reducing sugars (glucose and fructose), speeding up cell division and consequently leading to an increase of the volume of cultured tissues (Gurel and Gulsen 1998). This indicates that increasing sucrose concentration to some point might be beneficial for plant growth. However, hydrolysis of sucrose may cause a

lowering of the pH in the medium, limiting uptake of nutrients by the plantlets (Rahman et al. 2010; Leifert et al. 1992). Deceleration of plant growth in our study could therefore be associated with sucrose addition at higher than optimal concentration for plant growth. This could explain less intensive growth after the addition of 90 g l^{-1} sucrose and an extreme growth-inhibiting effect of 120 g l^{-1} sucrose, resulting in the death of the plants. Our results correspond to those of da Silva (2004), where the effect of various levels of sucrose on the morphogenic response in *Dendrathema grandiflora* was investigated and it was found that a sucrose concentration higher than 60 g l^{-1} was inhibitory to shoot development.

MH as a culture medium supplement efficiently slowed down ulluco plant growth and provided the highest total survival among all treatments at 17 °C. These results confirm that MH acts as a growth regulator, inhibiting cell division by mitotic disruption (Venezian et al. 2017), and as an anti-auxin and anti-gibberellin (Hoffman and Parups 1964). Probably due to its inhibiting activity (Hu et al. 2016), the re-growth ability of plants after ulluco conservation on MH-medium was relatively low. By contrast, in *Tetrastigma hemsleyanum* microplants (Peng et al. 2015), 0.2 mg l^{-1} MH was successfully used for 10-month conservation, and microplants preserved on MH recovered normally. In the mentioned study, growth inhibitors ABA and CCC were tested for *T. hemsleyanum* conservation as well, providing lower survival percentages compared to MH treatments, and browning of plants in the case of ABA and leaf etiolation after CCC-conservation. Our study corroborates these results, as CCC and ABA supplementation led to low survival percentages and weak plant growth with a low number of meristems, especially for ABA treatments.

7.2.3. Effect of the slow-growth conservation on genetic fidelity

Tuberous crops are usually maintained in gene banks as *in vitro* propagated microplants under disease-free tissue culture conditions (Gopal et al. 2002). Vegetative propagation ensures their genetic integrity; however, some changes may occur as a result of somaclonal variation among *in vitro* plants. Thus, in our study, plants after conservation were subjected to ISSR analysis, which detected no genetic variation among *in vitro* regenerants.

Genetic stability assessment using molecular markers has recently been considered as a crucial approach to evaluate true-to-typeness of meristem-derived microplants after their conservation; for example, genetic fidelity of *Tetrastigma hemsleyanum* plants after 10-month conservation was confirmed using ISSR and sequence-related amplified polymorphism (SRAP) markers (Peng et al. 2015), in *Curcuma longa*, periodical ISSR and random amplified

polymorphic DNA (RAPD) monitoring of plants conserved for 7 years with 90-day sub-cultures, revealed no variation (Nayak et al. 2011), plants regenerated from artificial seeds containing shoot tips, and conserved for 12 months, displayed no genetic variation as confirmed by RAPD (Kaminska et al. 2018), while in *Pistacia lentiscus* plants after 6-month conservation at 4 °C, genetic variation was detected using amplified fragment length polymorphism (AFLP) and inter retrotransposon amplified polymorphism (IRAP) marker systems (Koc et al. 2014).

8. Conclusion

The present study aimed at testing the efficiency of PVS2 and PVS3 osmotic dehydration methods to develop a long-term preservation protocol for yacon, as well as to determine the optimal medium for post cryopreservation regrowth using full-strength MS media with or without 0.1 mg l^{-1} BA. The results in the optimization phase using the ECU 41 genotype proved the feasibility of both PVS2 and PVS3 plant vitrification techniques for the long-term storage of yacon. With 60 min treatment time duration at difference temperatures (at 0 °C PVS2 and 22 °C PVS3) in combination with MS medium without 0.1 mg l^{-1} BA supplementation as recovery media being optimal for yacon cryopreservation providing the highest rate of shoot tip regrowth post thawing with minimal morphological abnormalities.

When applied to the other four yacon genotyped originated from Bolivia and Peru (BOL 22, BOL 23, PER 12 and PER 14) both methods were effective in ensuring high survival and recovery rates of these genotypes post cryopreservation.

Despite comparable results of these two PVS treatments, the use of PVS3 treatment, unlike PVS2, does not contain DMSO which may lead to cytotoxicity and thus, can be considered as most suitable.

The high survival and regrowth rates of these five yacon genotypes after cryopreservation using both methods provide a promising alternative to safeguard the yacon accessions currently being preserved by traditional methods.

The study also aimed at optimizing a medium-term *in vitro* conservation protocol for ulluco by testing half-strength MS medium supplemented with either mannitol (10-30 g l^{-1}), sorbitol (10-30 g l^{-1}), sucrose (10-120 g l^{-1}), chlorcholinchlorid (CCC; 300-700 mg l^{-1}), abscisic acid (ABA; 1-3 mg l^{-1}), or maleic hydrazide (MH; 0.1-0.5 mg l^{-1}) as osmotic agents or growth inhibitors and two cultivation temperatures either at 5 °C or 17 °C.

The study results showed the limiting temperature for growth of tubers, i.e. 5 °C was not effective in increasing the efficiency of the slow-growth conservation conditions for ulluco. This temperature had an adverse effect on the survival of the conserved plants leading to senescence. On the contrary, 17 °C proved to be the more suitable cultivation temperature providing very promising results in terms of ulluco survival post conservation. The most suitable treatment of the tested growth inhibitors and osmotic agents for ulluco *in vitro* conservation by slow-growth proved to be half-strength MS medium supplemented by mannitol at 30 g l^{-1} concentration and maintained at a cultivation temperature of 17 °C with a 16/8 light/dark regime. This treatment enabled 18-month slow-growth conservation while

maintaining the vitality and genetic stability of the plant. The 30 g l^{-1} sorbitol treatment at 17 °C, the 0.3 mg l^{-1} MH at 17 °C and the 60 g l^{-1} sucrose at 17 °C also provided promising results in terms of reducing the growth of ulluco, however, they were not as effective as the mannitol 30 g l^{-1} treatment. The other treatments including all the chlorcholinchlorid and abscisic acid treatments were not effective in reducing the growth of plantlets and did show potential for the medium-term conservation of ulluco.

The development of reliable *in vitro* conservation protocols for both ulluco and yacon will contribute to the general and specific knowledge of these species and will provide suitable biotechnological tools for gene banks maintaining these species.

9. Practical use of the thesis research results

The results obtained in the present research on yacon are of vital importance for the long-term storage of the species genetic material, as the protocol developed ensures high survival and regrowth rates post cryopreservation as shown in the five tested yacon genotypes (BOL 22, BOL23, PER 12 and PER14). The developed cryopreservation protocol for yacon also provides valuable technical supports for a promising alternative to safeguard yacon germplasm for the long-term by cryo-banking using either PVS2 or PVS3 vitrification solution. Due to the presence of HH in surviving plantlets, further research should be carried out to determine what goes on in the yacon shoot tips at an anatomical level and to further understand why there was the occurrence of HH and contribute to how it can be prevented.

In the case of ulluco, the protocol developed within the present study represents a reliable conservation system, suitable for maintaining ulluco plantlets under slow-growth conditions, with minimal risk of somaclonal variation occurrence and ensuring the immediate availability of healthy plant material free of pathogens throughout the entire year regardless of the climatic season. The developed slow-growth protocol within our research may also be used for longer conservation of ulluco (longer than 18 months), as our research showed that the 18-month old conserved plantlets on the optimal conservation media (mannitol at 30 g l^{-1}) cultivated at the most suitable temperature (17 °C with a 16/8 light/dark regime) led to high survival rates mainly in the form of live shoots, meaning that the plants may be able to be conserved for a longer time until they produce MT and reach senescence.

Within the Andean region, the ARTCs are mainly cultivated by ancestral methods of cultivation that also contribute to preserving the diversity on-farm conservation of the ARTCs (Luziatelli et al. 2020). However, the farmer's collections are at risk of genetic erosion which is caused by various factors including habitat destruction, increased human population, land degradation, environmental changes, extreme cultivation conditions (grown at high altitudes under extremely difficult conditions of drought, freezing temperatures, and UV exposure) government subsidies to plantation crops and the introduction of modern crop varieties (Luziatelli et al. 2020, Rao & Sthapit 2013). Hence, it is necessary to complement the on-farm conservation method with *ex situ* conservation approaches (Luziatelli et al. 2020, Rao & Sthapit 2013; Panta et al. 1999; Tay 2013).

In the case of yacon, within the Andean region other than the traditional method of conservation (on-farm collections), and the *in vitro* slow-growth protocols reported by Skalova et al. (2013) and Panta et al. (1999) for medium-terms conservation. There is no conservation

strategy that ensures the long-term conservation of the species genetic resources. Therefore, the protocol developed within the present research is of vital importance as it provides valuable insights on developing a cryobank of this species to ensure its long-term conservation.

For ulluco on the other hand, in addition to the traditional conservation methods used in the Andean region (on-farm or in fields collections) cryopreservation has also been developed for the long-term conservation of the species as reported by Sanchez et al. (2011) who used PVS2 droplet-vitrification method to develop their protocol. Zamecnikova et al. (2011) also reported the development of a cryopreservation protocol for ulluco using the preparation of shoot tips with sucrose and dehydration pre-treatment, as well as Arizaga et al. (2017) who developed a cryopreservation protocol for ulluco using the D cryo-plate system. However, until now, there was no effective medium-term *in vitro* conservation technique that can serve as a complementary method to maintain the vitality of the species, free from pest and diseases, and provide plant material immediately available at and at a lower cost than in field preservation (Tyagi et al. 2006; Kulus 2018). Therefore, the medium-term conservation protocol developed within our research not only serves as a complementary conservation method to the already existing conservation techniques for ulluco but also serves as a cost-effective *in vitro* cultivation technique that ensures the immediate availability of high-quality plant material free from pest and diseases for both researchers and farmers alike.

10. References

Altieri M, Merrick L. 1998. Agroecology and *in situ* conservation of native crop diversity in the third world. Biodivers. Conserv. 361p.

Arbelaez PE. 1956. Plantas utiles de Colombia. Bogota: Libreria Colombiana. 141p. (Italian).

Arbizu C. 2004. Clasificación y morfología (Capítulo 2). En: López G & M Hermann (eds.). El cultivo del ulluco en la Sierra Central del Perú. Serie: Conservación y uso de la biodiversidad de raíces y tubérculos andinos: Una década de investigación para el desarrollo (1993-2003). No.3. Centro Internacional de la Papa, Universidad Nacional del Centro, Instituto Vida en los Andes, Universidad Nacional Agraria La Molina, Agencia Suiza para el Desarrollo y la Cooperación. Lima, Perú. 5-11p.

Arbizu C. 2009. *Ex situ* conservation of underutilized Andean roots and tubers. The International Potato Center. 15th Triennial ISTRC Symposium.

Arizaga MV, Yamamoto SI, Tanaka D, Fukui K, Nohara N, Nishikawa T, Watanabe K, Niino T. 2017. Cryopreservation of *in vitro* shoot tips of ulluco (*Ullucus tuberosus* cal.) using D cryo-plate method. CryoLetters. 38:419-427.

Ashmore SE. 1997. Status report on the development and application of *in vitro* techniques for the conservation and use of plant genetic resources. International Plant Genetic Resources Institute, Rome.

Baker CM, Muñoz-Fernandez N, Carter CD. 1999. Improved shoot development and rooting from mature cotyledons of sunflower. Plant Cell Tiss. Organ Cult. 58: 39-49.

Barrera V, Espinosa P, Tapia C, Monteros A y F Valverde. 2004. Caracterización de las raíces y tubérculos andinos en la ecoregión andina del Ecuador (Capítulo 1). En: Barrera V, Tapia C, A Monteros (eds). Raíces y Tubérculos Andinos: Alternativas para el uso sostenible en el Ecuador. Serie: Conservación y uso de la biodiversidad de raíces y tubérculos andinos: Una década de investigación para el desarrollo (1993-2003). No.4. Instituto Nacional Autónomo de Investigaciones Agropecuarias, Centro Internacional de la Papa, Agencia Suiza para el Desarrollo y la Cooperación. Quito, Ecuador - Lima, Perú. 3-30p.

Bekheet SA, Sota V, El-Shabrawi HM, El-Minisy AM. 2020. Cryopreservation of shoot apices and callus cultures of globe artichoke using vitrification method. J. Genet. Eng. Biotechnol. 18:2.

Bello-Bello JJ, Garcia-Garcia GG, Iglesias-Andreu L. 2015. *In vitro* conservation of vanilla (*Vanilla planifolia* Jacks) under slow growth conditions. Rev. Fitotec Mex. 38:165-171.

Bellon MR, Pham JL, Jackson MT. 1997. Genetic conservation: a role for rice farmers. In: Maxted N, Ford-Lloyd BV, Hawkes JG (eds) Plant Genet. Resour. Chapman and Hall, London. 263-289p.

Benson EE, Harding K, Debouck D, Dumet D, Escobar R, Mafla G, Panis B, Panta A, Tay D, Van den houwe I, Roux N. 2011. Refinement and standardization of storage procedures for clonal crops - Global Public Goods Phase 2: Part I. Project landscape and general status of clonal crop in vitro conservation technologies. System-wide Genetic Resources Programme, Rome, Italy.

Benson EE, Reed BM, Brennan RM, Clacher KA, Ross DA. 1996. Use of thermal analysis in the evaluation of cryopreservation protocols for *Ribes nigrum* L. germplasm. CryoLetters. 17:347-362.

Berk Z. 2018. Freeze drying (lyophilization) and freeze concentration. In Berk Z. (ed) Food Process Engineering and Technology. J Food Sci Technol. 3:567-581.

Bessembinder JJE, Staritsky G, Zandvoort EA. 1993. Long-term in vitro storage of *Colocasia esculenta* under minimal growth conditions. Plant Cell Tiss Organ Cult. 33:121-127.

Bhatia S, Sharma K. 2015. Technical Glitches in Micropropagation. In: Modern Applications of Plant Biotechnology in Pharmaceutical Sciences. 452p.

Bi WL, Hao XY, Cui ZH, Pathirana R, Volk GM, Wang QC. 2018. Shoot tip cryotherapy for efficient eradication of grapevine leafroll-associated virus-3 from diseased grapevine *in vitro* plants. Ann. Appl. Biol. 173:261-270.

Bordallo PN, Silva DH, Maria J, Cruz CD, Fontes EP. 2006. Somaclonal variation on *in vitro* callus culture potato cultivars. Hortic. Bras. 22:300-304.

Bradai F, Sanchez-Romero C, Martín C. 2019. Somaclonal variation in olive (*Olea europaea* L.) plants regenerated via somatic embryogenesis: Influence of genotype and culture age on genetic stability. Sci. Hortic. 251:260-266.

Brush SB. 2010. *In situ* conservation of landraces in centers of crop diversity. Crop Science. 35:346-354.

Busch JM, Sangketkit C, Savage GP, Martin RJ, Halloy S, Deo B. 2000. Nutritional analysis and sensory evaluation of ulluco (*Ullucus tuberosus* Loz) grown in New Zealand. J Sci Food Agri. 80:2231-2240.

Calvino M. 1940. A new plant *Polymnia edulis* for forage or alcohol. Industria Saccharifera Italiana. 33:95-98.

Campos D, Betalleluz-Pallardel I, Chirinos R, Aguilar-Galvez A, Noratto G, Pedreschi R. 2012. Prebiotic effects of yacon (*Smallanthus sonchifolius* Poepp. & Endl), a source of fructooligosaccharides and phenolic compounds with antioxidant activity. Food Chemistry. 135:1592-1599.

Campos D, Noratto G, Chirinos R, Arbizu C, Roca W, Cisneros-Zevallos L. 2006. Antioxidant capacity and secondary metabolites in four species of Andean tuber crops: Native potato (*Solanum sp*.), mashua (*Tropaeolum tuberosum* Ruiz & Pavón), Oca (*Oxalis tuberosa* Molina) and ulluco (*Ullucus tuberosus* Caldas). J. Sci. Food Agri. 86:1481-1488.

Chaquilla G. 1997. Obtencion de azucar a partir de yacon (*Polymnia sonchifolia*) y su potencial. IX Congreso Internacional de Cultivos Andinos. Cusco, Peru. (Spanish). 56p.

Chauhan R, Singh V, Quraishi A. 2019. In Vitro Conservation Through Slow-Growth Storage. In: Faisal M, Alatar A. (eds) Synthetic Seeds. Springer, Cham. 397-416pp.

Coste A, Suteu D, Bacila I, Deliu C, Valimareanu S, Halmagyi A. 2015. Genetic integrity assessment of cryopreserved tomato (*Lycopersicon esculentum* Mill.) genotypes. Turk. J. Biol. 39:638-648.

Cruz LG, Gomez ZR. 1995. Conservacion de germoplasma *in vitro*: Experiencias en Oca, ulluco y mashua en Ayacucho. 1. Congreso Peruano de Cultivos Andinos. Ayacucho, Peru.

Cruz-Cruz CA, Gonzalez-Arnao MT, Engelmann F. 2013. Biotechnology and conservation of plant biodiversity. Resour. 2:73-95.

Cuesta F, Chacón-Moreno E, Peralvo M, Tovar A. 2011. Physical Geography and Ecosystems in the Tropical Andes. In: Herzog SKH, Martínez R, Holm JPM. (eds) TiessenClimate Change and Biodiversity in the Tropical Andes. MacArthur Foundation. 10:152-169.

D'amato F. 1985. Cytogenetics of plant cell and tissue cultures and their regenerates. CRC. Crit. Rev. Plant Sci. 3:73-112.

Da Silva Cordeiro L, Simões-Gurgel C, Albarello N, Engelmann F. 2015. Cryopreservation of *in vitro* grown shoot tips of *Cleome rosea* Vahl (Cleomaceae) using the V cryo-plate technique. In Vitro Cell Dev. Biol. Plant. 51:688-695.

da Silva JA. 2004. The effect of carbon source on *in vitro* organogenesis of chrysanthemum thin cell layers. Bragantia. 63:165-177.

De-Qiang D, Fang T, Ying-Kun Q, Zheng X, Bi XX, Ting GK. Feng D. 2010. Studies on chemical constituents of the leaves of *Smallantus sonchifolius* (yacon): Structures of two new diterpenes. Nat. Prod. Res. 24:40-47.

Doyle JJ, Doyle JL. 1989. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bulletin. 19:11-15.

Dulloo ME, Guarino L, Engelmann F, Maxted N, Newbury JH, Attere F, Ford-Lloyd BV. 1998. Complementary conservation strategies for the genus *Coffea*: A case study of Mascarene *Coffea* species. 45:565-579.

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

Ellis D, Skogerboe D, Andre C, Hellier B, Volk G. 2006. Implementation of garlic cryopreservation techniques in the national plant germplasm system. CryoLetters. 27:99-106.

Engelmann F. 1997. *In vitro* conservation methods. In: Callow JA, Ford-Lloyd BV, Newbury HJ (eds) Biotechnology and Plant Genetic Resources. Conservation and Use. CAB International, Rome. 119-160p.

Engelmann F. 2004. Plant cryopreservation progress and prospects. In Vitro Cell. Dev. Biol. Plant. 40:427-433.

Engelmann F. 2011. Use of biotechnologies for the conservation of plant biodiversity. In Vitro Cell. Dev. Biol. Plant. 47:5-16.

Eriksson R. 2007. A synopsis of Basellaceae. Kew Bulletin. 62:297-320.

Espasandin FD, Brugnoli EA, Ayala PG Ayala LP, Ruiz OA, Sansberro PA. 2019. Long-term preservation of *Lotus tenuis* adventitious buds. Plant Cell Tiss. Organ Cult. 136:373-382.

Espin S, Villacres E, Brito B. 2004. Caracterización físico-química, nutricional y funcional de raíces y tubérculos andinos (Capítulo 4). En: Barrera V, Tapia C & A Monteros (eds.). Raíces y Tubérculos Andinos: Alternativas Para el Uso Sostenible en el Ecuador. Serie: Conservación y uso de la biodiversidad de raíces y tubérculos andinos: Una década de investigación para el desarrollo (1993-2003). No.4. Instituto Nacional Autónomo de Investigaciones Agropecuarias, Centro Internacional de la Papa, Agencia Suiza para el Desarrollo y la Cooperación. Quito, Ecuador - Lima, Perú. 91-116p.

Espinoza N, Lizarraga R, Siguenas C, Buitron F, Bryan J, Dodds JH. 1992. Tissue culture micropropagation, conservation, and export of potato germplasm. Specialized technology document 1. International Potato Center, Lima, Peru. 20p.

FAO. 1989. Utilization of tropical foods: roots and tubers. FAO Food and Nutrition Paper. 47:56-63.

Fauguel CM, Vega TA, Nestares G, Zorzoli R, Picardi LA. 2008. Anatomy of normal and hyperhydric sunflower shoots regenerated in vitro. Helia. 48:17-26.

Fernandez CE, Kucera L. 1997 Determination of number of chromosomes in select ekotypes of yacon (*Polymnia sonchifolia* Poeppig and Endlicher) cultivated *in vitro*. Agricultura Tropica et Subtropica. 30:89-93.

Fernandez CE, Viehmannova I, Lachman J, Milella L. 2006. Yacon [*Smallanthus sonchifolius* (Poeppig & Endlicher) H. Robinson]: a new crop in the Central Europe. Plant Soil Environ. 52:564-570.

Fernandez CE. 2007. The cultivation and phenological growth stages of yacon [*Smallanthus sonchifolius* (poepp. et endl.) h. robinson. Agricultura Tropica et Subtropica. 40:71-88.

Fisher TR, Wells JR. 1962. Heteromorphich pollen grains in *Polymnia*. Rhodora. 64:336-339. (French)

Flores HE, Flores T. 1997. Biology and Biochemistry of Underground Plant Storage Organs. Functionality of Food Phytochemicals. 31:113-132.

Flores HE, Walker TS, Guimarães RL, Bais HP, Vivanco JM. 2003. Andean root and tuber crops: Underground rainbows. 38:161-167.

Frias AM, Grau A, Lozzia E, Caro S. 1997. Estudio citologico del yacon (*Polymnia sonchifolia*) y el yacon del campo (*P. macroscypha*). 28 Congreso Argentino de Genetica, Tucuman, Argentina. (Spanish)

Garcia MB, Sanchez YA, Malaurie B, Jerez YH, Pupo JJS. 2011. Conservación in vitro de *Dioscorea alata* L. clon caraqueño (Dioscoreaceae). Rev. peru. biol. 16:203-208.

Golmirzaie A, Panta A. 1997. Tissue culture methods and approaches for conservation of root and tuber crops. In: Razdan MK, Cocking EC (Eds.) Conservation of Plant Genetic Resources *In vitro*. General aspects. 1:123-152.

Golmirzaie AM, Panta A, Toledo J. 1999. Advances in the conservation of root and tuber crops. In: Benson EE (Ed.) Plant Conservation Biotechnology. Taylor and Francis LTD, London. 1:165-178.

Gonzalez-Arnao MT, Panta A, Roca WM, Escobar RH, Engelmann F. 2008. Development and large scale application of cryopreservation techniques for shoot and somatic embryo cultures of tropical crops. Plant Cell Tiss. Organ Cult. 92:1-13.

Gopal A, Chamail A, Sarkar D. 2004. *In vitro* production of microtubers for conservation of potato germplasm: effect of genotype, abscisic acid, and sucrose. In Vitro Cell. Dev. Biol. Plant. 40:485-490.

Gopal J, Chauhan NS. 2010. Slow growth *in vitro* conservation of potato germplasm at low temperature. Potato Res. 53:141-149.

Gopal, J, Chamail A, Sarkar D. 2002. Slow-growth *in vitro* conservation of potato germplasm at normal propagation temperature. Potato Res. 45:203-213.

Gopal, J, Chauhan N. 2010. Slow growth in vitro conservation of potato germplasm at low temperature. Potato Research 53:141-149.

Grau A, Rea J. 1997. Yacon [*Smallanthus sonchifolia* (Poepp. et Endl.) H. Robinson]. Andean roots and tuberous roots: ahipa, arracacha, maca and yacon. In: Promoting the conservation and use of underutilized crops. International Plant Genetic Resources Institute. 66p.

Grau A, Slanis A. 1996. Is *Polymnia sylphioides* var. *perennis* a wild ancestor of yacon? Resumos I Congresso Latino Americano de Raizes Tropicais. CERAT-UNESP, Sao Pedro, Brasil. (Portuguese).

Grospietsch M, Stodůlkova E, Zamecník J. 1999. Effect of osmotic stress on the dehydration tolerance and cryopreservation of *Solanum tuberosum* shoot tips. CryoLetters. 20:339-346.

Grout BWW. 1990. In vitro conservation of germplasm. In: Bhojwani SS (ed) Plant tissue culture: applications and limitations. Elsevier, Amsterdam. 394-423 pp.

Gurel S, Gulsen Y. 1998. The effects of different sucrose, agar and pH levels on *in vitro* shoot production of almond (*Amygdalus communis* L.). Turk. J. Bot. 22:363-373.

Hammond HSD, Faltus M, Zamecnik J. 2019a. Methods of thermal analysis as a tool to develop cryopreservation protocols of vegetatively propagated crops [Online First], IntechOpen, DOI: 10.5772/intechopen.89723.

Hammond HSD, Viehmannova I, Zamecnik J, Hlasna Cepkova P, Panis B. 2019b. Efficient slow-growth conservation and assessment of clonal fidelity of *Ullucus tuberosus* Caldas microshoots. Plant Cell Tiss. Organ Cult. 138:559-570.

Harding K, Johnston J W, Benson EE .2009. Exploring the physiological basis of cryopreservation success and failure in clonally propagated *in vitro* crop plant germplasm. J. Sci. Food Agric. 18:103-116.

Heiser C. 1963. Numeracion cromosomica de plantas ecuatorianas. Ciencia y Naturaleza. 6: 2-6.

Hermann M, Heller J, Engels JMM, Hammer K. 1990. Andean roots and tubers: ahipa, arracacha, maca and yacon. Rome: IPK and IPGRI. 21p.

Hermann M, Heller J. 1997. Andean roots and tubers: ahipa, arracacha, maca and yacon. Rome: IPGRI. 204p.

Hermann M. 1992. Andean Root and Tubers Research Priorities for Neglected Food Resource. CIP. 36p.

Hernandez Bermejo JE, Leon J. 1994. Neglected crops: 1492 from a different perspective. Plant Production and Protection Series. FAO, Rome, Italy. 26: 65-179.

Herrera FL. 1943. Plantas alimenticias y condimenticias indigenas del departamento del Cuzco. Bol. DGAP. 14:48-51.

Hirai D, Sakai A. 1999. Cryopreservation of *in vitro* grown meristems of potato (*Solanum tuberosum* L.) by encapsulation-vitrification. Am. Potato. J. 42:153-160.

Hirai D. 2011. Gelled droplet vitrification improves recovery of cryopreserved potato germplasm. CryoLett. 32:287-296.

Hoffman I, Parups EV .1964. Mode of action of maleic hydrazide in relation to residues in crops and soils. In: Gunther F.A. (eds) Residue Reviews/Rückstands-Berichte. Rev. Environ. Contam. Toxicol. vol 7. Springer, New York, NY.

Hu QJ, Fu YY, Guan YJ, Lin C, Cao DD, Hu WM, Sheteiwy M, Hu J. 2016. Inhibitory effect of chemical combinations on seed germinaion and pre-harvest sprouting in hybrid rice. Plant Growth Regul. 80:281-289.

Ignacio S, Camarena F, Baudoin J, Blas R. 2017. Ethno-Botany and *in situ* conservation of the genetic diversity of arracacha (*Arracacia xanthorrhiza* Bancroft), yacon (*Smallanthus sonchifolious* H.Robinson), and wild relatives. Peruvian Journal of Agronomy. 1:21-31.

INEI. 2013. Compendio estadístico del Perú 2013; Tomo 2. INEI. Lima, Perú.

Ishiki K, Salgado M, Arellano J. 1997. Revision of chromosome number and karyotype of Yacon (*Polymnia sonchifolia*). Resumenes del Primer Taller Internacional sobre Recursos Fitogeneticos del Noroeste Argentino. INTA, Salta, Argentina.

Izquierdo J, Roca W. 1997. Under-utilized Andean food crops: Status and prospects of plant biotechnology for the conservation and sustainable agricultural use of genetic resources. ISHS Acta Hortic. Symposium on plant biotechnology as a tool for the exploitation of mountain lands. In: Hermann M, Heller J. (eds.) Andean Roots and Tubers: Ahipa, arracacha, maca, yacon. IPGRI. Rome. 457:202-242.

Jarret RL, Gawel N. 1991. Abscisic acid-induced growth inhibition of sweet potato (*Ipomoea batatas* L.) in vitro. Plant Cell Tiss Organ Cult. 24:13-18.

Jean GDM. 2015. Experimenting with growing ulluco as a niche crop for fun and profit. Hort. J. 2:2.

Jin S, Mushke R, Zhu H, Tu L, Lin Z, Zhang Y, Zhang X. 2008. Detection of somaclonal variation of cotton (*Gossypium hirsutum*) using cytogenetics, flow cytometry and molecular markers. Plant Cell Rep. 27:1303-1316.

Josse C, Cuesta F, Navarro G, Barrena V. 2009. Ecosistemas de los Andes del Norte y Centro. Bolivia, Colombia, Ecuador, Perú y Venezuela. Secretaría General de la Comunidad Andina, Programa Regional ECOBONA-Intercooperation, CONDESAN-Proyecto Paramo Andino, Programa BioAndes, EcoCiencia, NatureServe, IAvH, LTA-UNALM, ICAE-ULA, CDC-UNALM, RUMBOL SRL. Lima. 7-9p.

Kaczmarczyk A, Rokka VM, Keller ERJ. 2011. Potato Shoot Tip Cryopreservation. A Review. Potato Res. 54:45-79.

Kakihara TS, Camara FLA, Vilhena SMC, Riera L. 1996. Cultivo e industrilizacao de yacon: uma experiencia brasileira. Resumos I Congresso Latino Americano de Raizes Tropicais. CERAT-UNESP, Sao Pedro, Brasil. (Portuguese).

Kalaiselvi R, Rajasekar M, Gomathi S. 2017. Cryopreservation of Plant Materials - A Review. IJCS. 5: 560-564.

Kaminska M, Gołębiewski M, Tretyn A, Trejgell A. 2018. Efficient long-term conservation of *Taraxacum pieninicum* synthetic seeds in slow growth conditions. Plant Cell Tiss. Organ Cult. 132:469-478.

Keller ERJ, Sunura A, Zanke Ch, Grube M, Kaczmarczyk A. 2011. Cryopreservation and in vitro culture-State of the art as conservation strategy for genebank. Acta Hort. 918:99-111.

Khajehei F, Niakousari M, Damyeh MS, Merkt N, Claupein W, Graeff-Hoenninger S. 2017. Impact of Ohmic-Assisted Decoction on Bioactive Components Extracted from Yacon (*Smallanthus sonchifolius* Poepp.) Leaves: Comparison with Conventional Decoction. Molecules. 22:2043.

Kim H, Lee JK, Yoon JW, Ji JJ, Nam SS, Hwang HS, Cho EG, Engelmann F. 2006b. Cryopreservation of garlic bulbil primordia by the droplet-vitrification procedure. CryoLetters. 27:143-153.

Kim HH, Lee YG, Shin DJ, Ko HC, Gwag JG, ChoEG, Engelmann F. 2009a. Development of alternative plant vitrification solutions in droplet-vitrification procedures. CryoLetters. 30:320-334.

Kim HH, Yoon JW, Park YE, Cho EG, Sohn JK, Kim TS, Engelmann F. 2006a. Cryopreservation of potato cultivated varieties and wild species: critical factors in droplet vitrification. CryoLett. 27:223-234.

King RS, Gershoff SN. 1987. Nutritional evaluation of three underexploited Andean tubers: *Oxalis tuberosa* (Oxalidaceae), *Ullucus tuberosus* (Basellaceae) and *Tropaeolum tuberosum* (Tropaeolaceae). Econ. Bot. 41:503-511.

Koc I, Akdemir H, Onay A, Ciftci YÖ. 2014. Cold-induced genetic instability in micropropagated *Pistacia lentiscus* L. plantlets. Acta Physiol. Plant. 36:2373-2384.

Kwiatkowski S, Brown CR, Martin MW, Sluis C. 1988. Serial microtuber formation as a long-term conservation method for *in vitro* potato germplasm. Am. J. Potato Res. 65:369-375.

Lachman J, Fernandez EC, Orsak M. 2003. Plant, Soil and Environment. 49:283-290.

Lachman J, Haveland B, Fernandez EC, Dudjak J. 2004. Saccharine of yacon [*Smallanthus sonchifolius* (Poeppig and Endlicher) Robinson] tubers and rhizomes and factors affecting their content. Plant Soil Environ. 50:383-390.

Lachman J, Havrland B, Hejtmankova A, Fernandez E, Pivec V. 2005. Content of polyphenolic antioxidants and phenolic acids in selected parts of yacon [*Smallanthus sonchifolius* (Poepp. et Endl.) H. Robinson]. SAB. 36:49-54.

Lambardi M, Fabbri A, Caccavale A. 2000. Cryopreservation of white poplar (*Populus alba* L.) by vitrification of *in vitro* grown shoot tips. Plant Cell Rep. 19:213-218.

Lata H, Chandra S, Mehmedic Z, Khan IA, ElSohly MA. 2012. *In vitro* germplasm conservation of high Delta(9)-tetrahydrocannabinol yielding elite clones of *Cannabis sativa* L. under slow growth conditions. Acta Physiol. Plant. 34:743-750.

Lebeda A, Doleyalova I, Doležal K. 2004. Variation in Morphological and Biochemical Characters in Genotypes of Maca and Yacon. Acta Hort. (ISHS). 629:483-490.

Lebeda A, Dolezalova I, Valentova K, Gasmanova N, Dziechciarkova M, Ulrichova J. 2003. Genetic resources, chromosome engineering and crop improvement: medicinal plants. Boca Raton: CRC. Press. 5: 49-56.

Leifert C, Pryce S, Lumsden PJ, Waites WM.1992. Effect of medium acidity on growth and rooting of different plant species growing *in vitro*. Plant Cell Tiss. Organ Cult. 30:171-179.

Lempiainen T. 1989. Germination of the seeds of ulluco (*Ullucus tuberosus*, Basellaceae). Econ. Bot. 43:456-463.

Leon E. 2013. 14,000 años de alimentación en el Perú. Universidad San Martín de Porres, Fondo Editorial. Lima, Perú. 661 p.

Leon J. 1964. Plantas alimenticias andinas. Lima: Instituto Interamericano de Ciencias Agricolas Zona Andina. 6:50-56p.

Leunufna S, Keller ER. 2005. Cryopreservation of yams using vitrification modified by including droplet method: effects of cold acclimation and sucrose. Cryo Letters. 26:93-102.

Leva AR, Petruccelli R, Rinaldi LMR. 2012. Somaclonal variation in tissue culture: A case study with olive. Recent Advances in Plant *in vitro* Culture. Intech. Croatia. 1:123-150.

Li M, Zhao X, Hong S, Zhang X, Li P, Liu J, Xie C. 2009. Cryopreservation of plantlet nodes of *Dioscorea opposita* Thunb. using a vitrification method. CryoLetters. 30:19-28.

Linnaeus C. 1751. Species plantarum. Vol 1. Impensis Laurentii Salvii. Stockholm.

Liu H, Yu W, Dai J, Gong Q, Yang K, Lu X. 2004a. Cryopreservation of protoplasts of the alga *Porphyra yezoensis* by vitrification. Plant Sci. 166:97-102.

Liu XX, Wen YB, Cheng ZH, Mou SW. 2017. Establishment of a garlic cryopreservation protocol for shoot apices from adventitious buds *in vitro*. Sci. Hort. 226:10-18

Liu Y, Wang X, Liu L. 2004b. Analysis of genetic variation in surviving apple shoots following cryopreservation by vitrification. Plant Sci. 166:677-685.

Lizarraga C. 1997. Progress in the identification of the virus infecting Andean root and tuber crops. Lima: International potato center. 44p.

Lizarraga R, Panta A, Espinoza N, Dodds JH. 1992. Tissue culture of *Ipomea batatas*: micropropagation and maintenance. CIP Research Guide 32, Lima, Peru. 20p.

Luziatellia G, Sorensena M, Jacobsenb SE (2020) Current uses of Andean Roots and Tuber Crops in South American gourmet restaurants. IJGFS. 22:100270.

Mafla G, Roa JC, Aranzales E, Debouck D. 2009. Handbook of procedures for *in vitro* germplasm conservation of the genus *Manihot*. CIAT, Cali, Colombia. 56 pp.

Malaurie B, Trouslot MF, Berthaud J, Bousalem M, Pinel A, Dubern J. 1998. Medium-term and long-term in vitro conservation and safe international exchange of yam (*Dioscorea spp.*) germplasm. J. Biotechnol. 1: 26-27.

Malice J, Bizoux JP, Blas R, Baudoin JP. 2010. Genetic Diversity of Andean Tuber Crop Species in the *in situ* Microcenter of Huanuco, Peru. Crop Sci. 50:1915-1923.

Malice M, Baudoin JP. 2009. Diversité génétique et conservation du germoplasme de trois tubercules andins mineurs. Biotechnol. Agron. Soc. 185 p.

Manrique I, Arbizu C, Vivanco F, Gonzales R, Ramírez C, Chavez O, Tay D, Ellis D. 2017. *Ullucus tuberosus* Caldas Colección de germoplasma de ulluco conservada en el Centro Internacional de la Papa (CIP). Centro Internacional de la Papa (CIP). Peru. 445 p.

Manrique I, Parraga A, Hermann M. 2005. Yacon syrup: principles and processing. Lima: International Potato Center, Universidad Nacional Daniel Alcides Carrion, Erbacher Foundation, SDC. 31p.

Martin RJ, Scheffer JJC, Deo B, Halloy SRP. 2005. Effect of planting date on Ulluco yield. Acta hortic. 670:181-187.

Martinez MM, Gonzalez-Arnao MT, Borroto-Nordelo C, Puentes-Diaz C, Engelmann F. 1998. Cryopreservation of sugarcane embryogenic callus using a simplified freezing process. CryoLetters. 19:171-176.

Martinez O. 2018. Selection of Molecular Markers for the Estimation of Somaclonal Variation. Methods in Molecular Biology. Plant Cell Culture Protocols. Sringer.1815:103-129.

Maryam A, Tariq R, Chuadhary S, Azmat R, Javed S, Khanam S. 2014. A review: role of tissue culture (in vitro) techniques in the conservation of rare and endangered species. Pac J Life Sci. 2:93-103.

Maxted N, Ford-Lloyd BV, Hawkes JG. 1997. Complementary conservation strategies, In. Plant Genetic Conservation: The in situ Approach, Chapman -Hall, London. 1:15-40.

Medina AM, Casas JL, Swennen R, Panis B. 2010. Cryopreservation of *Thymus moroderi* by droplet vitrification. CryoLetters. 31:14-23.

Mendez M, Arbizu C, Orrillo M. 1994. Niveles de ploidía de los ullucus cultivados y silvestres. Universidad Austral de Chile. Resúmenes de trabajos presentados al VIII Congreso Internacional de Sistemas Agropecuarios y su proyección al tercer milenio. Valdivia (Chile). Agro. Sur. 12p.

Meza ZG. 1995. Variedades nativas de Llacon (*Polymnia sonchifolia* Ker Gawler) en Cusco. Kayra: UNSAC-CICA, CIP-COTESU.

Michaels J, Michaels F. 2007. Yacon Growing Information. Green Harvest, [cit. 2019-26-08]. On line from http://www.greenharvest.com.au/Plants/yacon_info.html.

Ming-Hua Y, Sen-Rong H. 2010. A simple cryopreservation protocol of *Dioscorea bulbifera* L. embryogenic calli by encapsulation-vitrification. Plant Cell Tiss Organ Cult. 101:349-358.

Morales F. 2007. Sociedades precolombinas asociadas a la domesticación y cultivo de la papa (*Solanum tuberosum*) en Sudamérica. Revista Latinoamericana de la Papa. 14:1-9.

Muñoz M, Diaz O, Waleska R, Winkler A, Quevedo R. 2019. Slow growth in vitro culture for conservation of Chilotanum potato germplasm. CHIL J AGR RES. 79:26-35.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Naqvi B, Abbas H, Ali H. 2019. Evaluation of *in vitro* tuber induction ability of two potato genotypes. Pak. J. Agri. Sci. 56:77-81.

National Research Council. 1989. Ulluco. En: Lost crops of the Incas: Little-known plants of the Andes with promise for worldwide cultivation. National Academy Press, Washington DC. 1:105-113.

Nayak S, Kaur T, Mohanty S, Ghosh G, Choudhury R, Acharya L, Subudhi E. 2011. *In vitro* and *ex vitro* evaluation of long-term micropropagated turmeric as analyzed through cytophotometry, phytoconstituents, biochemical and molecular markers. Plant Growth Regul. 64:91-98.

Niino T, Arizaga MV. 2015. Cryopreservation for preservation of potato genetic resources. Breed Sci. 65:41-52.

Niino T, Tanaka D, Daisuke, Ichikawa S, Takano J, Seguel I, Shirata K, Uemura M. 2003. Cryopreservation of *in vitro* grown apical shoot tips of strawberry by vitrification. Plant Biotechnol. J. 20:75-80.

Nishizawa S, Sakai A, Amano Y, Matsuzawa T. 1993. Cryopreservation of asparagus (*Asparagus officinalis* L.) embryogenic suspension cells and subsequent plant regeneration by vitrification. Plant Sci. 91:67-73.

Noor NM, Kean CW, Vun YL, Mohamed-Hussein ZA. 2011. *In vitro* conservation of Malaysian biodiversity-achievements, challenges and future directions. In Vitro Cell. Dev. Biol. Plant. 47:26-36.

Ozudogru EA, Benelli C, Dradi G, Lambardi M. 2017. Effect of culture container and carbohydrate content on *in vitro* slow growth storage of the cherryrootstock '*Gisela* (R) 5'. Acta Physiol. Plant. 39:94.

Panis B, Helliot B, Strosse H, Remy S, Lepoivre P, Swennen R. 2005. Germplasm conservation, virus eradication and safe storage of transformation competent cultures in banana: The importance of cryopreservation. Acta Hortic. 692:51-59.

Panta A, Golmirzaie A, Toledo J. 1999. Advances in the conservation of root and tuber crops. In: E. Benson (ed). Plant Conservation Biotechnology. In Advances in the conservation of root and tuber crops., Publisher: Taylor and Francis Ltd. London. 1:165-178.

Panta A, Panis B, Ynouye C, Criel B, Swennen R, Roca W. 2006. Improvement of potato cryopreservation for the long-term conservation of Andean landraces at the International Potato Center (CIP). Cryobiology. 120:117-125.

Peng X, Ji Q, Wu H, Li Y. 2015. Slow-growth conservation and clonal fidelity of *Tetrastigma hemsleyanum* microplants. In Vitro Cell. Dev. Biol. Plant. 51:463-470.

Pietila L, Jokela P. 1990. Seed set of ulluco (*Ullucus tuberosus* Loz.). Variation between clones and enhancement of seed production through the application of plant growth regulators. Euphytica. 47:139-145.

Poeppig EF, Endlicher SFL. 1845. *Polymnia sonchifolia* Poepp. Nov. Gen. Sp. Pl. (Poeppig & Endlicher).3:47.

Rahman MH, Islam R, Hossain M, Islam MS .2010. Role of sucrose, glucose and maltose on conventional potato micropropagation. J. Agric. Tech. 6:733-739.

Rai MK, Jaiswal VS, Jaiswal U. 2008. Encapsulation of shoot tips of guava (*Psidium guajava* L.) for short-term storage and germplasm exchange. Sci. Hortic. 118:33-38.

Rao R, Sthapit B. 2013. Conservation of Tropical Plant Genetic Resources: In Situ Approach. In: Normah M, Chin H, Reed B. (eds). Conservation of Tropical Plant Species. Springer, New York. 1:3-26.

Rea J. 1995. Conservacion y manejo in situ de recursos fitogeneticos Agricolas en Bolivia. Taller electronic sobre conservacion in situ. Lima, Peru. 24p.

Reed BM, Dumet D, Denoma JM, Benson EE. 2001b. Validation of cryopreservation protocols for plant germplasm conservation: a pilot study using *Ribes* L. Biodivers. Conserv. 10:939-949.

Reed BM, Gupta S, Uchendu EE. 2012. *In vitro* genebanks for preserving tropical biodiversity. Conservation of Tropical Plant Species. 1:77-106.

Reed BM. 2001a. Implementing cryogenic storage of clonally propagated plants. CryoLetters 22:97-104.

Reed BM. 2002. Photoperiod improves long-term survival of *in vitro* stored strawberry plantlets. HortScience. 37:811-814.

Reed BM. 2008. Plant cryopreservation: a practical guide pp 3-13. In: Reed BM (ed.) Plant Cryopreservation: A Practical Guide, Springer, New York. 3358pp.

Robinson H. 1978. Studies in the Heliantheae (Asteraceae). XII. Re-establishment of the genus *Smallanthus*. Phytologia. 39:47-53.

Roca WM, Angel F, Sarria R, Mafla G. 1992. Future initiatives in biotechnology research for tropical agriculture: the case of cassava. In: McCorwick DK, editor. Advances in Gene Technology: Feeding the World in the 21st Century, 1992 Miami Bio/technology Winter Symposium, Miami, FL, USA. 87p.

Roca WM, Espinoza NO, Roca MR, Bryan JE. 1978. A tissue culture method for the rapid propagation of potatoes. Am. J. Potato Res. 55:691-701.

Sakai A, Engelmann F. 2007. Vitrification, encapsulation-vitrification and droplet-vitrification: a review. CryoLetters. 28:151-172.

Sakai A, Kobayashi S, Oiyama I. 1990. Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis Tanaka*) by vitrification. Plant Cell Rep. 9:30-33.

Salgado M. 1996. Evolucion y Caracterizacion citogenetica de 16 estradas de Jicama (*Polymnia sonchifolia* Poeppig and Endlicher) de Sudamerica. Tesis de Grado Universidad Central del Ecuador.

San Jose MCS, Valladares S, Janeiro LV. 2014 Cryopreservation of *in vitro*-grown shoot tips of *Alnus glutinosa* (L.) Gaertn. Acta Physiol. Plant. 36:109.

Sanchez DF, Panta A, Tay D, Roca W. 2011. Cryopreservation of Ulluco (*Ullucus tuberosus* Cal.) and oca (*Oxalis tuberosa* Mol.) shoot tips using the PVS2 droplet-vitrification method. Acta Hortic. 908: 339-346.

Sarasan V. 2010. Importance of *in vitro* technology to future conservation programmes worldwide. 65:549-554.

Sarkar D, Chakrabarti SK, Naik PS. 2001. Slow-growth conservation of potato microplants: efficacy of ancymidol for long-term storage in vitro. Euphytica. 117:133-142.

Seminario J, Valderrama M, Manrique I. 2003. El yacón: fundamentos para el aprovechamiento de un recurso promisorio. Centro Internacional de la Papa (CIP), Universidad Nacional de Cajamarca, Agencia Suiza para el Desarrollo y la Cooperación (COSUDE).

Senula A, Büchner D, Keller ERJ, Nagel M (2018) An Improved Cryopreservation Protocol for Mentha spp. Based on Pvs3 as the Cryoprotectant. CryoLetters. 39:345-353.

Sestak J, Zamecnik J. 2007. Can clustering of liquid water and thermal analysis be of assistance for better understanding of biological germplasm exposed to ultra-low temperatures. J. Therm. Anal. Calorim. 88:411-416.

Skalova I, Viehmannova I, Vitamvas J. 2013. *In vitro* Conservation of *Smallanthus sonchifolius* under Slow-Growth Conditions. Agricultura Tropica et Subtropica. 45:147-150.

Sperling CR, King RS. 1990. Andean tuber crops: Worldwide potential, In: J. Janick and J. E. Simon (eds.), Advances in New Crops. Timber Press, Portland. 1:428-435.

Sperling CR. 1987. Systematic of the Basellaceae. Ph.D. Thesis, Harvard Univeristy, Cambridge, Massachusetts. 279p.

Steinitz B. 1999. Sugar alcohols display nonosmotic roles in regulating morphogenesis and metabolism in plants that do not produce polyols as primary photosynthetic products. J. Plant Physiol. 155:1-8

Suzuki M, Tandon P, Ishikawa M, Toyomasu T. 2008. Development of a new vitrification solution, VSL, and its application to the cryopreservation of gentian axillary buds. Plant Biotechnol. Rep. 2:123-131.

Svenson J, Smallfield BM, Joyce NI, Sansom CE, Perry NB. 2008. Betalains in red and yellow varieties of the andean tuber crop ulluco (*Ullucus tuberosus*). J. Agric. Food Chem. 56:7730-7737.

Talledo D, Escobar M. 1996. Caracterizacion cariotipica de germoplasma RTA. Laboratorio de Biologia Celular y Genetica. Memorias.

Tay D. 2013. Tropical and subtropical root and tuber crops, In Normah MN, Chin HF. (eds) Conservation of Tropical Plant Species. Springer, New York, NY. 1:249-292.

Thakur S, Tiwari KL, Jadhav SK. 2015. *In vitro* approaches for conservation of *Asparagus racemosus* Willd. In Vitro Cell Dev. Biol. Plant. 51:619-625.

Towill LE, Jarret RL. 1992. Cryopreservation of sweet potato (*Ipomoea batatas* [L.] Lam.) shoot tips by vitrification. Plant Cell Reports. 11:175-178.

Towill LE. 1990. Cryopreservation of isolated mint shoot tips by vitrification. Plant Cell Rep. 9:178-80.

Uragami A, Sakai A, Nagai M, Takahashi T. 1989. Survival of cultured cells and somatic embryos of *Asparagus officinalis* cryopreserved by vitrification. Plant Cell Rep. 8:418-21.

Valentova K, Frcek J, Ulrichova J. 2001. Yacon (*Smallanthus sonchifolius*) and maca (*Lepidium meyenii*), traditional Andean crops as new functional foods on the European market. Chemické Listy. 95:594-601.

Valentova K, Ulrichova J. 2003. *Smallanthus sonchifolius* and Lepidium meyenii - prospective Andean crops for the prevention of chronic deceases. Biomedical Papers of the Medical Faculty of the University Palacky, Olomouc, Czech Republic. 147:119-130.

Venezian A, Dor E, Achdari G, Plakhine D, Smirnov E, Hershenhorn J. 2017. The influence of the plant growth regulator maleic hydrazide on Egyptian broomrape early developmental stages and its control efficacy in tomato under greenhouse and field conditions. Front. Plant Sci. 8:691.

Villaluz ZA, Arradaza CC. 2012. Development of In vitro Slow Growth Culture for Yam (*Dioscorea alata* L.). Annals of Tropical Research. 34:79-95.

Volk GM, Caspersen AM. 2017. Cryoprotectants and components induce plasmolytic responses in sweet potato (*Ipomoea batatas* (L.) Lam.) suspension cells. In vitro Cell Dev. Biol. Plant. 53:363-371.

Vollmer R, Villagaray R, Egusquiza V, Espirilla J, Garcia M, Torres A, Rojas E, Panta A, Barkley NA, Ellis D. 2016. The potato cryobank at the International Potato Center (CIP): A model for long term conservation of clonal plant genetic resources collections of the future. CryoLetters. 37:318-329.

Walker T. 2013. Plant conservation: Why tt matters and how it works. Timber Press. pp 304.

Wang M, Zhang Z, Zamecnik J, Bilavcik A, Blystad DR, Haugslien S, Wang QC. 2020. Droplet-vitrification for shoot tip cryopreservation of shallot (*Allium cepa* var. *Aggregatum*): effects of PVS3 and PVS2 on shoot regrowth. Plant Cell Tiss. Organ Cult. 140:185-195.

Weddell HA. 1857. Notice sur l'ahipa et l'aricoma, plantes alimentaires du Haut-Perou. Ann. Sci. Nat. Bot. IV. (French). 7:114p.

Wells JR. 1967. A new species of *Polymnia* (Compositae): Heliantheae from Mexico. Brittonia. 19:391-394.

Wells JR. 1971. Variation in Polymnia pollen. American Journal of Botany. 38:124-130.

William M R, Ynouye C, Manrique I, Arbizu C, Gomez R. 2007. Indigenous Andean Root and Tuber Crops: New Foods for the New Millennium. Chron. Horticult. 47:13-19.

Wilms H, Fanega Sleziak N, Van der Auweraer M, Brands M, Verleije M, Hardeman D, Andre E, Panis B. 2020. Development of a fast and user-friendly cryopreservation protocol for sweet potato genetic resources. Scientific Reports. 10:14674.

Yamamoto S, Rafique T, Fukui K, Castillo MCR, Niino T. 2013. Application of V-Cryo-plate method to in vitro shoot tips of potato varieties. Abst. of 2nd International Symposium on Plant Cryopreservation. Fort Collins, Colorado, USA. 31p.

Yaseen M, Ahmad T, Sablok G, Standardi A, Hafiz IA. 2013. Review: Role of carbon sources for *in vitro* plant growth and development. Mol. Biol. Rep. 40:2837-2849.

Yin ZF, Bi WL, Chen L, Zhao B, Volk GM, Wang QC. 2014. An efficient, widely applicable cryopreservation of Lilium shoot tips by droplet vitrification. Acta Physiol. Plant. 36:1683-1692.

Zamecnik J, Faltus M, & Bilavcik A. 2007. Cryoprotocols used for cryopreservation of vegetatively propagated plants in the Czech cryobank. Adv Hortic Sci. 21:247-250.

Zamecnik J, Faltus M, Kotkova R, Hejnak V. 2011. Glass transition determination in Allium shoot tips after dehydration. Acta Hortic. 908:33-38.

Zamecnik J, Faltus M. 2009. Evaluation of thermograms from differential scanning calorimeter. CryoLetters. 30:388-388.

Zamecnikova J, Fernandez E, Viehmannova I, Zamecnik J, Faltus M. 2011. Preparation of shoot tips by sucrose and dehydration pre-treatment of *Ullucus tuberosus* Cal. for cryopreservation. Acta Hortic. 908:331-338.

Zardini E. 1991. Ethnobotanical notes on "yacon" *Polymnia sonchifolia* (Asteraceae). Econ. Bot. 45:72-85.

Zee FT, Munekata M. 2019. *In Vitro* storage of pineapple (*Ananas spp*.) Germplasm. HortScience. 27:57-58.

Ziv M. 1991. Quality of micropropagated plants-vitrification. In Vitro Cell Dev Biol - Plant 27:64-69.