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CONSERVATION OF GENETIC RESOURCES – *SMALLANTHUS SONCHIFOLIUS* (POEPP. *ET* ENDL.) H. ROBINSON AND *ULLUCUS TUBEROSUS* (LOZ.)

Ph.D. Thesis

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Certification

I, Jana Mandíková, declare that this thesis and its intellectual content is my own work unless otherwise referenced. The document has not been submitted for qualifications at any other academic institute and I agree with its publication.

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List of abbreviations

ABA	Abscisic acid
ARTC	Andean root and tuber crops
BAP	Benzylaminopurine
BOL	Bolivia
CBD	Convention on Biological Diversity
CGIAR	Consultative Group on International Agricultural Research
CIP	Centro Internacional de la Papa (International Potato Center)
CR	The Czech Republic
CULS	Czech University of Life Sciences
DEU	Germany
DMRT	Duncan Multiple Range Test
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DSC	Differential Scanning Calorimetry
DTA	Differential Thermal Analysis
ECU	Ecuador
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IPGRI	International Plant Genetic Resources Institute
LN	Liquid nitrogen
LS	Loading solution
LSD	Least Significant Difference test
MP	Melting point
NAA	1-naphthaleneacetic acid
NZL	New Zealand
NZL2	New Zealand 2
PGR	Plant genetic resources
PROINPA	Promocion e Investigacion de Productos Andinos (Foundation for the Promotion and Investigation of Andean Products)
PVS2	Plant vitrification solution 2
PVS3	Plant vitrification solution 3
Tg	Glass transition temperature
TG	Thermogravimetry
TMA	Thermomechanical Analysis
UV-VIS	Ultraviolet-visible
WC	Water content

ABSTRACT

Conservation of Genetic Resources – *Smallanthus sonchifolius* (Poepp. et Endl.) H. Robinson and *Ullucus tuberosus* (Loz.)

Smallanthus sonchifolius (Poepp. *et* Endl.) H. Robinson (yacon) and *Ullucus tuberosus* (Loz.) (ulluco) are vegetatively propagated root and tuber crops of Andean region making their long-term storage for keeping their biodiversity difficult.

Appropriate methods of ulluco and yacon conservation were tested with the aim for long-term storage. This study evaluated the best method for long-term storage of ulluco and yacon genetic material by field conservation, *in vitro* conservation and cryopreservation. The research was focused on temperature influence physiology of plant material.

The field conservation was efficient for yacon with an average yield of rhizomes 1.6 kg per plant, which were further stored at 8–10 °C and 70–75 % moisture content for 7 months. It is possible to obtain 5.7 yacon plants by propagation of rhizomes from a single plant in one year. Ulluco field conservation cannot be recommended because of low yields of tubers which was low in the yield and also used for propagation. On the other hand, the *in vitro* storage is highly effective especially for ulluco.

Ulluco can be conserved up to 15 months by microtuberization or 11 months by appropriate temperature (17 °C) on hormone free medium with the possibility of fast micropropagation. The micropropagation of ulluco had high propagation coefficient (7.7 from a single plant in 30 days) and also it had better response to *in vitro* growing in comparison with yacon propagation coefficient (3.4).

It was possible to prolong the conservation of yacon for 2 months only by changing the growing temperature from 21 °C to 17 °C. Cold acclimatization of ulluco plantlets was studied with the further aim to improv cryopreservation protocol and for deeper understanding of the influence of temperature to the plant material. Using the UV-VIS Spectroscopy the betalains with a higher concentration of betacyanins in the leaves and betaxanthins in the stems were found. Monitoring of betalain leakage after different freezing temperatures was supported by data of electrolyte leakage and shows lower injury of cold hardened ulluco plants.

Cryopreservation of ulluco had a high water content, dangerous in ice formation. The water was removed from shoot tips using two methods: desiccation over silica gel and osmotic dehydration in plant vitrification solution (PVS3). Plant shoot tips were able to

recover from water stress caused by removing up to 82 % of water. PVS3 pre-treatment decreases the percentage of crystallized water to less than 2 % of water. The great effect of sucrose pre-treatment of shoot tips on their dehydration was found. The tolerance to PVS3 dehydration was improved by sucrose pretreatment. The ice nucleation was decreased by desiccation from -30 °C to -36 °C. This decreased temperature can help narrow the dangerous ice nucleation temperature range before biological glass formation.

The combination of sucrose pre-treatment and 2 hour desiccation over silica gel allowed only 10 % of the shoot tips to survive and regrow following cryopreservation. In comparison, the percentage reached 52.5 % in the case where shoot tips were treated with PVS3 for 1.5 hour regeneration. According to results the theoretical potential for improvement of regeneration after LN is still 21 %. This makes cryopreservation a promising method for long-term storage of ulluco.

According to results the field and *in vitro* conservation of yacon and *in vitro* conservation and cryoconservation for ulluco are the best methods for conservation of these crops for medium-term and long-term. The highest propagation rate for both plants was in *in vitro* conditions. Cryopreservation proved to be the best method for long-term conservation of ulluco. This study of conservation methods improves the storage security of yacon and ulluco germplasm.

Key words: ulluco, yacon, *in vitro*, field conservation, cryopreservation, temperature

ABSTRAKT

Uchovávání genetických zdrojů – *Smallanthus sonchifolius* (Poepp. *et* Endl.) H. Robinson a *Ullucus tuberosus* (Loz.)

Smallanthus sonchifolius (Poepp. *et* Endl.) H. Robinson (jakon) a *Ullucus tuberosus* (Loz.) (melok) jsou okopaniny pocházející z andských oblastí. Vzhledem k tomu, že jsou to vegetativně množené rostliny, cílem práce byl výzkum možností uchovávání genotypů těchto rostlin. V této studii byly sledovány vybrané metody pro dlouhodobé uchovávání genetického materiálu: v polních podmínkách, v *in vitro* podmínkách a pomocí kryoprezervace.

V polních podmínkách se rostliny jakonu množí pomocí rhizomů, u pokusných rostlin průměrný výnos rhizomů představoval 1,6 kg na rostlinu. Rhizomy pak byly skladovány 7 měsíců při teplotě 8–10 °C a vlhkosti 70–75 %. Po 7 měsících skladování bylo možné získat 5,7 rhizomů z jedné mateční rostliny. Pro jakon je tedy tento způsob uchovávání efektivní. Naopak pro melok se uchovávání v polních podmínkách České republiky neprokázalo jako vhodné, vzhledem k nízkým výnosům hlíz, které jsou využívány pro množení.

V podmínkách *in vitro* se podařilo uchovat melok pomocí mikrotuberizace, kdy byly rostliny kultivovány po dobu 15 měsíců na modifikovaném MS mediu se sníženým obsahem dusíku v dusičnanové formě a 16/8 (den/noc) hodinové fotoperiodě. Mikrotuberizace byla indukovaná nízkou teplotou (-4 °C) a následně při 21 °C po 6 měsíců. Další možností střednědobého uchovávání byla kultivace meloku po dobu 11 měsíců při teplotě 17 °C s možností následného rychlého namnožení.

Mikropropagační koeficient je pro melok vysoký 7,7 nové rostliny z jedné původní rostliny za 30 dní a při porovnání s jakonem (mikropropagační koeficient 3,4). Při sledování působení teploty při uchování jakonu v *in vitro* podmínkách bylo možné prodloužit uchovávání o 2 měsíce snížením teploty z 21 °C na 17 °C.

Dále byla studována aklimace na chlad pro melok v *in vitro* s dalším cílem vylepšení kryoprezervačního protokolu a pro důkladné pochopení vlivu teploty na rostlinný materiál.

Měřením pomocí UV-VIS spektroskopie byly nalezeny betalainy s vyšší koncentrací betakyanů v listech a betaxanthinů ve stoncích. Jako exaktní (objektivní) kritérium

poškození buněk mrazem bylo zavedeno měření výtoku betalainů ze zmrzlých částí rostlin a bylo doplněno měřením výtoku elektrolytů z pletiv.

Kryoprezervace meloku je limitována vysokým obsahem vody, který je nebezpečný tvorbou ledových krystalů. Voda byla odstraněna pomocí dvou metod: desikací nad silikagelem nebo osmotickou dehydratací vyvolanou sacharózou nebo vitrifikačním roztokem (PVS3). Vzrostné vrcholy byly schopné tolerovat dehydrataci až do 82 % obsahu vody čerstvé hmotnosti. Na základě termických vlastností pletiv měřených pomocí diferenčního skenujícího kalorimetu bylo možné upřesnit limity kryoprezervačního protokolu. Vitrifikační roztok PVS3 snížil procento krystalické vody na méně než 2 %. Působení sacharózy mělo velký vliv na vzrostné vrcholy a zlepšila se tím i tolerance k PVS3. Dehydratace snížila teplotu nukleace ledu z -30 °C na -36 °C, což může pomoci zmenšit nebezpečné rozmezí teplot nukleace a růstu ledových krystalků ledu před tvorbou biologického skla.

Kombinace působení sacharózy a dvou hodin desikace nad silikagelen přežilo 10 % vzrostných vrcholů, které byly schopny po následující kryoprezervaci v tekutém dusíku při -196 °C zregenerovat v nové rostliny. V případě ponoření vzrostných vrcholů do PVS3 na 1,5 h regenerace meloku po kryoprezervaci dosahovala 52,5 %. Regenerace proběhla v *in vitro* podmínkách na MS mediu s přidáním růstových látek. Podle našich výsledků teoretický potenciál pro zlepšení regenerace po tekutém dusíku je o dalších 21 % regenerujících rostlin skladování v tekutém dusíku. Na základě získaných dat je metoda kryoprezervace doporučena pro dlouhodobé uchování meloku.

Shrnutí: Podle dosažených výsledků nejúspěšnější metodou pro konzervaci jakonu je uchování v polních podmínkách nebo v *in vitro* podmínkách. Pro melok je výhodná *in vitro* konzervace a kryokonzervace. Nejvyšší propagační koeficient byl pro oba genotypy v *in vitro* podmínkách. Pomocí moderních metod byly stanoveny nejvhodnější způsoby uchování genofondu pro jakon a melok.

Klíčová slova: melok, jakon, *in vitro*, polní uchování, kryoprezervace, teplota

PREFACE

Through the centuries, the Andean farmers selected and bred their plants to create an incredible diversity of properties (King, 1986). Plant species that are vegetatively propagated are traditionally maintained in field collections but maintaining plants in the field is costly and carries high risks of loss (Reed *et al.*, 2004). Andean root and tuber crops fed the pre-Columbian Incas and still feed their modern descendants, but only the potato has travelled to other parts of the world. Who knows what rivalry the potato might have had if the other had received the same attention in efforts toward improvement (Hodge, 1951)? There are increasing opportunities for using *in vitro* methods for the conservation of crops normally conserved in the field, and there is a need to develop strategies and procedures for managing *in vitro* collections as routine and integral part of the overall conservation strategy of a crop gene pool or collection (Reed *et al.*, 2004). Conservation and sustainable use of genetic resources are essential to meet the demand for future food security (Kameswara-Rao, 2004). Future collecting of germplasm is a high priority in many crops and regions worldwide (Khoury *et al.*, 2010). The commitment of international research to the ARTC will not only aid in the introduction of these crops to other global regions but will enhance the current production of these crops in their native Andean region. The potential for introducing several ARTC as food crops for other areas of the world is significant (Flores *et al.*, 2003). Also introducing of ulluco and yacon to the Czech Republic seems to have significant potential as they have unique characteristics, which can enrich diet. Because of this unique germplasm it is needed to find the best way of a conservation strategy, which could be crucial for further breeding and use of these plants.

So this work was focused to three different ways of conservation which are currently going on at the Czech Republic.

1 INTRODUCTION

1.1 Andean Root and Tuber Crops

Andean root and tuber crops (ARTC) are neglected root and tuber-bearing crop species, native to the Andean highlands (Malice and Baudoin, 2009). These crops have developed the highest diversity of below ground crops in the most marginal climatic conditions anywhere in the world (close to 20 species of tubers and roots, e.g. potato, oca, ulluco, mashua, yacon, arracacha, achira, maca, sweet potato; Table 1, Flores *et al.*, 2003). ARTC have been cultivated for thousands of years for their edible tubers, through which they are propagated. For many centuries, they have continuously contributed to the food security of the Andean populations and are part of their culture and social expression (Malice and Baudoin, 2009). Andean people use a complex system of linked mechanisms to even out supply and buffer against risk: temporal (long-term storage), spatial and diversity (numerous plots in different conditions, numerous varieties and species, long distance exchanges) and social reciprocity (Halloy *et al.*, 2005).

Yacon and ulluco, together with potato, belong to the group of edible tuber crops indigenous to the Andean mountains, where they are a food staple in rural communities (Malice and Baudoin, 2009; Zardini, 1991). Potatoes were voted the most important crop; oca and ulluco were joint third (Terrazas *et al.*, 1999). Compared to potatoes, which are now cultivated in 130 countries around the world, these roots and tubers are essentially still unknown outside of the Andean region. The requirement of short days for tuber formation may limit potential production areas. The existing variation of different clones in response to day length gives promise that new cultivars adapted to long days or day neutral clones might be selected (Sperling and King, 1990). Biotic and abiotic pressures of the Andes, coupled with anthropic selection for food purpose and crop husbandry, have resulted in a large morphological variation (Malice and Baudoin, 2009).

Rapid erosion of the Andean tuber diversity indicates the importance of identifying and conserving Andean tuber cultivars throughout the Andes (King and Gershoff, 1987). Andean Countries of Latin America face the need to strengthen the Advanced biotechnologies which can be successfully used to cope genetic erosion, to reinforce *ex-situ* collections and in *in-situ* conservation, to upgrade the supply of planting materials to farmers and to integrate a new approach into the development programmes for food production and food security in mountain lands (Izquierdo and Roca, 1997). Research on

plant breeding and crop management on under-exploited Andean crops is not new and not starting from zero level. The great deal of biotechnological work on ARTC was done by CIP (International Potato Center). The application of plant biotechnology for the propagation, characterisation and conservation of promising genetic resources (cultivars) is still not significant (Izquierdo and Roca, 1997). Techniques like *in vitro* culture and cryopreservation have made it easy to collect and conserve genetic resources, especially of species that are difficult to conserve as seeds (Kameswara-Rao, 2004). Plant biotechnology applications for the conservation and sustainable use of germplasm of yacon and ulluco are developed for *in vitro* conservation and micropropagation (Table 4), but there is a little knowledge about research on cryopreservation (Izquierdo and Roca, 1997). Thus, this thesis will be focused to this not well known biotechnological research.

Numerous other root and tuber crops have been domesticated in the Andes. Typical and indigenous are nine Andean tubers and roots: *Ullucus tuberosus, Oxalis tuberosa, Tropaeolum tuberosum, Arracacia xanthorrhiza, Smallanthus sonchifolius, Mirabilis expansa, Canna edulis, Pachyrhizus ahipa, Lepidium meyenii* (Table 1). No discussion of Andean crop resources would be complete without mentioning the global potential of these and other crops that are part of the agricultural heritage of the Andean region. These tubers, grain legume and other crops have been among the primary food sources in the highland Andean region for centuries (Sperling and King, 1990).

Table 1 Main roots and tubers species originating in the Andes（Lizarraga *et al.*, 1997）

Crop (the common name)	**Latin name**	**Altitude**	**Agro-ecological zone**
Tubers			
Mashwa, añu	*Tropaeolum tuberosum*	3 500 - 4 100	Suni, puna
Oca	*Oxalis tuberosa*	2 300 - 4 000	High quechua, suni
Bitter potato	*Solanum x curtilobum*	3 900 - 4 200	Suni, puna
Potato	*Solanum indigenum*	1 000 - 3 900	Yunga, quechua, suni
Ullucu, oca quina	*Ullucus tuberosus*	2 800 - 4 000	High quechua, suni
Roots			
Achira, Queensland arrowroot	*Canna edulis*	1 000 - 2 500	Yunga, low quechua
Arracacha, apio, Peruvian parsnip	*Arracacia xanthorrhiza*	1 000 - 2 800	Yunga, low quechua
Mauka, chago	*Mirabilis expansa*	1 000 - 2 500	Yunga, humid quechua
Maca, pepper grass, pepper weed	*Lepidium meyenii*	3 900 - 4 200	Puna
Leafcup yacón	*Smallanthus sonchifolius*	1 000 - 3 000	Yunga, low quechua

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

Taxonomy

Botanical name: *Smallanthus sonchifolius* (Poepp. *et* Endl.) H. Robinson

Family: *Asteraceae*

Common names: *Polymnia sonchifolia, English:* yacon, leafcup; *Spanish:* yacón, yacuma, jícama (Ecuador, Bolivia), arboloco (Colombia). jícama (Lizarraga *et al.*, 1997). Other names are Peruvian ground apple, strawberry jicama, Bolivian sunroot, llacon (Michaels and Michaels, 2007) and jakon (Czech) (Michl, 2002)

The yacon is a perennial herb, 1.5–3 m tall. The root system is composed of 4–20 fleshy tuberous storage roots that can reach a length of 25 cm by 10 cm in diameter, and an extensive system of thin fibrous roots. Roots have an adventitious nature, growing from a developed and ramified stem system formed by short, thick sympodial rhizomes or rootstock (‘corona’, crown). The yacon rhizomes could be used both as plant cultivation material, as well as a rich source of polyphenolic antioxidants (Lachman *et al.*, 2005).

In Peru, yacon can be harvested throughout the year in areas that are frost free and are well irrigated. In the high Andes, only one growing season is possible, and planting is done at the beginning of the rainy season. In the high jungle region of Peru planting can take place at any time of year. The yacon root system forms fleshy rhizomes and yacon is harvested from six to twelve months after sowing. Location and altitude most affect the length of the growing season. In Peru, for example, it can be from 6 to 10 months depending on the altitude. To obtain oligofructan rich derivates (e.g. syrup) roots should be processed as soon as possible after harvest (Manrique *et al.*, 2005).

Cultivation practices

Different ways of propagation were observed: by seeds, stem cuttings, layering and proliferation of rhizomes, rhizome parts and by tissues cultures. Material of rhizomes is optimal for planting. Therefore, the good way for propagation is the usage of whole rhizomes or rhizomes parts (Viehmannova *et al.*, 2007a). The yacon root system forms fleshy rhizomes as well as tuberous roots. On the surface of each rhizome, there are many buds or points of growth. A mature rhizome can be broken into ten or twenty parts, each of

which are traditionally used as seed and have between 3 and 5 growing points (Seminario *et al.*, 2003). It has expenses arise for treatment of cutting wounds. Layering has little propagation coefficient, and it cannot be therefore used for commercial propagation (Plchova, 1997). There is no possibility to propagate yacon by seeds because of high sterility of seeds (Viehmannova *et al.*, 2007a). Propagation *in vitro* (nodal and apical segments) proved to be the best way of propagation with a high percentage of plants taken up (99%) (Plchova, 1997); however, it is highly economically demanding (Viehmannova *et al.*, 2007b).

Genetic background

Different numbers of chromosomes were found for yacon from 32 (Leon, 1964) to 87 (Ishiki *et al.*, 1997). For yacon ecotypes sustained in the Czech Republic it was published 58 (2n) chromosomes (Fernandez and Kucera, 1997) also (Frias *et al.*, 1997) and (Salgado Moreno, 1996) indicated 58 (2n) chromosomes.

Uses and nutritional value

The main product is tubers which are used for eaten by their up to 20 % sugars (FAO, 1989). Yacon tubers contain as storage compounds mainly fructans with low glucose content. Their structure is of the inulin type, i.e. (2→1) fructofuranosylsaccharose (Goto *et al.*, 1995). It is used mainly as a vegetable and may be eaten raw cooked or dried. Because it is easy to digest, it is used in invalids' diets in the usual areas of cultivation. The average sugar content increases as it becomes concentrated in the roots which are exposed to the sun for two weeks (Hernandez Bermejo and Leon, 1994). It can be also boiled, steamed or baked. The leaves have a protein content of 11–17% and when cut the foliage sprouts again from the underground stems (Michaels and Michaels, 2007). The main phenolic components are chlorogenic acid and coffeic acid, present both in the form of their esters and in the free form. Rhizomes and leaves were observed to be the richest sources of polyphenolic antioxidants. Phenolic acids derived from cinnamic acid contained in yacon are dominant yacon antioxidants (Lachman *et al.*, 2005).

1) Medicinal properties

In medicine yacon tubers are used for centuries by original Peruvian populations as a traditional folk medicament to treat hyperglycemia, kidney problems and for skin rejuvenation. In Brazil, medicinal properties have been ascribed to yacon leaves that are used to prepare a medicinal tea. In Japan, yacon leaves and stems are mixed with tea leaves

(Aybar *et al.*, 2001). The suitability of yacon foodstuffs for diabetic dishes, diets for weight reduction and for patients with chronic liver diseases has been shown in a clinical study performed at Olomouc Faculty Hospital (Valentova *et al.*, 2001). Polyphenol contents in different parts decreased in order rhizomes > leaves > stems = tuberous roots. Compared with potato, the yacon tuberous roots are 3–10 times richer in polyphenolic antioxidants. These results suggest that the yacon tuberous roots is a rich source of phenolic antioxidants in human nutrition as well as its leaves as an active component in dietary supplements for the prevention of chronic diseases (Lachman *et al.*, 2005). It was also demonstrated that yacon leaf extracts reduce glucose production in hepatocytes via both gluconeogenesis and glycogenolysis pathway, and their insulin-like effect (Valentova and Ulrichova, 2003). Yacon has exceptional qualities for low-calorie diets (Grau and Rea, 1997).

Some health effects associated with the consumption of fructooligosaccharides FOS (Manrique *et al.*, 2005):

- Low Caloric value (4,2–6,3 kJ g^{-1})
- Reduces the risk of colon cancer
- Reduces the triglycerides and cholesterol levels
- Reduces constipation (fiber)
- Promotes the synthesis of folic acid and B-complex vitamins
- Does not increase the glucose level
- Improves calcium assimilation
- Strengthens the immune system

2) Products

Other potential of yacon in the agro-industrial field – the sugar content of yacon is so high that it has potential for alcohol production (IPGRI, 2001). Potential use as a forage for both underground and aerial parts, wide range of processing alternatives (Grau and Rea, 1997). Cattle and pigs can eat the tuberous roots together with the foliage (Hernandez Bermejo and Leon, 1994) as inulin is rapidly metabolized by ruminants (Michaels and Michaels, 2007).

3) Environmental influence

The high agronomic potential of yacon may be appreciated. It is also used as a soil protector because of its ability to maintain itself as a perennial species, especially in dry agro-ecological areas (Hernandez Bermejo and Leon, 1994). Yacon grows well beneath a

canopy of trees. In this case yield of tubers should not be expected but it should be grown for animal forage.

4) Other advantages of yacon

High fresh weight productivity, potential good fit in agro-forestry systems, adaptability to a wide range of climates and soils, erosion control and good post-harvest life, if managed properly (Grau and Rea, 1997).

Biodiversity conservation

Yacon has been successfully cultivated in several different regions with varying climates including Brazil, Czech Republic, China, Korea, Japan, New Zealand, Russia, Taiwan and United States. The geographical distribution of yacon to greater latitudes and altitudes has been limited because of the combination of its long growing season (>180 days) and its susceptibility to frost (Manrique *et al.*, 2005).

It would be important to evaluate their presence in the existing national parks and reserves. As plants adapted to invade vegetation gaps, *Smallanthus* may also prefer disturbed habitats created by human intervention (Grau and Rea, 1997).

1.1.2 *Ullucus tuberosus* (Loz.)

Taxonomy

Botanical name: *Ullucus tuberosus* Lozano. Synonyms *Ullucus kunthii* Moq.*, Basella tuberosa* HBK, *Melloca tuberosa* Lindl., *Melloca peruviana* Lindl.

Family: *Basellaceae*

Common names: *English:* ulluco (Lempiainen, 1989); *Spanish:* michurui, tiquiño (Venezuela), chigua, chugua, rubas (Colombia), melloco (Ecuador), lisa, papalisa (Bolivia) and olloco, ulluca, ulluma (Argentina) (Lizarraga *et al.*, 1997), melok hlíznatý (in Czech) (Michl, 2002).

Ulluco of the *Basellaceae* family is one of the most widely grown and economically important tuber crops in the Andean region. As in its close relative, the tropical Malabar spinach (*Basella alba*) also the leaves are edible (CIP, 2011). Ancient in origin, it is likely that its cultivation extended from the Andes of Venezuela (lat. 10°N) to north-western Argentina and North-Eastern Chile (lat. 25°S) in pre-Hispanic times. However, the exact region of its domestication is not known (Hernandez Bermejo and Leon, 1994). The succulent ulluco leaves can be used as a vegetable and are similar in texture to spinach. They are known to contain high levels of protein, calcium and carotene; a spoonful of

cooked leaves can provide a considerable part of a child's daily requirements of essential nutrients (Hermann, 1992). Somewhere ulluco is maintained without greater economic yield and its production is almost entirely for farmers own consumption (Parra Quijano *et al.*, 2005).

Ulluco tubers come in a variety of colours and shapes. They are also popular in lowland cities far away from production zones. Yield-reducing viruses often infect the ulluco plant (Parra Quijano *et al.*, 2005) and some varieties such as the ulluco "Llausa lisa" used for chuño, have been lost recently (Terrazas *et al.*, 1999). Thermotherapy and meristem culture have been used to obtain pathogen-tested material for distribution of ulluco (Anonym, 2002).

Botanical description

The ulluco is an erect, compact plant which reaches a height of 20 to 50 cm. At the end of its growth, it is prostrate. Tuber shapes vary from spherical to cylindrical (Hernandez Bermejo and Leon, 1994) and colours range from green, cream and red (color of the tubers change with time and exposure to sunlight) (Parra Quijano *et al.*, 2005). On extremely rare occasions, it forms fruit; the seed then has the form of an inverted pyramid, with highly prominent angles and a corrugated surface.

Cultivation practices

The origin and development of the ulluco in the cold climates of the Andes suggests that it is one of the crops most suited to the complex agro-ecology of areas between 3 000 and 4 000 m asl. Although the precise role of hybridisation, introgression and mutation in the ulluco is not known, these must have acted – along with natural and human selection pressure – to favour the plant's distribution and adaptation to the various types of Andean climate and soils. Ulluco requires 11–13.5 hours of day length for stolon formation and tuberization (Sperling and King, 1990). The cultivation of ulluco was found closely related to *Tropaeolum tuberosum*, *Oxalis tuberosa* and *Solanum tuberosum* ssp. Andigena (Parra Quijano *et al.*, 2005). Cytological studies of ulluco indicate that abnormal meiosis resulting in incomplete microsporocyte formation may account for the rarity of seed formation (Sperling and King, 1990). Asexual reproduction through ulluco tubers determines the proper way to collect and preserve (Parra Quijano *et al.*, 2005) because here is genotypic variation in the ability of ulluco to set fruit. After open pollination, there were significant differences between clones in the total number of fruits per plant and in the number of

fruits produced per number of open flowers (Pietila and Jokela, 1990, Rousi *et al.*, 1989). The most probable reason for the poor seed set of ulluco is general lowered sexual fertility due to a long period of vegetative reproduction (Pietila and Jokela, 1990).

Eight viruses infect ulluco ulluco, an Andean tuber crop.

Ulluco Virus C (UVC), Ullucus Mild Mottle Virus (UMMV3), Ulluco Mosaic Virus (UMV) (CIP, 2002), three viruses also infecting potato (*Solanum tuberosum* L.) – potato leaf roll virus (PLRV), Andean potato latent virus, and potato virus T. Arracacha virus A and papaya mosaic virus, ulluco isolate (PapMV-U) infect other crops (Lizarraga *et al.*, 1997).

Storage

Careful selection of tubers free of mechanical and insect damage is a key to keeping loses at a minimum. They should be stored in boxes of about 50 kg or less with eucalyptus leaves at the base and at the top and kept in well-ventilated rooms where temperatures usually range from 8 to 12°C (Tupac, 1999).

Genetic diversity

The wild ulluco would seem to indicate a sympatric distribution with the cultivated ulluco since up to now it has been found from the Andes of La Libertad in Peru (lat. 8°S) to north-western Argentina (lat. 25°S). Cultivated ulluco are diploid and triploid, with a basic number of 12 (Hernandez Bermejo and Leon, 1994).

Biodiversity conservation

Germplasm accessions of ulluco in Andean gene banks is in Ecuador (156) Peru (255) (Sperling and King, 1990). Ulluco collections in South America shortages which limit the knowledge and promotion of the ulluco:

- Duplication of accessions: In clonal propagation crops such as the ulluco, there is a high probability of repeatedly collecting one and the same clone in different localities; also, the exchange of germplasm between national programmes without identifying data has meant that one and the same clone can be recorded under different numbers in various banks.
- Incomplete documentation: No standardized, internationally accepted descriptors exist for the characterization of the ulluco; there is a lack of specimens from herbaria such information would be extraordinarily useful in the event of living collections being lost.

- A lack of wild plant collections: There is an almost total absence of wild material; such material would help to understand the variation patterns of the cultivated forms and could provide valuable characteristics for improvement (Hernandez Bermejo and Leon, 1994).

1.2 Conservation of Yacon and Ulluco

Farmers have managed germplasm for thousands of years under complex and changing conditions. The term "conservation" fails to capture the dynamics of adoption and selection, however. Technological interventions that target highly specific problems outside the context of the local agro ecosystem do not allow the full utilisation of available biodiversity (Terrazas *et al.*, 1999). Crop genetic resources are managed at the intersection of agriculture, food security, environment and trade. Genetic erosion and genetic vulnerability continue to threaten crop diversity, but they can be overcome with good science, adequate resources, cooperation and political will (Fraleigh, 2006), and collection-level information (Khoury *et al.*, 2010). No doubt those primitive and wild gene pools will continue to serve as valuable sources of genes for resistance to parasites. They are important for characteristics indicated by advances in science of technology or by changing demands of the consumer. In the case of species, which are already used by human beings as crops, it is crucial to have a broad genetic base for improvement existing genotypes when necessary (Hammer and Teklu, 2008). For many crops, but particularly the vegetatively propagated species, the development of new technologies, or extension of technologies to more genotypes, is needed for better conservation systems (Khoury *et al.*, 2010). The conservation of plant genetic resources (PGR), on the other hand, is primarily concerned with genetic diversity within crops and related species, while diversity spread out of their natural centers to all areas where these crops are now grown. The Convention on Biological Diversity (CBD) was primarily designed and negotiated to deal with natural biodiversity. In the final stages of these negotiations, developing countries in particular insisted on more attention to biodiversity in crops crucial to food and agriculture (Hardon, 1996). Conservation of the biodiversity of crop genetic resources is not enough: their potential uses and values need to be understood by characterizing, evaluating and documenting them (Fraleigh, 2006). The importance of conservation of agro biodiversity for the future of global food security lies, in its potential, to supply crop breeders and other users' future needs for germplasm (Jarvis *et al.*, 2000).

Greater biodiversity security results from the application of a range of *ex situ* and *in situ* techniques applied in a complementary manner, one technique acting as a backup to the other techniques (Hammer and Teklu, 2008).

Genetic erosion

The richness and range of the diversity of these Andean landraces is now under threat because of the changing nature of agricultural production. One key factor is the widespread adoption of modern varieties that are the products of formal plant breeding (Tripp and van der Heide, 1996). Customers contribute to the genetic erosion by preferring certain varieties and types of crops (Chloupek, 2008). Much diversity has been lost with increasing market integration. For example, farmers in Candelaria and elsewhere in Bolivia, concentrate production on the few varieties of potatoes widely accepted by the market (Terrazas *et al.*, 1999).

Second, the widespread use of modern varieties raises questions about the stability of crop production (Tripp and van der Heide, 1996) especially by pest and disease outbreaks (Hammer and Teklu, 2008). For example, potatoes, oca and ulluco tuber seed gradually becomes infected with viruses that lower yields (Terrazas *et al.*, 1999). The very success of modern plant breeding now threatens the source of genetic diversity on which further progress depends as farmers find it less rewarding to maintain the diverse mixture of landraces developed by their ancestors. Hence the advent of plant variety protection lends added urgency to search for solutions to the conservation of plant genetic diversity (Tripp and van der Heide, 1996).

An alarming loss of genetic variability of ulluco has been observed during the last decades due to many factors such as the introduction of new varieties, droughts, changes in food habit, encroaching agriculture, deforestation and the migration from rural to urban areas. Ulluco showed an average loss of variability of 37.6 % in three provinces in Ecuador (Tapia and Estrella, 2001).

Yacon is also disappearing, for example, is not grown anymore in Salta province in northwestern Argentina though it was formerly widely cultivated, the same would apply to Peru, where the roots have been seen only in the markets of Tarma and Huaraz (Zardini, 1991). Yacon is a rare crop in northwestern Argentina, present only in a few localities. Cultivation was probably more widespread in the past, but at present the number of clones available in the area is reduced to two or three, and at risk of further losses. While genetic

erosion has probably also happened in Bolivia, cultivation of the species is still remarkably common in most Andean departments[1] of the country, Tarija, Chuquisaca, Cochabamba and La Paz. La Paz department, particularly the Camacho and Sud Yungas provinces, is most likely the one with the largest cultivated area and the largest germplasm diversity. However, Cochabamba, Chuquisaca and Tarija departments have been poorly researched up to now and may hold valuable material (Grau and Rea, 1997). There is a need for conservation, because of genetic erosion (Hammer and Teklu, 2008).

1.2.1 Conservation strategy

There are two basic types of conservation, namely, *in situ* and *ex situ* conservation (Tao, 2003), each of which includes a range of different techniques that can be implemented to achieve the aim of the strategy (Fig. 1). The products of conservation activities are primarily conserved germplasm, live and dried plants, cultures and conservation data. To ensure safety, conservation products should be duplicated more than one location (Hammer and Teklu, 2008). The sustainable utilization of plant diversity can be considerably assisted by the application of direct and indirect biotechnological procedures (Benson, 1999). Very little of the Andean root and tuber germplasm is conserved in gene banks as botanical seed since the flowers rarely produce viable seeds (Zandstra, 1994).

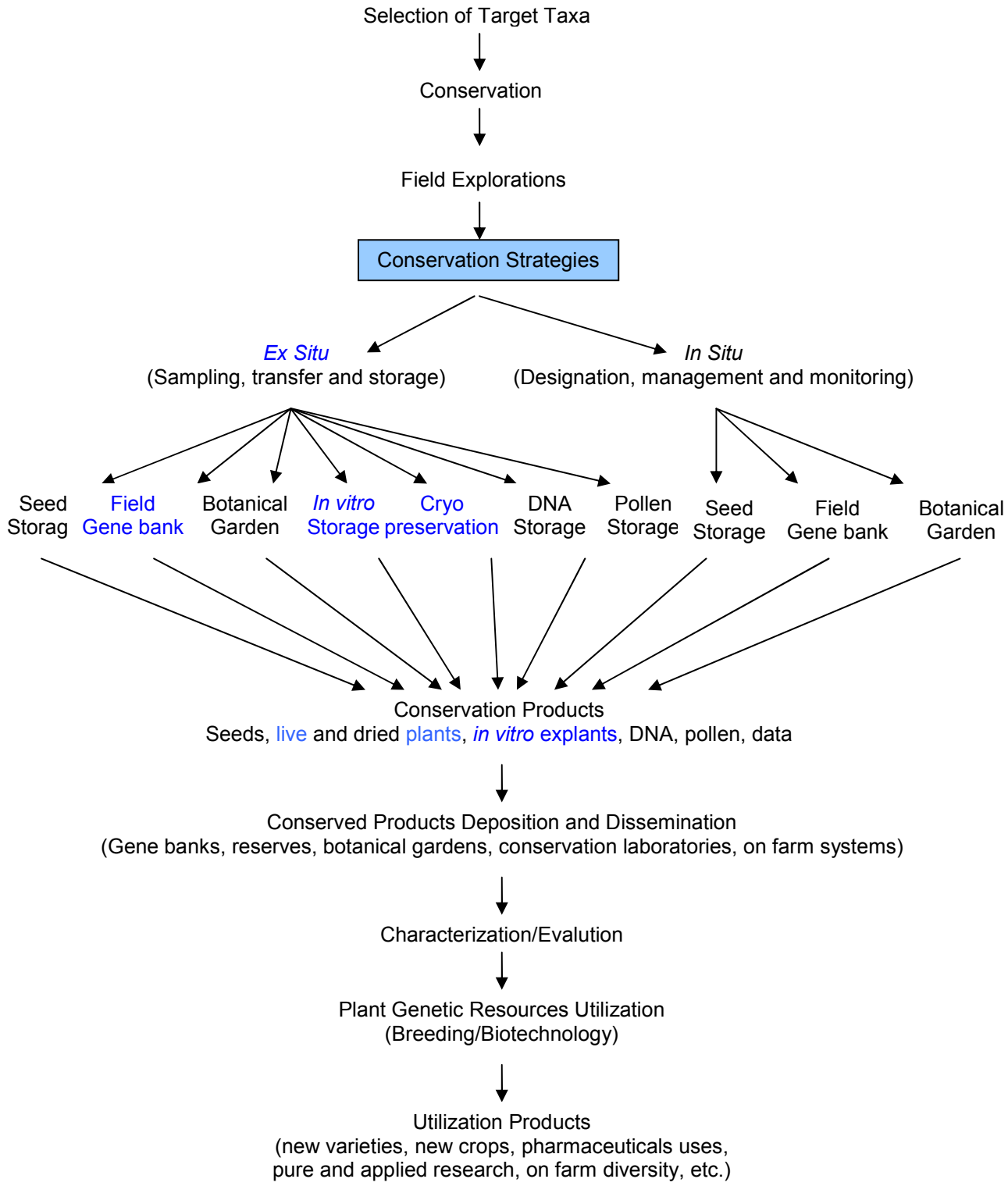

Fig. 1 Proposed model of plant genetic resources conservation adapted from (Hammer and Teklu, 2008, Maxted *et al.*, 1997) (blue color is used for methods tested in this thesis)

In situ conservation

The *in situ* conservation methods include genetic reserve area, home gardens and on-farm conservation (Fig. 1) (Hammer and Teklu, 2008). It is focused on farmers' fields as part of existing agro ecosystems while other types of *in situ* conservation are concerned with wild plant populations growing in their original habitats (genetic reserves) (Jarvis *et al.*, 2000). Andean native use a complex system of linked mechanism to even out supply and buffer against risk: temporal (long-term storage), spatial and diversity (numerous plots in different conditions, numerous varieties and species, long distance exchanges) and social reciprocity (Halloy *et al.*, 2005). On-farm conservation involves the maintenance of traditional crop varieties, especially landraces or of cropping systems by farmers within traditional agricultural or silvicultural systems (Tao, 2003). These elements show high resilience and adaptation to external climate change (Halloy *et al.*, 2005). Each season, the farmers keep a portion of harvested seed for re-sowing in the following year. Therefore, crop germplasm could be conserved and further evolve over time as in the history (Tao, 2003).

Farmers in the Andes usually renew their tuber seed every 3–5 years. For this reason, there is a continuous tuber seed flow within and between communities, mainly at harvest and planting. Furthermore, in traditional Andean systems, farmers usually consider the rotational system and follow the key factors in reducing pests and diseases. Selection of the normal tuber shapes of varieties with clean shiny tuber surfaces, understanding that the planting material is viral- and bacterial-disease free which, to a certain extent, is positive selection. This system has met most of the annual tuber seed requirements of oca, ulluco and mashua of the Andean rural families (Arbizu, 2010). Yacon is being grown in many localities scattered throughout the Andes, from Ecuador to northwestern Argentina. In most cases, just a few yacon plants are cultivated for family consumption. Less frequently yacon is grown as a cash crop, to be marketed at the local level. Even in this situation, farmers rarely cultivate yacon, the main crop and seldom dedicate a high-proportion of their arable land to it (Grau and Rea, 1997).

Advantages of *in situ* conservation

There are significant advantages to *in situ* conservation. One is its conservation of both genetic material and the processes that give rise to diversity. The long-term sustainability of breeding efforts may depend on the continued availability of the genetic variation that can be maintained and developed in farmers' fields. In addition, *in situ* conservation can

address the conservation of a large number of species at a single site, while this might be difficult for *ex situ* conservation owing to species' different requirements for *ex situ* maintenance (e.g. different seed storage behaviour, multiplication requirements, etc.) (Jarvis *et al.*, 2000). The Foundation for the Promotion and Investigation of Andean Products (PROINPA) is custodian of the Bolivian Andean tuber germplasm collection. Its focus has shifted from *in situ* conservation and resolving specific production constraints to promoting the sustainable use of Andean tubers in the context of a complex local social, economic and political environment (Terrazas *et al.*, 1999). *In situ* conservation programm also have significant potential to improve the livelihoods of farmers at the local level. On-farm conservation will allow the processes of evolution and adaptation to continue in crop plants, ensuring that new germplasm is generated over time, rather than limiting conservation to a finite set of genetic resources conserved in gene banks (Jarvis *et al.*, 2000).

Disadvantages *in situ* conservation

However, there are also distinct problems associated with *in situ* approaches to conservation. It may be difficult for scientists to identify and access the genetic material being conserved, which can be a problem for plant breeders who wish to use material with characteristics for their work. Moreover, on-farm approaches rarely allow the close control of germplasm by scientists that *ex situ* approaches facilitate. The same factors that allow for dynamic conservation may serve to threaten the security of landraces. Genetic erosion can still occur due to unforeseen circumstances like war and natural disasters while social and economic change may either foster or hinder on-farm biodiversity conservation over time.

Indeed, one of the challenges of *in situ* conservation research is to evaluate how economic development is affecting farmer maintenance of diversity so as to account for this process in the implementation of conservation initiatives (Jarvis *et al.*, 2000). Mono cropping tendencies and poor use of modern agricultural inputs have affected agro ecosystem viability and increased pest damage (Terrazas *et al.*, 1999). Abiotic components of agro ecosystems include temperature, soil, water, relative humidity, light and wind. Biotic factors include parasitic and herbivorous pests, competition from other plants, and favourable (symbiotic) relationships with other organisms (Jarvis *et al.*, 2000). Ulluco is attacked by roya (*Aecidium ulluci*), a disease farmers call "tojtu", a term also used to

describe potato blight. Because ulluco does not store well, it has to be marketed immediately after harvest when prices are low (Terrazas *et al.*, 1999).
Because of this disadvantages and implementation of ulluco and yacon plants at the conditions of Czech Republic this work is focused to *ex situ* conservation.

Ex situ conservation

Ex situ conservation is the conservation of components of biological diversity outside their natural habitats. The methods of *ex situ* conservation include seed gene bank, *in vitro* storage, cryopreservation, DNA storage, pollen storage, field gene bank and botanical garden. However, the DNA and pollen storage are still in a developmental stage (Tao, 2003). Biotechnology has generated new opportunities for genetic resources conservation. Techniques like *in vitro* culture and cryopreservation have made it possible to collect and conserve genetic resources, especially of species that are difficult to conserve as seeds (Hammer and Teklu, 2008).

Main groups of *ex situ* conservation:
a) seeds gene bank (Jarvis *et al.*, 2000) – for example The Svalbard Global Seed Vault opened in 2009, and had received accessions from the majority of CGIAR Centers, and from a number of national gene banks (Khoury *et al.*, 2010);
b) in the field（Huaman *et al.*, 1995, Jarvis *et al.*, 2000);
c) *in vitro* – micropropagation was the new technology most widely applied (Fraleigh, 2006). The needs continually ensure disease-free conditions, slow-growth storage of vegetative material *in vitro* has become increasingly prominent (Khoury *et al.*, 2010);
d) botanical garden (Jarvis *et al.*, 2000);
e) cryopreservation – new biotechnology for long-term conservation.

Ex situ conservation for yacon and ulluco is not enough studied so there is a need for deeper research. Yacon and ulluco are in *ex situ* maintained in the field (Frček *et al.*, 1995), (Douglas *et al.*, 2005; Martin *et al.*, 2005) *in vitro* (Estrella and Lazarte, 1994; Golmirzaie *et al.*, 1999) and by cryopreservation (Sanchez *et al.*, 2009).

Disadvantages *ex situ* conservation

There are some disadvantages associated with *ex situ* conservation. Foremost among these is the inevitable fact that *ex situ* conservation removes genetic material from its natural environment. This halts the ongoing evolutionary processes which help to make landraces unique and adaptable to changing environments. Moreover, *ex situ* conservation can be a highly expensive endeavour, making it unsustainable in some settings. These costs affect the choice of which crops are collected for *ex situ* conservation, as only major crops or those of high economic value as determined by breeders and scientists are likely to receive attention (Jarvis *et al.*, 2000).

Advantages *ex situ* conservation

Ex situ conservation has several important advantages for plant genetic resources conservationists (Table 2). It is relatively easy to identify the genetic diversity conserved in a gene bank, as the material is usually fully documented for the use of plant breeders and other scientists. Moreover, the genetic diversity maintained by these methods is directly controllable: as long as accessions are kept in suitable conditions and regenerated periodically, the likelihood of loosing material is relatively low (Jarvis *et al.*, 2000). Together the global crop and regional strategies demonstrate a number of main trends and needs in *ex situ* conservation of plant genetic resources (Khoury *et al.*, 2010)

Table 2 Advantages of germplasm conservation methods（Reed *et al.*, 2004）revised.

Character	**Field**	**Slow-growth *in vitro***	**Cryopreservation**
Medim-term	+	+	–
Long-term	–	–	+
Characterize	+	–	–
Evaluate	+	–	–
Virus elimination	–	+	+/–
Distribution	+	+	+/–
Base collection	+	+	+
Core collection	+	+/–	+
Safety duplication	+	+	+

+ = applicable – = not applicable

1.3 Field planting

Andean tubers are vegetatively propagated species, so the most appropriate method for *ex situ* conservation remains the establishment of gene banks in the field (Malice and Baudoin, 2009). The principle aim of any field gene banks is to conserve the maximum diversity of germplasm using a minimum sample size and number (Reed *et al.*, 2004). The conservation of germplasm in the field gene bank involves the collecting of plant materials and planting in the orchard or field in another location (Tao, 2003). Management of field collections and conservation costs are important in the case of vegetatively propagated species, mainly because of the need to regenerate varieties in the field and to maintain them as tubers (Malice and Baudoin, 2009).

Research on genetic diversity can help define core collections for field collections, thereby reducing collection size and costs. Such research can also help better characterize the field collection and identify gaps in such collections (Reed *et al.*, 2004). Tuber plant are most prominent crops at high altitudes, above 3500 m potatoes alone represent close to 40 % of all cultivated areas in Chrazani (Bolivia), while together with other tubers they cover 49 % of the area (Schuelte and Torrico, 1998). Genetic studies can also help determine the causes for genetic instability, including mutations, drifts and shifts in field collections. Better means for a reliable identification (fingerprinting) of accessions, which are relatively easy and cheap to run (kits), are also needed (Reed *et al.*, 2004). Adequate funding for regeneration is necessary in order to support labour, grow-out costs, materials and infrastructure purchases, replanting of field collections, the transfer of field collections to *in vitro* and cryopreservation storage, production of disease-free vegetative material, and related activities. New research and technologies are needed in order to develop or refine successful regeneration techniques, especially for vegetative crops and for wild species, in order to regenerate difficult germplasm, extend the time interval between regenerations, and lower maintenance requirements (Khoury *et al.*, 2010).

Chemical composition of three Andean root and tuber crops grown under native conditions and grown in temperate conditions in the Czech Republic (CR) were similar (Landa *et al.*, 2004).

Germplasm maintained in field gene banks may be lost to disease epidemics. The development of low-input management strategies can help reduce cost of maintaining field collections. Research on alternative methods for conservation (e.g. seed, *in vitro*, cryopreservation or *in situ* conservation) should be promoted. Studies on seed physiology

may help defining conditions to allow conserving seeds rather than whole plants and thus reduce the necessity of maintaining field collections (Reed *et al.*, 2004).

Maintaining germplasm in the field is expensive, and risks the loss of germplasm due to infectious diseases or unfavourable climatic conditions. Biotechnology through *in vitro* methods offers a valuable alternative which helps to safeguard these genetic resources (Golmirzaie and Salazar, 1995b).

The loss of accessions was reaching a maximum of 5 %, but these losses were not definitive because they were replaced by duplicates in the field or from *in vitro* in each case (Parra Quijano *et al.*, 2005).

1.4 *In vitro* conservation

For conservation of vegetatively propagated species, biotechnological approach is being considered to be valuable adjunct to field gene banks. *In vitro* conservation and cryopreservation are recognized as viable options for respectively medium and long-term conservation options. In response to the projected demand for grains and other starch producing tubers due to the increasing world population, more intensive crop improvement and development activities need to be carried out in root and tuber crops (Edison *et al.*, 2005). Acclimatization to cold and freezing is initiated exposure to low positive temperature (Pearce *et al.*, 1996).

The greatest need identified in the strategies for research or new technologies involves the development or further refining of conservation techniques for *in vitro* storage and cryopreservation for vegetative crops (Khoury *et al.*, 2010). Curators of collections with a heavy virus load, or those at risk of loss in the field from biotic or abiotic factors, should consider the advantages of *in vitro* storage. Crops with difficult quarantine problems may require distribution as *in vitro* plants. Perennial or tree crop collections may reduce the number of field replicates and thus land costs by duplicating the collection and storing it in tissue culture. Any germplasm curator with interest in long-term storage of crop germplasm should consider cryopreservation for a base collection, especially for irreplaceable accessions such as those that are endangered extinct elsewhere, habitat destroyed, etc. (Reed *et al.*, 2004).

A number of chemical and physical factors affecting cultivation these factors include media components, phytohormones (growth regulators), pH, temperature, aeration, agitation, light, etc. This is the most fundamental approach in plant cell culture technology (Misawa, 1994).

The process of micropropagation is usually divided into the following stages (Kovac, 1995), (Lynch, 1999):

Stage 0 – Preparative stage – selection and pre-treatment of suitable plants

Stage I – Establishment stage – initiation of aseptic culture

Stage III – Multiplication stage – proliferation of tissue culture

Stage IV – Plantlet production – rooting of the explants

Stage V – Establishment under *in vivo* conditions – rooting *in vivo* and acclimatization

The *in vitro* growth and development of a plant is determined by a number of complex factor (Pierik, 1987):

- The genetic make-up of the plant
- Nutrients: water, macro- and micro-elements, and sugars
- Physical growth factors: light, temperature, pH, O_2 and CO_2 concentration
- Some organic substances: for example hormones, vitamins

Sucrose and glucose are the preferred carbon source for plant tissue cultures. (Misawa, 1994). To improve the root system formation the medium could be supplemented with grow hormone of auxin character (NAA) (Acuna *et al.*, 2007). Regeneration activities complement a range of other gene bank functions, including multiplication, gathering of characterization data, and ridding accessions of disease. Characterization of accessions not only produces valuable agronomic and breeding data, but also is useful for the identification of duplicates within and between collections, contributing to the potential for rationalization (i.e. elimination of unnecessary duplication) of collections, which in turn can help ensure that the limited resources available for regeneration are used most efficiently and effectively (Khoury *et al.*, 2010).

Table 3 Plant biotechnology applications for the conservation and sustainable use of germplasm of yacon and ulluco (Izquierdo and Roca, 1997).

Biotechnology applications	**Yacon**	**Ulluco**
Micropropagation	++	+++
In vitro conservation	++	++
Transfer to soil	+ + +	+++
Pathogen eradication	0	+++
Virus diagnostics	0	+++
Cryo-conservation	0	+
Biochem. characterization	+	+
Callus culture	0	++
Organogenesis	0	0
Embryogenesis	0	0
In vitro selection/clone variants	0	+
Protoplast culture	0	++
In vitro tuberization	0	++
Embryo rescue	0	0
Cell suspensions	0	0
Transgenic plants	0	+

Legend: +++ = routine ++ = available + = starting 0 = not available

Ulluco growth *in vitro* is well explored (Table 3), even with a single culture medium for its introduction, rooting, propagation and conservation. Moreover, its acclimatization to *ex vitro* proved to be a simple process with a rapid adaptation of plants. The loss of accessions was reaching a maximum of 5 % in *in vitro* conditions (Parra Quijano *et al.*, 2005).

Yacon was also successfully introduced to *in vitro* conditions (Table 3) (Estrella and Lazarte, 1994), thousands of plants can be obtained in one year from one single tip. Continuous axillary shoot formation is achieved by repeating stem node culture every 2 months in MS medium (Matsubara, 1997). *In vitro* propagation of yacon was investigated by (Estrella and Lazarte, 1994) with the use of growth regulators BA and IBA. The micropropagation procedure established through this research has subsequently permitted the preservation of more than 28 collected yacon genotypes in INIAP´s Ecuadorin germplasm bank.

Conservation of yacon and ulluco *in vitro* is routinely done at CIP at the conditions for ulluco on MS medium (Murashige and Skoog, 1962) supplemented by 2% sucrose, 3%

mannitol, and for yacon supplemented by 3%, 4% mannitol and temperature 18–22°C (Golmirzaie *et al.*, 1999).

Establishment of aseptic culture

The establishment of aseptic cultures is feasible in vegetatively propagated crops, by the use of actively growing buds or nodes (Golmirzaie *et al.*, 1999). Medium was sterilized by autoclaving at 121 °C with a pressure of 15 psi (1.06 kg m^{-2}) (Sharma, 2003).

Propagation

The type as well as physiological stage of explants is very important. From various explants used for germplasm conservation apical and axillary meristem has many advantages; lowest frequency of somaclonal variation, it is easy to propagate, true-to-type compared with parent material, and it is genome independent (Tyagi and Yusuf, 2003).
Micropropagation of yacon is achieved by apex, node and callus cultures for mass propagation of virus – or disease-free plants (Matsubara, 1997) on MS medium supplemented by NAA and BA (Matsubara *et al.*, 1990).

Germplasm maintenance

Combining growth restricting media with a relatively low incubation temperature reduces the number of transfers of *in vitro* plantlets (subcultures). At CIP, potato germplasm can be maintained for up to two years between subcultures by applying this system (Golmirzaie and Salazar, 1995b).

Slow-growth storage lowers the risk of losing germplasm through handling errors, such as contamination and media errors; decreases mislabeling; decreases the risks of genetic instability; cuts down on labour; and reduces the overall cost of maintaining the germplasm (Reed *et al.*, 2004). *In vitro* conservation of potato, sweet potato, and some Andean root and tuber crops is maintained by using a combination of osmotic stress agents, low temperature and low light intensity (Golmirzaie *et al.*, 1999). Increasing sucrose concentration provides reduce plant growth (Bonnier and van Tuyl, 1997). Slow-growth techniques are in a more advanced state of development than cryopreservation techniques, which still require improvement before they can be used on a routine basis in a number of species (Kameswara-Rao, 2004). Storage may be *as in vitro* cultures in warm or cold conditions, or as meristems, pollen or dormant buds in liquid nitrogen. The selection of the

method or methods will depend on the plant genotype and the available techniques. Storage *in vitro* decreases labour requirements, cost and the chance for contamination of cultures. The longer the plants can be stored without transfer or subculture the lower cost and the more secure the accessions. The main goal is the recovery of a healthy plant with good propagation potential (Reed *et al.*, 2004).

Slow-growth can be achieved by (Tyagi and Yusuf, 2003), (Golmirzaie *et al.*, 1999):

- Temperature or light intensity
- Use of growth retardants – it is not preferred s it is likely to cause genetic variation
- Use of decreased contents in the culture medium
- Use of osmotic regulators
- Type of enclosure
- Size and type of culture vessels
- Induction of storage organs
- Reduction in oxygen concentration
- Modification of the gaseous environment
- Combined treatments

It is possible to maximize the interval between transfers (subcultures) of *in vitro* plantlets by using both growth restriction media and reduced incubation temperature. CIP can maintain potato germplasm for an average of two years between transfers by applying this system (Golmirzaie and Salazar, 1995a). The most appropriate protocols have been developed for slow growth of oca and ulluco by using low temperature (6–8°C) combined with sorbitol osmotic stress complemented with light intensity of 30 µmol m^{-2} s^{-1} for 16 hours, giving, as a result, the storage of oca and ulluco for at least one year before transferring to fresh medium. Arracacha, yacon, and achira conservation have been performed by *in vitro* subculture every 3–4 months (Panta and Roca, 2008) and according to Toledo et al. (1994) up to 6 months at 18–22 °C on MS with 4 % mannitol and 3 % sucrose.

Microtuberization

Microtubers offer several advantages for *in vitro* propagated plants: they can be stored for a long time and directly transplanted without an acclimation stage, unlike *vitro* plants. They allow a reduction in the number of field generations in the multiplication of high quality seed tubers. They guarantee a safe in vivo transfer, and they facilitate

commercialisation (Manzelli *et al.*, 2004). Tuberization *in vitro* for conservation is a complex physiological process regulated by many factors. Factors known to influence *in vitro* tuberization include sucrose concentration (Keram and Al-Majathoub, 2000), environmental factors, hormonal factors, nitrogen supply, source of explants. For example, jasmonic acid would act as a chemical signal for processes like tuberization, which take place after a sufficient vegetative development of the potato plant (Pelacho, 1991). Also, modified MS medium (3–6 % sucrose) without growth regulators is used for potato microtuberization (Horackova and Domkarova, 2005).

Ulluco response in terms of microtuber production is in opposition to potato – high sucrose levels in a medium can slow, reduce or inhibit the tuberization process (Manzelli *et al.*, 2004). *In vitro* produced microtubers have advantages compared with conventional field grown tubers; they are small, light, and aseptic (Espinoza *et al.*, 1986). Many studies about microtuberization have been done. Microtubers of potato are produced during 2–3 months and can be stored at 10 °C for 21 months (Kwiatkowski *et al.*, 1988).

Simultaneous conservation of accessions *in vitro* provides protection from pests, pathogens and climatic hazards, and increases their availability for distribution if the materials are maintained virus free (Reed *et al.*, 2004). However, the technology is capital, labour and energy intensive. Although, labour is cheap in many developing countries, the resources of trained personnel and equipment are often not readily available. In addition, energy, particularly electricity, and clean water are costly. The energy requirements for tissue culture technology depend on day temperature, day-length and relative humidity, and they have to be controlled during the process of propagation. Individual plant species also differ in their growth requirements. Hence, it is necessary to have low cost options for weaning, hardening of micropropagated plants and finally growing them in the field (Ahloowalia *et al.*, 2004).

It is not clear how some factors, such as dark/light, presence and concentration in the medium of sucrose affect tuber formation (Manzelli *et al.*, 2004).

Aim of *in vitro* conservation studies was to find out influence of temperature on ulluco and yacon shoot tips for propagation and conservation as these factors are very important also for cryopreservation.

1.5 Cryopreservation

Cryopreservation is storage of viable cells, tissues, organs and organisms at ultra low temperatures usually in liquid nitrogen (LN) and/or its vapour phase, at temperatures of -196°C to -140°C (Benson *et al.*, 2007). Over the last decades, plant cryopreservation technologies have been evolving rapidly, opening the door to the possibility of long-term storage of valuable genetic resources of many crops (Panis and Lambardi, 2006). Significant progress has been made during the last 10 years in the area of plant cryopreservation, with the development of various efficient vitrification-based protocols (Sakai, 2004).

Methods of cryopreservation:

- Two-step cooling (Bajaj, 1977), (Towill, 1981)
- Encapsulation – dehydration (Fabre and Dereuddre, 1990)
- Vitrification (Luo and Reed, 1997)
- Ultra rapid freezing – droplet method (Schafer-Menuhr *et al.*, 1997)

Vitrification is manipulated using two main approaches: (1) Addition of penetrating cryoprotective additives at high concentrations, and (2) The removal of water by evaporative desiccation and/or osmotic dehydration (Benson, 2008).

Application of cryopreservation to the shoot-tip meristems of crop plant germplasm droplet-vitrification is highly successful. This method is now one of the most widely used cryopreservation protocols to date. In this capacity, it has facilitated overcoming the bottleneck of differential genotype response which is a serious problem in the large-scale cryobanking of crop plant genetic resources (Benson, 2008). The success of the droplet-vitrification (PVS2) and droplet-freezing (DMSO) methods is critically dependent upon using ultra rapid cooling and rewarming and which necessitates direct exposure to LN, making containment one of its main drawbacks (Benson, 2008). In encapsulation-vitrification protocol, samples are encapsulated in alginate beads, osmoprotected and then dehydrated with a highly concentrated vitrification solution for 2 or 3 h before a plunge into LN (Hirai and Sakai, 1999). If cryopreservation is available, it can provide a secure backup at little continuing operational cost. Initial costs appear high, but cryopreservation procedures fit well with an *in vitro* facility and require little additional expenditure other than labour. A cryopreserved duplicate should not be considered as the only form for a collection, rather it should be a base collection as insurance against loss of field or *in vitro* stored accessions (Reed *et al.*, 2004).

Some plant genetic resources constituencies may view cryobanking as an alternative or replacement to *ex situ* conservation in field gene banks, rather than as a parallel conservation strategy that provides additional security and safety, the latter being the preferred option to enhance the overall security of valuable genetic resources. Keeping all conservation options open is important and to this end two main approaches have been used to both secure and use plant genetic resources *in vitro* (Benson, 2008). Cryopreservation studies conducted over the last decades have enabled the regeneration of entire plants from cells, protoplasts, meristems, embryos, endosperm etc. of a variety of genera belonging to diverse families. Notable examples are the vegetatively propagated crops (potato, cassava) (Bajaj, 1995b).

Basic steps, which are involved in the freeze preservation of *in vitro* cultures (Bajaj, 1995b):

1. Establishment of *in vitro* cultures (chapter 3.4)
2. Addition of appropriate cryoprotectant
3. Subjection of cultures to super low temperatures
4. Storage of frozen cultures
5. Thawing of retrieved cultures
6. Removal of cryoprotectant by washing
7. Determination of viability
8. Induction of growth and regeneration of plants

Vitrification is a process by which water is solidified, not into a crystal, but into an amorphous glass. The glass incorporates all of the dissolved solutes present in the water and hence vitrified cells are not under osmotic, ionic strength, or volume stresses as frozen ones are. In the laboratory, the requirements for achieving a glass transition are daunting and include the need for extremely high concentrations of solutes (about 40 % solutions), rapid cooling to the glass transition temperature (Tg) that is often well below -30 °C, and warming rates in the order of 30–50 °C min^{-1} to prevent devitrification, the instantaneous crystallization of ice that can occur during warming at any temperature between the Tg and the melting point (MP) of the solution (Storey and Storey, 2005). Cryopreservation techniques based on the vitrification have been developed mainly in the 80´s or early 90´s. In these methods, cell desiccation is performed either by exposure of samples to concentrated cryoprotective solutions or air desiccation. This is followed by rapid freezing through direct immersion of the samples in LN. These methods have practical advantages

over classical techniques such as slow rate cooling. Vitrification based procedures are simpler than the classical technique they obviate the need for a programmable freezer and they have greater potential for broader applicability (Mandal *et al.*, 2003). At CIP is used vitrification method (Steponkus *et al.*, 1992) for potatoes.

The routes leading to the creation of the total vitrified state involve different permutations of the following treatments (Benson, 2008):

- Slow freezing (freeze dehydration) (Sakai, 2004)
- Evaporative desiccation only (air, chemical desiccants such as silica gel) (Benson, 2008, Sakai, 2004)
- Osmotic dehydration (Benson, 2008), (Sakai, 2004)
- Osmotic dehydration, combined with alginate bead encapsulation and evaporative desiccation (encapsulation-dehydration)
- Chemical additive vitrification using high concentrations of cryoprotectants used in traditional controlled (2-step) cooling (DMSO, ethylene glycol, polyethylene, glycol, sucrose, glycerol), the most common being PVS2
- Chemical additives combined with alginate bead encapsulation (encapsulation-vitrification) (Benson, 2008, Sakai, 2004)
- Droplet-vitrification (micro-droplets of vitrification solution are cooled at ultra rapid rates by direct exposure to LN) (Benson, 2008)

An important advantage of these new techniques is their operational simplicity and efficiency (Sakai, 2004). Vitrification unlike freezing is not physically destructive of tissue organization or subcellular architecture and, hence, the technique has been pursued as the method for the ultralow storage of cells, tissues and organs (Storey and Storey, 2005). Resistance to dehydration is very important for higher regeneration of thawed plants from ultralow temperatures (Zamecnik and Faltus, 2011). Low tolerance for substantial dehydration, the requirement for extremely high amounts of cryoprotective agents (such as dimethylsulfoxide, ethylene glycol, glycerol), which may have cytotoxic effects, and the need for very fast and even cooling and warming to induce vitrification during cooling and avoiding devitrification during warming (Storey and Storey, 2005). Vitrification involves treatment of the culture with higher concentrations of cryoprotectants. The main advantage of vitrification is that controlled-rate is not required; thus the time needed for cooling is reduced. Vitrification, in fact, is the formation of glass-like, noncrystalline solid at the freezing point of an aqueous solution (Bajaj, 1995b). No ice forms when materials are

vitrified, even at cryogenic temperatures. The formation of ice is prevented by the presence of high concentrations of chemicals that interact strongly with water and, therefore, prevent water molecules from interacting to form ice. Vitrification is potentially applicable to all biological systems (Taylor *et al.*, 2004).

Material used for cryopreservation

Shoot-meristematic tissues are the most commonly used explants for the cryopreservation of vegetatively propagated species, such as fruit trees and many root and tuber crops. Also in view of the lower chance for somaclonal variation, organized tissues such as meristems are often preferred over non-organized tissues, such as calli and cell suspensions (Panis and Lambardi, 2006).

Cryoprotectants

The function of the cryoprotectant is to act as an antifreeze; it avoids intracellular freezing of cells, when occurs, is invariably lethal (Bajaj, 1995a).

- **Sugars** Sucrose is an important pre-growth additive for the acquisition of desiccation tolerance during cryopreservation of shoot apices by vitrification (Sakai and Naik, 1998).
- **Glycerol** poly-hydroxylated solute with a high solubility in water, and a low toxicity during short-term exposure to living cells (Fuller, 2004). Glycerol is highly concentrated cryoprotective solution which is highly viscous is easily supercooled below -70 °C allow to be vitrified on rapid cooling (Rasmussen and Luyet, 1970).
- **DMSO –** dimethyl sulfoxide – can penetrate the plasma membrane (Fuller, 2004)
- **PVS3** solution is 50 % w/v of sucrose and 50 % w/v glycerol prepared in standard liquid culture medium (Nishizawa *et al.*, 1993). Modification of PVS3 is 40 % w/v of sucrose and 40 % w/v glycerol (Sakai *et al.*, 2008)
- **PVS2** – solution is 30 % glycerol (w/v), 15 % ethylene glycol (w/v) and 15 % dimethyl sulfoxide (DMSO; w/v) in basal culture medium (Sakai and Kobayashi, 1990) – the most common approach of treatment (Reed, 2008) but it can have toxic effects (Shambhu *et al.*, 2009).

Tolerance to dehydration is critical in plants. Freeze drying, solute dehydration and air desiccation all require either to innate or can acquire dehydration tolerance (Sakai, 2004).

Recovery of cryopreserved plants can be improved by modifying plant culture, pretreatments, cryoprotectant type, cryoprotectant exposure time, freezing or thawing rates and recovery medium. Methods to improve the health of the cultures will also improve survival rates since plants in poor condition seldom survive cryopreservation. Most meristems are stored in the liquid phase (Reed *et al.*, 2004).

Preculture

The culture of meristems on a medium containing low concentrations of cryoprotectants for a couple of days before freezing has been observed to be beneficial (Bajaj, 1995a). Preconditioning of meristem donor plants can be achieved simply by supplementing their growth medium with rising concentrations of sucrose over a period of a month before vitrification (Sakai, 2004).

Cooling and warming

During the cooling and warming procedures, rapid heat transfer is needed to avoid freezing injury (Kim *et al.*, 2006). Higher cooling rates form smaller internal ice crystals, and small crystals appear to be less damaging than large ones. However, if subsequent warming is slow those small crystals can enlarge to damaging size by the process of recrystallization. Another related possibility is that even at low cooling rates, not all the cell water freezes – a portion may be converted to a glass. If subsequent warming is too slow, this glassy water may devitrify (freeze), with lethal results. This partial glass formation is an introduction to purposeful attempts to induce vitrification of all the cell water – attempts that are being increasingly pursued in the cryopreservation of tissues and organs that are damaged by extracellular and intracellular ice (Mazur, 2004).

Storage of frozen culture

At ultra low temperatures, all metabolic activities of cell cease, and theoretically the cell or tissue can be stored for an indefinite period (Dhillon and Saxena, 2003). Also according to (Mix-Wagner *et al.*, 2003) and (Volk *et al.*, 2008) potato apices and *Malus* dormant buds after 7–10 years of storage in LN did not change in either survival or recovery.

Table 4 Cryopreservation of root and tuber crops, revised from (Sakai, 2004, Reed, 2008)

Root and tuber crops	**PVS based vitrification**	**Encapsulation-dehydration /vitrification**	**References**
Manihot esculenta	+	-	(Charoensub *et al.*, 1999)
Colocasia esculenta	+	-	(Takagi *et al.*, 1997)
Dioscorea sp.	+	+	(Kyesmu and Takagi, 2000), Malaurie *et al.*, 1998)
Solanum tuberosum	+	+	(Hirai and Sakai, 1999, Bouafia *et al.*, 1996, Shambhu *et al.*, 2009)
Oxalis tuberosa	+	-	(Gonzales-Benito *et al.*, 2007)
Smallanthus sonchifolius	-	-	-
Ullucus tuberosus	+	-	(Sanchez *et al.*, 2009, Zamecnikova *et al.*, 2011)

Note: + applied; - not applied

1.5.1 Thermal analysis

Conventional methods (Cernoskova and Cernosek, 2009)**:**

- Differential Thermal Analysis (DTA)
- Differential Scanning Calorimetry (DSC)
- Thermogravimetry (TG)
- Thermomechanical Analysis (TMA)

DSC is the most widely used of the thermal techniques available to the analyst (Fig. 2). DSC is a well established measuring method which is used on a large scale in different areas of research, development, and quality inspection and testing. Measurement values obtained by DSC allow heat capacity, heat of transition, kinetic data, purity and glass transition to be determined. DSC curves serve to identify substances, to set up phase diagrams and to determine degrees of crystallinity (Hohne *et al.*, 2003). DSC is used for the direct estimation of glass transition temperature (Zamecnik and Faltus, 2011).

The Glass transition temperature (T_g), is a fundamental property of all glass-forming materials, and a significant change in mechanical properties occurs at this temperature. Below T_g, an amorphous material is a glass, and above T_g it behaves more like a rubber and is defined as being in a rubbery state. The T_g is a second-order process and will, therefore, depend upon how it is measured. Unlike a melting point, there is no unique value for the T_g process since its value depends upon how it is measured and how it has been conditioned. The T_g manifests itself as a change in the coefficient of thermal expansion, which is usually lower for the glassy state than the rubbery state (Gabbott, 2008).

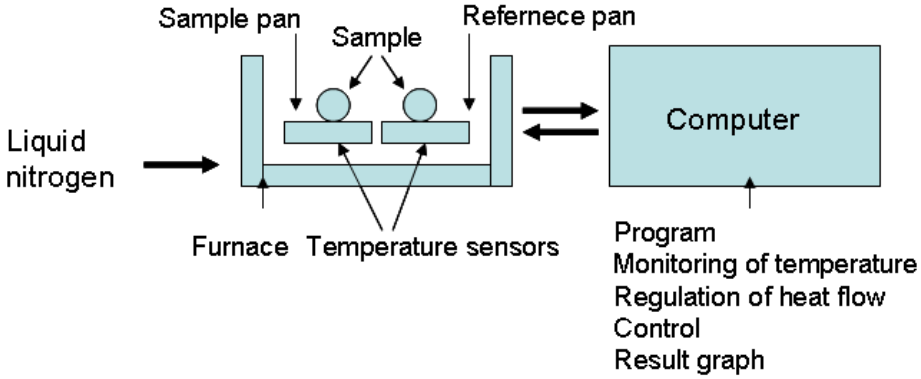

Fig. 2 Scheme of differential scanning calorimeter (Mandikova unpublished)

1.5.2 Institutions involved in genetic conservation for yacon and ulluco

The International Plant Genetic Resources Institute (IPGRI) is an independent international scientific organization that seeks to advance the conservation and use of plant genetic diversity for the well-being of present and future generations. It is one of 15 Future Harvest Centers supported by the Consultative Group on International Agricultural Research (CGIAR), an association of public and private members who support efforts to mobilize cutting-edge science to reduce hunger and poverty, improve human nutrition and health, and protect the environment. The international status of IPGRI is conferred under an Establishment Agreement which, by January 2003, had been signed by 48 states including Czech Republic (Reed *et al.*, 2004).

Systematic collecting of yacon germplasm began in the 1980s, sponsored by the International Board for Plant Genetic Resources. It focused mainly on crops of global importance such as potato. However, some work was diverted to secondary crops such as the other Andean tubers and yacon. The collecting effort lasted about 5 years and concentrated on Ecuador and Peru. A second collecting period began in 1993, guided by the Programme of Roots and Tubers in the Andes, administered by the CIP and funded by Swiss Technical Cooperation for a 5-year period. More emphasis was placed on secondary Andean crops and the collecting activities were expanded to Bolivia, aiming mainly at *in situ* conservation (Grau and Rea, 1997).

Institutions holding germplasm of yacon and ulluco are mainly concentrated in south America: in 1971, the CIP assumed a mandate to safeguard Andean Root and Tuber Crops including yacon and ulluco (Golmirzaie *et al.*, 1999). Number of root and tuber crops *in vitro* accessions in gene bank in CIP is shown at the Table 5.

Another institutions are The National University of Cajamarca, The University of Ayacucho. Material from southern Peru is concentrated in Cusco. The CICA (Centro Internacional de Cultivos Andinos) of the University of Cusco maintained under field conditions and *in vitro*. Ecuadorian Andean root and tuber germplasm is managed by the INIAP (Instituto Nacional Autónomo de Investigaciones Agropecuarias) (Grau and Rea, 1997).

Table 5 Number of *in vitro* accessions in gene bank in CIP（Golmirzaie *et al.*, 1999）

Crop	Species	Collection	Accession number
Potato	*Solanum tuberosum*	World	4218
		Pathogen tested	938
		Research	2247
		Total	7403
Sweet potato	*Ipomoea batatas*	World	4996
		Pathogen tested	414
		Research	7
		Total	5417
Oca	*Oxalis tuberosa*	World	476
Ulluco	*Ullucus tuberosus*	World	439
Mashua	*Tropaeolum tuberosum*	World	48
Yacon	*Smallanthus sonchifolius*	World	38
Arracacha	*Arracacia xanthorrhiza*	World	9
Achira	*Canna edulis*	World	36

Andean root and tuber crops has been successfully cultivated and maintained in three localities of the Czech Republic – yacon and ulluco at the Czech University of Life Sciences in Prague and yacon at the Potato Research Institute in Havlickuv Brod and Palacky University in Olomouc-Holice (Lebeda *et al.*, 2004).

2 OBJECTIVES

The aim of this thesis is to find proper way of genetic diversity conservation of two Andean crops *Smallanthus sonchifolius* (Poepp. *et* Endl.) H. Robinson (yacon) and *Ullucus tuberosus* (Loz.) (ulluco) – to compare different ways of their *ex situ* conservation – field conservation, *in vitro* conservation and cryopreservation.

The environmental conditions of the Czech Republic for conservation of both tuber crops in the field were tested. The second possibility in *in vitro* conservation is focused to maintain of ulluco and yacon by slow growth for medium-term conservation or short-term conservation and repeated propagation. Cryopreservation as a modern biotechnological method is based on vitrification compares different sucrose pre-treatment and its efficiency towards optimal dehydration and desiccation for ulluco and yacon cryopreservation.

I. **Hypothesis** – ulluco and yacon are possible to propagate and to store in *in vitro* conditions
II. **Hypothesis** – ulluco and yacon are possible to conserve by cryopreservation
III. **Hypothesis** – for long-term conservation *in vitro* conservation is more effective and safe than field conservation

3 MATERIALS AND METHODS

3.1 Plant material

During the field experiments, five different landraces of yacon were used (NZL, DEU, ECU, BOL, NZL2) which were collected at the Czech University of Life Sciences (CULS) during 1993–2005. Landraces are maintained by the Institute of Tropics and Subtropics, Prague at field conditions, and they are stored and propagated by rhizomes. For *in vitro* conservation was used yacon landrace (17) obtained from Potato Research Institute Havlickuv Brod. Plantlets were further propagated under *in vitro* conditions.

ulluco cultivar (´Gallo blanco´) originated from Bolivia was imported to Czech University of Life Sciences in Prague in 2007, and it is maintained by the Institute of Tropics and Subtropics. Meristem shoot tips cultures were cultivated *in vitro*.

This research work was carried out at fields and laboratory of the Institute of Tropics and Subtropics (CULS) and at the laboratory of the Crop Research Institute, Prague.

3.2 Field conservation

3.2.1 Conditions

Plants were cultivated on the experimental fields of the Czech University of Agriculture in Prague, which are situated in an average altitude of 286 m, on 50°04' north latitude and 14°26' east longitude and have loamy soils. The climatic area is mildly warm and mildly dry with average year temperature 9 °C and total precipitation of 500 mm.

3.2.2 Germplasm maintenance

Five different landraces of yacon were studied: NZL (New Zealand), NZL2 (New Zealand 2), DEU (Germany), ECU (Ecuador) and BOL (Bolivia). The planting on a field site was operated during the time, when the spring frosts were definitely over (mostly in the second half of May). The organic fertilizer (cattle manure, 20 t ha^{-1}) to soil had been applied before. Plants were planted in ridges in spacing of 0.70 × 0.70 m. Yacon's beginning growth is somewhat slow and that is why its resistance to weeds is remarkably small. Hoeing was performed approximately 30 days after the out planting, so the inter-rows were ploughed, and the weeds were destroyed. The ridging was performed approximately two months after the helped to get rid of the weeds. Once the growth was fully involved, there was no need for other cultivation actions as yacon was resistant to weeds, and further interventions could seriously harm the developing tubers. At the beginning of growth and

during the development of the storage organs, it was necessary to water sufficiently the plants, especially during the dry seasons. It is advisable to shift the harvest as far as possible; however the above ground biomass, and especially the root tubers, must not be damaged by the autumn first frosts (October). The above ground part was removed before the harvest, so the under ground parts were manipulated better, and it also facilitated the manual harvest. For the purpose of mechanical harvest, a truncated puller constructed specially for the harvest of yacon was used. After the harvest of the under ground part, the root and stem tubers were separated from each other. All cut parts on the stem tubers were treated with charcoal or at least they were letting dry. Bigger cut parts on the root tubers were treated in the same way. The tubers determined for consummation were placed in boxes and stored in a cold and moist room at the temperature of around 10 °C; this prevented the tubers from loosing water and from shrinking (Fernandez *et al.*, 2007).

Rhizomes were further stored in boxes with peat in 8–10 °C and moisture content 70–75%. From one rhizome, it is possible to get around 6 parts with 5 buds with the weight approximately 250–280 g. Weight (kg per plant) and survival of rhizomes after storage was evaluated, and propagation coefficient was counted.

Ulluco was planted during the year 2006 with the spacing between plants 0.4 × 0.8 m at the same conditions as for yacon. Plants were planted from May till November and tubers were harvested manually and number and weight was investigated. Statistica 7, Duncan Multiple Range Test (DMRT), was used for statistical evaluation.

3.3 *In vitro* cultivation

3.3.1 Initiation of aseptic culture

Plant material (axillary and apical buds) of yacon and ulluco was sterilized by 70% ethanol for 1 minute. They were washed with sterile distilled water, then sterilized in 10% SAVO (commercial bleach solution with 10 % active chlorine Na-hypochlorid) with 0.1 ml l^{-1} Tween 20 and washed with sterile distilled water three times. After sterilization explants were transferred to Petri dishes, where segments of the size 3–5 mm were separated. These were further cultivated in hormone-free MS medium (Murashige and Skoog, 1962) with 30 g l^{-1} sucrose and pH 5.7. The cultures were placed at 25 °C under 16-hour photoperiod and photosynthetic active radiation of 80 ± 5 μmol m^{-2} s^{-1}. The aseptic cultures were visually evaluated after 3 weeks.

3.3.2 Propagation and regeneration

A high number of plants is essential for long-term storage of the plant. Study of temperature influence to root, shoot and leaves was applied. Comparison of ulluco and yacon propagation coefficient was set out by formula:

The theoretical number of plant

$$N = c^P$$

where N – is a theoretical number of plants/time, c – micropropagation coefficient and P – time of subcultivation (30 days).

Yacon propagation and short-term conservation

Explants 5–10 mm were excised from apical and axillary buds and placed in 12 × 150 mm test tubes containing 2.5 ml culture media MS + 30 g l^{-1} sucrose at three different temperatures 17, 21 and 25 °C. Nodal segments of *in vitro* cloned plants were placed inside test tubes under 16 hours photoperiod and photosynthetic photon flux of 80 ± 5 μmol m^{-2} s^{-1}. Each plant parts (number of roots, leaves and plantlets high) were studied for 2 month and in 1 week´s intervals and visually evaluated. For statistic evaluation Statistica 7 was used with the use of Richards´ function (Richards, 1959). Experiments were 3 times repeated with 20–40 test tubes for each temperature.

Ulluco propagation and short-term conservation

Explants 3–10 mm were excised from both apical and axillary buds and placed in 12 × 150 mm test tubes containing 2.5 ml culture media. Shoot tips were micropropagated and transferred to modified solid MS medium (Murashige and Skoog, 1962) according to (Grospietsch *et al.*, 1999) without casein and myo-inositol, with a lower amount of nitrogen (25 % of NH_4NO_3 and 50 % of KNO_3 of the original MS medium), with 30 g l^{-1} glucose and pH 5.6. Nodal segments in test tubes were placed at different temperatures 17°C, 21°C, 23 °C under 16 hours photoperiod and photosynthetic photon flux of 80 ± 5 μmol m^{-2} s^{-1}.

Influence of different temperatures (17, 20, 23 °C) to each plant parts were observed for two months in one week´s intervals and visually evaluated (number of roots, leaves and plantlets high). For statistic evaluation Statistica 7 was used with the use of Richards´ function (Richards, 1959). Experiments were 3 times repeated on 20–40 test tubes for each temperature.

3.3.3 *In vitro* conservation (slow-growth)

Ulluco and yacon conservation was induced by different temperatures (5–25°C) under 16 hours photoperiod and photosynthetic photon flux of 80 ± 5 μmol m^{-2} s^{-1}. Yacon explants were cultivated on MS medium and 30 g l^{-1} sucrose at different temperatures 5–25 °C.

Nodal segments of *in vitro* cloned plants were placed inside test tubes and cultivated under 16 hours photoperiod and photosynthetic photon flux of 80 ± 5 μmol m^{-2} s^{-1}.

Ulluco explants were cultivated on a modified MS medium according (Grospietsch *et al.*, 1999) without casein and myo-inositol, with a lower amount of nitrogen (25 % of NH_4NO_3 and 50 % of KNO_3 of the original MS medium) and 30 g l^{-1} sucrose, at different temperatures 5–23 °C. Survival, viability (in the scale 9 – the best condition to 1 majorly bad) and number of leaves were counted in one month intervals and statistically evaluated by Statistica 7 with the use of Richards´ function (Richards, 1959).

Ulluco microtuberization

Plantlets were grown in Erlenmeyer flask at 21 °C under 16 hour photoperiod and photosynthetic photon flux of 80 ± 5 μmol m^{-2} s^{-1} at the same medium used for conservation. After two months the Erlenmeyer flasks were transferred to 4/3°C day/night temperature for other two month and then plantlets were placed to the temperature below zero (-4 °C) for 2 days. After freezing temperatures the Erlenmeyer flasks were returned back to 21 °C for 6 months and further stored at 17 °C for 5 months. Afterward microtubers were harvested, counted, weighted and transferred to *ex vitro* conditions. Microtubers were placed into commercial available soil substrate for seeding and propagation (moisture max 65 %, pH 5.5–7) and grown at temperature 20 °C. After three weeks the survival percentages of the plantlets were determined.

3.3.4 Cold hardening of ulluco

Cold acclimatization

Plantlets were grown for 2 months at the same medium used for conservation at 21°C with 16-hour and photosynthetic photon flux photoperiod of 80±5 μmol m^{-2} s^{-1}. The explants were cold-acclimated at 4/3°C day/night for one month and photosynthetic photon flux of 80 ± 5 μmol m^{-2} s^{-1}. Plantlets were pretreated by liquid nitrogen (LN) – ice induction in cultivation medium (short dip Erlenmeyer flask to LN) or without pretreatment and

transferred to freezer with different freezing temperatures ranged from -1 to -10°C for 39 hours. Freezing tolerance was evaluated visually by counting of survive plants. Influence of LN treatment and survival of cold hardened and non-hardened plantlets were compared.

UV-VIS Spectroscopy and electrolyte leakage measurements

Injury was evaluated by electrolyte leakage and UV-VIS Spectroscopy (by visible betalains and comparison with *Beta vulgaris* L.). Cold acclimatized leaves and stems were used for measurements and nine different temperatures (ranged of -1 to -8 °C and one without cold acclimatization).

Measurements were done at 10 leaves and 100 mm of stem in 3 ml of distilled water. First measurements were done after one hour of shaking and second after warming (100 °C for 1 h) of the material. The measurements were three times repeated.

3.3.5 Acclimatization to *ex vitro* conditions

Plantlets with shoots 4–5 cm long and ulluco microtubers were transfer from *in vitro* to *ex vitro* conditions. First, roots were washed in running water to clear off agar and then plantlets were placed into commercial available soil substrate for seeding and propagation (moisture maximum 65 %, pH 5.5–7). They were covered with a perforated plastic cover which was taken off every day 2 times for 30 min to acclimate plant to *ex vitro* conditions. They were grown at temperature 22 °C. After three weeks, the survival percentages of the plantlets were determined.

3.4 Cryopreservation methods

In vitro grown ulluco and yacon cultivated for 4 weeks on MS medium (Murashige and Skoog, 1962) were used in this study. Shoot tips were used as starting material for cryopreservation experiments, and pre-treatment media with different sucrose concentrations were used. Three different vitrification cryopreservation methods were tested and survival, regeneration rates were evaluated.

Shoot tips were micropropagated and transferred to MS basal medium without growth regulators in plastic cultivation boxes (9 × 10 × 10 cm) with 100 plantlets.

After one week of cultivation plantlets were overlaid by 25 ml of 0.7 M (1st trials) or 2 M sucrose solution (2nd trials) (Table 6).

After five days cultivation shoot tips were excised from nodal cuttings and transferred to Petri dishes with liquid MS medium containing 0.7 M sucrose for 12 hours in the dark. For sucrose experiments, shoot tips were placed on aluminium foil strips and desiccated over silica gel for 0.5, 1, 1.5, 2, 2.5 or 3 hours at 22 °C. In 3rd trials shoot tips were dehydrated with sucrose in combination with glycerol plant vitrification solution (PVS3) – 50 % (w/v) sucrose and 50 % glycerol (Nishizawa *et al.*, 1993).

One week cultivated plantlets were pre-treated as in the previous experiment and overlaid by 25 ml of 2 M sucrose solution after one week of cultivation. After five days cultivation shoot tips were excised from nodal cuttings and transferred to Petri dishes with liquid MS containing 0.7 M sucrose for 12 hours in the dark. Shoot tips were then transferred to loading solution (LS) (Sakai and Kobayashi, 1990) for 20 min and afterwards immersed to PVS3 for up to 120 min. Aluminium foil strips with 10 shoot tips were directly plunged into LN and in LN placed into cryovials. Shoot tips were immersed in liquid nitrogen at least one hour (Sarkar and Naik, 1998) and after that, the cryovials were rapidly immersed into a 40 °C water bath for 30 seconds for thawing (Bajaj, 1995a). Afterwards, shoot tips were transferred on MS medium supplemented with IAA (0.5 mg l^{-1}), kinetin (0.5 mg l^{-1}) and GA3 (0.2 mg l^{-1}). Survival of shoot tips was observed a) after loading of shoot tips in cryoprotectant solution b) dehydration by PVS3 c) desiccation over silica gel d) without immersion to LN. Evaluation of regeneration was made after 4 weeks. Each cryopreservation treatment was repeated 4 times and at least ten shoot tips were used for each trial.

Table 6 Design of cryopreservation experiments

Material	Pretreatment/ Treatment	**Trials**					
		1st	Duration	2nd	Duration	3rd	Duration
Plantlets	Sucrose solution	0.7 M	5d	2 M	5d	2M	5d
Shoot tips	Sucrose solution	0.7 M	12h	0.7 M	12h	0.7 M	12h
Shoot tips	Dehydration/ desiccation	Silica gel	0.5–3h	Silica gel	0.5–3h	PVS3	1–2 h

Five weeks after regeneration of shoot tips, plantlets were transferred to MS medium without growth regulators. Plantlets without cryopreservation and after cryopreservation were morphologically compared.

Yacon and ulluco shoot tips regeneration on recovery medium

Recovery medium was used to stimulate regeneration after cryopreservation treatment and it was the same for yacon and ulluco. Recovery medium is based on MS medium (Murashige and Skoog, 1962) modified according to (Grospietsch *et al.*, 1999) without casein and myo-inositol, with a lower amount of nitrogen (25% of NH_4NO_3 and 50% of KNO_3 of the original MS medium), with 0.5 mg l^{-1} IAA, 0.5 mg l^{-1}, KIN and 0.2 mg l^{-1} GA_3 with 30 g l^{-1} glucose and pH 5.6. Temperature conditions for yacon were 23 °C and for ulluco 21 °C with low light intensity (covered by white paper). Plant growth was evaluated after 7 and 30 days.

3.5 Differential scanning calorimetry

Differential scanning calorimetry (cooling and warming rate 10 °C min^{-1}) was used for thermal analysis for evaluation of sucrose and PVS3 effect on water behavior during the freeze/thaw cycle and glass transition temperature. The data were measured from the heating curve. The amount of crystallized water and glass transition temperature was evaluated by the TA Universal Analysis software 2000.

Ten shoot tips were placed into pans and hermetically sealed. Samples were pre-treated in the same way as for cryopreservation. Measurements were made twice on 10 shoot tips. Water content (WC) of shoot tips was calculated on fresh weight basis as follows: the shoot tips were weighed before and during desiccation in 30 min intervals up to 3 h. They were further desiccated in an oven at 105 °C until two successive weights gave the same value. For statistical data evaluation Statistica v 6.0 software was used.

4 RESULTS AND DISCUSSION

4.1 Conservation in the field conditions

Cultivation of yacon and ulluco in field condition with further aim of genetic resources conservation was investigated during 3 years.

Yacon plants growing in field condition were used for further conservation of yacon genetic resources. Experiments were evaluated according to weight of rhizomes survival of plant material during storage. Other parameters were evaluated such as yield of tuberous roots, which is also crucial parameter, influencing the rhizomes weight (Fig. 4).

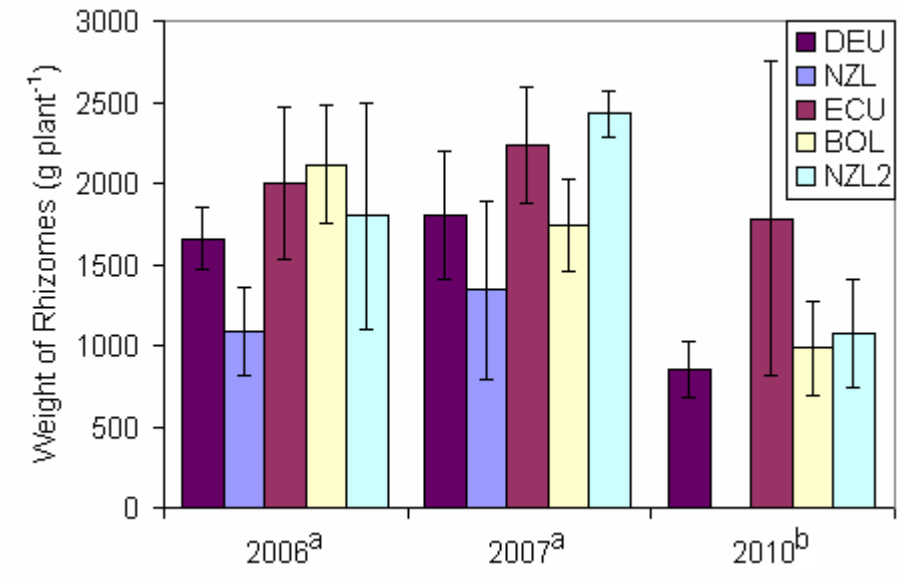

Fig. 3 Yield of rhizomes (g per plant) of 5 yacon landraces (DEU, NZL, ECU, BOL and NZL2) during three years. Bars represent standard deviations. Different letters per years are significantly different at $P<0.05$.

The yield of rhizomes (Fig. 3) ranged from 0.9 ± 0.18 kg per plant (DEU) and to 2.4 ± 0.14 kg per plant (NZL2). The average weight of rhizomes was 1.6 kg per plant.

The highest yield of rhizomes was obtained in the year 2007 for most of the landraces in contrary to the year 2010 when the yield of rhizomes was the lowest. The highest yield of rhizomes was found in the year 2007 for DEU landrace within the studied years. Yields in years 2006 and 2007 were statistically different from 2010.

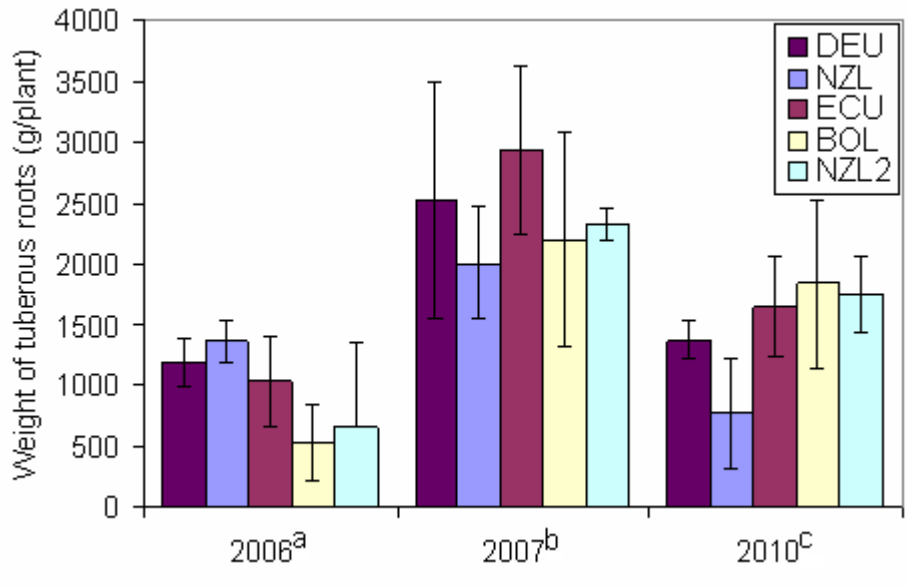

Fig. 4 Yield of tuberous roots (g per plant) of 5 different landraces during years 2006, 2007, 2010. Means of 3 to 6 different yacon plans ± SE. Means with different letters per years are significantly different at $P \leq 0.05$. Years were significantly different.

The weight of yacon tuberous roots (Fig. 4) was from 0.5 ± 0.2 kg per plant (BOL) to 2.94 ± 0.69 kg per plant (ECU). The highest yield for studied landraces was in the year 2007 when average yield of studied landraces of yacon was 2.4 ± 0.36 kg per plant and the lowest yield was in the year 2006 when average was 1.0 ± 0.35 kg per plant. The yacon landraces with highest average yield of tuberous roots were ECU and DEU within studied genotypes.

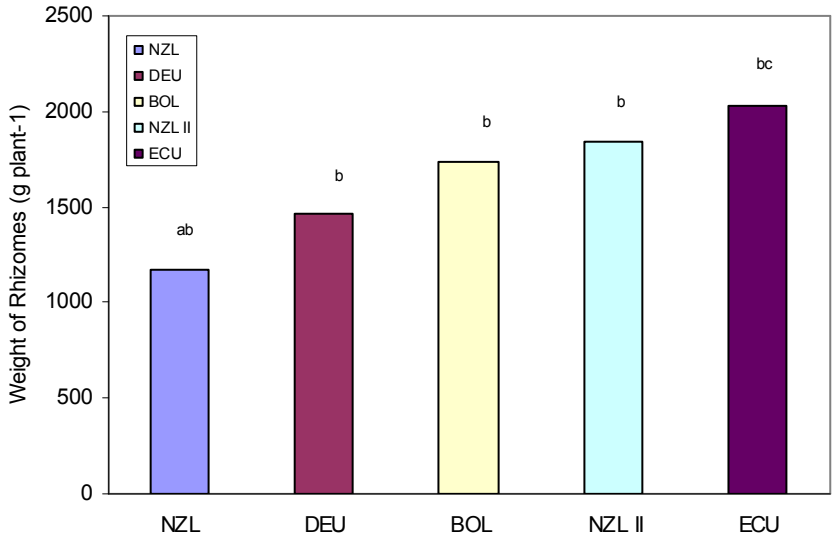

Fig. 5 An average yield of yacon rhizomes (g per plant) of different genotypes over three years. Different letters are significantly different at $P \leq 0.05$ using ANOVA Scheffe test.

The average yield of rhizomes during 2006, 2007 and 2010 (Fig. 5) ranged from 1.2 ± 0.18 kg per plant (NZL) and to 2 ± 0.23 kg per plant (ECU). Difference between genotypes were not significantly different, and there was not found a correlation between weight of rhizomes and other yield characteristics like plant height, leaves number and sugar content (Table 7).

Table 7 Weight of tuberous roots and rhizomes and other yield characteristics. The mean and standard deviation of yield and yield parameters evaluation during 3 year period.

	Plant height (cm)	Leaves No	Weight of tuberous roots (kg plant^{-1})	Weight of rhizomes (kg plant^{-1})	Sugar content (°Brix)
DEU	102.4 ± 5.74	5.8 ± 0.41	1.7 ± 0.73	1.4 ± 0.51	6.5 ± 0.07
NZL	121.4 ± 18.28	6.3 ± 1.51	1.4 ± 0.62	1.2 ± 0.18	9.8 ± 1.75
ECU	159.6 ± 12.43	7.5 ± 1.52	1.9 ± 0.97	2 ± 0.23	7.5 ± 0.99
BOL	170.6 ± 6.75	5.7 ± 0.52	1.5 ± 0.87	1.6 ± 0.58	8.1 ± 1.63
NZL2	147.9 ± 8.75	6.3 ± 0.82	1.6 ± 0.84	1.7 ± 0.70	6.8 ± 0.92

Table 8 Averages of five studied landraces – weight of tuberous roots and rhizomes in the three years (mean ± STD)

	2006		2007		2010	
Weight of rhizomes (kg plant^{-1})	1.73	± 0.40	1.91	± 0.43	1.16	± 0.42
Weight of tuberose roots (kg plant^{-1})	0.95	± 0.35	2.39	± 0.36	1.47	± 0.43

The calculated average number of plants per 1 ha over three years was 20 408. The average losses of planting material after three winters were 20 ± 8% in average. It was calculated for each year theoretical storage coefficients (number) as the average number of plants per 1 ha. From a single plant, we could get 1.7 ± 0.4 kg in average per plant rhizomes in the year 2006, 1.9 ± 0.4 kg per plant in 2007 and 1.1 ± 0.4 kg per plant in 2010 (Table 8). The average yield of rhizomes from the experimental years (2006, 2007, 2010) was 1.6 ± 0.39 kg per plant, which can be finally divided into the smaller parts with at least 5 buds with weight 180–280 g. This means that from a single plant we can theoretically get 5.7 plants in average after one year planting. Also previous study (Viehmannova *et al.*, 2007b) confirms that it is possible to obtain about 20 buds from one kilogram of rhizomes. From one kg of rhizomes we can get in average 4 new yacon plants during one year (Plate 1 B, C). It is possible to obtain up to 6 planting stock from one rhizome after one year of planting.

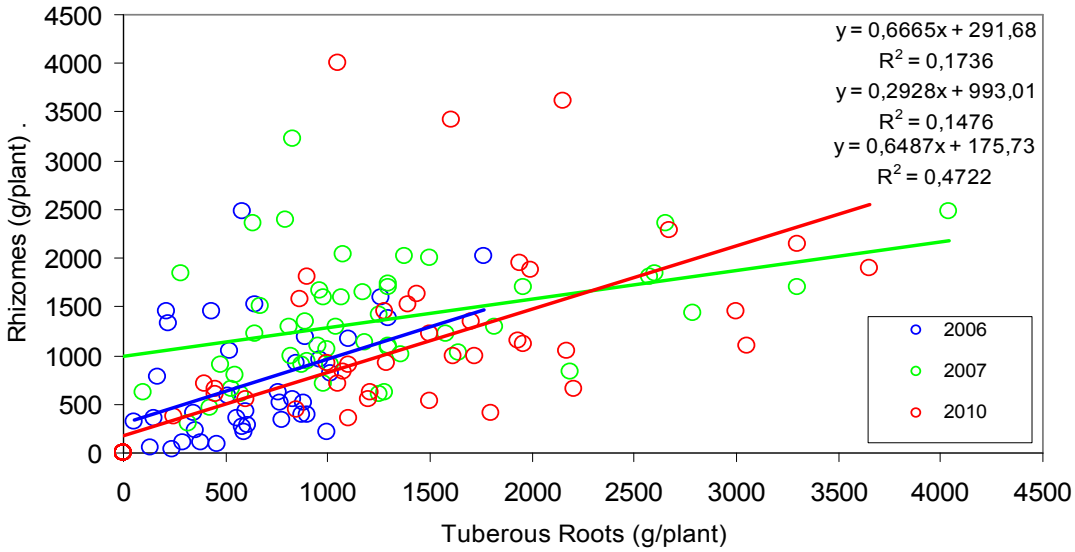

Fig. 6 Relation between weight of rhizomes (g plant^{-1}) and weight of tuberous roots (g per plant) for three years 2006 (blue colour), 2007 (green colour) and 2010 (red colour). Linear regression lines are fit to the experimental point. Correlation coefficients are significant at $P < 0.01$, $P < 0.05$ and $P < 0.001$ for the years 2006, 2007 and 2010 respectively.

Correlation between tuberous roots and rhizomes was in the year 2006 and in the year 2010 nearly the same according to linear regression test where no differences at the 0.95 probability level were found (Fig. 6). The relationship between yield of tuberous roots and rhizomes was for the year 2007 and other two years (2006, 2010) significantly different. According to the positive correlation (Fig. 6), the higher weight of tuberous roots prerequisites the higher weight of rhizomes.

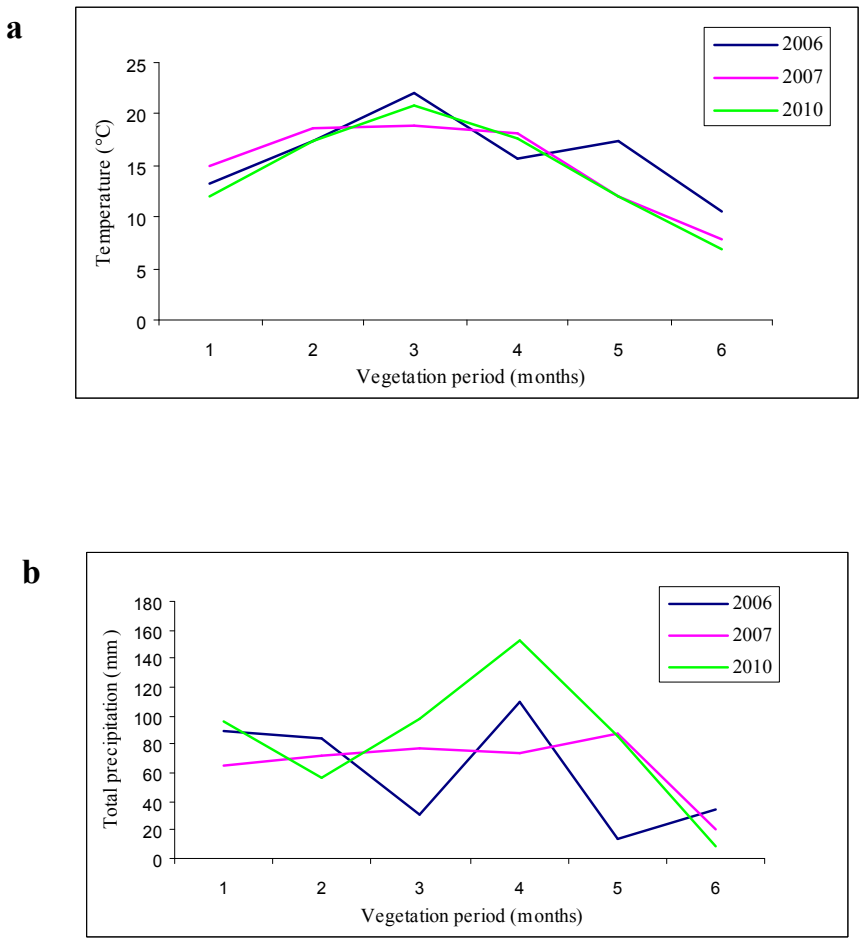

Fig. 7 Meteorological data measured during the growing season (6 months from May till October) vegetation of yacon during three years a) An average temperature at 280 m above the sea level b) Total rainfall precipitation (data obtained from the Meteostation of the Czech University of Life Sciences Prague, months averages).

Although yacon root yields up to 100 t ha^{-1} have been reported in the literature, yields under Andean conditions typically range 20–40 t ha^{-1} (Manrique *et al.*, 2004).

Our results of yields for yacon in the Czech Republic show average yields similar to those under *in situ* conditions (Plate 1 D). Yield of rhizomes 32.7 t ha^{-1} was nearly the same as tuberous roots – 32.6 t ha^{-1}. Yields in conditions of the Czech Republic were also reported

by (Fernandez *et al.*, 2006) and long-term studies show also close correlation between yields of tuberous root and rhizomes. It was found during the long-term studies (2001–2005), that yacon had a high yield of the biomass, in average 86.5 t ha^{-1}, from which tuberous roots form 25.5 t ha^{-1}, rhizomes 24 t ha^{-1} and aboveground part forms 36.9 t ha^{-1}.Our studies during 2006, 2007 and 2010 show in comparison to this higher yields of rhizomes (8.6 t ha^{-1} higher) and tuberous roots (7 t ha^{-1} higher). According to Frček *et al.* (1995) results the yields in the Czech Republic ranged between 38–66 t ha^{-1}. Thus our studies were in the range of the two previous studies.

Similar yields of tuberous roots and rhizomes, with the highest production 3.8 kg tuberous root per plant (Lebeda *et al.*, 2004) were achieved also in another part of the Czech Republic in the field conditions of the Haná region (altitude 210 m, day mean temperature 16.3 °C, precipitation during cultivation period 271.4 mm). Similar yield was reported, for example, in Japan (Ogiso *et al.*, 1990), Brazil (Amaya, 2000) and Korea (Doo *et al.*, 2001). Colder temperatures and a shorter growing season limit yacon production. Time of harvest trials showed that root yields increased throughout autumn until winter frosts halted their growth. Yields of tuberous root at the commercial plantings were 30–40 t ha^{-1} after 6–7 months in New Zealand. (Douglas *et al.*, 2005).

The yield of yacon varies according to the ecotypes, area of cultivation, soil type, environmental conditions, method of cultivation, cultivation period, the length of growing period and the size of planting material (Lebeda *et al.*, 2011). There is a variation in the yield between cultivars, but the environment can significantly modify yields (Manrique *et al.*, 2004).

Weather affected the final yields of yacon rhizomes or tubers during investigated years (Fig. 7) mainly by total precipitation. Also according to Fernandez *et al.* (2006) the main factor influencing the formation of the yield of yacon is the sum of precipitation during the vegetation period and not the length and temperature of the growing season. Low precipitation was at the field conditions of the Czech University of Life Sciences supported by irrigation, but higher average temperature and sunshine during planting increased the sucrose content. The significant differences in ecotype × year of cultivation were found for glucose and inulin levels (Lachman *et al.*, 2005). It was found a correlation between yield of tuberous roots and rhizomes and the higher weight of tuberous roots prerequisite the higher weight of rhizomes (Fig. 6).

Yacon is a crop tolerant of the oscillation of temperatures because it can withstand high temperatures in the summer as well as low temperatures about the point of freezing in autumn. The first frosts in autumn can cause yacon damage (being burnt by frost) beginning on the overground part, which is 1 meter above the ground (at -1°C). Basal parts of the stem resist at -2 °C and underground organs at -3 °C. Below lower temperatures the tuberous roots crack (Fernandez *et al.*, 2007). An ideal solution for the cultivation of yacon would be to select the clones with a rapid growth and early development (i.e. with short growing season) and to provide sufficient irrigation for the crop (Fernandez *et al.*, 2006).
Ulluco is a short day plant, requiring 11–13.5 hours of day length for the initiation of tuber formation (Sperling and King, 1990). It is recommended that the crop is planted as soon as the danger of spring frosts has passed (Martin *et al.*, 2005). Morphotypes differ in the time it takes to reach maturity, varying from 5–9 months. Ulluco requires 700–800 mm of precipitation to sustain productive yield, but it is considered well adapted to dry conditions and slightly acidic soil (5.5 to 6.5) (Vega, 1997). The underground tuber yield of 20 t ha^{-1} at Pukekohe New Zealand and average tuber size was extremely small – 5 g suggests that this plant may be very indeterminate in its tuber setting unless prevented by frost (Martin *et al.*, 2005).

Ulluco plants were planted at field conditions during the year 2006 with low average yield 39 ± 17 g per plant, and the average number of tubers were 8.2 ± 4.4 tubers per plant. The unsufficient yield at the field conditions was probably caused by short vegetative period in the Czech Republic and by their day length sensitivity according to (Landa *et al.*, 2004) who also reported ulluco low yield in the conditions of the Czech Republic (just 50 g per plant). Andean farmers have traditionally used 3–5 small tubers (5–10 g each) per planting station for ulluco as opposed to the 20–30 g which is recommended for good quality tuber seed. As the small tubers are set at the late (senescent) stage of plant development, virus particles probably would not have transported to the last-developed tubers making some of the small tubers virus free (Chuquillanqui, personal communication). For this reason, the cultivated ulluco did not become extinct during their 3 000–5 000 years of continuous cultivation (FAO, 2010). Also according to (Martin *et al.*, 2005) ulluco sets tubers late yields are likely to be restricted by autumn frosts. Despite this disadvantage with growing it was found that the chemical composition is the same as in tubers grown under native

conditions (Landa *et al.*, 2004), therefore this plant has potential to enrich diet and furthered studies are needed.

There has been a long-term observation of storage of yacon and ulluco carried out in the Czech Republic.These plants are vegetatively propagated which makes them difficult to store them for a long time.

Ulluco yield was very low (39 ± 17 g per plant), therefore further field conservation of ulluco is not recommended in the conditions of the Czech Republic. Yacon storing by rhizomes is well studied with a good average yield of rhizomes (32.7 t ha^{-1}). From a single plant can be getting in average 5.7 plants in one year. No correlation was found among climatological data for the growing season and yield for the locality near Prague. The difference in yields is caused mainly by genotype and partly possibly caused by irrigation during the the growing season.

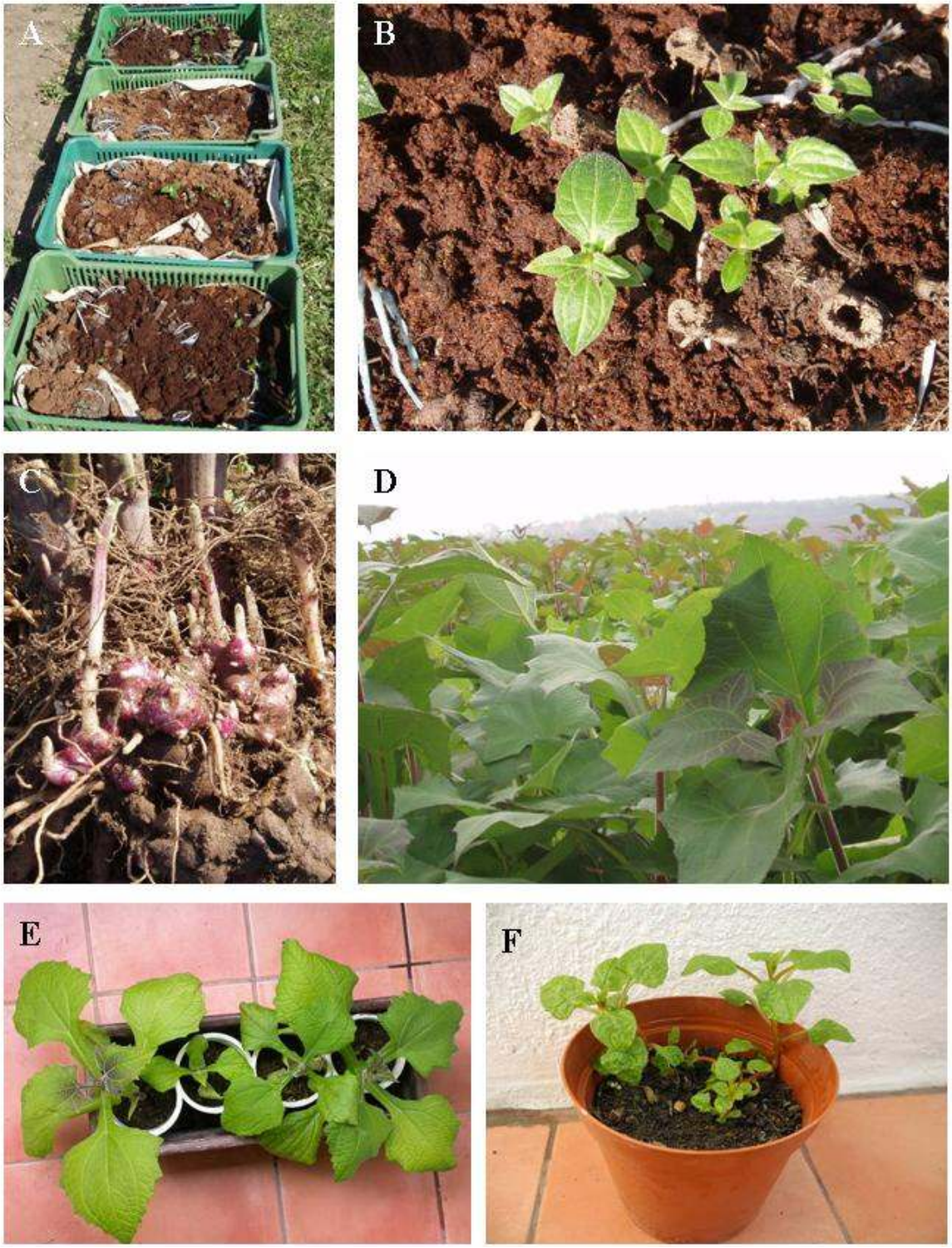

Plate 1. *Ex situ* conservation o yacon at the Czech Republic **A.** yacon rhizomes after storing for 7 months at 10 °C – starting sprouting, **B.** sprouting of rhizomes (in detail), **C.** sprouting of rhizomes, **D.** plants before harvest, **E.** yacon from *in vitro* after acclimatization, **F.** ulluco growth from microtuber

4.2 Conservation in *in vitro* conditions

In vitro cultivation was focused on the influence of temperature on propagation and conservation of *in vitro* grown plantlets, study of recovery medium for regeneration of shoot tips after cryopreservation and cold acclimatization of plantlets.

The main advantage of tissue culture technology lies in the production of high quality and uniform planting material that can be multiplied on a year-round basis under disease-free conditions anywhere irrespective of the weather (Ahloowalia *et al.*, 2004) and it is easy access for evaluation and utilisation (Tao, 2003).

4.2.1 Micropropagation and short-term conservation

The culture rooms generally have a temperature range similar to those needed for growing a given crop in the field. That is called short-term conservation (Golmirzaie and Toledo, 1998). Micropropagation is prerequisite to *in vitro* conservation (Tyagi and Yusuf, 2003) and further propagation of stored plantlets.

The aim of the present study was to investigate the influence of different temperatures in order to determine a proper protocol for *in vitro* propagation for further cryopreservation of ulluco and yacon.

4.2.1.1 Micropropagation and short-term conservation of ulluco

The aim of the present study was to investigate the influence of different temperatures in order to determine a proper protocol for *in vitro* propagation for further cryopreservation of ulluco.

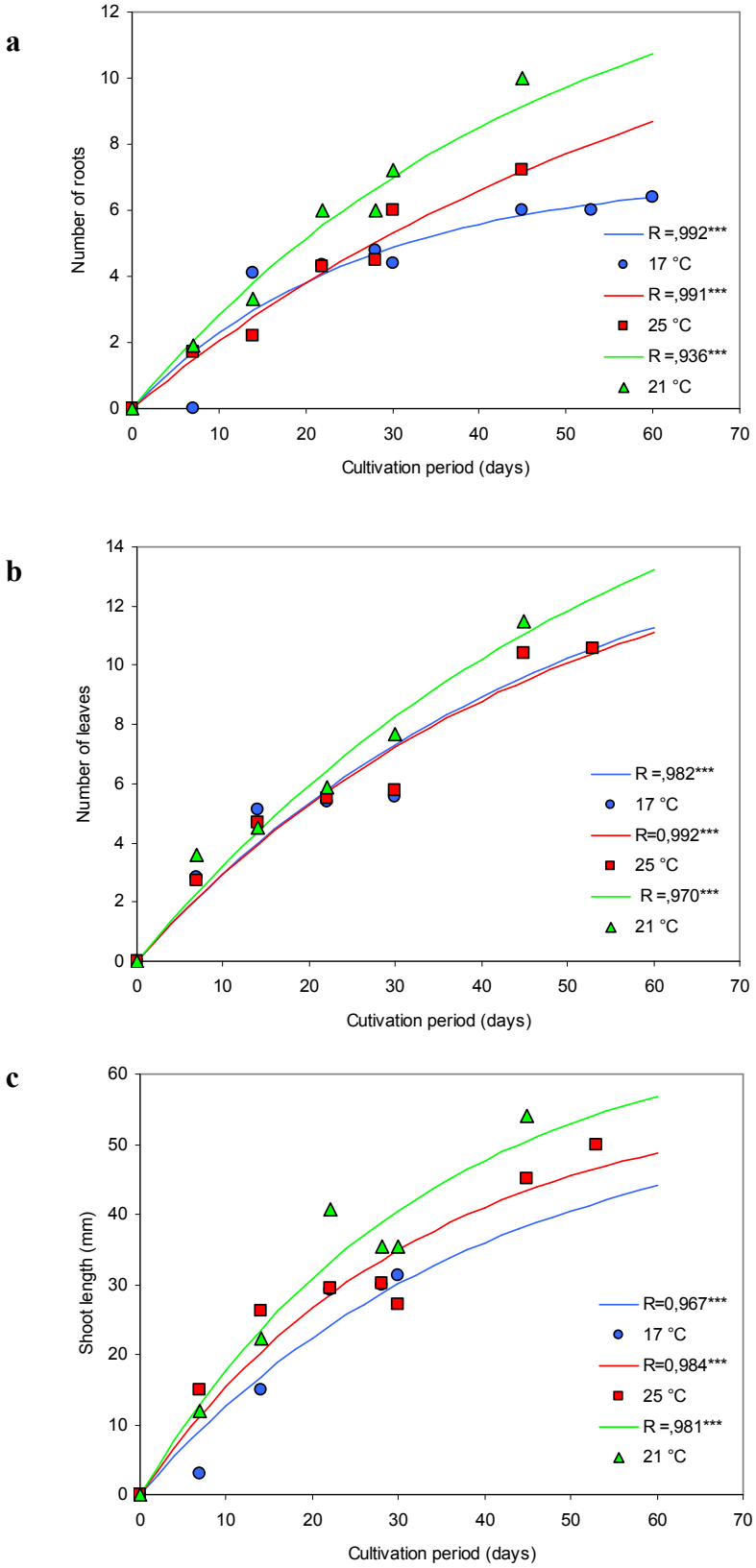

Fig. 8 *In vitro* growth of ulluco at 17, 21, 25 °C during two months. Different temperatures influenced; a) the root formation; b) the number of leaves; c) the length of shoots.

Table 9 Comparison of different temperatures for ulluco multiplication and theoretical number of obtained plant after one year of cultivation

Temperature	**Subcultivation** (days)	**Multiplication coefficient** (c)	**Number of subcultivation per year (n)**	**Theoretical number of obtained plants per year (N)**
25 °C	30	1:5.8	12	1 449 mil
21 °C	30	1:7.7	12	43 439 mil
17 °C	30	1:5.6	12	951 mil

Growth of ulluco *in vitro* is very fast, and after 60 days of planting it is possible to get 10.2 ± 3.23 roots, 13.1 ± 2.1 leaves and length of shoots up to 55 mm (Fig. 8). Also according (Parra Quijano *et al.*, 2005) ulluco showed excellent adaptation to *in vitro*.

Propagation of ulluco was observed for two months in 3 different temperatures (25 °C, 21 °C and 17 °C) on different plantlet characteristics. For all characteristics the first month was more effective than the second. In the second month is still observed fast growth at all characteristics, so for rapid propagation is can be recommended one month planting but two month growth when we get more nodal segments of healthy plantlets is more economically advantageous.

The most important characteristic is the leaves number (Fig. 8 B) which is the same number as for nodal segments we can obtain from a single plant. The highest average leaves number after one month of planting was 7.7 ± 1.93 at the temperature 21 °C. The temperatures significantly affect the leaves number when 21 °C was optimal and 17 °C and 25 °C reduced the number of nodal segment by 1.9–2.1 nodal segments after 30 days cultivation.

Plants exhaust the nutrients in medium in 2–3 months; therefore, *in vitro* plants have to be transferred frequently to fresh medium (Golmirzaie and Toledo, 1998). According to our results nutrients for ulluco plantlets in medium were exhausted after 2 months.

Also, another characteristics - root number and shoot length were reduced by high and low temperature and supported by temperatures 21 °C. Also according (Bensalim *et al.*, 1998) temperature increase had the most dramatic effect on potato root development. An average shoot to root ratio decreased from 3.7 at 20/15 °C to 1.7 at 33/25 °C. Some studies have been done to ulluco microropagation on different growth regulators at temperature 22 ± 1 °C (Jordan *et al.*, 2002), but the effect of temperature to *in vitro* propagation have not been reported yet. According to our study the propagation can be also improved by

appropriate temperature which is 21 ±1 °C and only change of temperature can significantly improve the production of plantlets to approximately 2 nodal segments per plantlet (Table 9).

Finally it can be concluded that the optimum temperature for ulluco propagation is 21 °C, which increased the length of shoots, number of leaves and root formation (Plate 3 A,B). This study is needed for rapid *in vitro* propagation after conservation methods and supports the I. hypothesis that the ulluco plant can be propagated *in vitro,* and it has uncommonly good results with fast growth which enables to get up to 7.7 ± 1.93 new plantlets from a single plant in 30 days of *in vitro* growing cycle (Plate 3 B).

4.2.1.2 Micropropagation and short-term conservation of yacon

The aim of the present study was to investigate the influence of different temperatures in order to determine a proper protocol for *in vitro* propagation for further cryopreservation of yacon.

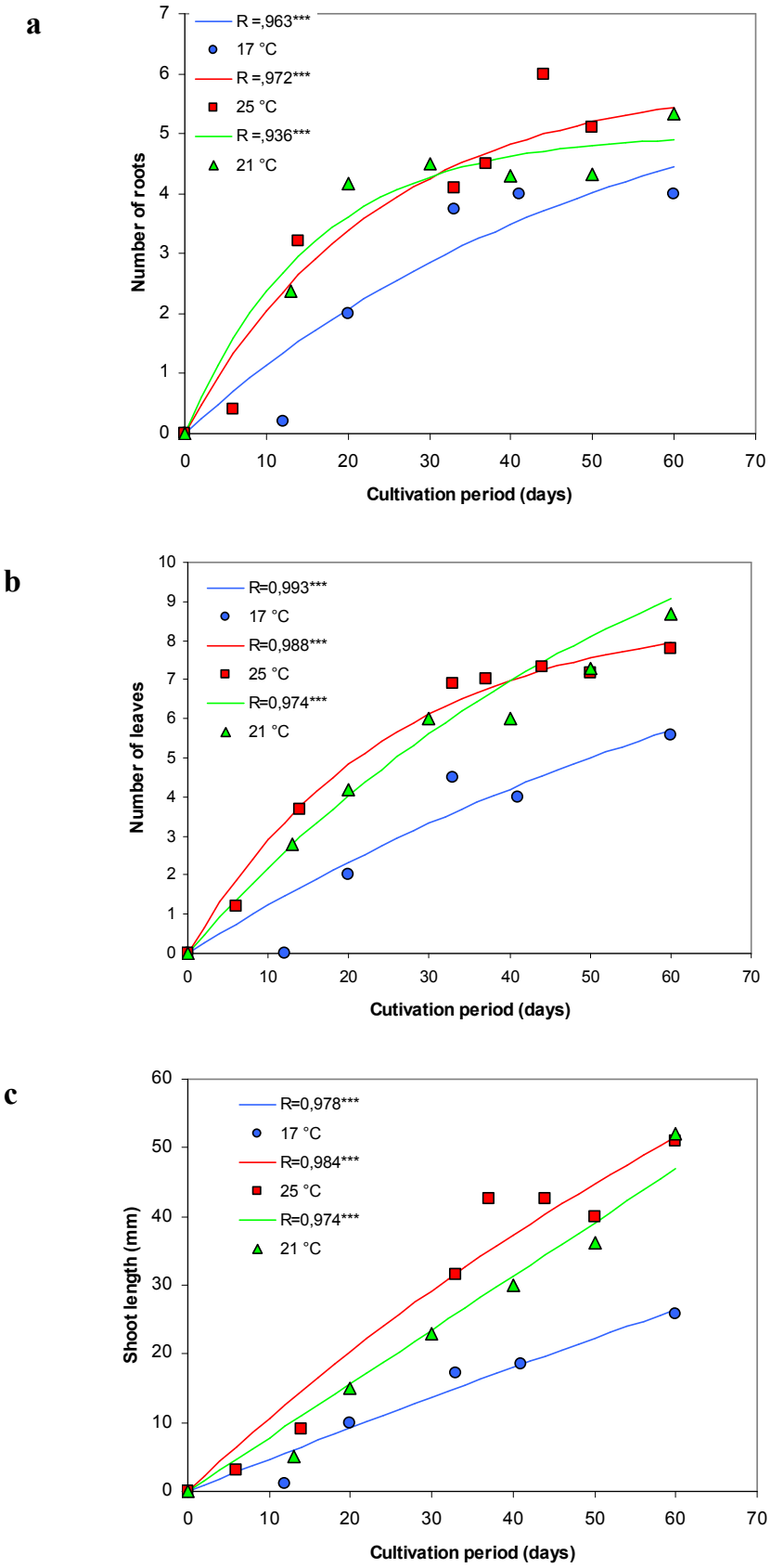

Fig. 9 *In vitro* growth of yacon in different temperatures during 2 months; a) number of roots; b) number of leaves; c) height of plant

Table 10 Comparison of different temperatures for yacon micropropagation and theoretical number of obtained plants after one year of cultivation

Temperature	**Subcultivation** (days)	**Micropropagation coefficient** (c)	**Number of subcultivation per year (n)**	**Theoretical number of obtained plants per year (N)**
25 °C	30	1:3.45	12	2 843 342
21 °C	30	1:3	12	531 441
17 °C	30	1:2.25	12	16 834

Yacon plantlets were propagated *in vitro* and growth was studied during 2 months at three different temperatures (Fig. 9). The growth was fastest during the first 30 days, so we recommend 30 days for rapid propagation. Main parameter is the number of leaves from which we can count the number of nodal segments (as one nodal segment contains 2 leaves). After 30 days of cultivation, it was possible to obtain 3.45 ± 1.5 nodes and after 60 days 4.5 ± 0.53. Temperature improved the propagation coefficient to up to 0.9 nodal segments in 30 days after cultivation at the temperature 25 °C.

Yacon can be propagated both from apical and axillary buds even on basic MS (Murashige and Skoog, 1962) medium, without growth hormones (Lebeda *et al.*, 2011) (Plate 2 B).

According our study there was also no need of plant growth regulators for yacon propagation even it can improve growth because it can induce some undesirable effect. In contrary, it was reported that BAP addition to the culture medium will be unnecessary (Mogor and Mogor, 2003).

Our results are in accordance of literature with the same temperature, higher coefficient of propagation can be caused by another genotype. Viehmannova *et al.* (2007b) obtained after 30 days of cultivation 2.5–2.8 nodes (coefficient of micropropagation) and so theoretical number of plantlets which can be obtain in one year from one single plant is 60 000 to 200 000. Also according (Lebeda *et al.*, 2011) the explants formed 2.4–2.9 nodes (micropropagation coefficient) per plant in 30 days. The optimum temperature for rapid propagation was 25 °C with 16 h photoperiod. The sucrose in a 3% (w/v) concentration was better as carbon and energy source than glucose and fructose in the same concentration. (Estrella and Lazarte, 1994) used lower temperature (20 ± 2°C) and growth hormones (IBA and BAP) for root and shoot induction. This micropropagation procedure has subsequently permitted the preservation of > 28 collected yacon genotypes in INIAP's Ecuadorian germplasm bank. According to Fernandez *et. al* (2007b) difference in plant

length was found (for 25 °C was the average 4.1 ± 0.18 cm and 3.6 ± 0.35 cm for 20 °C), but for nodes formation (coefficient of micropropagation) it was almost the same (2.6 ± 0.49 for 20 °C and 2.5 ± 0.5 for 25 °C). This corresponds with our results (Table 10).

According to our study, optimum temperature for yacon propagation was 25 °C and cultivation for 30 days (Plate 2 A,C,D). This supports the I. hypothesis of *in vitro* propagation of yacon which enables fast obtaining of a new plant material. Also according (Viehmannova *et al.*, 2007a) *in vitro* micropropagation is the most effective one for obtaining planting material; however, it is highly economically demanding and may be lost because of mite infestations or microbial infections (Reed *et al.*, 2004).

Propagation of yacon and ulluco was successful. For ulluco, we recommend temperature 21 °C and 30 or 60 days of cultivation and for yacon 25 °C and 30 days of cultivation.Comparing these two plants, ulluco growth *in vitro* is easier with higher propagation coefficient.

4.2.2 Conservation *in vitro* (slow-growth)

Short-term conservation can be extended to medium-term conservation by prolongation of interval between subcultures, however, can be extended through growth rate reduction by modification to the environment or changes in some media components (Golmirzaie and Toledo, 1998). *In vitro* storage is excellent for medium-term storage and is used as the active collection in some gene banks. It may be a duplicate for field collections, or the main collection with a second culture collection or a field collection as the duplicates (Reed *et al.*, 2004). The addition of osmotics or growth retardants to the medium has proved efficient for reducing growth rates of different plant species (Golmirzaie and Toledo, 1998), but growth retardants can produce some physiological changes or generate mutation which can threaten the genetic stability of the materials conserved *in vitro* (Golmirzaie and Toledo, 1998). Osmotic stress and low temperature of 6 °C for potato and 18 °C for sweet potato appears to be the best and least costly way to maintain the germplasm collection for a long-term storage (Golmirzaie and Salazar, 1995a). In the Czech Republic (Potato Research Institute Havlíčkův Brod) potato is maintained at 10 °C and subcultivated each 14–18 months (Horackova and Domkarova, 2005). Growth reduction for yacon and ulluco was achieved by modifying the temperature.

4.2.2.1 *In vitro* yacon conservation

The aim of the present study was to investigate the influence of different temperatures in order to determine a proper protocol for *in vitro* conservation and for further cryopreservation of yacon.

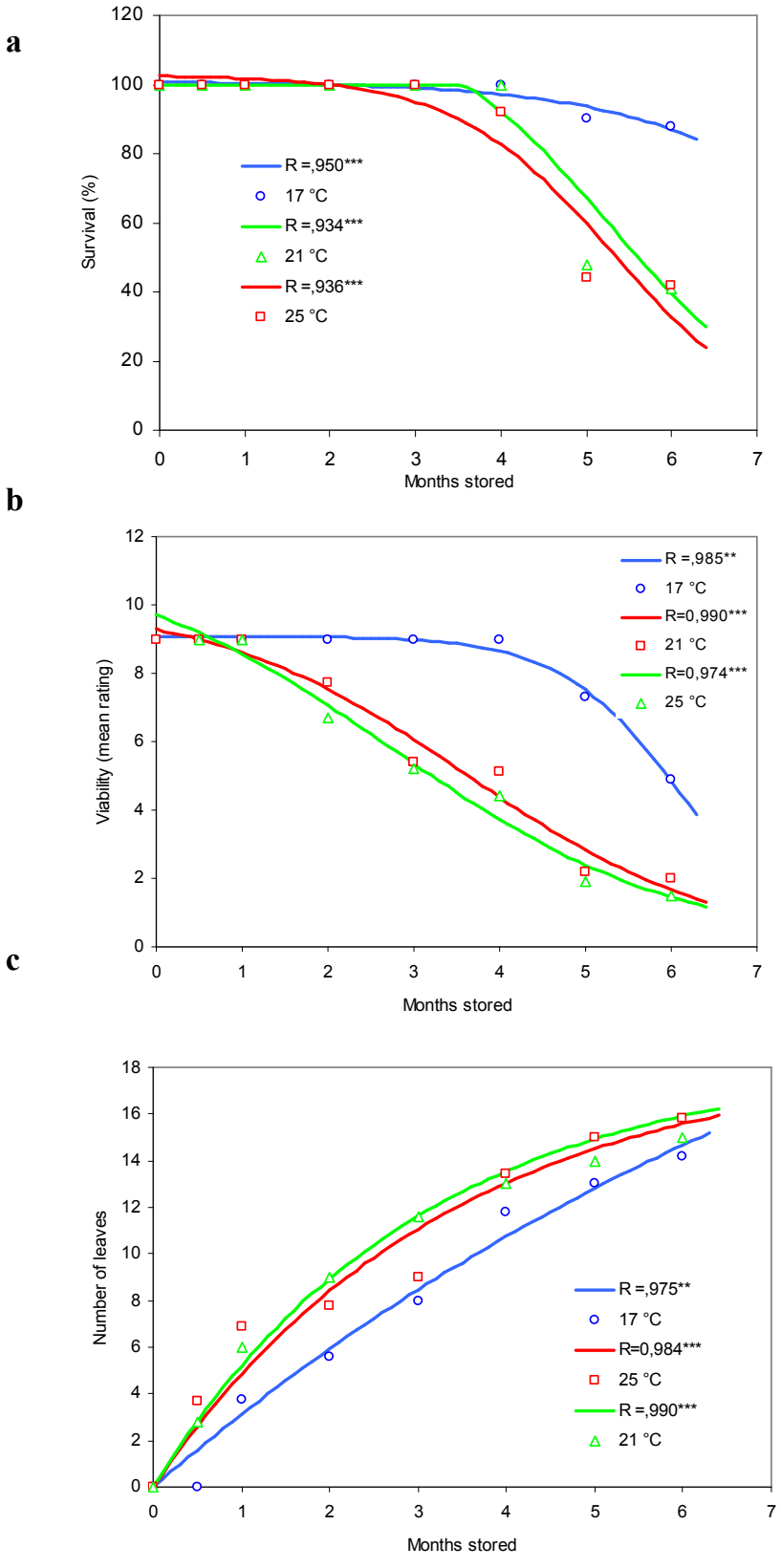

Fig. 10 Conservation of yacon plantlets *in vitro* in three different temperatures for 6 months; a) percentage of survive plantlets; b) viability (rated on the scale from 1 to 9); c) number of leaves per plant.

Conservation of yacon plantlets in different temperatures was studied (Fig. 10). The effect of temperature was significant at all studied measurements. Low temperature (17 °C) significantly increased survival and viability and decreased plant growth and leaves growth. The main factor in conservation of planting material is viability of stored plantlets (Plate 2 A-E). Difference of 4 °C increased the best viability up to 2 months (for temperatures 17 °C and 21 °C). Survival was also supported by 17 °C and the plantlets were able to survive more than 6 months with 90 % of survival.

After 30 days, there was found 3.7 ± 0.41 leaves (which in comparison with propagation temperature are 3.15 lower values) and 14.2 ± 2.1 leaves at 17 °C were found after 180 days.

Yacon at CIP is routinely stored at temperature 18–22 °C and average storage duration is 3 months (minimum 3 and maximum 4 months) (Panta *et al.*, 2009) and according to Toledo *et al.* (1994) up to 6 months.

The same conditions for conservation are used for arracacha and achira (Panta and Roca, 2008).

CIP research on sweet potato has shown that temperatures of 16–18 °C lengthen the storage period up to 1 year in cultures containing 2% sorbitol. Storage at 23–25 °C and 90 μmol m^{-2} s^{-1} (Lizarraga *et al.*, 1990) successfully maintained the sweet potato collection at CIP 4–6 months without sub culturing (Golmirzaie and Toledo, 1998).

4.2.2.2 *In vitro* ulluco conservation

The aim of the present study was to investigate the influence of different temperatures in order to determine a proper protocol for *in vitro* conservation and for further cryopreservation of ulluco.

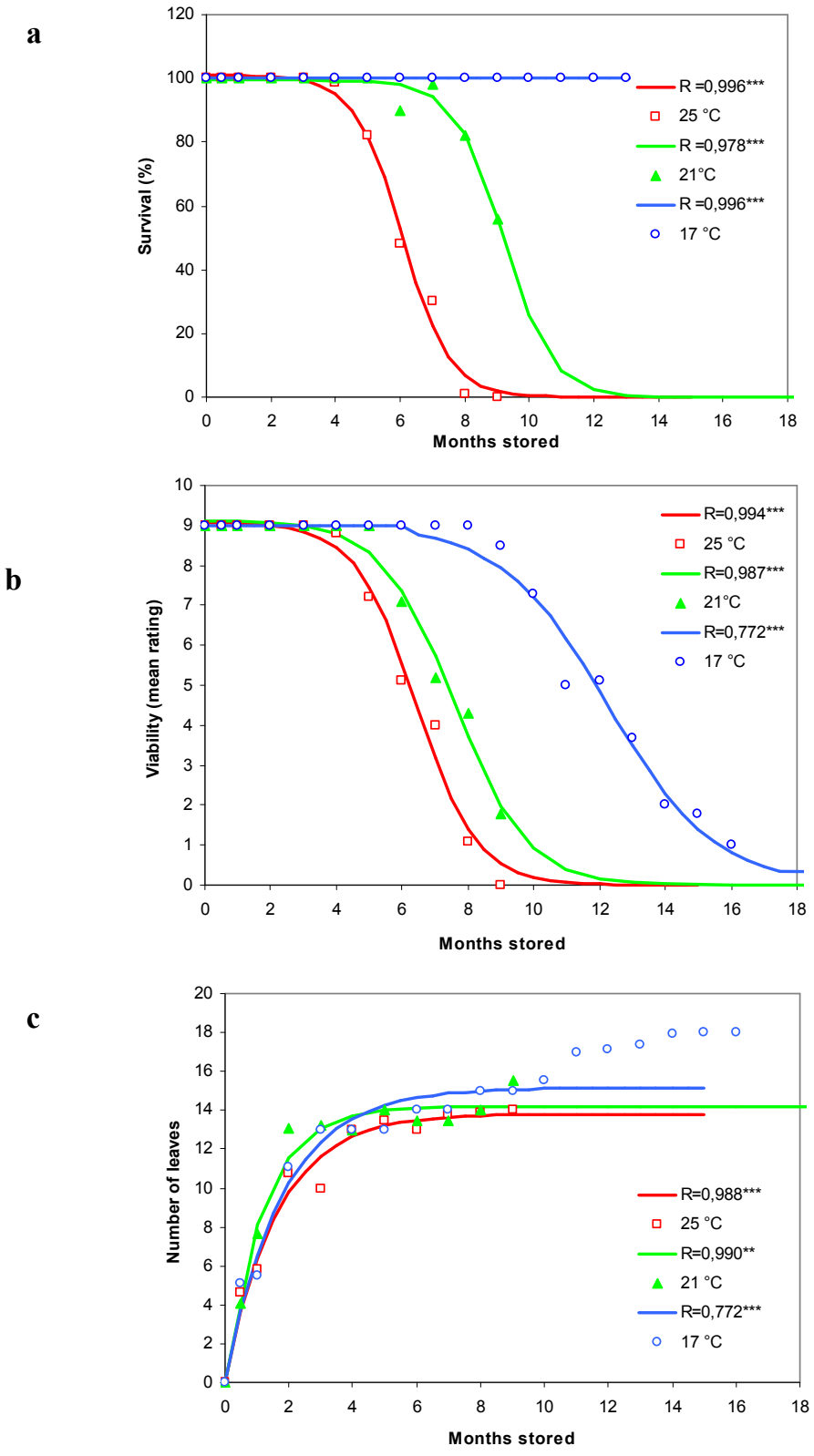

Fig. 11 *In vitro* conservation of ulluco in three different temperatures up to 16 months; a) percentage of survived plantlets; b) viability (rated on the scale from 1 to 9); c) number of leaves per plant.

Effect of temperature was studied for ulluco conservation. The best results were obtained with 17 °C for plantlets with best viability (11 months with the mean rating of viability 5 and survival up to 16 months) (Fig. 11). The longest conservation *in vitro* was however

achieved when nodal segments were cultivated in 5 °C when plantlets were able to survive up to 18 months with plant height 3 ± 0.65 without roots and after their removal to 20 °C they started to grow next 6 months with little progress and afterward 3 polynodal segments (with 5 small leaves) were afterwards used for further propagation.

The temperature range between 17 and 25 °C does have a significant role on leaves growing. But, growth was still enough at the temperature of 17 °C (10.8 ± 1.3 leaves after 2 months) so the temperature 17 °C could be recommended for both – long-term conservation and also propagation of plant material which can be extraordinarily useful for further experiments.

The potato cultures were maintained at 10 °C with a light intensity of 60 μmol m^{-2} $s^{-1}$and 16 hours-day and can be stored under these conditions up to three years (Mix-Wagner, 1999). *Solanum demissum* Lindl. is a short-day dependent, tuber-bearing species. *In vitro* propagated genetically identical plants were grown under long-day (16 h) conditions at 20 °C day and 15 °C night temperatures (Helder *et al.*, 1993)

CIP can maintain the potato germplasm for an average two years between transfers by applying growth restriction media and reduced incubation temperature (6 °C) (Golmirzaie and Salazar, 1995a). Also ulluco is routinely stored at temperature 6–8 °C and average storage duration is 18 months (minimum 12 and maximum 20 months (Panta *et al.*, 2009)**,** (Panta and Roca, 2008).

Potato collection at CIP is conserved in conservation medium containing 4 % sorbitol at a temperature 6–8 °C and light intensity 30 μmol m^{-2} s^{-1}. That extends the *in vitro* conservation of the potato collection for 2–4 years without subculture. After several years of *in vitro* culture, plantlets can recover normal growth after one to two subcultures (Golmirzaie and Toledo, 1998). The advantage of higher storing temperature (17 °C) which was used at this study was the immediate growth without waiting for one or two subcultures, so this temperature is not limiting for further rapid propagation.

Plants exhaust the nutrients in medium in 2–3 months; therefore, *in vitro* plants have to be transferred frequently to fresh medium (Golmirzaie and Toledo, 1998). Nutrients in the medium were exhausted after one month for yacon plantlets and after two months for ulluco plantlets.

4.2.3 Ulluco microtuberization

Production of microtubers in *in vitro* culture is an alternative method for long-term conservation of potato. Once the microtubers sprout, growth can be retarded as with *in vitro* plants, for 2–4 years by storing them embedded in a conservation medium. (Golmirzaie and Toledo, 1998). The potato plants ability to produce tubers, derived from cryopreserved shoot-tips, was not affected by the cryopreservation process (Harding and Benson, 1994).

Using the alternating temperature for tuber induction was reported also for potato microtuberization (25 °C for multiplication, 18 °C for microtuber induction). Microtubers of 6 different potato cultivars were stored at 4 °C for 3 years and planted in soil. It was observed that tubers retained their viability under low temperature when tuber diameter was > 5 mm. Such tubers produced healthy plantlets upon transfer to soil (Prematilake and Mendis, 1999). (Harvey *et al.*, 1992) had reported about microtuber formation by single node stem segments of *in vitro* grown potato plantlets when continuous incubation at 26 °C strongly inhibited tuberisation and exposure to 26 °C for only 1 week before transfer to 20 °C significantly reduced microtuber fresh weight.

Microtuberization of *in vitro* plantlets is decidedly useful for medium term conservation, but it is usually supplemented by growth inhibitors or lowering of nutrients, for example, ABA stimulated tuberization (Xu *et al.*, 1998). These supplements can influence plant material so in our experiment tuber induction was supported by different temperatures on conservation medium. Two months old plantlets were used as material for tuber induction. They were stored for 2 months at 4/3 °C day/night temperature. After cold acclimatization, they were transferred to freezing temperature (-4 °C) for 2 days and further grown at 21 °C. According (Akita and Takayama, 1994) the number of tubers and the total tuber weight clearly decreased under the lower temperature (17 °C), the weight decrease was partially prevented by changing the temperature from 17 °C to 25 °C after 2 weeks. Lower temperatures influenced the localization and size distribution of tubers.

First microtuberization occurs 1–2 month after hardening, and after six months, it was possible to harvest microtubers from 90 % of plantlets (Plate 3 C-E). Plant height was in average 11 ± 2.86 cm (Plate 3 B), with the highest number of 2 tubers per plant. The average weight of microtuber was 0.06 ± 0.02g and biggest tuber had 0.1 g (Plate 3 D).

Optimum temperature for the microtuberization of potato is 20 ºC. Temperature below 12 ºC and over 28 ºC are strongly inhibitory (Rajan and Markose, 2007). However, our result for ulluco shows that short influence of low temperature can induce microtuberization.

Conservation with this method lasted for 10 months, and microtubers were further stored for 5 months at 17 °C so it was possible to store ulluco for 15 months together without changing of growth medium. Microtubers were further grown in *in vitro* or *ex vitro* conditions without losses of plant material.

So we can conclude that this method is the best from medium-term storage for ulluco plantlets. Microtuberization of ulluco confirms the I. hypothesis about medium-term conservation of ulluco not only by *in vitro* conservation but also by microtuber production.

4.2.4 *In vitro* cold hardening

The possible way how to increase low regeneration after cryopreservation is by acclimation at low temperatures, by alternating of hardening temperatures.

In vitro hardening of plantlets helps understanding of plant material and cold hardening of plantlets can be beneficial for further cryopreservation. For example, hardened plantlets increase the survival rate for potatoes (Kaczmarczyk *et al.*, 2008). This means that cold acclimatization can play an important role for long-term conservation.

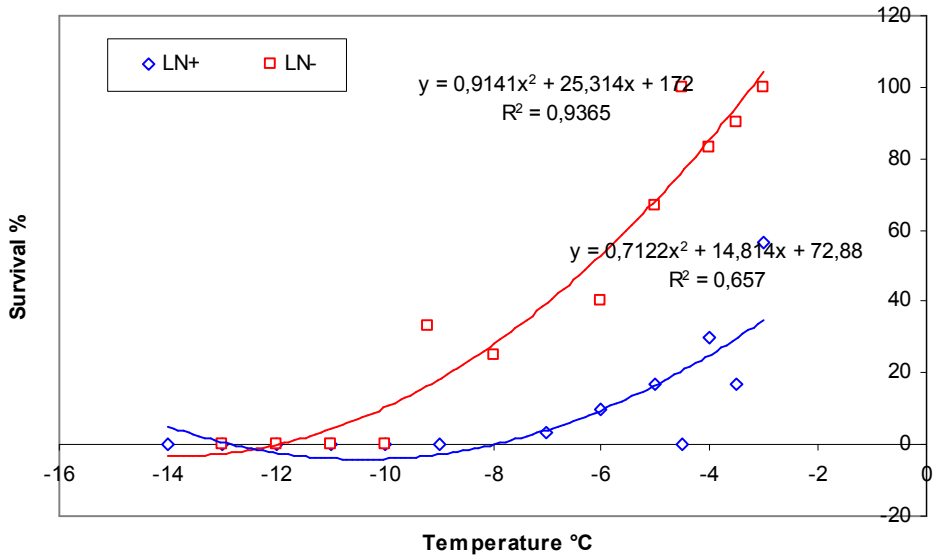

Fig. 12 Cold hardening and survival of ulluco plant in low temperatures with ice induction in agar by LN (blue line) or without LN pretreatment (red line). Correlation coefficients are significant at $P < 0.01$ and $P < 0.001$, for LN and without LN respectively.

Comparison of survival of ulluco plantlets after ice nucleation by LN or without ice nucleation is in the Fig. 12. Recent study has shown that the ice nucleation did not increase survival of plantlets. Ice nucleation was induced by short contact of agar with LN and the ice occurs immediately. Afterwards the plants stay at low temperature for 39 hours (Fig. 12). Second control observation (LN-) of survival of ulluco was done without LN induction and ice spontaneously occured between -4 and -6 °C after 39 hours. Plantlets were further grown at 21 °C. After one month of growing at 21 °C survival of the plant was evaluated.

The plants without LN treatment were able to survive lower temperatures with better survival rate. The plantlets without LN were able to surviveand the lowest temperature which was 9.5 °C.

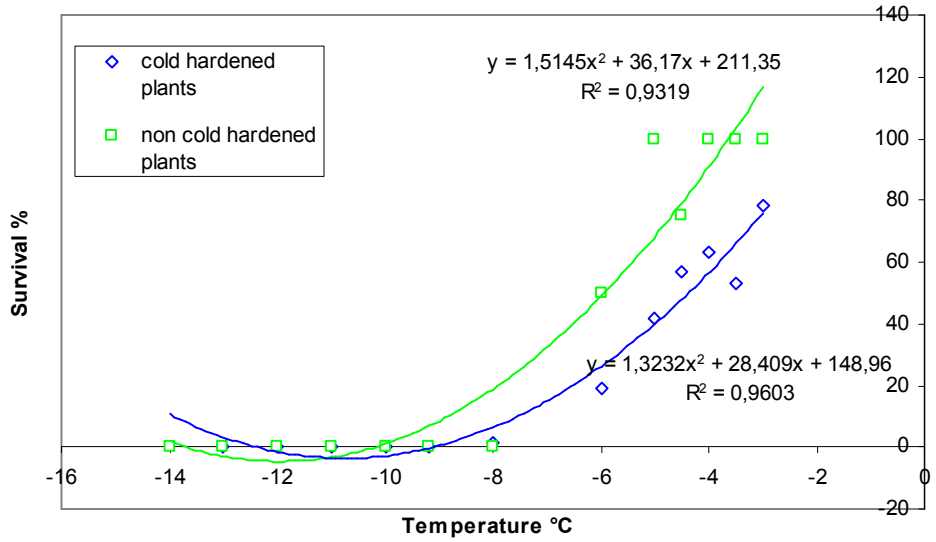

Fig. 13 Differences in survival of cold acclimated and non acclimated ulluco plantlets at temperatures below zero. The polynomial curve was fitted to the experimental data. The curve of cold hardened plants significantly represents (r = 0,965) the measured data on the $P < 0.001$. The curve of non-cold hardened plants was significant on $P < 0.001$ level (r = 0,980).

According to our results the cold hardening (Fig. 13) of plants improved survival at temperature between -2 °C to -7 °C but did not increase survival at temperatures below -7 °C. For both cold acclimated and non acclimated plantlets were the lowest temperature was -7 °C.

Also according (Sanchez *et al.*, 2009) research on ulluco and oca the constant low temperature (6 °C) did not increase the recovery percentage after cryopreservation, but there was a small increase of recovery (5 % in ulluco and 4 % in oca plantlets) when alternating temperatures (18 °C for one week and 6 °C second) were applied.

Ulluco hardening before cryopreservation can thus be recommend by alternating temperature even though it is not necessary for high improvement of cryopreservation protocol.

4.2.4.1 Determination of cold tolerance by UV-VIS Spectroscopy

Experimental studies of cold hardening have been made by UV-VIS Spectroscopy and electrolyte leakage for deeper understanding of cold hardening process also inside plant material.

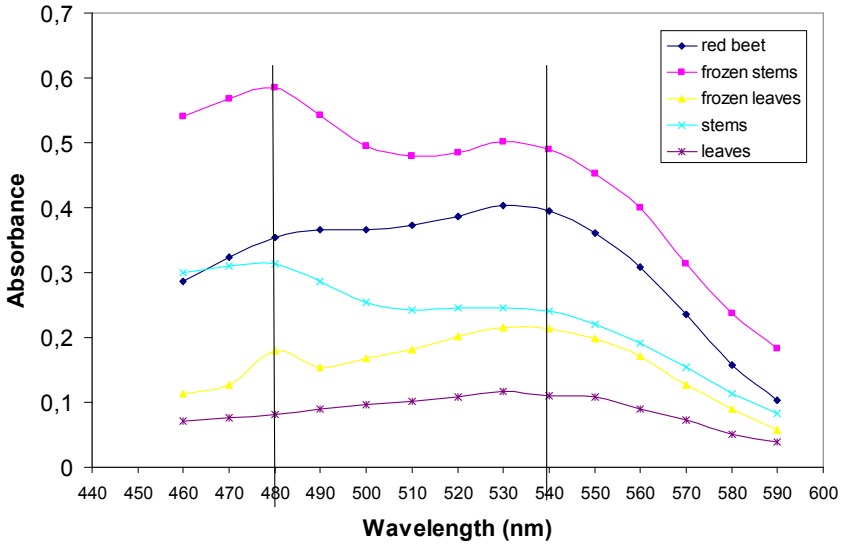

Fig. 14 Differences in absorption spectra of betalains for different part (frozen and non-frozen) of ulluco and comparison with red beet. Samples were measured by spectrophotometer after 2 hours of shaking in distillated water. The measured points were connected by spline lane. The absorption maximum at 480 nm (yellow, betaxanthins) and at 540 nm (violet, betacyanins) according Strack *et al.*, (2003) is equal for maxima of tested plant samples.

Frozen and non-frozen leaves and stems of ulluco was used for UV-VIS Spectroscopy study and red beet were used as a control. The results (Fig. 14) show increase of absorbance after freezing temperature and injury of plants. This method also confirms the presence of betalains in ulluco as it is mentioned in the study of (Svenson *et al.*, 2008).

Betalains are water-soluble nitrogen-containing pigments, which are synthesized from the amino acid tyrosine into two structural groups: the red-violet betacyanins and the yellow-orange betaxanthins (Azered, 2009). Measured absorbance was 0.1 higher for leaves and 0.25 for stems at 280 nm and 0.13 higher for leaves and 0.1 for stem. Thus, it can be

concluded that stems contains higher amounts of betaxanthins and leaves have a higher level of betacyanins.

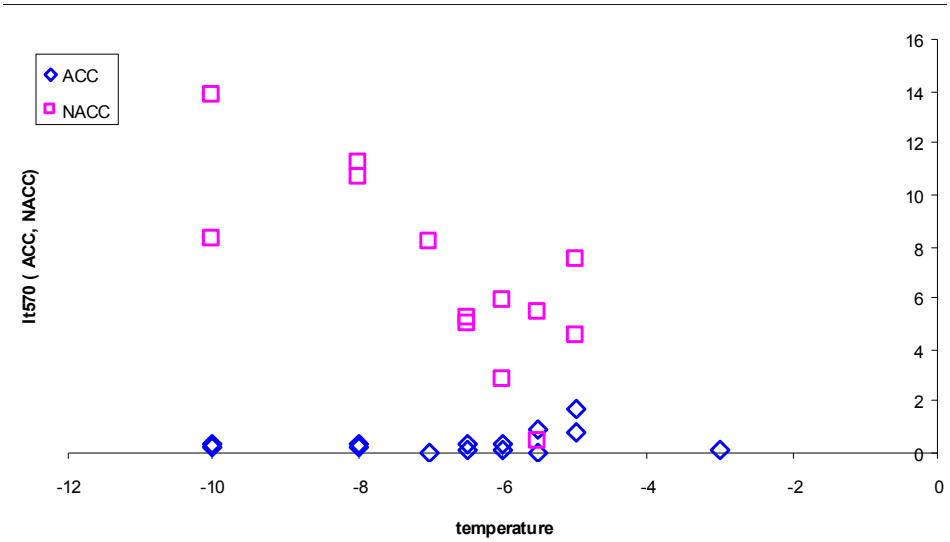

Fig. 15 Injury index of acclimated x non-acclimated leaves of ulluco at freezing temperature by UV-VIS Spectroscopy. Explants were cold acclimated for 1 month at 4 °C (blue colour) or grown at 21°C (purple colour).

Comparison of injury index for ulluco cold acclimated and non-acclimated leaves was measured by UV-VIS Spectroscopy (Fig. 15). Non-acclimated ulluco leaves were destroyed by the temperature under -5 °C. Cold acclimated plantlets were able to survive in better condition lower temperatures than non-acclimated plantlets up to -10 °C. The lower temperature for index injury measured by UV-VIS Spectroscopy can be caused by lower sensitivity of this method when compared to survival of cold hardened plantlets and measurements by electrolyte leakage (Fig. 16).

4.2.4.2 Determination of cold tolerance by electrolyte leakage

The conductivity method, based on the measurement of the amount of electrolytes leaked out from injured tissues into deionized water, is widely used for the determination of the degree of freezing injury in plants.

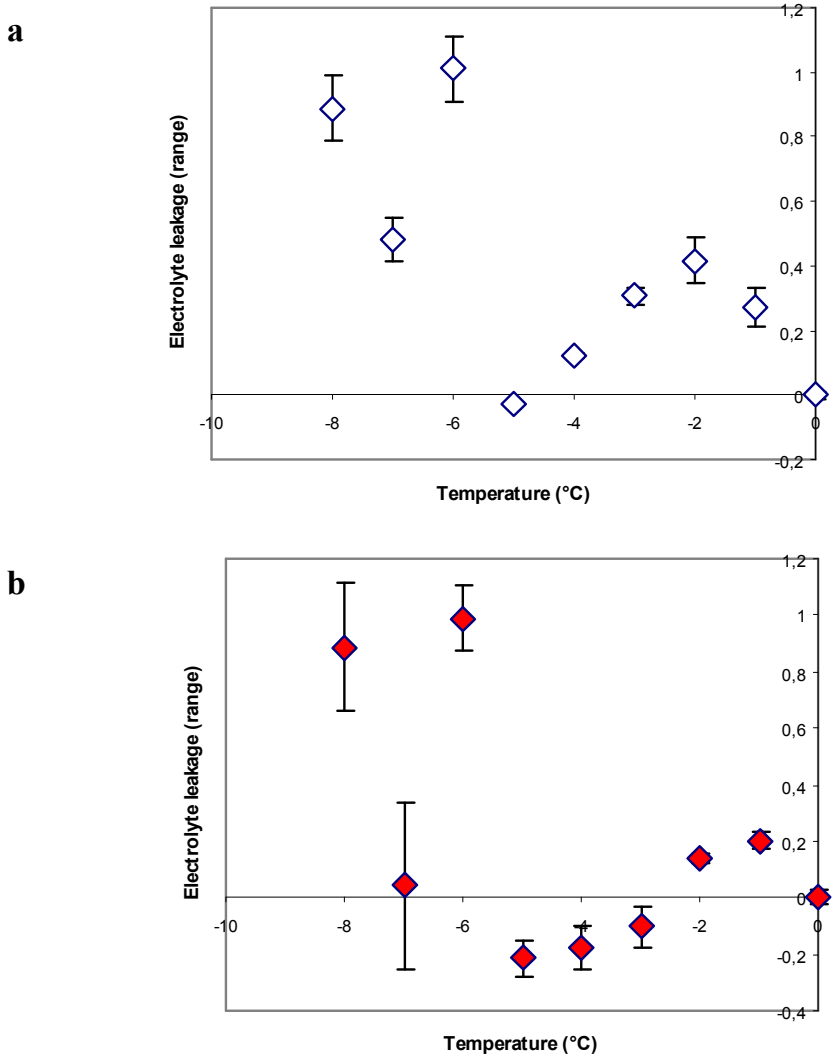

Fig. 16 Effect of low temperatures to ulluco by electrolyte leakage of *in vitro* grown plants (hardened in 4 °C for 5 weeks) a) Electrolyte leakage from leaves at the range (0 – no injury, 1 – damaged) at freezing temperatures; b) Electrolyte leakage from stems at the range (0 – no injury, 1 – damaged) at freezing temperatures.

There was no ice occurance at temperatures from -1 to -5 °C and -7 °C was without ice occurrence and temperatures -6 °C, and -8 °C destroyed plants by ice. The ratio of the

conductivity was counted from the result before treatment by boiling water to that after boiling. The conductivity after boiling represents the complete electrolyte leakage.

Cold hardened plantlets are able to survive till -7 °C according the electrolyte leakage (Fig. 16). This supports the data of plantlets´ survival of after cold hardening. Effect of cold hardening to ulluco was found, but freezing tolerance did not increase by the hardening of plants by 5 weeks in 4 °C. There could also be found higher electrolyte leakage at -2 °C (especially for leaves) which corresponds also to the field research (Martin *et al.*, 2005) where ulluco tops were usually killed by two to three radiation frosts of over -2 °C.

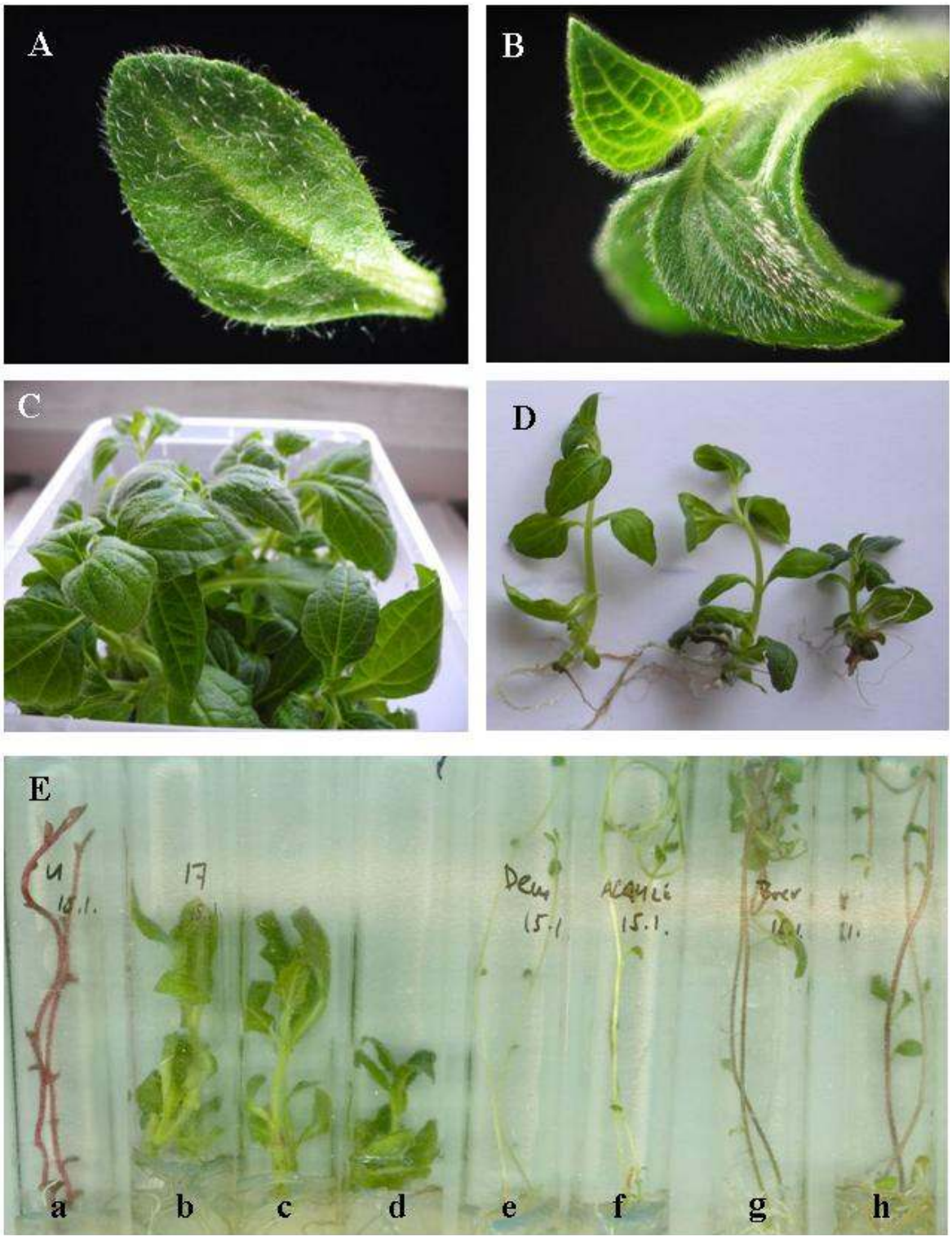

Plate 2. Growth of yacon *in vitro* conditions **A.** detail of leaf, **B.** yacon plantlet, **C.** yacon *in vitro* – storing in boxes short term conservation, **D**. yacon plantlets after 40 days of growing at 21°C, **E**. comparison of growth after 40 days of storing at 21°C (**a.-** ulluco, **b.c.d.-**yacon, **e.f.g.h.** – wild *Solanum* species – *demissum, acaule, brevidens* and *vernei*)

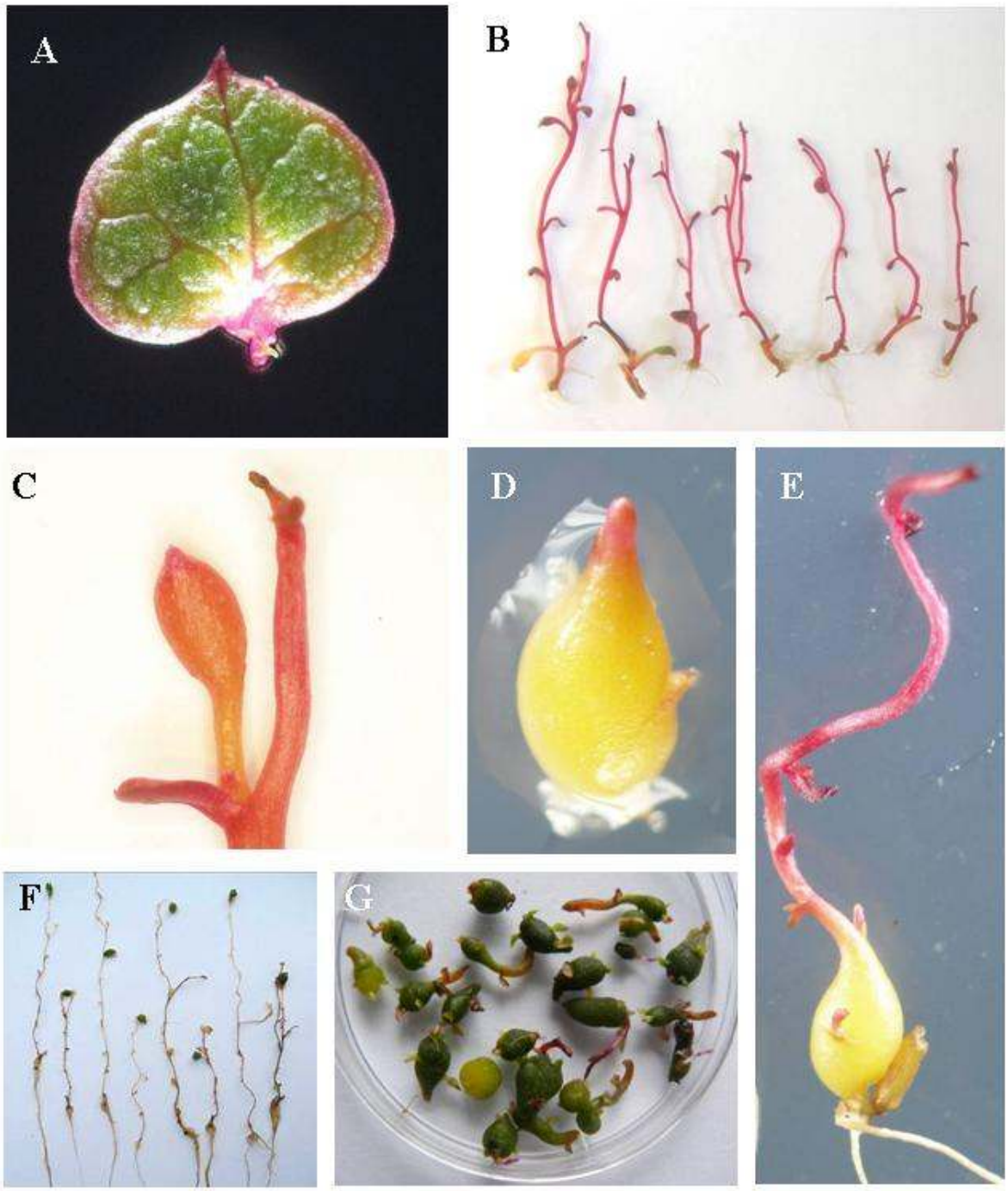

Plate 3. Growth of ulluco *in vitro* conditions **A.** leaf detail, **B.** ulluco plantlets after 5 week cultivation at 21°C, **C.** microtuber development on plant, **D.** harvested microtuber, **E.** microtubers at mother plants after 6 months of storage, **F.** shoot proliferation from microtuber, **G.** i*n vitro* sprouting microtubers

4.3 Cryopreservation

In vitro conservation entails the risk of losing material due to cooling equipment failure, contamination of cultures or mislabeling accessions (Golmirzaie and Toledo, 1998). From all investigated methods, cryopreservation is the type of conservation which enables longest storing of plant material. Cryopreservation of ulluco and yacon has not been studied enough yet, which is why this study was focused on cryopreservation and study of freezing temperature to plant material.

4.3.1 Ulluco cryopreservation

Three different vitrification methods were used for cryopreservation of ulluco by different sucrose content and air desiccation and by PVS3 treatment (Table 13).

Table 13 Regeneration of shoot tips of ulluco after cryopreservation using vitrification methods.

Treatment hours	1st Trial Air desiccation (%)		2nd Trial Air desiccation (%)		3rd Trial PVS3 dehydration (%)	
	LN-	LN+	LN-	LN+	LN-	LN+
0	90	-	92.5	-	80	-
0.5	72.5	0	82.5	0	80	10
1	65	0	80	0	70	23
1.5	62.5	0	75	0	73.3	52.5
2	52.5	0	80	10	67.5	25
2.5	50	0	42	0	-	-
3	30	0	45	0	-	-

Note: - not examined

4.3.1.1 Sucrose pretreatment

Sucrose pretreatment – 1st trial (0.7 M sucrose)

Non-frozen desiccated shoot tips regeneration decreased from 90 % to 30 % after 3 hours of desiccation and were used as a control to immersed samples to LN. There was no regeneration of shoot tips after immersion to LN (Table 13).

Sucrose pretreatment – 2nd trial (2 M sucrose)

Regeneration of non-frozen desiccated shoot tips decreased from 92.5 % to 45 % after 3 hours of desiccation and was used as control for immersed treatment (Table 13). The decreasing of regeneration without LN treatment is the response only to the desiccation. The regeneration of cooled shoot tips in LN was zero, the exception was found in shoot tips with low regeneration up 10 % after two hours of desiccation before LN immersion (Table 13).

Ice nucleation temperature of shoot tips decreased from -30 °C in non-desiccated to minimum -36.1 °C in shoot tips after 2.5 hours of desiccation according to the onset of heat exothermic reaction. The desiccation decreases the ice nucleation by 6 °C which can help to narrow the dangerous ice nucleation temperature range before glass formation.

Pretreatment of 2M sucrose increases significantly regeneration in comparison to 0.7 M sucrose treatment after cryopreservation; therefore it was used for other trials with PVS3. 2M sucrose pretreatment show also good results for another tuber crop *Solanum tuberosum* (Faltus *et al.*, 2006).

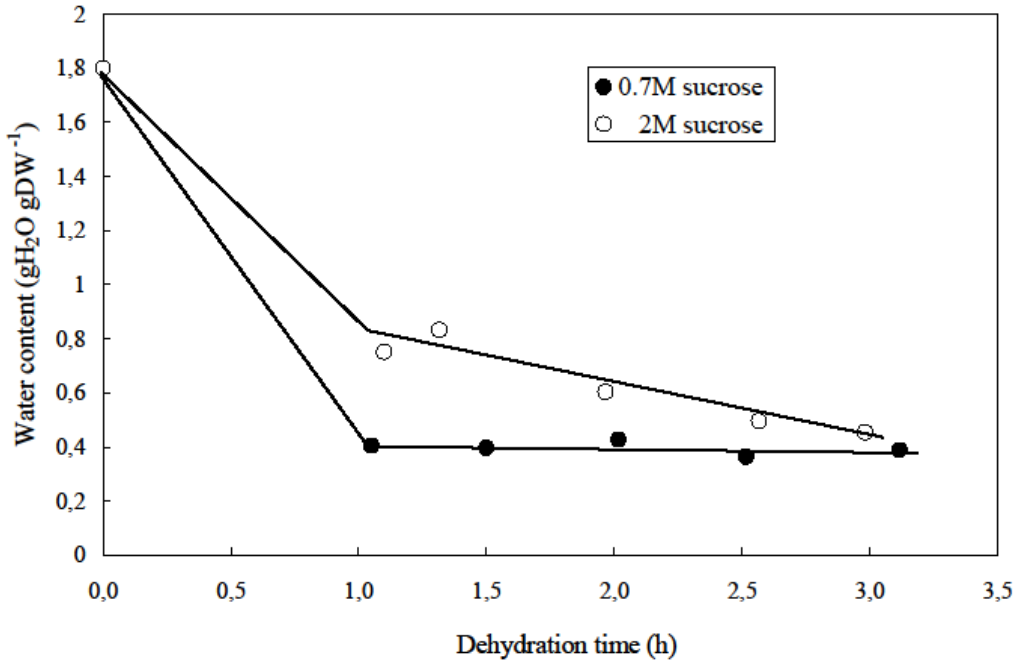

Fig. 17 Water content/desiccation rate after 0.7M and 2M sucrose pre-treatment and desiccation over silica gel.

The water content during air desiccation decreased dramatically in the first hour from 1.8 to 0.4 g H_2O g^{-1}DW in the first trial and to 0.8 g H_2O g^{-1}DW in the second trial. During further air desiccation, the water content of shoot tips decreased slowly in 2M of sucrose treatment and was constant when we used 0.7M sucrose treatment (Fig. 17). Ten percent of shoot tips recover after exposure to LN temperature after 2M sucrose pre-treatment of nodal segments following two hours desiccation over silica gel. The desiccation of shoot tips does not help the regeneration of shoot tips after LN treatment.

4.3.1.2 PVS3 pretreatment

Sucrose in combination with glycerol pretreatment – 3rd trial (PVS3)

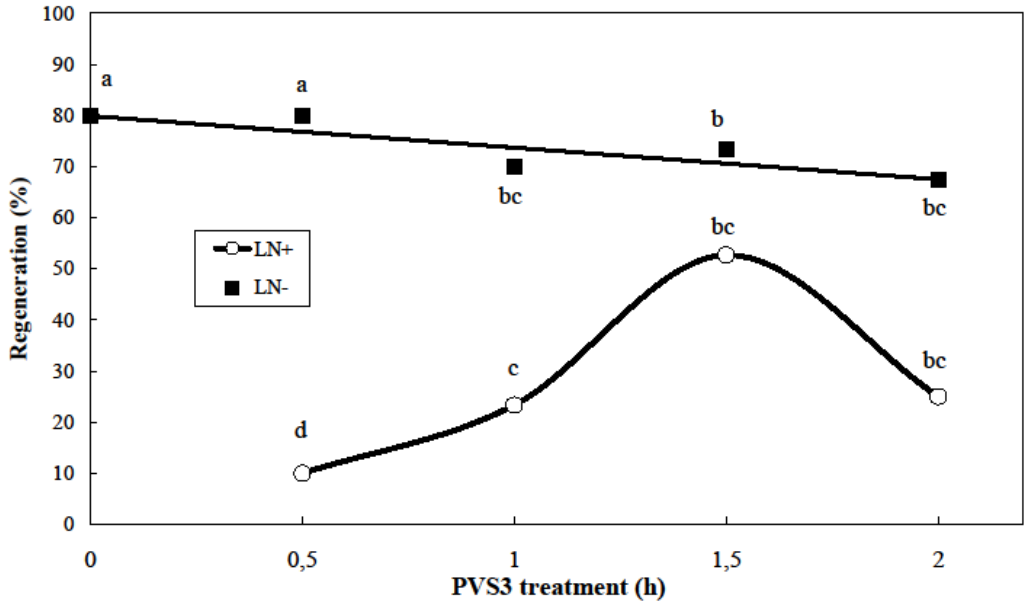

Fig. 18 Percentage of ulluco shoot tips regeneration without liquid nitrogen treatment is taken as a control (full square) for regeneration of shoot tips from liquid nitrogen after different time of PVS3 pre-treatment (open circles). The same letters mean no difference on $p < 0.05$ according to LSD test. The curves are guide for the eye.

Non-frozen dehydrated shoot tips decrease their regeneration rate from 80 % to 67.5 % after 2 h of dehydration in PVS3 and were used as a control for regeneration after immersion in liquid nitrogen. The regeneration of immersed trials increases from 10 % to 52 % between the 0.5 h and 2 h in PVS3 after LN exposure (Fig. 18, Plate 5 A–F).

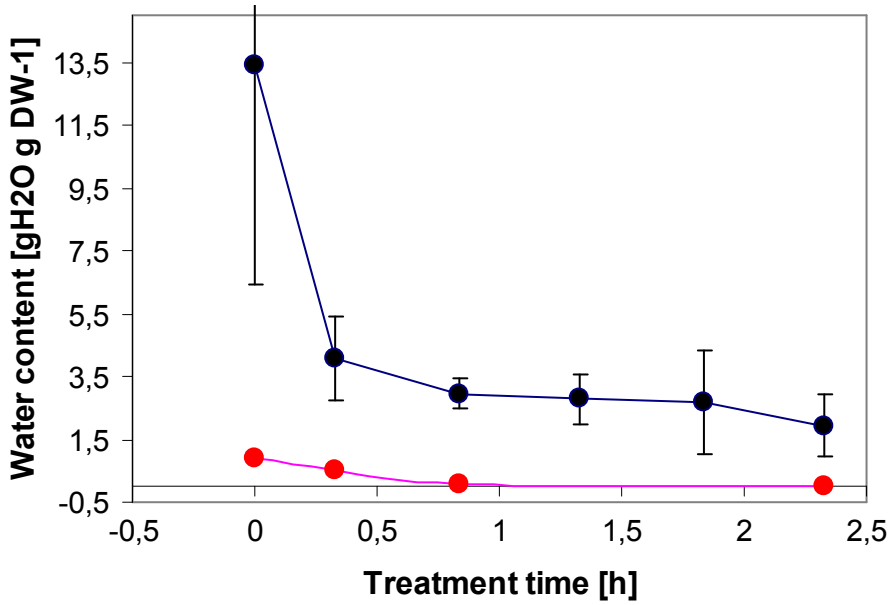

Fig. 19 Water content during dehydration of shoot tips by PVS3. Total water content (black points and black line) during dehydration of shoot tips in PVS3 – cryoprotectant mixture and content of crystallized water (red points and red line).

Samples measured by differential scanning clorimetry were calculated as area of the melting peak dividing by heat capacity of water. Total amount of water (black line) and amount of crystallized water (red line) in shoot tips treated by PVS3 (Fig. 19) rapidly descend during first 30 min and further slowly decreased. Crystallized water reached close to 0 after 1 h of PVS3 treatment.

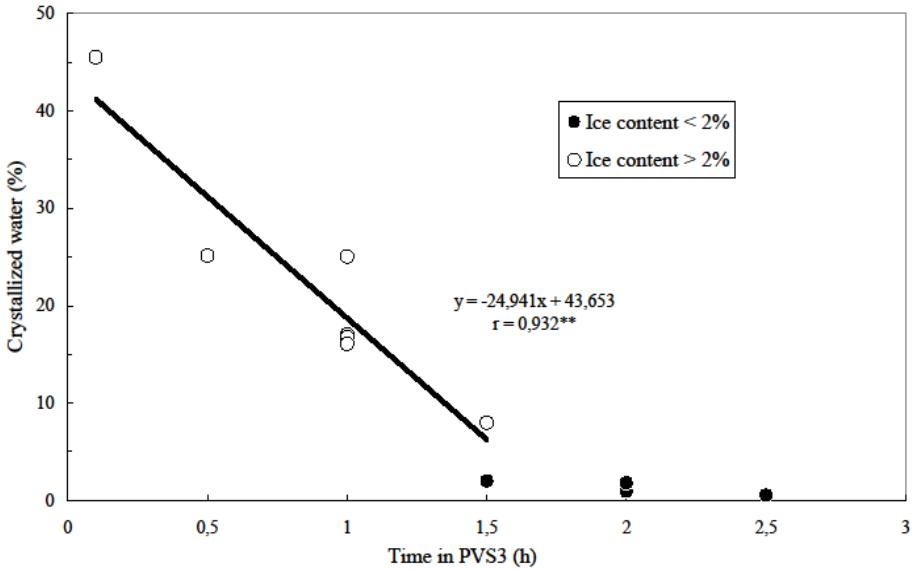

Fig. 20 Ice crystals content and dehydration rate after 2M sucrose and PVS3 pre-treatment. Linear regression of ice content for more than 5 % of crystallized water is highly significant at the level of $P < 0.05$.

For investigating crystallized water inside ulluco shoot tips differential scanning calorimeter measurements were performed. Percentage of water crystallization was less than 2 % after 1.5 h PVS3 pre-treatment (Fig. 20).

4.3.1.3 Thermal analysis of cryoprotected shoot tips

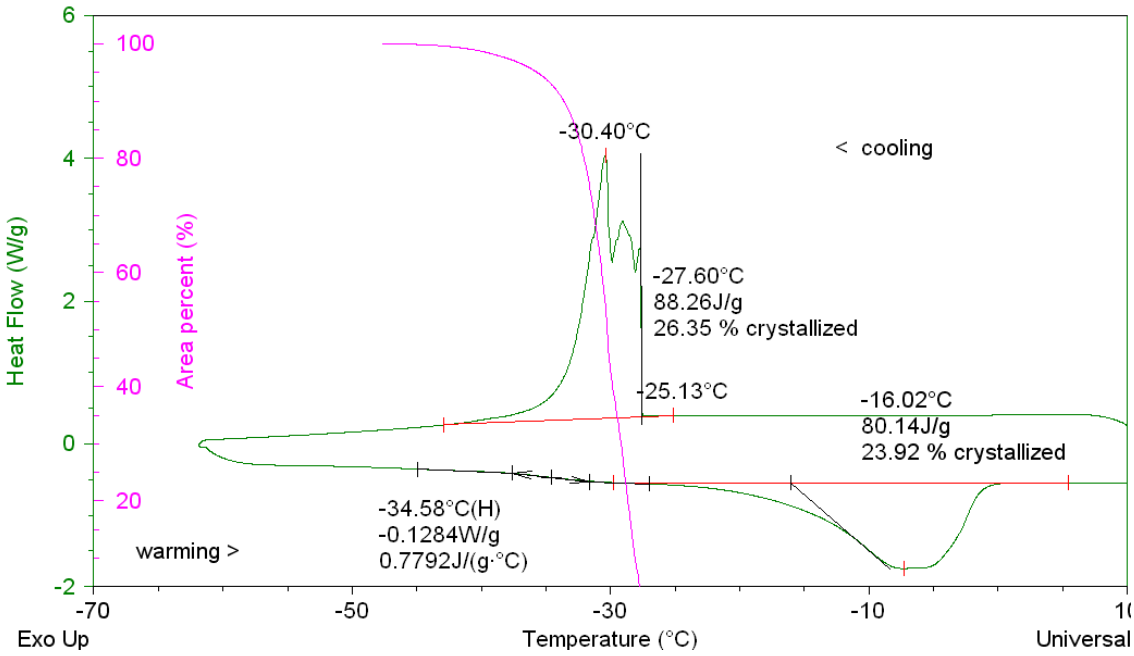

Fig. 21 The thermogram of 10 ulluco shoot tips in PVS3 after sucrose and LS pretreatment. Measurements were made by DSC with colling and warming rate 10 °C min^{-1}.

Glass transition occurs at -34.6 °C, but there was found 26.4 % of crystallized water and 24.0 % of melted water. The temperature of ice nucleation was -27 °C and 50 % of shoot tips were frozen at -30.4 °C.

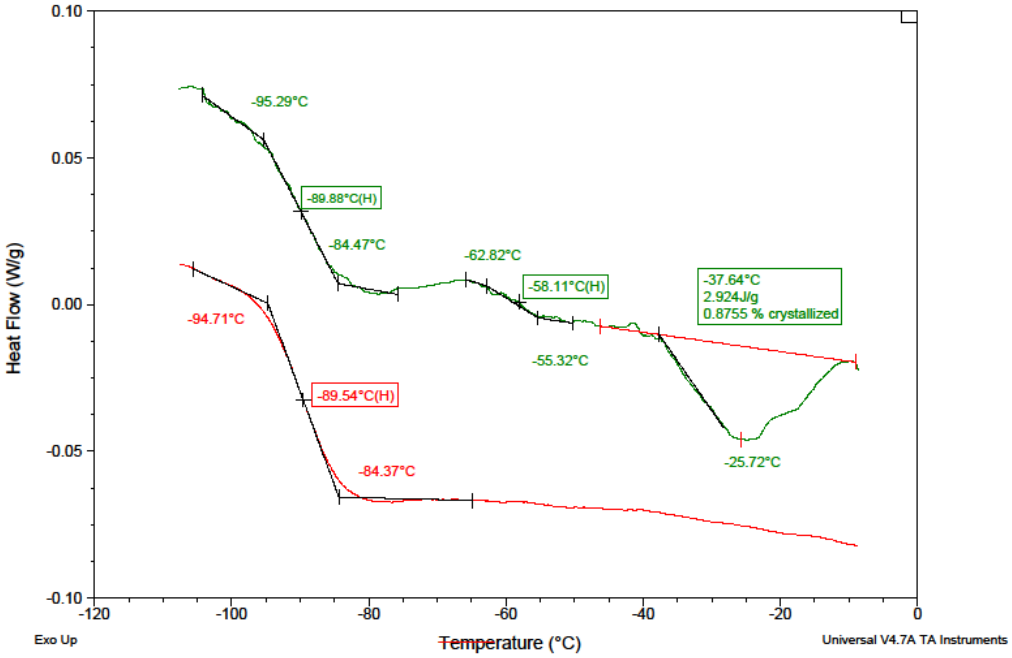

Fig. 22 Differential Scanning Calorimetry measurement of heat flow during warming, glass transition detection. The green curve for PVS3 with ulluco shoot tips has two glass transitions and endothermic peak with less then 1 % of water thawed. The red curve is for PVS3 with only one glass transition.

The upper curve is a thermogram of 10 shoot tips during warming (10 °C min^{-1}) after controlled freezing (10 °C min^{-1}). Shoot tips were incubated in loading solution for 20 min and treated 1 h in PVS3. Lower curve stands for a PVS3 without meristems. The glass transition of PVS3 is obvious at -89 °C on the upper curve, and the second glass transition is at -58 °C with thawing endothermal peak representing thawing of approximately 1 % of water (Fig. 22).

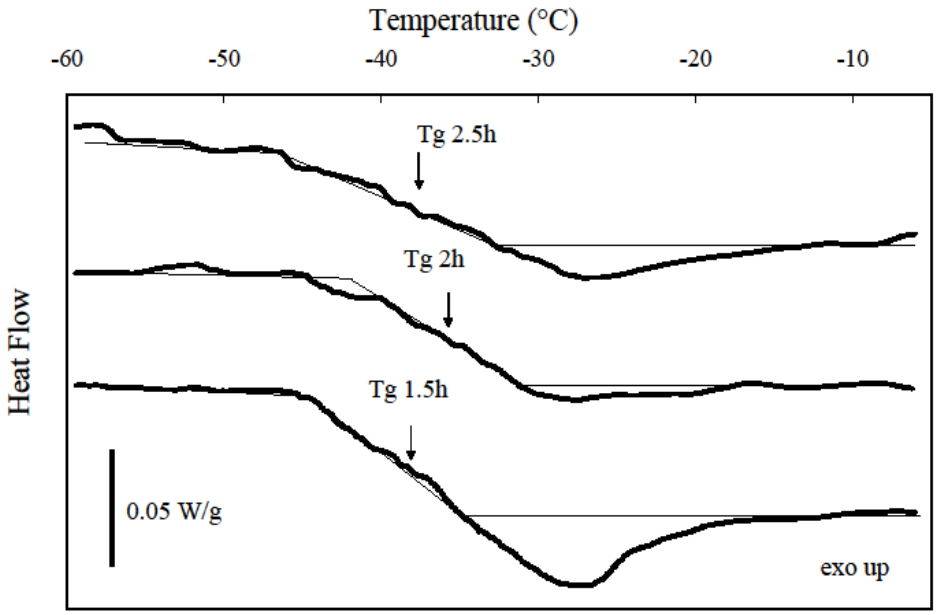

Fig. 23 Thermogram from differential scanning calorimeter during warming of 10 shoot tips after 1.5, 2 and 2.5 hours PVS3 treatments. Curves are shifted along y-axis. The arrows show inflection point of glass transition temperature. The bar represents the scale of heat flow. Endothermal undershoot represents negligible amount of melted ice, less than 2% (Zamecnikova *et al.*, 2011).

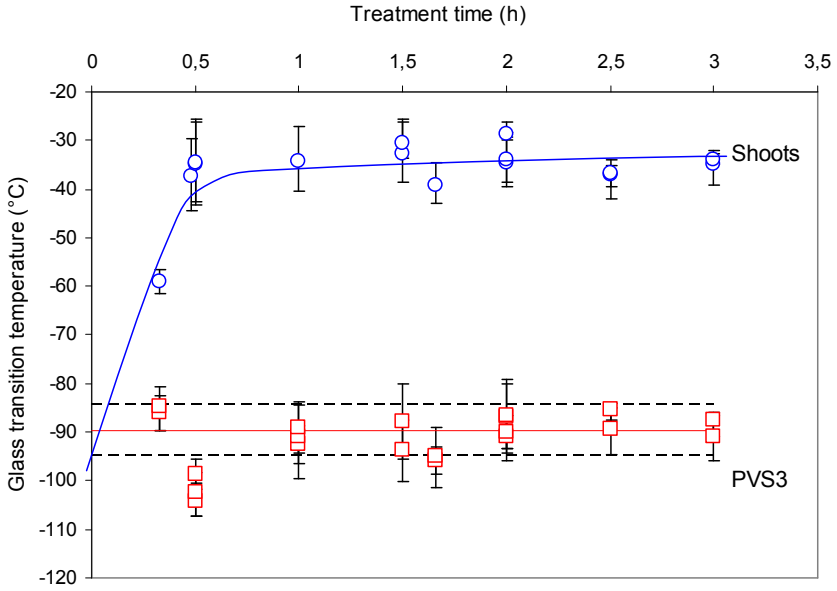

Fig. 24 Glass transition of ulluco shoot tips after different time in PVS 3 (blue line) and glass transition of PVS3 alone (red line) – measured by differential scanning calorimeter Circles show glass transition in shoot tips and bars show onset and endset of glass transition. Squares show glass transition of PVS3 in shoots. Red line is for glass transition of PVS3 without shoots, dashed line above is the onset of glass transition and below endset of glass transition for PVS3. The curves are guide for the eye. Adapted from Zamecnikova *et al.*, (2011).

Two glass transitions were detected during warming in ulluco shoot tips (Fig. 24) in average at -89 °C and the second glass transition at -58 °C with thawing endothermal peak representing thawing of approximately 1 % of water. PVS3 without meristems had glass transition at -89 °C.

The first colder glass transition temperature detected in PVS3 with ulluco shoot tips belonging to PVS3 on the shoot tips surface and the warmer glass transition temperature is for the shoot tip saturated by PVS3 as is obvious from these results. Thus the second glass transition, which occurs at higher temperatures, is essential for regeneration of shoot tips after thawing.

Shoot tips immersed in PVS3 were saturated after 0.5 h and detectable glass transition temperature was found between -25 °C and -45 °C. Glass transition for PVS3 was found at -89,6 °C with onset and endset -95 °C and -84 °C respectively and was constant in time after 0.5 h. Glass transition of PVS3 in shoots were found between -85 °C and -105 °C.

The glass transition was probably not evident after 1 h though the regeneration was 23 %. the glass transition was masked by dominant melting peak of endogenous heat flow corresponding to melting of ice crystals. The first detectable glass transition was after 1.5 h in PVS3 and the regeneration rate increased up to 52.5 % at the same time.

There is still 21 % difference between regeneration of shoot tips immersed in LN and control samples after 1.5h in PVS3. This difference in regeneration is theoretical potential for future improvement of this method. The PVS3 solution positively affected regeneration of ulluco shoot tips after liquid nitrogen immersion. PVS3 treatment was successfully used also for garlic (Makowska *et al.*, 1999), apple (Wu *et al.*, 2001), pineapple (Martinez *et al.*, 2002). It is difficult to compare the ulluco results with the literature due to the obvious lack of information about ulluco cryopreservation. In contrary to the published methods, not all authors use for shoot tips in PVS3 foils strips as in our experiments, but their shoot tips were directly transferred to 1 ml of PVS3 and submerged in cryotubes in LN. Fast freezing in our experiment should protect shoot tips from water crystallization better than using 1 ml PVS3 which slow down the freezing rate (data not shown). The large range interval for PVS3 treatment ranging in hours (from 0.5–2.5 hours) has an advantage for application and for repetition of this method in contrary to e.g. PVS2 cryoprotectant treatment which is more time and temperature dependent. The advantage of PVS3 is also in the absence of DMSO potential toxic substances.

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4.3.2 Yacon treatment preparation for cryopreservation

Effect of PVS3 treatment on shoot tips of yacon was studied. Increasing duration of PVS3 treatment decreases the survival of yacon shoot tips.

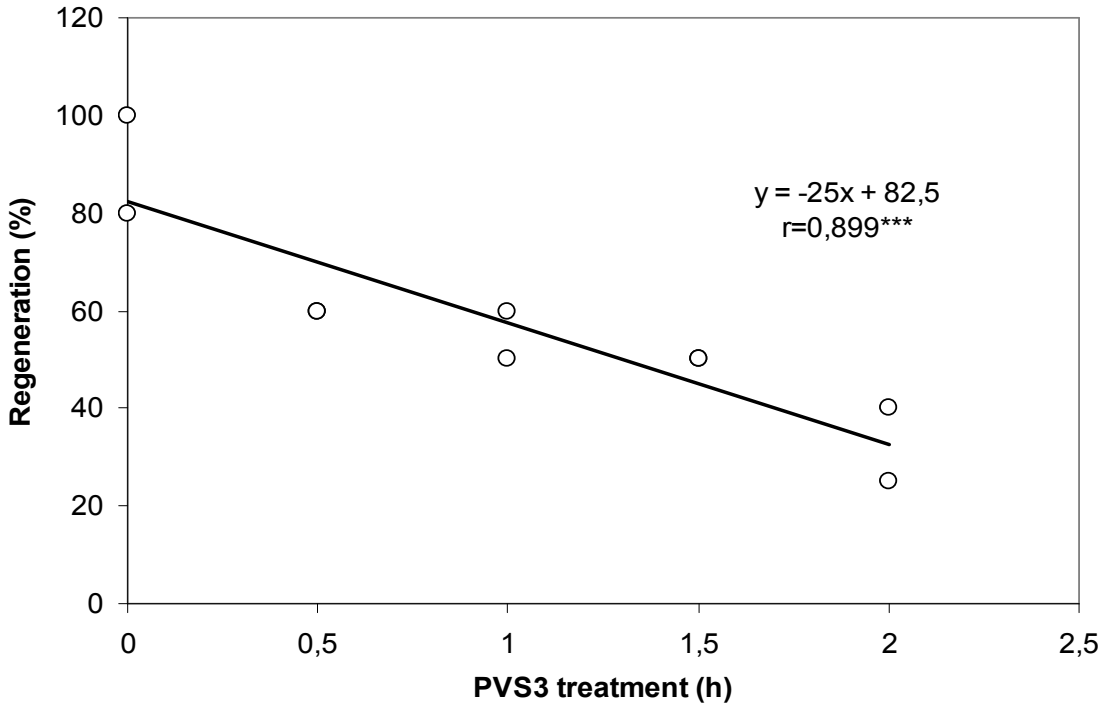

Fig. 25 Regeneration of yacon shoot tips as a percentage of all shoot tips after sucrose and LS pretreatment and after different time in PVS3 treatment. The linear line was significant on $P < 0.001$ level.

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Regeneration of yacon shoot tips (Fig. 25) was observed for different time in PVS3 0.5–2.5 hours. Control samples without LN had after 1 hour 60 % regeneration, while it decreases to 35 % after 2 hours (Plate 4 C-E). There was no survival after cryopreservation.

The hypothesis of yacon cryopreservation was partly supported by survival after PVS3 treatment, but there is needed further study for cryopreservation protocol which could lead to long-term conservation. Pre-treatment by PVS3 seems to be promising method, but more research is needed. In comparison with ulluco which was successfully conserved by cryopreservation disadvantages of yacon for cryopreservation are following: bigger size of shoot tip (Plate 4 A-C) with higher water content, needs of higher temperature to survive and worse response to *in vitro* growing. This study was needed for better understanding of pre-treatment of yacon shoot tips for furthered vitrification protocol for cryopreservation.

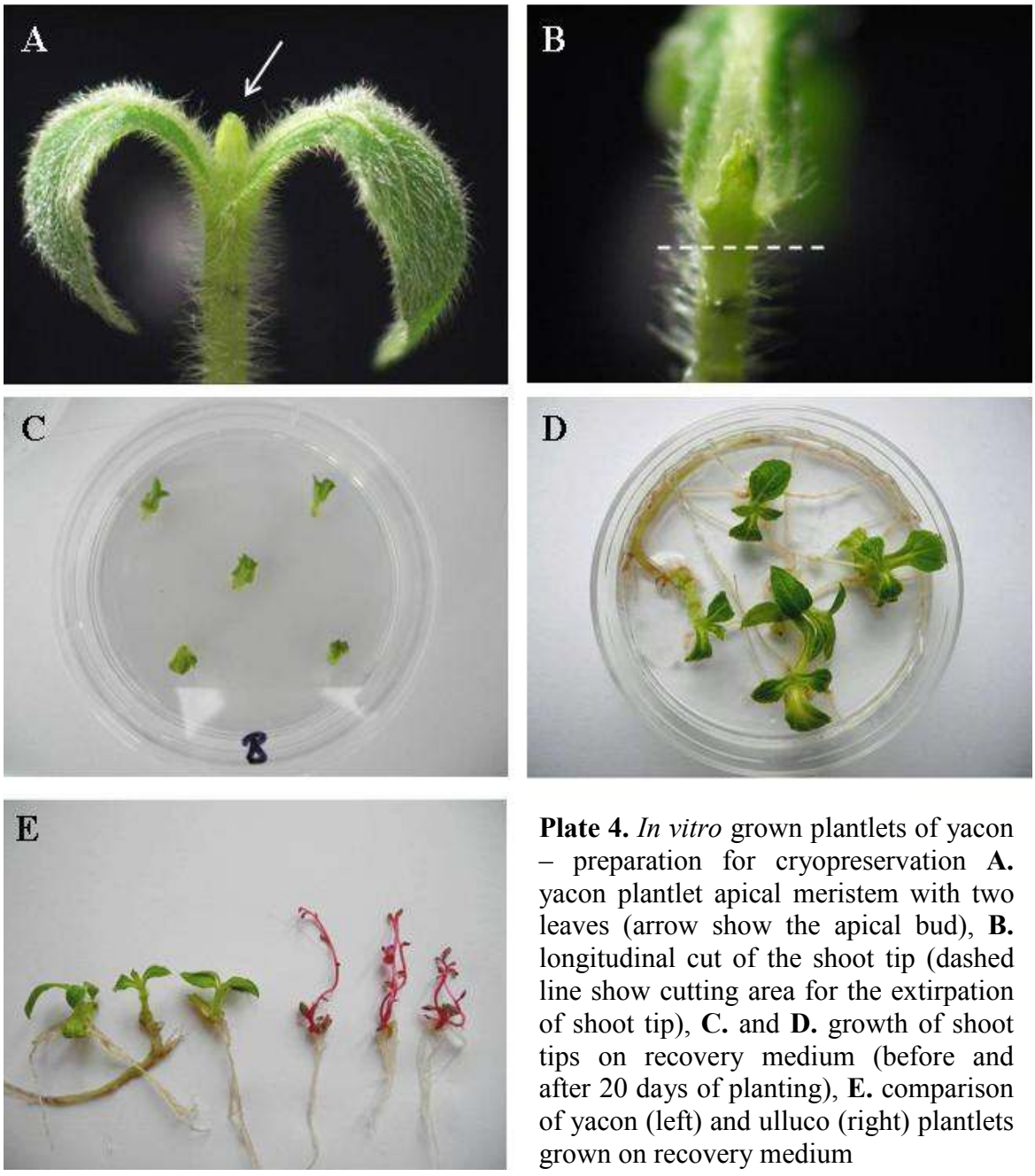

Plate 4. *In vitro* grown plantlets of yacon – preparation for cryopreservation **A.** yacon plantlet apical meristem with two leaves (arrow show the apical bud), **B.** longitudinal cut of the shoot tip (dashed line show cutting area for the extirpation of shoot tip), **C.** and **D.** growth of shoot tips on recovery medium (before and after 20 days of planting), **E.** comparison of yacon (left) and ulluco (right) plantlets grown on recovery medium

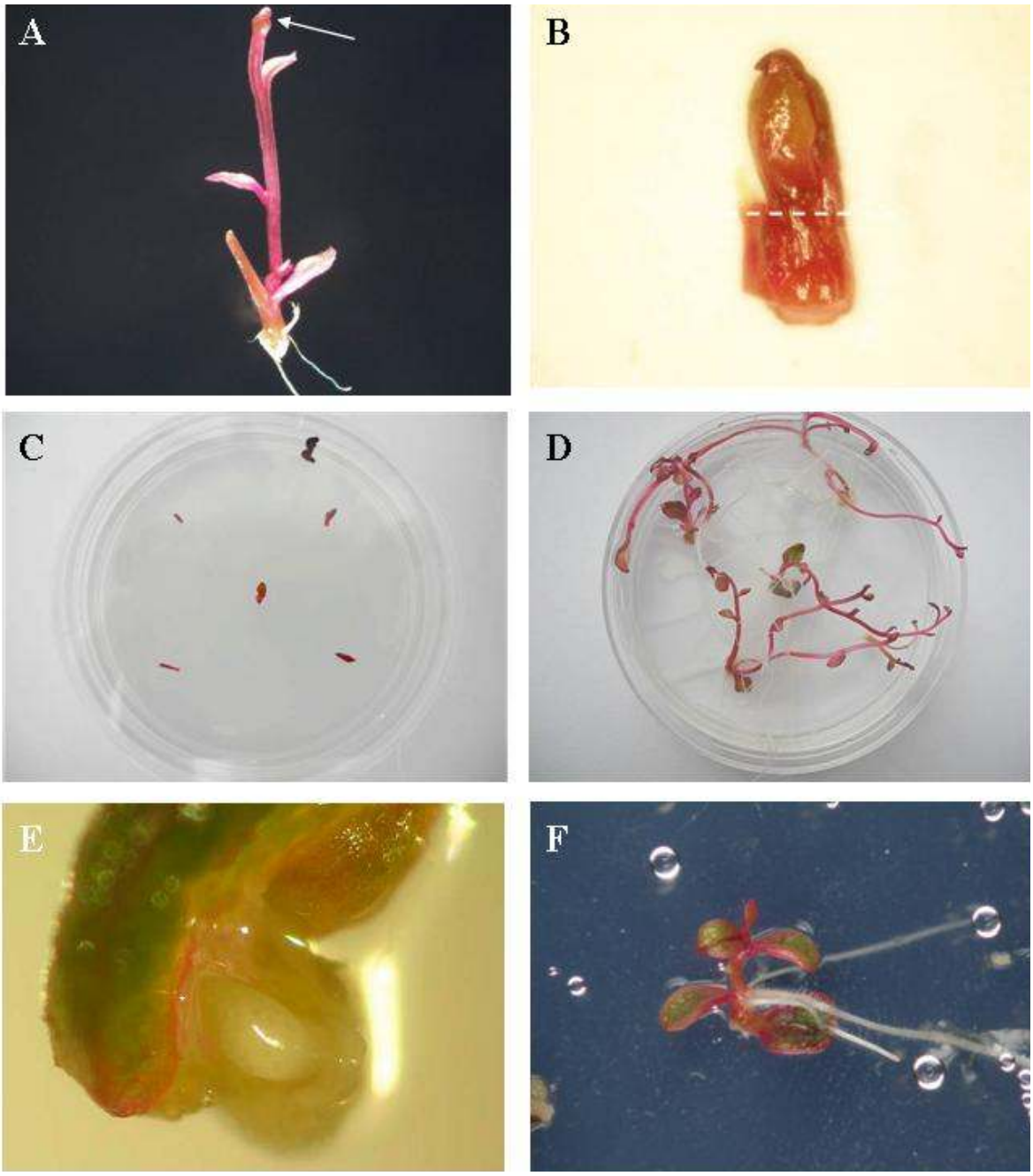

Plate 5. *In vitro* grown plantlets of ulluco - preparation for cryopreservation and regeneration after cryopreservation, **A.** ulluco plantlet (arrow show the apical bud), **B.** longitudinal cut of the shoot tip (dashed line show cutting area for the extirpation of shoot tip), **C.** and **D.** growth of shoot tips on recovery medium (before and after 20 days of planting), **E.** regeneration after cryopreservation, **F.** ulluco plantlet after cryopreservation.

4.3.3 Micropropagation on recovery medium

The addition of growth regulators can positively influence growth of roots in regenerated plants. (Lebeda *et al.*, 2011). In the context of a working gene bank, ability to synchronize growth of early post-thaw plantlets and their development to mature plants may be critical considerations in choosing freezing and recovery strategies for the conservation of potato genetic resources (Harding and Benson, 1994).

Growth of yacon and ulluco was studied on recovery medium supplemented by IAA, kinetin and GA_3 for further aim of recovery of plant material after cryopreservation.

Table 11 Growth of yacon shoot tips on recovery medium during one month without pretreatment or cryopreservation

Growth (days)	Root (No)	Leaves (No)	Plant height (cm)
0	0	0	0.3 ±0.1
7	0	0	0.9 ±0.2
30	2.6±1.51	4±1.41	1.1±0.43

Table 12 Growth of ulluco shoot tips on recovery medium during one month without pretreatment or cryopreservation

Growth (days)	Root (No)	Leaves (No)	Plant Height (cm)
0	0	0	0.2 ± 0.10
7	1.8 ± 1.69	2.8 ± 0.33	0.6 ± 0.19
30	5.0 ± 1.0	7.2 ± 1.92	2.4 ± 0.41

Recovery medium is routinely used for regeneration of shoot tips after cryopreservation for potatoes MS medium (Murashige and Skoog, 1962) modified according to (Grospietsch *et al.*, 1999) without casein and myo-inositol, with a lower amount of nitrogen (25 % of NH_4NO_3 and 50 % of KNO_3 of the original MS medium), with 0.5 mg l^{-1} IAA, 0.5 mg l^{-1}, KIN and 0.2 mg l^{-1} GA_3 with 30 g l^{-1} glucose and pH 5.6. It is suitable also for yacon and ulluco growth (Table 11, 12, Plate 2 E). Also, according to (Estrella and Lazarte, 1994) there were no developed shoots or roots when 1–2 mm explants were planted on medium lacking plant growth regulators and cultured at 20 ± 2 °C under a 16-h photoperiod, but

they achieved rooting and shoot development by IBA and BA (Lebeda *et al.*, 2011) use IAA (together with IBA) for rooting of yacon explants.

Cryopreserved shoot-tips of potato recovered in a medium containing zeatin, gibberellic acid and indole-acetic acid showed relatively rapidly, synchronous rates of plant regeneration and maturation, whereas shoot-tips regenerated on hormone-free medium, or media containing auxins and/or gibberellic acid developed asynchronously (Harding and Benson, 1994).

Yacon was able to produce 2 ± 0.7 nodal segments and ulluco 7.2 ± 1.92 nodal segments after 30 days of cultivation. They were used for further propagation. This partly supported II. hypothesis that yacon and ulluco should be possible to conserve by cryopreservation and to obtain healthy plant material which can be further propagated.

4.4 Comparison of conservation methods

Three methods, which are currently used for conservation and further propagation, were observed for yacon and ulluco (Table 14). Temperature alternation led to very good results for prolongation of storage period *in vitro* and cryopreservation was succusfull for ulluco plant. Also other methods were examined and will be useful for further conservation of genetic resources.

Table 14 Summarising data of yacon and ulluco conservation at the field, *in vitro* and by cryopreservation with evaluation of storage risk

Type of conservation	Plant	Theoretical number of obtained plants per year (from one mother plant)	Micropropagation coefficient / subcultivation (months)	Length of storage (months)/ Temperature (°C)	Risk of storage
Field	Yacon	5.7	5.7 / 6	7 / 10	xxx
	Ulluco	-	8.2 / 6	-	xxxx
In vitro	Yacon	2 843 342	3.45 / 1	6 / 17	xx
	Ulluco	43 439 mil	7.7 / 1	11 / 17 18 / 5	xx
Microtuberization	Yacon	-	-	-	-
	Ulluco	-	-	15 / 17°C	xx
Cryopreservation	Yacon	-	-	-	-
	Ulluco	-	-	An indefinite period	x

Note: - no data; x – low; xx – middle; xxx – high; xxxx – very high

The longest possibility of conservation was found for ulluco by cryopreservation and by *in vitro* storing. On the other hand, field conservation in the conditions of the Czech Republic was connected with particularly high risk of storage because of exceptionally low yields. The most appropriate method for yacon maintanance is field conservation, even though it has low propagation coefficient since there should be a safe backup by *in vitro* storage yacon plants which has lower storage risk and is suitable for rapid propagation of stored material.

5 CONCLUSIONS

Research was focused on three different ways of *ex situ* conservation – field conservation, *in vitro* conservation and cryopreservation. Research of *Smallanthus sonchifolius* (Poepp. *et* Endl.) H. Robinson (yacon) and *Ullucus tuberosus* (Loz.) (ulluco) conservation is extraordinarily limited. There is an attempt to introduce them also to the Czech Republic. There is a considerable need for improvement of conservation methods. Especially long-term conservation by cryopreservation could be advantageous for saving these unique genetic resources.

According to this study yacon and ulluco was reacting differently to the various *ex situ* conservation methods. Yacon was successfully conserved in the field conditions for three years with very good yield of tuberous roots, (average 1.6 ± 0.6 kg per plant). It was found that the higher weight of tuberous roots correlates with the higher weight of rhizomes. Rhizomes (average 1.6 ± 0.32 kg per plant) are routinely used for storage of plant material during winter at 8–10 °C and at moisture content 70–75%. Survival of rhizomes after storage was 80–90 %. Propagation coefficient for yacon field conservation is 5.7 in average which means that from a single plant. We can get 5.7 new plants in average in one year. The *ex situ* storage for preservation of germplasm is rather risky due to the biotic and abiotic stresses which can be lethal and lead to the loss of genotype.
Ulluco growth is very limited at the field conditions with the average yields of 39 ± 17 g per plant of tubers. Because of low yields of ulluco the *ex situ* conservation in the field conditions in the Czech Republic is not further recommended. Therefore is recommending conserving ulluco germplasm in *in vitro* conditions.

Ulluco growth *in vitro* is remarkably fast, and it has good response for medium-term conservation by both methods slow-grow and microtuberisation. This study was focused on the temperature influence on plantlets conservation. As a result, it can be recommend temperature 21 °C for ulluco propagation and also for short-term conservation (it is possible to obtain in average 7.7 ± 1.93 plants from a single plant after 30 days). The temperature 17 °C was found to be the best temperature for medium-term conservation (up to 11 months) with the possibility of consequent fast propagation and temperature 5 °C for long-term conservation (up to 18 months) with the requirement of subsequent subcultures.

Microtuberization was inducted by decreasing temperature in two-step during growth (2 months at 3/4 °C and 2 days of low temperature -4°C). By this method, it was possible to conserve ulluco for 15 months. The *in vitro* conservation according to this study seems to be a promising method – for both microtuberization and medium-term conservation, in comparison with the field conservation where the number of produced tubers was very low.

Yacon's growth and conservation in *in vitro* conditions had also exceptionally good results but in comparison with ulluco differences were found. We recommend temperature of 25 °C for propagation and short-term conservation. At this temperature, it was possible to obtain 3.5 ± 1.50 nodal segments in average after 30 days of cultivation. The reduction of incubation temperature to 17 °C was remarkably effective to prolong subcultures and plantlets which could be stored up to 6 months with uncommonly good viability at hormone free agar medium.

Both species grown *in vitro* have a high rate of multiplication coefficient. According to these results the plants can be stored in *in vitro* conditions. Moreover, the plant can be multiplied in *in vitro* condition for other conservation method e.g. cryopreservation. A multiplication coefficient in *in vitro* conditions was higher for ulluco than for yacon.

Cold hardening ability was studied at ulluco plantlets which is important for cryopreservation method. It was found that ulluco after cold hardening had a higher regeneration rate at higher temperatures, but the lethal temperature was not change. These results were supported by experimental studies by UV-VIS Spectroscopy and electrolyte leakage. UV-VIS Spectroscopy confirmed the presence of betalains in ulluco leaves and stems with high leakage rate from frozen parts. Higher amounts of betaxanthins in stems and betacyanins in leaves were confirmed.

New biotechnological approach of conservation – cryopreservation – was successfully applied to ulluco. The excess of water, dangerous in ice formation, was removed from shoot tips by two methods: by desiccation over silica gel and by osmotic dehydration in plant vitrification solution (PVS3). Plant shoot tips were not able to recover from water stress caused by removal up to 82 % of water. The effect of sucrose pre-treatment on shoot tips improved tolerance to PVS3 dehydration. Shoot tips lost their water at a rate of 1.4 g H_2O g^{-1}DW during the first hour over silicagel and reached 22 % of their initial water content fresh weight basis. The combination of sucrose pre-treatment and 2 hour desiccation over silica gel allowed 10 % of the shoot tips to survive and regrow following

cryopreservation. In the case where shoot tips were treated with PVS3 for 1.5 hour regeneration the percentage reached 53%. According to our results the theoretical potential for improvement of regeneration after LN is 21 %. This makes cryopreservation a promising method for the ulluco plant.

Long-term conservation of yacon was supported by high survival of shoot tips after pre-treatment by sucrose and PVS3 even though there was not detected survival after the storage by cryopreservation. PVS3 treatment for 1 hour seems to be promising, but more research is needed to overcome the problem with regeneration after storage in LN. Changing the pre-treatment steps and using other cryoprotocols will be the basic for future improvement of yacon cryopreservation.

The safest method for long-term storage without the risk of loosing the genotypes by biotic and abiotic stresses is the cryopreservation method.

According to our results, which were implemented in the Czech Republic, the best methods for conservation of yacon were field and *in vitro* conservation and for ulluco *in vitro* conservation and cryoconservation. The highest propagation rate was in *in vitro* conditions for both plants. Cryopreservation was the best method for long-term conservation as was demonstrated on ulluco.

Research on key conservation methods was successful and led to an improvement of conservation methods for both plants. Introduction of obtained information will also help to further research and saving of this unique germplasm.

Recommendations

(1) The tuber of yacon harvested from the field conditions is suitable for short-time (up to one year) as a seed yacon for the next year. Ulluco is not able to form tubers in The Czech Republic environment. The ulluco progeny is, therefore, not recommended for multiplication in the field condition because of its low yield.

(2) It is recommended to prolong the storage period *in vitro* up to 6 months of yacon by lower temperature 17 °C. Also, ulluco showed remarkably good response to conservation *in vitro* in low temperatures prolonged at temperature 5 °C.

(3) The *in vitro* plantlets are recommended for medium–time storage of both species. The lower the temperature (up to 17 °C), the longer time can be yacon and ulluco stored without passage.

(4) The optimal temperature 25 °C for yacon micropropagation with multiplication coefficient 3.45 and optimal temperature 21 °C for ulluco micropropagation with

multiplication coefficient 7.7 in *in vitro* condition is recommended. The plantlets of yacon and ulluco grown *in vitro* at these optimal temperatures are suitable for slow-growth storage as well as for multiplication for cryopreservation.

(5) *In vitro* grown plantlets are suitable for short-time and medium-term storage for clone repository. The micropagation cycles one month for yacon and two months for ulluco respectively made this method laborious.

(6) Involvement of microtubers *in vitro* is most effective for medium-term storage of ulluco for 15 months.

(7) The cryopreservation method is recommended as the most effective method for long-term storage of ulluco germplasm. The vitrification state of plants involved by Plant Vitrification Solution No 3 is recommended as optimized for non-lethal thermal events.

(8) Because the yacon cryopreservation is still not as successful as ulluco cryopreservation, the alternative way *in vitro* slow-growth method is recommended for saving the biodiversity of yacon.

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